SOME STUDIES CONCERNING PYRIMIDINE BIOSYNTHESIS

By

Robert L. Herrmann

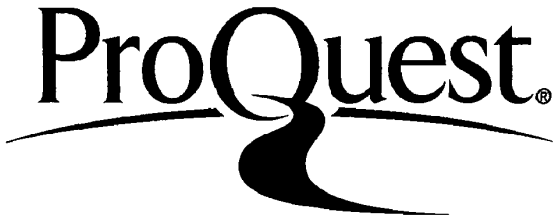
A THESIS

Submitted to the School of Graduate Studies
of Michigan State University of
Agriculture and Applied Science
in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1956



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VITA

The author was born July 17, 1928 in New York City, and received his secondary education at Bayside High School, Bayside, New York. He enrolled at Purdue University for the spring semester, 1946, and entered the United States Navy in August of the same year. After two years as a naval electronics technician the author resumed his studies at Purdue University and was graduated in June of 1951 with a Bachelor of Science degree. He was then recalled to naval service for fifteen months. In September of 1952 he enrolled in the Graduate School of Michigan State University. In the course of his graduate training the author served two years as a Graduate Teaching Assistant in Chemistry and two years as a Special Graduate Research Assistant under an Atomic Energy Commission Grant.

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ABSTRACT

The following investigations were carried out to study several aspects of pyrimidine biosynthesis: 1) A study of methionine methyl group utilization for thymine biosynthesis in the rat; 2) A study of the possibility of alpha-aminobutyric acid utilization for pyrimidine biosynthesis in Neurospora; 3) A similar study of aminobutyric acid utilization in the rat.

Methionine-methyl-C^{14} was administered to rats and the purines and pyrimidines of deoxyribonucleic acid were isolated and their specific activities determined. Adenine, guanine and thymine were found to be appreciably labeled, presumably by way of the "one-carbon pool". The radioactivity of thymine appeared to be in the methyl carbon, since cytosine was not labeled appreciably and the iodoform - representing the methyl carbon - obtained on degradation of thymine was highly radioactive. The data suggests that formic acid is not an intermediate in conversion of the methionine methyl group to thymine, but rather that a hydroxymethyl derivative is involved. The results indicate that the methyl group of methionine is not a significant precursor of the ureide carbons of the purines, but its important position in biosynthesis of the methyl carbon of thymine is definitely established.

A pyrimidine-requiring mutant of Neurospora crassa, strain 1298, which had previously been shown to utilize

alpha-aminobutyric acid for growth, was grown on the 3-C^{14} labeled amino acid in an effort to determine the reason for its pyrimidine-replacing ability. The isolated ribonucleotides were hydrolyzed to the free purine and pyrimidine bases, and the pyrimidines were found to have five times the specific activity of the purines. A similar distribution of activity was found in the acid-soluble nucleotide fraction. A number of amino acids isolated from the soluble protein fraction were found to have specific activities even lower than the purines, with the exception of isoleucine, which was labeled to about the same extent as the pyrimidines. The fact that labeled aminobutyric acid - a known intermediate in isoleucine biosynthesis - gave rise to a similar degree of labeling in isoleucine and the pyrimidines establishes the importance of aminobutyric acid as a pyrimidine precursor in Neurospora. A ten-fold dilution of the labeled precursor was found to occur on conversion to the pyrimidines, but a pool of diluted aminobutyric acid was found to exist in the mold, suggesting that once growth begins the organism is able to synthesize aminobutyric acid. This phenomenon and the nature of the mutation involved are discussed. A possible pathway of utilization of aminobutyric acid for pyrimidine biosynthesis involving homoserine and beta-aspartyl phosphate is also suggested.

A study of the possible utilization of aminobutyric acid for pyrimidine biosynthesis in the rat was also begun. The cytosine from isolated deoxyribonucleic acid was found to be four times as radioactive as adenine from the same source, suggesting that aminobutyric acid may also be a pyrimidine precursor in the rat. The low labeling of thymine indicates that a different pathway for its biosynthesis may exist. However, the generally low level of radioactivity of the various isolated compounds makes the interpretation of the data of questionable value.

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INTRODUCTION

INTRODUCTION

The biosynthesis of the pyrimidine bases of the nucleic acids - the major constituents of the genetic material of the cell and the active principle of virus particles - has become an increasingly important subject for research studies. A clear picture of the method of pyrimidine formation by the cell has become a necessary goal for any general understanding of nucleic acid biosynthesis.

Early work on the origin of the carbon atoms of the pyrimidine ring was carried out by Heinrich and Wilson (1) who demonstrated the origin of the carbon atom at position 2 from carbon dioxide in the rat. Similar results were also obtained by Reichard and Lagerkvist (2).

Work by Mitchell and Houlahan (3) pointed to a role for the 4-carbon acids of the citric acid cycle, since oxalacetic acid and aminofumaric acid were found effective in stimulating the growth of pyrimidineless *Neurospora* mutants. The findings of Loring and Pierce (4), showing that nucleosides were utilized more effectively than the free pyrimidine bases by these mutants, suggested that an acyclic intermediate may be combined with ribose at some step prior to ring closure. Orotic acid and uracil were visualized as being utilized by way of ring rupture and subsequent ribosidation, and Mitchell *et al.* (5),

after genetic investigations, arrived at the conclusion that orotic acid was not a normal intermediate, but arose in a side reaction during pyrimidine biosynthesis.

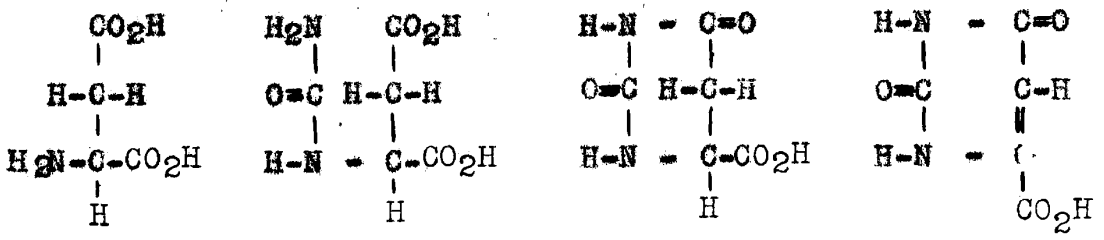
However, in other organisms the importance of orotic acid seems well established. Reichard (6) found that N^{15}-labeled orotic acid was extensively incorporated into the pyrimidines of polynucleotides of several organs of the rat, whereas purines were not labeled. Later work by Weed and Wilson (7) corroborated these findings, since orotic acid labeled in the 6 position with C^{14} was found to be utilized similarly in the rat. It is interesting to note that the orotic acid pathway apparently exists also in yeast, as shown by Edmonds, Delluva, and Wilson (8). Experiments were also carried out with Lactobacillus bulgaricus 09 by Wright et al. (9, 10) which demonstrated the requirement of this organism for orotic acid; the requirement could not be replaced by any other pyrimidine. In addition, further understanding of the pyrimidine biosynthetic pathway in this organism was afforded by the finding that DL-ureidosuccinic acid labeled in the ureide carbon was as effective a precursor for nucleic acid pyrimidines as was orotic acid. This relationship was also demonstrated to exist in the rat by Weed and Wilson (11) who found that DL-ureidosuccinic acid was incorporated into polynucleotide pyrimidines.

A role for aspartic acid as a precursor of the carbon chain of pyrimidines was suggested by the work of

Lagerkvist et al. (12), who tested aspartic acid-3-C^{13}-4-C^{14} in rat liver slices. The labels were incorporated into the polynucleotide pyrimidines, though the methylene carbon was utilized to a greater degree than the carboxyl carbon which suggested cleavage of the carbon chain in the course of utilization. The authors felt, however, that the molecule was used as a whole, since the pyrimidines were not degraded and there was thus some question as to the validity of the C^{13} to C^{14} ratio. The importance of aspartic acid was also suggested by the experiments of Woods, Ravel and Shive (13) with Lactobacillus arabinosus 17-5, an aspartic acid-requiring mutant which was shown to be able to utilize pyrimidines, as well as threonine and lysine, as a means of sparing the aspartic acid requirement. This was taken as proof that aspartic acid was involved in pyrimidine biosynthesis.

The conversion of L-aspartic acid to L-ureidosuccinic acid in rat liver mitochondria has recently been demonstrated by Reichard (14). Aspartic acid, carbon dioxide and ammonia are converted to ureidosuccinic acid in the presence of carbamylglutamic acid, adenosine triphosphate, and magnesium ion. The reaction appears to require several enzymes, and the initial step may be formation of carbamyl phosphate. The latter has been demonstrated as involved in the conversion of aspartic acid to ureidosuccinic acid in S. fecaelis extracts by Jones, Spector, and Lipmann (15).

The entire sequence of reactions from aspartic acid to pyrimidines has been demonstrated by Lieberman and Kornberg (16, 17, 18) for Zymobacterium orotigum. An enzyme called ureidosuccinase converts L-ureidosuccinic acid (II) to L-aspartic acid (I) with the liberation of carbon dioxide and ammonia. The next step is reversible and involves cyclisation and dehydration to L-dihydro-orotic acid (III) with the involvement of an enzyme called dihydroorotase. The dihydropyrimidine is then oxidized to orotic acid (IV) by the action of dihydroorotic dehydrogenase. The conversion of aspartic acid to ureidosuccinic acid appears to require a separate enzyme system, perhaps involving an enzyme such as that found in rat liver extracts by Lowenstein and Cohen (19). This enzyme may convert carbon dioxide, ammonia, and adenosine triphosphate to a reactive carboxamide-group donor such as carbamyl phosphate, which then reacts with aspartic acid to form ureidosuccinic acid.



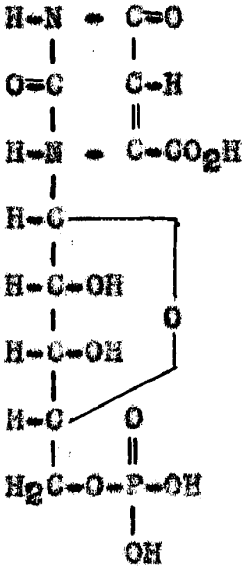
L-aspartic acid — I

L-ureidosuccinic acid — II

L-dihydroorotic acid — III

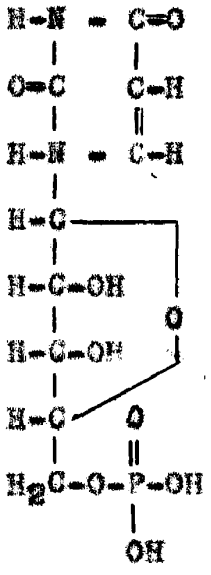
orotic acid — IV

The existence of the orotic acid pathway implies the initial formation of the pyrimidine ring followed by ribosidation, and this sequence of reactions was recently shown by Lieberman, Kornberg, and Simms (20). Yeast enzymes were able to convert orotic acid to orotidine-5'-phosphate (V) by reaction with 5-phosphoribosylpyrophosphate, and then, by decarboxylation, to convert orotidine-5'-phosphate to uridine-5'-phosphate (VI).



orotidine-5'-phosphate

V



uridine-5'-phosphate

VI

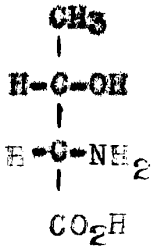
In contrast to this rather well-defined biosynthetic pathway in several bacteria, in yeasts, and in the rat, pyrimidine biosynthesis in Neurospora is not clearly understood. The work of Mitchell and Houlahan (3) and of Loring and Pierce (4) combine to suggest a role for some acyclic ribose derivative. The occurrence of a second possible pathway does not seem unlikely since the same situation

exists in purine biosynthesis, where much work by Buchanan (21) and by Greenberg (22) has established an acyclic ribose derivative as an intermediate in purine biosynthesis in pigeon liver. However, Kornberg and coworkers (23) have recently reported the reaction of purines with 5-phosphyribosylpyrophosphate to form the nucleotide in yeast extracts. The two mechanisms, ribosidation before and after ring closure, therefore do occur, and it seems reasonable to suspect that a similar situation exists in pyrimidine biosynthesis.

Furthermore, a number of growth studies also suggest that some organisms may possess a pyrimidine biosynthetic route distinct from that involving orotic acid. Woods and coworkers (13) were unable to replace the aspartic acid requirement of Lactobacillus arabinosus 17-5 by either orotic acid or ureidosuccinic acid. In addition, Fairley (24) has demonstrated that several pyrimidineless Neurospora mutants may utilize threonine (VIII) or alpha-aminobutyric acid (VIII) for growth. Aspartic acid was ineffective, and later work (25) demonstrated that ureidosuccinic acid was likewise not utilized.

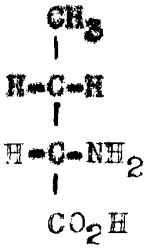
Later, evidence was obtained for the involvement of the "1-carbon-pool" in the biosynthesis of the 5-methyl carbon atom of thymine by Totter et al. (26), who found activity in this group using formic acid-C^{14} as precursor, and by Elwyn and Sprinson (27), who demonstrated the utilization of serine-3-C^{14} and glycine-2-C^{14} for this methyl carbon. The involvement of methionine (IX) in the

"1-carbon pool" was indicated by the work of Berg (28), which demonstrated the conversion of formic acid to the methyl group of methionine in pigeon liver extracts, and suggested an S-hydroxymethyl derivative as an intermediate. In addition, methionine has been shown to be a very important donor of intact methyl groups in transmethylation reactions in animals (29).



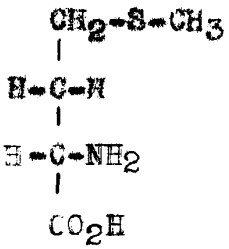
L-threonine

VII



L-alpha-amino-n-butyric acid

VIII

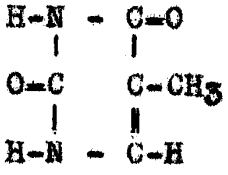


L-methionine

IX

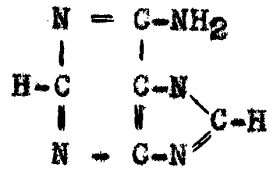
It therefore appeared likely that methionine might be a significant precursor of the methyl group of thymine (X), by way of the "1-carbon pool", and the possibility also existed that a transmethylation to the 5 carbon of the pyrimidine ring might be a significant source of the thymine methyl group. In the present work the possible utilization of C^{14}-methyl-labeled methionine for the biosynthesis of the thymine methyl carbon of deoxyribonucleic acid in the rat has therefore been investigated. The labeling of the purine bases, adenine (XI) and guanine (XII), of deoxyribonucleic acid was also studied as a possible means of comparison of the importance of the two possibilities -

transmethylation, or oxidation to a 1-carbon intermediate - and formic acid-C^{14} was run in parallel experiments to have a means of comparison with other published work.



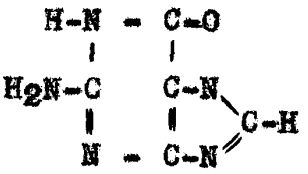
thymine

X



adenine

XI



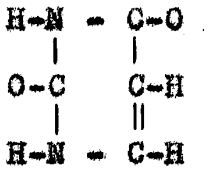
guanine

XII

The previously-mentioned growth studies in which alpha-aminobutyric acid was found to support the growth of a pyrimidineless mutant, N. crassa strain 1298, suggested that this amino acid might be involved in pyrimidine biosynthesis in Neurospora. The fact that the generally accepted pathway - involving conversion of aspartic acid to the pyrimidine nucleotides by way of ureidosuccinic acid, dihydroorotic acid, and orotic acid - does not appear to exist in this organism seems to support this possibility. The present work therefore also includes a study of the labeling of the pyrimidines - uracil (XIII) and cytosine (XIV) - and related compounds after growth of N. crassa 1298 on 3-C^{14} alpha-aminobutyric acid. A similar study has also been started with the rat.

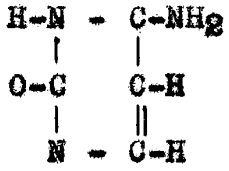
Growth studies with Neurospora were also carried out to test a number of possible relationships suggested by this work and by recent reports of Fink, et al. (30) and of Grisolia and Wallach (31) that a biosynthetic

pathway involving conversion of beta-alanine (XV) to beta-ureidopropionic acid (XVI) and subsequent cyclisation may be involved in pyrimidine biosynthesis.



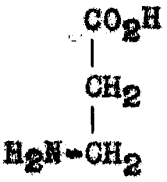
uracil

XIII



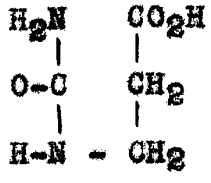
cytosine

XIV



beta-alanine

XV



beta-ureido propionic acid

XVI

EXPERIMENTAL AND RESULTS

EXPERIMENTAL AND RESULTS

The Utilization of Methionine for Thymine Biosynthesis

Materials

Two male albino rats weighing about 160 g. were injected intraperitoneally with 1 ml. of a water solution containing 0.1 mc. of methionine-methyl-C^{14}, and a second pair of rats were injected with 0.1 mc. of formic acid-C^{14}*. This amount of activity was supplied by 20 mg. of radioactive methionine and by 2.61 mg. of formic acid-C^{14}.

The synthesis of methionine-methyl-C^{14} was carried out according to the procedure of du Vigneaud, Dyer, and Harmon (32). This consisted of the reduction of 1 millimole of homocystine to homocysteine by reaction with sodium in liquid ammonia. The mixture was contained in a glass tube through which nitrogen gas was slowly bubbled to afford stirring and to maintain an inert atmosphere. A temperature of approximately -70°C was maintained by use of a dry ice - acetone bath. One millimole of C^{14}-methyl iodide (1 mc./mM. activity) was added to the reaction mixture after warming to room temperature. The mixture was concentrated to a small volume, and the resulting methionine-methyl-C^{14} crystallized out. The crystals were washed with

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*Obtained from the Isotopes Specialties Company, Glendale, California

cold ethanol, then with ether, and dried in vacuo. The yield after recrystallization from 80 per cent ethanol was 90 per cent of theoretical. The compound melted at 279-280°C.

Isolation of Deoxyribonucleic Acid

The rats were killed by etherization 24 hours after injection. The total viscera, including heart, lungs, kidneys, liver, spleen, stomach, intestines and testes, was then removed, cleaned, frozen on solid carbon dioxide, and stored in the deep freeze for 12 hours. The frozen tissue was diced and then homogenized in a Waring Blendor with 200 ml. of cold absolute ethanol for five minutes. The homogenate was transferred to 250 ml. Pyrex centrifuge bottles and centrifuged at -10°C for 20 minutes at 5000 r.p.m., using a Model V International centrifuge. The supernatant was discarded and the residue was extracted three times with a boiling 3:1 (v/v) ethanol-ether mixture for five minutes. The residue from these lipid extractions was washed twice with ethanol, three times with ether, and was then air dried.

The dried, lipid-extracted viscera was then placed in a mortar and mixed with ten per cent (w/v) sodium chloride solution to make a paste. Carborundum of 120 mesh size was then added and grinding carried out for 15 minutes. The mixture was transferred to a 250 ml. Pyrex centrifuge bottle, using a sufficient amount of the sodium chloride solution to make a final volume of 150 ml. The mixture

was then heated on the steam bath for 20 minutes, and thereafter stirred slowly for 24 hours at room temperature. After centrifugation, the remaining solid was re-extracted with a 100 ml. portion of fresh sodium chloride solution for 12 hours, and the extracts combined. The residue from the re-extraction was washed with 20 ml. of the sodium chloride solution and the washing added to the combined extracts in a 1 liter beaker. Two and one-half volumes of ethanol were then added to precipitate the crude sodium nucleates, which were centrifuged down in 250 ml. centrifuge bottles. After washing with ethanol and ether, the material was air dried and weighed. A typical yield was 1 g. of mixed nucleates.

Deoxyribonucleic acid was isolated from the mixed sodium nucleates by the method of Hammarsten (33). Sufficient 0.1 N sodium hydroxide was added to the dried nucleates in a 200 ml. flask to make a 1 per cent solution. This was then heated in a boiling water bath for two hours and acidified to pH 2 with 2 N hydrochloric acid. One-tenth volume of 0.1 M lanthanum nitrate was then added to form the insoluble lanthanum salt of deoxyribonucleic acid, and the mixture was centrifuged in 250 ml. centrifuge bottles. The precipitate was washed twice with small amounts of 0.01 M lanthanum nitrate, transferring the solid to a 15 ml. centrifuge tube in the process. The lanthanum deoxynucleate precipitate was finally treated for separation from contaminating protein by the following procedure. One

ml. of 1 M potassium carbonate and four and one-half ml. of water was added to the solid, and the mixture heated in a hot water bath for 5 minutes. After centrifugation, the solution was placed in a 100 ml. beaker and the precipitate re-extracted twice with 0.5 ml. of water. The combined supernatants were acidified to pH 5.7 or below with glacial acetic acid, and then boiled for five minutes to remove carbon dioxide. The deoxyribonucleic acid was precipitated by the addition of four volumes of ethanol. The mixture was placed in the refrigerator for 12 hours, after which it was centrifuged, and the greyish-white precipitate washed with ethanol, ether, and then air dried and weighed. The average yield from a 160 g. rat was about 100 mg. of deoxyribonucleic acid.

Isolation of Purine and Pyrimidine Bases

The deoxyribonucleic acid was placed in a 10 ml. volumetric flask and hydrolyzed by the method of Marshak and Vogel (34). Two ml. of 7 N perchloric acid was added and the mixture was heated on the steam bath behind an explosion shield for one hour with occasional shaking. The contents of the flask were then transferred to a 15 ml. centrifuge tube with 1 ml. of water, and the charred residue washed with one 0.5 ml. portion of water. The combined supernatants were placed in a second 15 ml. centrifuge tube and basified to a pH of 11 by the addition of 5 N potassium hydroxide. The precipitate of potassium perchlorate was

removed by centrifugation, and the supernatant containing the free purine and pyrimidine bases, usually an amber-colored liquid due to the presence of suspended colloidal carbon particles, was placed on a 2.5 by 27 cm. Misco* column packed with Dowex 1 anion exchange resin of 50 to 100 mesh size and twelve per cent crosslinking. The resin had previously been converted to the chloride form by treatment with 1 N hydrochloric acid and subsequent washing to remove excess acid. After the solution of bases had filtered into the resin, a chromatographic procedure similar to that suggested by Cohn (35) was carried out. The pyrimidine bases were eluted in separate peaks by elution at the rate of two and one-half ml. per minute with 0.015 M ammonium formate buffers of successively lower pH. The first buffer to be passed through the column was of pH 10.1, and served to elute cytosine in a 75 ml. peak after about 100 ml. of buffer had passed through the column. The samples were collected in 50 ml. beakers located on the turntable of a Misco automatic fraction collector, and the elution of the samples was followed by reading a portion of the contents of each beaker in a Beckman Model DU Spectrophotometer**. The ratio of optical density (absorbancy) at 260 mμ to that at 280 mμ was found most reliable as a means of following the elution of the bases

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*Microchemical Specialties Co., Berkeley, California

**National Technical Laboratories, S. Pasadena, California

from the column. After elution of cytosine, the column was next treated with about 150 ml. of buffer of pH 9.1, which was then followed by addition of buffer of pH 8.25 for the removal of thymine. After the passage of about 150 ml. of the buffer, the pyrimidine appeared as a broad, symmetrical peak in 200 to 250 ml. of effluent. Finally, adenine and guanine were recovered by addition of 200 ml. of 1 N hydrochloric acid. The acid effluent was taken to dryness several times in vacuo to remove excess hydrochloric acid, and the residue, taken up in a few ml. of 0.1 N hydrochloric acid, was allowed to filter into the resin bed of a 1 by 12 cm. column. The resin used was a Dowex 50 cation exchanger of twelve per cent crosslinking and 100 to 200 mesh particle size which had been placed in the hydrogen form by successive acid and water washes as described for the preparation of the Dowex 1 column. The purine bases were eluted with 3 N hydrochloric acid, guanine coming off in a 200 ml. volume after 30 ml. of eluting agent had passed through the column, and adenine following - after efflux of an additional 40 ml. of acid - in a 200 ml. volume. The elution of the bases was followed by observation of the ratio of optical densities at 249 and 260 mu for each fraction.

Cytosine Purification

The ratio of optical densities at two wavelengths of the cytosine fraction, when compared to the ratio for known cytosine, indicated considerable contamination, and the eluate was therefore evaporated to a small volume, acidified with 2 drops of concentrated hydrochloric acid, and placed on a 1.5 by 23 cm. column packed with Dowex 50 resin in hydrogen form. After allowing the sample to filter in, the column was treated with 2 N hydrochloric acid, again following elution of the cytosine by observation of the optical densities at 260 and 280 mμ. A value of 1.55 for the ratio of optical density at 280 mμ to that at 260 mμ indicated that the cytosine was satisfactorily pure.

Isotope Measurement

One ml. aliquots of the thymine fractions obtained from the original Dowex 1 separation and similar aliquots of the cytosine fractions obtained from the Dowex 50 purification were plated on platinum dishes. Half-milliliter aliquots of the purine fractions were similarly plated. In all cases the solvents were removed by slow evaporation over an infrared lamp, and the radioactivity of the samples determined at infinite thinness in a Nuclear internal flow Geiger counter. Since the concentration of each aliquot was known from optical density measurements and molar extinction coefficients, the specific

activity, expressed as counts per minute per micromole, could be calculated. The molar extinction coefficients for thymine in 0.015 M ammonium formate buffer and for adenine and guanine in 4 N hydrochloric acid and 3 N hydrochloric acid respectively were determined as outlined in the appendix. The molar extinction coefficient of cytosine in acid solution was obtained from the literature (36).

Thymine Degradation

In an attempt to establish that the activity of the thymine molecule actually resided in the methyl carbon, the degradation of the molecule was undertaken.

The method of Baudisch and Davidson (37), was first tried. This involved conversion of thymine to 5-bromo-4-hydroxyhydrothymine with bromine water, followed by hydrolysis in the presence of silver oxide to thymine glycol and further hydrolysis with sodium bicarbonate to urea plus acetol. The acetol, representing carbons 4 and 5 and the methyl carbon, was distilled out of the reaction mixture and converted by iodine in sodium hydroxide to glycolic acid and iodoform. The latter represented the original methyl carbon of thymine. In practice the thymine glycol was not isolated, the hydrolysis being carried out directly to the acetol stage. Some difficulty was experienced in obtaining favorable yields on the millimolar scale by this method, and a more direct procedure,

suggested by Dr. John C. Speck of the Chemistry Department, was found more useful. This consisted of direct reaction of thymine with iodine in sodium bicarbonate solution, and gave yields of eighty to eighty-five per cent of theoretical.

Isolation of thymine from the column effluent was found necessary as a preliminary to the degradation to iodoform. Formate ion was found somewhat inhibitory, probably by competition for hypoiodite ion, and ammonium ion was strongly inhibitory at the concentrations of the buffer, though studies of the latter effect indicated no general inhibition of the iodoform reaction. Experiments to determine conditions under which sodium formate buffers could be substituted for ammonium formate proved fruitless. The eluate was also shaken with Dowex 50 resin in hydrogen form in an attempt to remove ammonium ion, followed by a similar treatment with Dowex 1 resin in iodide form to remove formate ion, but thymine still would not give iodoform. It was finally decided to use a column of Dowex 50 resin, in the event that shaking of the resin with the eluate did not bring about complete removal of interfering ions, which were therefore present in sufficient quantity to prevent reaction. The procedure was successful in removing ammonium ion, as evidenced by a negative test with Nessler's reagent, and evaporation of this column eluate to dryness served to remove formate ion as volatile formic

acid. The thymine was transferred to a 10 ml. volumetric flask with 5 ml. of half-saturated sodium bicarbonate solution and the total of 2.71 mg. was diluted with twice this amount of unlabeled thymine to give a total of 8.13 mg. One ml. of a solution of iodine in potassium iodide consisting of 12.7 g. of resublimed iodine in 25 ml. of water containing 40 g. of potassium iodide was added. The mixture was incubated at 37°C. for one week, and the iodoform, which precipitated as yellow platelets, was filtered off on a fritted glass filter. The precipitate was washed with saturated potassium iodide solution to remove contaminating iodine, and the crystals then dissolved from the filter with chloroform and the solution evaporated on a tared watch glass. The total yield was 2.1 mg. of iodoform. Attempts to purify the iodoform by sublimation had proved unsuccessful in trial experiments due to thermal decomposition to iodine, so without further purification the compound was taken up in chloroform and made up to 5 ml. in a glass-stoppered volumetric flask.

Aliquots ranging from 100 to 500 ul. were plated on platinum dishes, evaporated to dryness, and immediately counted in the internal flow counter. It was discovered that iodoform had a quenching action on the counting rate of such magnitude that the larger samples had less activity than the smaller samples. Since the largest sample plated was only 0.21 mg., the results could not be attributed to

self-absorption, so an attempt was made to obtain a quenching curve of the amount of iodoform versus the activity of the sample by plating known quantities of radioactive sodium carbonate mixed with known amounts of unlabeled iodoform, and determining the effect of the added iodoform on the counting rate. This curve should supply a factor by which the activities of corresponding amounts of labeled iodoform could be multiplied to obtain the actual activity. However, when this procedure was applied to the radioactive iodoform from the thymine of the methionine-fed rat, the specific activity determined was double the largest value theoretically possible. As an alternative, the remaining labeled iodoform, amounting to about 0.5 mg., was plated and an attempt was made to count the sample with an end-window Geiger counter, where no quenching is possible. However, the count obtained was not sufficiently above background to be significant. A similar result was found for iodoform from thymine isolated from a formate-fed rat. The solution to this problem thus appeared to lie only in an increase of the scale of the experiments.

Criteria of Purity

The fractions obtained in the column separations of the various bases were found to be satisfactorily pure as judged by the ratio of optical densities at two wavelengths. In the case of both cytosine and thymine the ratio of optical density at 280 mμ to that at 260 mμ was used.

For cytosine the value is 1.55, and for thymine the value is 0.56. In the case of the purines the ratio of optical density at 249 mμ to that at 260 mμ was employed, and the values obtained were 1.3 for guanine and 0.81 for adenine.

In addition, a comparison of radioactivity and concentration was also made. Aliquots of the chromatographic fractions which contained the labeled bases and also aliquots of several fractions devoid of these compounds were plated on platinum dishes and counted. The activities of these various fractions were plotted on the same axes as a curve of concentration versus volume of eluant. The coincidence of the radioactivity and concentration curves substantiated the purity of the isolated purines and pyrimidines in each case.

Results

The radioactivity of the isolated bases of the deoxyribonucleic acid of the rat after injection of formic acid-C^{14} and methionine-methyl-C^{14} is shown in Table I. The specific activity was expressed as counts per minute per micromole, the calculation of which is shown in the appendix.

It can be seen that appreciable amounts of isotopic carbon appeared in the adenine, guanine and thymine of deoxyribonucleic acid after administration of either precursor. The utilization of methionine was poorer than that of formic acid, as indicated by the dilution values

TABLE I

INCORPORATION OF FORMIC ACID-C^{14} AND METHIONINE-METHYL-C^{14} INTO RAT DNA COMPONENTS

Precursor	Exp. No.	Compound Isolated: Adenine c.p.m./μM	Dil'n*	Guanine c.p.m./μM	Dil'n.	Thymine c.p.m./μM	Dil'n.	Cytosine c.p.m./μM
Formic acid -C^{14}	1	10,700	106	9,000	126	1,300	870	100
	2	13,100	86	10,300	110	3,000	378	100
	Average	11,900	96	9,650	118	2,150	624	
Methionine-Methyl-C^{14}	3	380	777	510	952	220	1,340	10
	4	290	1,020	430	687	260	1,140	10
	Average	335	898	370	819	240	1,240	

*Dilution - specific activity of precursor/specific activity of compound isolated.

in the table. However, it is seen that the methionine precursor suffered a tenfold greater dilution on entering the purines than did formic acid, but only a two-fold greater dilution upon entering the thymine molecule. If the distribution of activity in the purines and thymine after methionine administration may be considered similar to that previously shown for formic acid, (3) the purines should be labeled in carbons 2 and 8 and the major activity of thymine should reside in the methyl carbon. The low activity of the cytosine lends support to the latter assumption. The results indicate that methionine does not serve as a significant precursor of the ureide carbons of the purines, and therefore does not contribute appreciably to the pool of "1-carbon units" at the oxidation state of formic acid. In contrast, the relatively higher incorporation of the methionine methyl group into thymine suggests that conversion to a "1-carbon unit" at the oxidation state of formaldehyde may readily occur, and that this derivative may then give rise to the methyl group of thymine. Transmethylation, though a possibility as a partial explanation of the data, cannot be a significant mechanism for thymine methyl formation because of the better utilization of formic acid.

The Utilization of Aminobutyric Acid for Pyrimidine Biosynthesis in Neurospora

Materials

Radioactive alpha-aminobutyric acid, labeled with C^{14} in the beta carbon atom, was kindly supplied by Dr. H. Tarver of the University of California. Paper chromatography and radioautography of the compound by procedures outlined in a subsequent section (see pages 33 and 54) indicated that the amino acid was essentially radiochemically pure, except for a slight indication of radioactive contamination which migrated as glycine and whose concentration was estimated at less than a tenth of 1 per cent of the total activity. The specific activity of the aminobutyric acid was given as 3.1 μc/mg., or 3.05×10^5 c.p.m./μM. However, the value actually obtained by quantitative ninhydrin (see page 64) and radioactivity (see page 16) determinations of the chromatographed sample was 4.41×10^5 c.p.m./μM. In order to obtain a pyrimidine labeling suitable for counting, 11 mg. of the radioactive compound was diluted with 89 mg. of recrystallized unlabeled aminobutyric acid, and the final activity of the precursor was therefore 4.84×10^4 c.p.m./μM.

Organisms

Neurospora crassa strain 1298, a pyrimidine-requiring mutant which had been isolated by Beadle and Tatum (38) and later described in detail by Loring and Pierce (4), was

used throughout the present study, except for several experiments where the wild, non-mutated strain was employed. The mold was maintained on culture slants consisting of basal medium containing 2 per cent agar and 1 mg. of uracil per ml.

Growth Procedure

The mold was grown in 125 ml. Erlenmeyer flasks to which was added 12.5 ml. of a basal nutrient medium having the composition shown in Table II. The composition of 500 ml. of the stock trace element solution, of which only 50 µl. are used per 10 l. of media, is also included in the table.

TABLE II

THE COMPOSITION OF THE BASAL NUTRIENT MEDIUM

Calcium chloride	1 g.	Trace element solution	50 µl.
Ammonium tartrate	50 g.	Sodium tetraborate	8.8 g.
Ammonium nitrate	10 g.	Ammonium molybdate	6.4 g.
Potassium dehydrogen phosphate	10 g.	Ferric chloride	50 g.
Magnesium sulfate heptahydrate	5 g.	Zinc sulfate heptahydrate	200 g.
Sodium chloride	1 g.	Cupric sulfate	27 g.
Sucrose	101 g.	Manganous chloride	4.5 g.
Biotin	26 µg.	Distilled water to	500 ml.
		Distilled water to	10 l.

Five mg. of the labeled alpha-aminobutyric acid having a specific activity of 48,000 c.p.m./µM and a total activity of 2.33×10^6 counts was added to each flask, and the flasks were then stoppered with cotton plugs and autoclaved

for 20 minutes. After cooling, the flasks were innoculated with 0.2 ml. of a spore suspension of the mold, made by dispersing two loopfuls of the spores in 10 ml. of sterile distilled water. Incubation was usually carried out at 25°C for 4 days, at which time sporulation was just commencing. The contents of the flasks were then filtered on a fritted glass funnel with suction, and the mycelial residue was washed with 5 ml. of water and then soaked in 100 ml. of acetone for 15 minutes. The acetone was removed and the mycelia placed in a desiccator for 24 hours. A brittle disc was thus obtained which was suitable for grinding.

Overall Distribution of Isotope

The amounts of activity contained in the mycelium, the trichloroacetic acid and ethanol-ether extracts, the ribonucleotides, and the residual growth medium were determined in the following manner.

The total amount of activity contained in the mycelium was determined by suspending 10 mg. of the total ground mycelium in 5 ml. of water in a volumetric flask. A 1 ml. aliquot was then diluted to 100 ml., and 1 ml. aliquots of this diluted sample were then plated and counted as described previously (see page 16). One milliliter portions of the residual growth medium, obtained by filtration of the mycelial pads at the end of the growth period, and similar portions of the trichloroacetic acid and ethanol-ether extracts, obtained as described in the following

section, were diluted to 100 ml. with water. One milliliter aliquots were again plated and counted. The ribonucleotide fraction was counted by diluting 100 µl. of the ribonucleotides - obtained by column purification as described in the next section - to 5 ml., and again plating and counting 1 ml. aliquots.

The results of the determination of the overall distribution of radiocarbon in the mutant and the wild strain are compared in Table III.

TABLE III

DISTRIBUTION OF CARBON-14 IN NEUROSPORA AFTER GROWTH IN THE PRESENCE OF AMINOBUTYRIC ACID-3-C^{14}

	Activity as 10^6 c.p.m.	
Fraction or Components	Mutant Strain	Wild Strain
Aminobutyric Acid-3-C^{14} supplied	47.0	47.0
Growth medium	8.0	2.4
Mycelium	20.0	15.9
TCA-soluble*	5.0	4.0
Lipids	0.7	0.2
Ribonucleotides	2.5	1.7
Residue (by difference)	11.8	10.0
Not accounted for (loss as CO_2)	19.0	28.7

*Soluble in 10 per cent trichloroacetic acid

It is interesting to note the much higher activity contained in the residual growth medium of the mutant, suggesting the accumulation of one or more radioactive compounds. Perhaps the two highly labeled amino acids located on radioautograms of chromatograms of the growth medium are these compounds.

Study of Ribonucleotides

Isolation of Ribonucleotides. The mycelium obtained from 10 flasks of the mold grown for 6 days on 3-C^{14}-aminobutyric acid was ground in a mortar with 120 mesh carborundum powder for 15 minutes. The resulting powder was extracted 3 times with 10 ml. of cold, 10 per cent (w/v) trichloroacetic acid transferring to a 12 ml. Pyrex centrifuge tube in the process. The solid residue was washed once with 4 to 1 (v/v) ethanol and was then extracted 3 times with boiling 3 to 1 (v/v) ethanol-ether. The extractions were carried out by suspending the centrifuge tube in a boiling water bath and stirring the contents occasionally. The process was carried out for a half-hour the first time, and for 5 minutes the remaining times. The lipid-extracted residue was then washed twice with ether and air dried. Five milliliters of 1 N potassium hydroxide was then added, and the mixture allowed to stand at room temperature for 24 hours. After centrifugation, the supernatant was transferred to a second 12 ml. centrifuge tube cooled on ice, and acidified to a pH of 3 with concentrated perchloric acid. The resulting precipitate of potassium

perchlorate and protein was allowed to coagulate for 10 minutes and then was removed by centrifugation. The resulting supernatant of ribonucleotides and suspended protein was filtered through a layer of Celite on a fritted glass filter, and basified to a pH of 11 with 1 N potassium hydroxide. The solution of ribonucleotides thus obtained was allowed to filter into a 1 by 27 cm. column containing Dowex 1 anion-exchange resin of 50-100 mesh size and 12 per cent crosslinking. The column was developed first with 200 ml. of water, and then with 200 ml. of 2 N hydrochloric acid. The optical density of the latter eluate was determined at 260 mμ, and an approximate extinction coefficient of 10,000 together with an average molecular weight of 350 was used to estimate the concentration of mixed nucleotides as 5 mg. The solution was then evaporated in vacuo to dryness several times to remove hydrochloric acid yielding a greyish-white residue.

Hydrolysis of Ribonucleotides. The mixed ribonucleotides were taken up in several ml. of 0.1 N hydrochloric acid and transferred to a glass-stoppered, 10 ml.-volumetric flask. The mixture was then blown to dryness by means of a stream of charcoal-filtered air. After careful addition of 0.5 ml. of concentrated perchloric acid, the flask was heated on the steam bath behind an explosion shield for 40 minutes. The contents of the flask were then transferred to a 12 ml. centrifuge tube with the aid of

several small portions of water, and, after centrifugation and transfer of the supernatant to a 5 ml. beaker, the precipitate was washed and the washings added to the beaker. The solution was then diluted to about 5 ml., to provide the perchloric acid concentration of about 1 N, preparatory to separation of the mixed bases.

Isolation of the Purine and Pyrimidine Bases. The solution of bases in 1 N perchloric acid was allowed to filter into the resin bed of a 1 by 27 cm. Dowex 50 column in hydrogen form. Elution with 100 ml. of water served to remove uracil mixed with perchloric acid. Cytosine came off in an 80 ml. volume after 160 ml. of 2 N hydrochloric acid had been passed through the column. Guanine was next eluted by 3 N hydrochloric acid in a 120 ml. volume after 60 ml. of the eluant had passed through the column, and adenine was then removed by 140 ml. of 4 N hydrochloric acid after a fore-run with 60 ml. of acid.

The uracil-perchloric acid solution was carefully taken to 3 ml. volume and basified to a pH of 11 with 5 N potassium hydroxide. After removal of the potassium perchlorate precipitate, the solution was placed on a 1 by 10 cm. Dowex 1 column in chloride form. After the solution had filtered into the resin bed, 100 ml. of 0.015 M ammonium formate buffer of a pH of 9.1 was passed through, and uracil was then removed in a 75 ml. volume after 75 ml. of 0.015 M ammonium formate buffer of a pH of 8.0 had

filtered through the column. It is worthy of note that it had been found that a slightly more acidic buffer was required to obtain a sharp peak on elution of the slightly more basic uracil than with thymine. The difference in pK_a values is very small, the value for uracil being 9.45 and that for thymine being 9.82. The effluent containing uracil was evaporated to a small volume and placed on a 1 by 27 cm. Dowex 50 column in hydrogen form for the removal of ammonium ion. The column was treated with 100 ml. of water which served to wash through all the uracil, the ammonium ion being retained on the resin. The water solution of uracil and formic acid was evaporated to dryness to remove the latter, leaving a white residue of about 0.5 mg. of uracil.

Purification of Purine and Pyrimidine Bases. Aliquots of the chromatographic fractions from the column separation of each base were plated and counted as described for the methionine-labeling studies, (see page 16) except that 1 ml. fractions of all the bases were plated. The procedures for quantitative determination of the bases by their absorption characteristics were likewise the same as those used for the previous study (see page 16).

Recrystallization. However, the various fractions proved to be grossly contaminated with other radioactive compounds, and rechromatography on both cation and anion-exchange resins proved of no avail in attempts at purification. The technique of recrystallization to constant

specific activity was therefore tried, making use of the general insolubility of these amphoteric substances at neutrality. The adenine, guanine, and cytosine were each evaporated to dryness several times to remove hydrochloric acid, and then all four of the bases were taken up in 0.1 N hydrochloric acid, transferred to 25 ml. volumetric flasks and made up to volume with the same solvent. The concentration of each solution was determined by measurement of its optical density at the characteristic absorption wavelength, and the compounds were then diluted with known amounts of the unlabeled bases. The pyrimidines were diluted with 9 parts of the unlabeled pyrimidine and the purines, because of their higher concentrations, were diluted with 19 parts of the unlabeled purines. In most of the experiments only cytosine and guanine were used, the other bases being retained for future degradation studies. The acid solutions of the diluted compounds were then neutralized with 0.1 N sodium hydroxide, and then evaporated to 2 ml. volume and placed in the cold overnight. The precipitate was centrifuged off, taken up in 0.1 N hydrochloric acid, the solution made up to 25 ml. volume, plated on platinum dishes, and counted. The procedure was repeated as many as four times, but the specific activity did not approach a constant value, and the ratio of optical densities at 260 and 280 mμ did not agree with the values determined for pure samples. However, the specific activity of the

pyrimidines generally increased with successive recrystallizations toward a maximum value of 6,000 c.p.m./μM, and the specific activity of the purines generally decreased with successive recrystallizations toward a minimum value of 500 c.p.m./μM.

One-dimensional paper chromatography. Success was finally obtained in purification of the purine and pyrimidine bases by the use of a one-dimensional paper chromatographic procedure. A solution of each base in 0.1 N hydrochloric acid was applied in a narrow band 3.5 inches from one end of a 7" by 19" strip of Whatman No. 1 filter paper. Careful application of a narrow band was possible using a 100 μl. micropipet attached to a hyperdermic syringe. Approximately 75 μl of solution was applied at a time, the papers being stretched between books and aired by a fan to promote rapid drying. After complete application of the solutions, the papers were folded 1 inch from the sample end and then in the opposite direction 2.25 inches from the same end. The 1-inch flaps of the paper strips were then placed in glass troughs and the papers passed up and over glass rods (each of which was above and to the side of a trough, parallel to it) such that the second folds coincided with the glass rods and the papers therefore hung straight down from this point. The two troughs were held at the top of an all-glass rack, and the entire system was contained in a 12 by 24 inch battery jar. The solvent used,

a mixture of 2-propanol, water and hydrochloric acid, was that described by Wyatt (39) for the separation of the purine and pyrimidine bases of the nucleic acids. In this solvent, the Rf values (the Rf value being defined as the ratio of the distance of migration of the compound being chromatographed to the distance traveled by the solvent) for the various bases are sufficiently different to obtain separation in the event that the contaminants are of this nature, and, in addition, the distances of migration are of intermediate value, making possible separations from materials which are either very soluble in the solvent, and would therefore migrate with the solvent front, or very insoluble in the solvent, remaining at the origin during development. The solvent was added to each trough in approximately 50 ml. amounts, and a 100 ml. portion was placed in the bottom of the jar, to aid in saturating the atmosphere. The jar was then covered with a glass plate and the solvent allowed to flow down the paper until it had almost reached the bottom, a process which required about 24 hours. The papers were then removed and dried, and viewed with ultraviolet light from a Mineralight* lamp; the so-called quenching technique. The pyrimidines and adenine appeared as dark blue bands on a light blue background, and guanine, which fluoresces slightly, appeared as a light blue band on the

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*Ultra-Violet Products, Inc., South Pasadena, California

blue background. The bands were cut out and eluted by means of attachment to a filter paper strip dipping into 0.1 N hydrochloric acid. The acid traveled down the paper and then through the band, and was collected in a 50 ml. beaker. The eluates were evaporated to dryness and rechromatographed by ion-exchange chromotography. On determination of concentration and radioactivity the fractions were found to be pure, as judged by the graphing method. (see page 21)

Specific Activity Determination. In subsequent experiments the bases were eluted from the paper chromatograms, transferred to 25 ml. volumetric flasks, diluted with 0.1 N hydrochloric acid, and the concentration and radioactivity determined. The concentration was ascertained by measurement of the optical density at the wavelength of maximum absorption. This value, when divided by the molar extinction coefficient for the appropriate compound in 0.1 N hydrochloric acid, gives the molar concentration. Radioactivity was determined by plating 1 ml. aliquots of the solutions and treating as outlined previously (see page 16). Uracil and Cytosine had specific activities of about 5000 c.p.m./μM, whereas the values for the purines were found to be about 900 c.p.m./μM.

Localization of Isotope. In an attempt to localize the radiocarbon of the uracil molecule, the degradation procedure of Heinrich and Wilson (1) was carried out with the diluted compound. In preliminary experiments 10 mg. of twice-

recrystallized uracil was dissolved in 5 ml. of hot, carbon dioxide-free water in the bottom half of a weighing bottle of about 50 ml. capacity. The bottle was then sealed with a greased rubber stopper which held a pair of pH electrodes connected to a portable Beckman model G pH meter*, a pair of 10 ml. burets, and two glass tubes. One of the tubes extended almost to the bottom of the bottle, and was connected to a scrubbing tower containing 10 N sodium hydroxide. Air from a compressed air line was slowly bubbled through the tower to be freed of carbon dioxide, and then passed into the bottle, serving to agitate the contents and also to carry air contained in the bottle out through the other glass tube. The outlet tube, which just barely extended into the bottle, served to carry the air to a capillary which extended to the bottom of a 7-inch test tube containing saturated barium hydroxide solution. After bubbling through this solution the air was allowed to escape through an outlet in the stopper which covered the absorption tube. After about ten volumes of air had passed through the reaction bottle, 0.4 N potassium permanganate was added dropwise by means of one of the burettes, and 0.5 N sulfuric acid was added through the other burette to maintain a pH of 6, as long as the permanganate was decolorized. Carbon dioxide, presumably

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*National Technical Laboratories, S. Pasadena, California

representing a mixture of carbon atoms 4 and 6 of the degraded pyrimidine (40, 41) was swept over into the absorption tube and trapped as barium carbonate. When no more precipitate was formed, the absorption tube was removed, the contents transferred to a 12 ml. centrifuge tube and the precipitate centrifuged off. The dried barium carbonate weighed 16 mg., or 80 per cent of the theoretical value. The reaction bottle was also removed and the contents treated with 3 per cent (v/v) hydrogen peroxide to remove excess permanganate. The precipitate of manganese dioxide was filtered off, washed with hot water and the combined filtrate and washings transferred to a 50 ml. centrifuge tube and made strongly alkaline with 5 drops of 10 per cent (w/v) sodium hydroxide. After heating in a boiling water bath for 5 minutes to hydrolyze the oxaluric acid, the tube was removed, the contents neutralized to a pH of 6 with 5 per cent (v/v) acetic acid, and calcium oxalate precipitated by the dropwise addition of 10 per cent (w/v) calcium chloride. After adjusting the pH of the solution to 7.5 with dilute ammonia, the mixture was centrifuged, and the washed precipitate transferred to the reaction bottle used previously and dissolved in 5 ml. of 2 N sulfuric acid. After attaching the bottle to the system used for the uracil oxidation and sweeping out all carbon dioxide, 0.4 N potassium permanganate was again added until no further discolorization occurred. The carbon

dioxide liberated, representing an average of carbon atoms 4 and 5 and carbon atoms 5 and 6 of the original uracil, was again trapped as barium carbonate. The yield was 20 mg., or 50 per cent of the theoretical value. The filtrate remaining from the precipitation of calcium oxalate was evaporated to 5 ml. volume, and a hydrolysis of the urea contained in this solution was carried out. The solution was placed in the reaction bottle and, after attaching the bottle to the system and sweeping out all carbon dioxide, a suspension of 25 mg. of urease powder* in 5 ml. of water was added through the outlet tube. The mixture was warmed in a 50°C. water bath for 15 minutes, and the resulting ammonium carbonate was then decomposed by the addition of 10 ml. of 2 N sulfuric acid. The liberated carbon dioxide was then trapped as barium carbonate in the absorption tube.

Prior to degradation of the labeled uracil, the samples from the various experiments were pooled, and the total quantity determined as 0.93 mg. and the specific activity as 2170 c.p.m./μM. One-half of the sample was diluted 20 times with unlabeled uracil, and the resulting 9.3 mg. then had a specific activity of 108 c.p.m./μM. This sample was then placed in the reaction bottle and degraded to carbon dioxide and oxaluric acid by the action of permanganate. The resulting barium carbonate was transferred to a 12 ml.

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*Armour and Company, Chicago, Illinois

centrifuge tube, centrifuged, and the barium hydroxide supernatant removed. The precipitate was suspended in 10 ml. of absolute ethanol and 1 ml. aliquots were plated on platinum dishes, evaporated over a heating lamp, and counted in the flow counter. The plates were then weighed and the specific activity calculated. The barium carbonate samples obtained in the oxalate oxidation and the urea hydrolysis were treated similarly. Compensation for self-absorption by the precipitates was effected by plating the same amount of barium carbonate for each step of the degradation. The results could thus be expressed as a percentage of the total activity of the uracil molecule.

The entire procedure was repeated on a larger scale, using 25 mg. of uracil. The other half of the labeled uracil (0.465 g.) was diluted 50 times by the addition of 22.8 mg. of unlabeled uracil, and the specific activity of the diluted compound was then 43 c.p.m./μM. One refinement, that of placing the calcium oxalate in the reaction vessel and flushing the system prior to dissolution in sulfuric acid, was carried out to obviate the possibility of isotope loss due to conversion of any side-products to carbon dioxide by the acid. Two mg. samples of barium carbonate were again plated in all steps.

The results of the uracil degradation are shown in Table IV. It can be seen that the distribution of activity calculated on the basis of the utilization of carbon atoms 2, 3 and 4 of aminobutyric acid for the carbon chain of the

TABLE IV

DISTRIBUTION OF CARBON-14 IN URACIL

Expt.	Compound	Mg. used	Mg. $BaCO_3$	Sp. act. c.p.m./μM	% Total Activity [d] found	% Total Activity [d] calc'd.
1	uracil (dil'd 20X)	9.3		108		
	carbon dioxide[a]		18.2	15.9	40.4	22.2
	oxalic acid[b]		22.0	15.4	39.2	55.6
	urea[c]		15.8	8.0	20.3	22.2
2	uracil (dil'd 50X)	23.3		43		
	carbon dioxide[a]		31.0	6.6	39.1	22.2
	oxalic acid[b]		40.5	6.1	36.1	55.6
	urea[c]		22.0	4.2	24.8	22.2

a. Average of Carbons 4 and 6
b. Average of Carbon 4 (or 6) and Carbon 5
c. Carbon 2
d. Total activity as sum of individual activities for carbon dioxide, oxalic acid, and urea for each run. The calculated values are those obtained from the assumption that carbon-3 of aminobutyric acid gave rise to carbon-5 of uracil.

pyrimidine does not agree with the pattern found. It was assumed that carbon atoms 2, 4 and 6 of the pyrimidine ring would possess the same degree of random labeling as that determined for the purines and the protein amino acids. The value determined for carbon 2 of the pyrimidine seems to be in agreement with this assumption, but the label of the aminobutyric acid appears to have been spread over carbons 4, 5 and 6 of the pyrimidine rather than specifically incorporated into carbon 5. These results suggest some type of cleavage and recombination of the precursor in such a way that two of the carbon atoms of the chain become highly labeled. The possibilities also exist that either the uracil contained a radioactive impurity or that the degradation did not occur as supposed.

Study of the Trichloroacetic Acid Extract

Isolation of Acid-soluble Nucleotides from the Trichloroacetic Acid Extract. It was of interest to study the solution obtained by extraction of the dried Neurospora mycelia with cold 10 per cent (w/v) trichloroacetic acid, since this fraction was found to contain one-quarter of the total radioactivity fixed. The solution was therefore placed in a 100 ml. separatory funnel and extracted 3 times with ethyl ether. The trichloroacetic acid-free aqueous solution was then evaporated to dryness, taken up in water, and a small portion chromatographed by a two-dimensional paper chromotographic procedure suggested by the work of Sanger and Tuppy (42)

and of Partridge and Westall (43). The solvents used were water-saturated phenol and butanol - acetic acid - water. The attempted separation of this total trichloroacetic acid extract by the ascending technique was unsuccessful, but the methods used, which were found very useful in later experiments, are described below.

Two-dimensional chromatography. Whatman No. 1 filter paper sheets (18 by 22 inches) were ruled 3.5 inches from the edge on two adjacent sides, and the origin marked at the point where the lines crossed. The origin was spotted with the sample while a fan was directed at the paper to hasten drying. In cases where unbuffered neutral solvents were to be used for development, it was found necessary to neutralize acidic samples by placing the still-moist spot over a beaker of moderately concentrated ammonia, and covering the area with a watch glass. The samples were applied in 10 µl. amounts by the use of micropipets or glass tubes drawn out to a fine capillary. After the sample had been applied, the papers were attached by one of the non-ruled edges to 24-inch glass rods by means of stainless steel clips. The papers were then hung into glass troughs, the bottom edge of each paper having been folded to form a one-inch flap which lay in the trough. A glass rod was placed on each flap, to supply tension and prevent the papers from touching each other since two papers were dipped into each trough. The entire system was

contained in a Chromatocab* type chromatography chest.

In subsequent studies of the trichloroacetic acid extract, a descending technique was found more convenient. The papers were folded about 1.75 inches above the flap, and the troughs were placed at the top of the cabinet, with the papers dipping into the troughs and then passing up and over 24-inch long glass rods and hanging down almost to the bottom of the chest. The chest was a Chromatocab Model B** which was provided with an inner glass cover with small holes through which solvent could be readily added to the troughs.

For reproducible results it was found advisable to equilibrate the chest for 18 hours prior to addition of the solvent to the troughs. This consisted of placing a dish of the solvent - with or without additions such as potassium cyanide, ammonia, etc. - in the bottom of the chest, and then preparing all the materials for addition of the solvent. The system was then closed and the atmosphere allowed to become saturated for the 18-hour period.

The solvent was then added to the troughs either by the use of a long tube which was passed in through a hole near the bottom of the chest - in the case of the ascending setup - or was added by passing the outlet of a separatory

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**Research Equipment Corporation, Oakland, California

funnel through holes in the inner cover of the chest, as in the descending technique. The solvent generally required about 24 hours to travel the length of the papers, at which time they were removed and dried in a forced draft of cool air. After rinsing to remove excess solvent if necessary, the papers were rotated through 90 degrees in order that the second ruled side could be folded and dipped into the troughs. After equilibration, the second solvent was added to the troughs and allowed to travel almost the full length of the papers. The papers were then removed and dried in a forced draft of cool air as before, preparatory to localization of spots by ultraviolet quenching of by spraying with the appropriate color reagents.

Ion-exchange Chromotography. In subsequent experiments the ether-extracted solution of the trichloroacetic acid extract was basified to a pH of 11 and placed on a 1 by 27 cm. column packed with Dowex 1 resin of 12 per cent crosslinking and 100 to 200 mesh size which had been previously converted to the chloride form. The column was eluted with 90 ml. of water, followed by 165 ml. of 2 N hydrochloric acid. Fractions of 15 ml. volume were collected, and the ultraviolet-absorbing materials were found to be eluted in the first 60 ml. of the 2 N hydrochloric acid effluant. The peak absorption of these compounds was in the 260 mμ range.

Separation of a Uridine Nucleotide. Since it appeared most probable that the purine nucleotides would predominate in this extract, it was decided to hydrolyze a portion with 1 N hydrochloric acid for one hour on the steam bath, a procedure suggested by the work of Smith and Markham (44). Since the pyrimidine nucleotides are much more stable to acid hydrolysis than the purine nucleotides, the former should be unaffected and the latter should appear as the free purine bases, which are readily separated from nucleotides in a variety of solvents. Accordingly, half of the residue obtained by evaporation of the 60 ml. volume containing the nucleotides was taken up in 2 ml. of 1 N hydrochloric acid and placed in a glass-stoppered 2 ml. volumetric flask. The mixture was heated on the steam bath for 1 hour, then evaporated to dryness to remove hydrochloric acid and both hydrolyzed and unhydrolyzed mixtures spotted on Whatman No. 1 sheets to be developed by the two-dimensional descending technique. The solvent used for the first direction was 2- propanol-hydrochloric acid - water, as described by Wyatt (39), and the second solvent was butanol - water - ammonia, as described by Hotchkiss (45) and applied to nucleotide separations by Wyatt (46). By ultraviolet quenching two dark blue areas were discernible in the chromatogram of the hydrolyzed extract, one of Rf .9 and the other of Rf .7 in isopropanol-hydrochloric acid. Both were located on the base

line of the butanol - water - ammonia development, and hence could be nucleotides, since these substances do not migrate in the latter solvent. Spots were also discernible at Rf values corresponding to adenine and guanosine, and several very faint spots were detected in the area to which free pyrimidines and their nucleosides would migrate. The two spots containing possible nucleotides were cut out and eluted with 0.1 N hydrochloric acid. Adjacent areas were also cut out and eluted with 0.1 N hydrochloric acid to serve as blanks for the determination of concentration. The ultraviolet absorption curve as recorded by the Beckman Ultraviolet Recording Spectrophotometer* indicated a peak absorption at 264 mμ for the material of Rf .7 in 2-propanol - hydrochloric acid, and indicated only end-absorbing material for the spot of Rf .9. In contrast, the chromatogram for the unhydrolyzed extract yielded four possible nucleotide spots at Rf values of .3, .4, .75, and .9 in isopropanol - hydrochloric acid, and the spot for adenine was of considerably decreased size. These results, coupled with approximate agreement of the Rf value with that of a known uridylic acid sample, strongly suggested that the nucleotide of Rf .7 was a uridine nucleotide. Since data was available on the behavior of the 5'-nucleotides of uridine,

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cytidine, and adenosine (47) in isobutyric acid - ammonia (the values being .23, .28, and .45 respectively), and because Magasanick et al. (48) had studied the nucleic acid nucleotides in this solvent, it was decided to apply one-dimensional strip chromatography, as described previously (see page 33), using this solvent. The nucleotide material of Rf .75 from the two-dimensional chromatogram of the unhydrolyzed extract was eluted with 0.1 N hydrochloric acid, the eluate was evaporated to dryness, and taken up in a small amount of 0.1 N hydrochloric acid. The solutions of the presumed uridine nucleotides were then spotted 2.25 inches in from the edge of the 7-inch paper strips, and known uridine, uridylic acid and adenylic acid were spotted 2.25 inches in from the opposite edge. Uridine-5'-phosphate was not available for chromatography at this time. The papers were developed until the solvent had almost reached the end of the strips and then removed and dried in a hood. By the ultraviolet quenching technique spots were discernible at Rf .18 for the unhydrolyzed nucleotide, and at Rf .19 for the hydrolyzed nucleotide. These compared with a published value of .23 for uridine-5'-phosphate, which appeared to be rather good agreement since the known compounds were found to have Rf values also slightly less than those in the literature. Other ultraviolet-absorbing areas were found at Rf values of .62 and .66. The various spots were cut out and eluted with 0.1 N hydrochloric acid.

The eluates were made up to 25 ml. and the optical densities determined for each over the range from 235 to 280 mμ. The compounds of Rf .18 and .19 were the only materials showing the typical nucleotide absorption curve, the peak in both cases being at 262 mμ, the maximum absorption characteristic of uridine nucleotides. Adjacent areas for these spots were cut out and eluted with 0.1 N hydrochloric acid to serve as blanks for comparison, and the optical density at this wavelength was used to determine the concentration of the compound, using the molar extinction coefficient for uridine-5'-phosphate. One ml. aliquots of the various solutions were also plated and counted as described previously (see page 16). The specific activity was found to be 4000 c.p.m./μM.

Characterization of Uridine-5'-phosphate. An attempt was also made to definitely establish the position of the phosphate group in this uridine nucleotide by means of the phosphate-liberating action of the specific 5' nucleotidase of rattlesnake venom* described by Gulland and Jackson (49). A sample of this crystalline venom was kindly supplied by Dr. H. A. Lillevik. Eight ml. of the solution of the nucleotide of Rf .18, containing 0.4 μM, or approximately 0.35 mg., was evaporated to dryness and taken up in 0.3 ml. of a sodium carbonate solution

*Ross Allen Reptile Institute, Silver Springs, Florida

containing 0.1 ml. of half-saturated sodium carbonate solution. A known sample of 0.5 mg. of uridine-5'-phosphate was treated similarly. Five mg. of snake venom was added to each sample in a 5 ml. glass-stoppered volumetric flask, and this was followed by addition of 3 ml. of 0.1 M sodium borate buffer of a pH of 8.7. Chloroform was then added to each flask in 250 µl. amounts, and the flasks were stoppered and incubated at 37°C for 3 hours. The mixtures were then evaporated to dryness in vacuo and the residues transferred to 12 ml. centrifuge tubes with the aid of 0.1 N hydrochloric acid. After centrifugation, the supernatants for known and unknown were spotted on separate Whatman No. 1 strips and developed with isobutyric acid - ammonia solvent. Known uridine-5'-phosphate and uridine samples were also chromatographed with and without added borate buffer. The latter had been concentrated to simulate the salt concentration of the enzyme-treated samples. The developed chromatograms, after drying, were found by ultraviolet quenching to contain ultraviolet-absorbing material only near the solvent front, suggesting that complete conversion to bases had somehow occurred. The poor definition of the spots on these chromatograms prompted a decision to repeat the process with the other pyrimidine nucleotide sample, of Rf .19. The results with the developed papers from this experiment were again inconclusive in the case of the unknown, (there being a

fluorescent spot at Rf .18 but no nucleoside spot) but were conclusive in establishing the activity of the enzyme and the validity of the procedure since the known uridine-5'-phosphate gave a strong spot at the Rf value for uridine and a faint spot for the residual, unhydrolyzed nucleotide. The known Rf values to which these results were compared were those which had been determined by addition of concentrated borate buffer to known uridine and uridine-5'-phosphate prior to chromatography. It seemed possible that some impurity in the isolated nucleotide might be either disrupting the normal course of reaction with snake venom 5'-nucleotidase or radically altering the rate of migration of the products.

Therefore, the entire isolation procedure was repeated, using a fresh trichloroacetic acid extract obtained from the mycelial growth from 20 flasks of the aminobutyric acid-fed mutant, as described in an earlier section (see pages 41 to 44). Two-dimensional paper chromatography yielded ultraviolet-absorbing spots of nucleotide character at Rf values .54 and .6 in isopropanol-hydrochloric acid. A fraction of each was again hydrolyzed, and the various portions rechromatographed on Whatman No. 1 strips in isobutyric acid - ammonia. After drying the developed chromatograms, they were viewed by ultraviolet quenching and the spot of Rf .18 again found in each case. The nucleotides were then removed

with 0.1 N hydrochloric acid and placed on other Whatman No. 1 strips for chromatography in tertiary butanol - hydrochloric acid - water, a solvent described by Smith and Markham (44). After development, spots were found at Rf values .36 and .75, the latter value comparing with a spot for known uridine-5'-phosphate. After elution, the material of the lower Rf value, which also contained a fluorescent fraction, was end-absorbing when studied as to ultraviolet absorption over the 235 to 280 mμ range. The nucleotide of Rf .75 read versus a blank obtained by elution of areas surrounding the spot showed a peak optical density reading of .360 at 262 mμ, and the typical absorption curve of uridine-5'-phosphate. Aliquots of the eluate were also plated and counted as described earlier (see page 16). The specific activity was found to be 1400 c.p.m./μM. The hydrolysis of the 5'-phosphate group by means of snake venom carried out with this purified nucleotide gave a faint spot for uridine on subsequent rechromatography in the same solvent. On the basis of chromatographic behavior, ultraviolet absorption and 5' nucleotidase treatment, it was therefore concluded that the unknown pyrimidine nucleotide was uridine-5'-phosphate.

Separation of an Adenine Nucleotide. Small portions of the original hydrolyzed and unhydrolyzed, trichloroacetic acid-free extracts were also banded on Whatman No. 1 strips and chromatographed in butanol - formic acid - water, a solvent described by Markham and Smith (50). After

development, ultraviolet quenching indicated a band at the origin which apparently consisted of nucleotides, since these compounds do not migrate in this solvent. Bands were also detected at Rf .08, which probably was due to action between hydrochloric acid and the paper, and at Rf .15 and .18, which was most probably a purine base or nucleoside band. The pattern was essentially the same for both hydrolyzed and unhydrolyzed samples.

To establish that the bands at the origin were indeed nucleotides, the phosphorus determination of Hanes and Isherwood (51) was applied to a portion of each chromatogram. The strips were sprayed at a rate of 1 ml. per 100 sq. cm., with a solution containing 5 ml. 60 per cent perchloric acid, 10 ml. 1 N hydrochloric acid, 25 ml. of 4 per cent (w/v) ammonium molybdate, and water to make 100 ml. The papers were then heated to 85°C in a chromatography oven for 7 minutes, and finally placed in a graduated cylinder containing hydrogen sulfide. Unfortunately, the entire paper turned black suggesting that metal ions, especially lead or copper, were present in the paper. An attempt was therefore made to use a stannous chloride spray for reduction of the phosphomolybdate complex, but the entire strip turned blue, making differentiation of the nucleotide band impossible. Apparently acid-washed papers were needed for a successful phosphorus analysis.

Characterization of Adenosine-5'-phosphate. The nucleotide band from the remaining portion of each chromatogram was therefore cut out and eluted with 0.1 N hydrochloric acid. The eluates were taken to dryness, the compounds banded on Whatman No. 1 strips, and the chromatograms developed with tertiary butanol-hydrochloric acid - water (44). After development, there appeared only one ultraviolet-absorbing band, at Rf .3, which corresponded to a purine nucleotide. In the chromatogram of the nucleotide fraction which had been hydrolyzed, the band was very much attenuated, compatible with the expected destruction of purine nucleotides under these conditions. Furthermore, the fact that this compound appeared to possess some degree of acid stability suggested that it was a 5'-nucleotide, since Ochoa (52) has demonstrated the much greater acid stability of the purine-5'-nucleotides under these hydrolytic conditions compared to purine nucleotides having phosphate attached at the 2' and 3' positions of the sugar moiety. Its ultraviolet absorption spectrum in 0.1 N hydrochloric acid showed a maximum at 255 mμ, and otherwise agreed exactly with the absorption curve of the adenine mononucleotides. From these several lines of evidence the conclusion was therefore reached that the isolated nucleotide was adenosine-5'-phosphate. Concentration and radioactivity measurements were carried out in the same manner as with the isolated uridine-5'-phosphate. The

specific activity was found to be 800 c.p.m./μM.

A parallel study was made of the nucleotides of the trichloroacetic acid extract after growing the mutant strain on unlabeled uridine rather than labeled aminobutyric acid. The pattern of nucleotides and bases showed no radical differences.

Radioautography of Paper Chromatograms. Sheets and strips to be radioautographed were taped to 14 by 17 inch sheets of Kodak Blue Brand X-ray film.* It was found helpful to place several spots of a radioactive solution at marked positions on the papers to serve as a key in order that the developed films and the corresponding chromatograms could later be correctly matched. The films, with attached papers, were placed between plywood boards which were then clamped tightly together to insure close contact of chromatograms and films. Pieces of thin cardboard were found sufficient as separators between the various radioautograms. After a period of 3 weeks the chromatograms were removed and the films were developed for four minutes with Kodak D-19 Developer, then placed in a stop bath of 1 per cent (w/v) acetic acid for 10 seconds, and left in a fixer solution of sodium thiosulfate for at least 10 minutes. The films were then washed in cold tap water for approximately 1 hour, and

*Eastman Kodak Company, Rochester, New York

then hung by clips to dry. An amber Kodak Safelight, Series 6-B, was found useful for the darkroom procedures.

Isolation of Amino Acids from the Trichloroacetic Acid Extract. Duplicates of the butanol - formic acid - water chromatograms of the trichloroacetic acid extracts of the mold which were prepared in studies of the acid-soluble nucleotides (see page 51) were sprayed with 0.02 per cent (w/v) ninhydrin in water-saturated butanol. Several ninhydrin-positive spots were obtained, one of which had the Rf value .27, the value for alpha-aminobutyric acid in this solvent. Since this spot was present in similar amounts in chromatograms of both the hydrolyzed and non-hydrolyzed extracts, it was probably not a peptide. This material was eluted from the paper and its concentration and radioactivity determined. The high specific activity suggested that this might indeed be the precursor, aminobutyric acid, and purification of this compound was therefore undertaken. On chromatography in both phenol - water - cupron and in butanol - water - ammonia, the Rf value of the compound compared precisely with known alpha-aminobutyric acid. The constancy of the specific activities of the eluates of the spots at each stage of purification indicated that the compound was essentially radiochemically pure after the final chromatography. The specific activity determined from the final measurements of concentration and radioactivity was found to be 2300 c.p.m./μM. Other

radioactive spots of the acid-soluble pool were tentatively identified by chromatographic procedures as isoleucine, methionine sulfone, and beta-alanine.

An investigation of the occurrence and quantity of the amino acids of the acid-soluble pool of the wild strain, grown without alpha-aminobutyric acid, was also carried out. Chromatography on long strips of Whatman No. 3 paper gave a separation of eight ninhydrin-positive spots, one of which coincided with a known spot of alpha-aminobutyric acid. The normal mold thus appears to possess an aminobutyric acid pool.

The smaller size of the spot for aminobutyric acid of the wild strain as compared to the corresponding spot for the mutant suggests that the pool size of this amino acid is normally small, but that a relatively large pool exists in the mutant when grown on aminobutyric acid.

Study of the Amino Acids of the Soluble Proteins

Isolation and Hydrolysis of the Soluble Proteins. The alkaline hydrolysis treatment for the separation of ribonucleotides (see page 28) also served to extract the soluble proteins from the mycelia. The precipitate obtained by acidification of this extract with concentrated perchloric acid in the cold therefore contained proteins, as well as potassium perchlorate and some polysaccharides. The proteins and polysaccharides were redissolved by

stirring the precipitate with 1 N sodium hydroxide for 12 hours. The residual potassium perchlorate was centrifuged off, and the dissolved materials reprecipitated with 1 N hydrochloric acid. On attempting to dry the protein by means of an ethanol wash, the major portion of the mixture dissolved, leaving a small amount of a light brown gelatinous precipitate. The latter was centrifuged down, and the supernatant was evaporated to dryness, yielding a white, amorphous precipitate weighing 75 mg., and presumably consisting mainly of alcohol-soluble protein.

This precipitate was next transferred to a 12 ml. centrifuge tube and hydrolyzed with 4 ml. of 6 N hydrochloric acid on the steam bath for 24 hours to bring about hydrolysis of the protein to the constituent amino acids. The mixture was then filtered, and the solution was evaporated to dryness several times to remove hydrochloric acid. The residue was taken up in water, transferred to a 12 ml. centrifuge tube, and evaporated to 0.5 ml. for chromotography.

Separation and Purification of Protein Amino Acids. Preliminary experiments, carried out with the hydrolysate from an unlabeled protein sample, demonstrated that a two-dimensional paper chromatographic technique was capable of resolving the amino acids of the crude hydrolysate without prior purification on ion exchange resins. The technique which was used followed very closely the procedure outlined

for the separation of the acid-soluble nucleotides (see page 42). The solvents used were phenol - water and butanol - acetic acid - water, both of which were described by Sanger and Tuppy (42). It is important to note that the hydrolysate had to be neutralized with ammonia, as mentioned previously, when using an unbuffered neutral solvent such as phenol - water, to prevent the streaking of the acidic amino acids. The equilibrating liquid consisted of 0.5 g. potassium cyanide and 20.8 ml. of concentrated ammonia in sufficient water to make 2 liters. After development in the phenol - water solvent, using the ascending technique, the sheets were removed, dried, and then rinsed with anhydrous ethyl ether to remove residual phenol. The sheets, while still clipped to the glass rods, were hung over a sink and rinsed with ether from a wash bottle, while a fan carried the vapors to a nearby hood. After the papers had dried, the bottom 2 inches of the papers were removed, and the chromatograms were then turned through 90 degrees and equilibrated for the second solvent. The equilibrating solution in this case was the lower layer from the butanol - acetic acid - water mixture. After 18 hours of equilibration, the solvent was added and the papers developed for the usual 24-hour period. After drying in an oven at 45°C for 6 hours in a forced draft, the papers were developed with a 0.02 per cent (w/v) solution of ninhydrin in water-saturated butanol. The solution was sprayed on the paper in an even film - but not enough to run -

while the papers hung, with the origin at the top, in a hood. The papers were then heated to 85°C. in a chromatography oven, and spots for a variety of amino acids became evident in 15 minutes. Several amounts of hydrolysate were tried. The heavier samples produced somewhat diffuse and elongated spots, but the lower concentrations gave reasonably clear separations, especially of the neutral and acidic amino acids. The concentration of proline appeared quite high, and corroborated a suspicion, prompted by the alcohol solubility, that the predominant base-soluble proteins of the Neurospora mycelia are prolamine in nature. Clear spots were also obtained for the isoleucine - leucine mixture, for aspartic and glutamic acids, and for valine. Spots for serine, glycine and threonine were somewhat diffuse. Radioautograms of these chromatograms, made as described in the previous section (see page 54) indicated high labeling of the isoleucine - leucine mixture, some labeling of the proline fraction - probably explained by its high concentration - and of the glutamic acid, but little activity in aspartic acid or in the serine - threonine - glycine mixture.

A long-strip paper chromatographic procedure was also found useful for the separation of the amino acids of the protein hydrolysate. The hydrolysate was first placed on a 2 by 30 cm. Dowex 50 column, which was then washed with water, and the amino acids then removed by elution with

2 N hydrochloric acid. The eluate was evaporated to dryness to remove hydrochloric acid, preparatory to paper chromatography. The 42-inch long papers were prepared as outlined for the paper strip chromatography used in connection with the studies of the purine and pyrimidine bases (see page 33). Heavy Whatman No. 3 paper was available, and, in conjunction with the banding technique, enabled the separation of much larger amounts of material. The long paper strips were dipped into troughs at the top of a double-length rack which stood in a 12 by 24-inch battery jar. A second jar, 12 by 18 inches, was inverted over the first after the solvent had been added, and the point of joining was then sealed with masking tape. The solvent in the troughs usually had to be replenished once in the course of development. This was accomplished by breaking the seal between the jars and raising the upper jar sufficiently to attach a funnel of solvent to tubes extending up to each trough. The solvent found most useful was butanol - formic acid - water (50) used earlier in connection with nucleotide separations. On development and comparison with known compounds, it was found that the basic amino acids, which were of low concentration, remained at the origin. Proceeding out from the origin, aspartic acid was obtained in a relatively well-separated band led by glutamic acid mixed with serine and glycine. A band of threonine was next, followed by a wide, heavy band of proline, yellow in color, and then, after a gap of several Rf

units, a wide band of valine and a wider band of mixed leucine - isoleucine. The valine and isoleucine - leucine bands were well separated.

The spots for the leucine - isoleucine mixture and for both aspartic and glutamic acids from the two-dimensional chromatograms used for the separation of the hydrolysate of the first isolated proteins were cut out and eluted with water.

The leucine - isoleucine mixture was treated by the method of Stein and Moore using cation-exchange chromatography (53). The method, though unsuccessful for the separation of the labeled mixture, was successful in separating a known sample of 1 mg. each of leucine and isoleucine. The following modification of the method is therefore presented because of its ease and simplicity. A 0.9 by 100 cm. column was completely jacketed with a 3 cm. wide glass tube which was in turn wound with a long heating tape. A variable resistance attached to the tape completed a simplified apparatus for control of the temperature of the column. This was found quite capable of maintaining the required temperature to within half a degree in the course of the separation. The column was packed with Dowex 50 cation-exchange resin of 12 per cent crosslinking and 100 to 200 mesh particle size by forming a slurry of the resin - previously placed in the sodium form by washing in 1 N sodium hydroxide and then in water - in 0.1 M

sodium citrate buffer of a pH of 3.41 and pouring this mixture into the column with gentle suction. A mixture of 1 mg. each of leucine and isoleucine in 1 ml. 0.1 N hydrochloric acid was mixed with 2 ml. of the citrate buffer to give a final pH of 2.5 to 3, and the amino acids were then allowed to filter into the column. Elution was begun at the rate of 4 ml. per hour with the same citrate buffer after the column temperature had been adjusted to 37°C. This slow rate of elution was controlled by a stopcock attached to the column outlet. After 215 ml. of the eluant had passed through the column, elution with 0.1 M sodium citrate buffer of a pH of 4.25 was begun, and the temperature was increased to 50°C. Two ninhydrin-positive peaks, as located by the quantitative determination of amino acids suggested by Moore and Stein (54) (see page 64), appeared after 55 fractions of 2 ml. each had been collected, using an automatic fraction collector. One amino acid-free tube occurred between the peaks, so the procedure was deemed useful for the separation of the labeled mixture of leucine and isoleucine. It is important to note that the buffers had to be made up with boiled water just prior to use in order to avoid bubble formation on passage into the heated column. The column was then repacked, fresh buffers were made, the labeled mixture was acidified and filtered into the resin, and elution with the first solvent begun. However, in the course of the night the eluant channeled through the stopcock and the resulting high flow rate apparently spread

the amino acids through the column. The mixture could not be located, but a second labeled sample, obtained by the strip chromatographic procedure, was successfully separated by paper chromatography.

The leucine - isoleucine band from the butanol - formic acid - water separation of the protein hydrolysate was located by the ninhydrin method and was then cut out and eluted with water. The solution was evaporated to 0.5 ml., banded on the 42-inch long Whatman No. 3 strips, and chromatographed in the double battery jar apparatus described for strip chromatography of the protein hydrolysate. (see page 59) The solvent chosen for the separation of these very similar amino acids was water-saturated butanol, (55), in which leucine has an Rf value of .46 and isoleucine a value of .41. The end of the papers had been cut to form a sawtooth edge and the solvent was allowed to drip from the end of the strips, thereby increasing the distance of travel of the bands and aiding the separation. The bottom of the tank was layered with a solution of the solvent containing 3 per cent (w/v) ammonia. After development the papers were dried and treated with ninhydrin. The bands of leucine and isoleucine were clearly defined, though not completely separated. Therefore, the trailing edge of the isoleucine band and the leading edge of the leucine band were cut out and eluted with water. The eluates were made up to 25 ml. and 0.1 ml.

samples taken for quantitative ninhydrin determination by the method of Moore and Stein (54). The samples, and isoleucine standards as well, were each mixed with 1 ml. of a ninhydrin reagent consisting of 0.8 g. of stannous chloride, 500 ml. of 0.1 M sodium citrate buffer of a pH of 5, 20 g. of ninhydrin, and 500 ml. of methyl cellosolve. The reagent was stored under nitrogen in the dark. The mixtures of samples and reagent, in 4-inch test tubes, were heated for 20 minutes in a boiling water bath and then transferred to the cells of a Klett-Summerson* colorimeter and diluted with 5 ml. of a 1 to 1 (v/v) mixture of water and n-propanol. The absorption of light, using a green filter (570 mμ), was measured for each sample versus a blank of distilled water and reagent which had been carried through the entire procedure. The absorption values of the known isoleucine samples were plotted against concentration to obtain a standard curve from which the concentration of the isolated amino acids could be determined. Aliquots of the eluates were also plated and counted as described earlier (see page 16). Isoleucine was found to have a specific activity of 7000 c.p.m./μM, whereas leucine was labeled to the extent of 1500 c.p.m./μM. The remainder of each eluate was evaporated to dryness and rechromatographed with butanol - formic acid - water. The

*Klett Manufacturing Company, New York

quantitative determination and radioactivity measurements were repeated for the eluates from these chromatograms. In addition, radioautographs were made of duplicates of the original leucine - isoleucine separation made with butanol - water. A portion of the isoleucine sample was also chromatographed with phenol - water - cupron, a solvent consisting of water-saturated phenol containing 0.1 per cent (w/v) alpha - benzoinoxime (cupron) (55), and a second portion was mixed with the known compound, chromatographed in ethanol - ether - water - ammonia (56) and radioautographed. The specific activity of isoleucine from the chromatography in phenol was found to be 1650 c.p.m./μM. The identity of the isolated compound with isoleucine was proved by migration of the mixture as one ninhydrin-positive spot which appeared as the only radioactive area on radioautography.

Aspartic and glutamic acids were obtained from two-dimensional chromatograms and also from strip chromatograms of the protein hydrolysates. The samples were banded on Whatman No. 3 strips and chromatographed in phenol - water - cupron. Concentration and radioactivity were determined as before. Aspartic acid had a specific activity of 300 c.p.m./μM, and glutamic acid has a specific activity of 550 c.p.m./μM.

Valine, obtained from the same sources, was treated similarly, but with an added purification by rechromato-

graphy in butanol - water in a 3 per cent (w/v) ammonia atmosphere. The specific activity of this amino acid was 200 c.p.m./μM.

Threonine from long strip chromatograms was purified by strip chromatography in phenol - water - cupron and its concentration and radioactivity likewise determined. The specific activity was determined as 300 c.p.m./μM.

Methionine was not located in the protein hydrolysate.

The results of the specific activity determinations for the various isolated compounds are summarized in Tables V and VI. Table V contains the values found for the compounds isolated from the Neurospora mycelium after the mold had been grown for 4 days on C^{14}-labeled alpha-aminobutyric acid. Table VI contains the specific activity values determined for compounds isolated from the mold mycelium after 6 days growth. The distinction is necessary since a considerable dilution of the labeled compound occurred in the longer growth period, and results were considered comparable only if the time of growth of the mold mycelia from which the compounds were isolated was the same.

It can be seen that the aminobutyric acid is indeed a pyrimidine precursor since a selective incorporation into the pyrimidine occurs, though the label is diluted 10 times in the course of growth. The pattern also

TABLE V

CARBON-14 CONTENT OF COMPOUNDS OF NEUROSPORA AFTER 4 DAYS GROWTH IN THE PRESENCE OF AMINOBUTYRIC ACID-3-C^{14}

Compound	Specific Activity (c.p.m./μM.)	
	Mutant Strain	Wild Strain
Nucleic Acid Components		
Adenine	850	600
Guanine	900	
Uracil	4800	1650
Cytosine	5000	
Nucleotides of Acid-Soluble Fraction		
Adenosine-5'-Phosphate	800	
Uridine-5'-Phosphate	4000	
Amino Acids of the Soluble Proteins		
Aspartic Acid	300	
Glutamic Acid	550	
Threonine	300	
Leucine		1000
Isoleucine		5000
Aminobutyric Acid Supplied	48000	48000

TABLE VI

CARBON-14 CONTENT OF COMPOUNDS OF NEUROSPORA 1298 AFTER 6 DAYS GROWTH IN THE PRESENCE OF AMINOBUTYRIC ACID-3-C^{14}

Compound	Specific Activity
Nucleic Acid Components	c.p.m./μM
Adenine	1000
Uracil	1700
Acid-Soluble Fraction	
Uridine-5'-phosphate	1400
Aminobutyric Acid	2300
Amino Acids of the Soluble Proteins	
Aspartic Acid	350
Glutamic Acid	650
Threonine	300
Valine	200
Isoleucine	1650
Aminobutyric Acid Supplied	48000

appears similar in the acid-soluble nucleotide fraction. Furthermore, the route of utilization appears to be a normal one, since the pyrimidines of the wild strain are also labeled.

The labeling of the isoleucine is not unexpected since Adelberg, Coughlin and Barratt (57) have shown that

aminobutyric acid is an intermediate in isoleucine biosynthesis in Neurospora. The low labeling of valine also lends support to the suggestion by these authors that aminobutyric acid is not involved in valine biosynthesis. The low labeling of aspartic acid precludes the possibility of its involvement as such in the conversion of aminobutyric acid to pyrimidines.

The finding that aminobutyric acid of a specific activity comparable to the pyrimidines is present in the acid-soluble fraction of Neurospora explains the observed dilution of the precursor on conversion to pyrimidine. It appears that once growth begins, the mold can then make additional aminobutyric acid, and this endogenous acid dilutes the precursor and therefore also the pyrimidines.

Study of the Final Nutrient Media

The nutrient medium remaining after growth of the mold on labeled aminobutyric acid was evaporated to dryness after the addition of caprylic alcohol as an antifoaming agent. That part of the residue which would dissolve in 0.1 N hydrochloric acid was transferred to a 1 by 30 cm. column containing Dowex 50 resin in hydrogen form. The column was eluted with water and then 2 N hydrochloric acid, and the eluates were evaporated to dryness, taken up in 0.1 N hydrochloric acid, and chromatographed on paper strips in butanol - formic acid - water. The chromatogram of the hydrochloric acid eluate showed no aminobutyric acid,

but ninhydrin-positive spots occurred at Rf values of .42 and .74. These spots were shown to be strongly radioactive on subsequent radioautography. The water eluate, which probably consisted largely of sugars, did not resolve on chromatography.

Study of the Saponifiable Lipid Fraction

The ethanol-ether extract of the mycelium was taken to dryness in a 100 ml. round-bottomed flask preparatory to saponification by the method of Weygand (58). It was estimated that the maximum amount of saponifiable lipids in 1 g. of mycelium was 100 mg., and the residue was therefore treated with 3 times the theoretical amount of alcoholic potassium hydroxide, or 60 mg. of the base. A reflux condenser was connected and the mixture was refluxed for one hour, at which time the contents of the flask were transferred to a 100 ml. separatory funnel with the aid of a small amount of ether, and extracted with water. The water extract was acidified with hydrochloric acid, then ether extracted, and the ether extract made up to a 200 ml. volume in a glass-stoppered volumetric flask. A 1 ml. aliquot was diluted to 25 ml. and 1 ml. aliquots of this diluted sample plated for counting. The remaining ether solution was evaporated to dryness and the weight of the remaining fatty acids determined. The procedure was carried out for the ethanol-ether extract of the mycelium of both the mutant and the wild strains after

growth of each on labeled aminobutyric acid. The total weight of saponifiable lipids in the case of the mutant was found to be 9.2 mg., and the specific activity was determined using this value in conjunction with the molecular weight of palmitic acid. The latter was assumed to approximate the average molecular weight of the fatty acids of the natural fats of this organism.

Study of Neurospora Deoxyribonucleic Acid

An attempt was made to isolate deoxyribonucleic acid with a view toward the study of the pyrimidine labeling in this fraction. It was considered probable that the basic hydrolysis step for the liberation of the ribonucleotides dissolved not only the soluble proteins but also deoxyribonucleic acid. Several attempts were therefore made to extract the latter compound from the precipitate of proteins and polysaccharides obtained after removal of potassium perchlorate (see page 28). Extraction with 10 per cent (w/v) sodium chloride and subsequent precipitation with 4 volumes of ethanol gave a white precipitate which was freed of protein as described earlier (see page 12) and then hydrolyzed with concentrated perchloric acid. The hydrolysate, when treated for the separation of purine and pyrimidine bases (see page 30) gave a large amount of end-absorbing material on elution with water and a very slight peak at 260 mμ after 20 ml. of 2 N hydrochloric acid had passed through the column. Since the bulk of the

material appeared to be polysaccharide in nature, an alternate procedure was therefore sought.

It was decided to start with the intact mycelium and to apply the method of deoxyribonucleic acid extraction suggested by Chargaff and Zamenhof (59) from bakers yeast. It was hoped that the content of deoxyribonucleic acid in Neurospora would be greater than in yeasts, where the overall yield is only 0.15 per cent. The mycelium, after soaking in 0.1 M sodium citrate buffer of a pH of 7.3 to inhibit deoxyribonuclease was ground with liquid nitrogen, and the resulting powder was suspended in 15 ml. of the citrate buffer and centrifuged at 4,000 r.p.m. for 2 hours in a Servall refrigerated centrifuge*. The procedure, called differential centrifugation, was expected to give three layers, the middle layer consisting of fragmented cells and nuclei. This situation was not realized, however, probably because of insufficient cell breakage during the grinding. The total solids were therefore centrifuged off at 10,000 r.p.m. and extracted with 1 M sodium chloride for 72 hours in the refrigerator. The supernatant obtained from a second centrifugation at 4,000 r.p.m. for 2 hours did not give the characteristic polymeric threads of deoxyribonucleic acid on addition of 4 volumes of ethanol. The apparent low concentration of deoxyribonucleic acid in the mycelium prompted a decision

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to forego the investigation of more efficient grinding methods.

The Effects of Related Substances on Growth of the Neurospora Mutant

In the course of the labeling studies a number of possible metabolic relationships suggested themselves. These were tested by growing the pyrimidine-requiring mutant in basal media, as described previously (see page 25), and adding the compounds to be studied to the flasks. Additions of heat-stable materials were made prior to sterilization by autoclaving, and heat-sensitive substances were added aseptically after filtration through a fritted glass bacteriological filter. The cultures, generally run in triplicate, were incubated at 25°C for 6 days. The mycelial mat of each flask was then removed, rinsed with distilled water, squeezed to remove most of the water, dried in a 55°C oven for 6 hours, and weighed.

Experiments were run in which alpha-hydroxybutyric acid was the only additive, in the event that alpha-aminobutyric acid might be utilized by way of the hydroxy-acid. Alpha-hydroxybutyric acid was synthesized from butyric acid by the Hell-Volhard-Zelinsky procedure (60). Twenty-five g. of butyric acid was placed in a 250 ml. round-bottomed flask equipped with a reflux condenser. Two grams of red phosphorus were then added followed by gradual addition of the theoretical amount of bromine.

The flask was cooled in an ice bath until the reaction subsided, and the mixture was then placed on the steam bath for the completion of the reaction. The entire bromination process required 4 hours. The resulting alpha-bromobutyric acid was slowly dropped with shaking into 100 ml. of hot water in a 250 ml. flask over a 1-hour period. The organic layer was distilled at 10 mm. and the 78 to 84°C fraction retained for hydrolysis. Ten g. of the alpha-bromobutyric acid was treated with 8.3 g. of potassium carbonate in 50 ml. of water. After 6 hours the mixture was acidified to a pH of 2, evaporated almost to dryness, extracted 4 times with ether, and the ether extract evaporated to small volume. The oily liquid resulting was distilled in vacuo and crystallized in the receiver as long white needles. Because of the hygroscopic nature of the compound, a melting point was not obtainable.

Another experiment was prompted by the studies of Jones, Spector, and Lipmann (15) on carbamyl phosphate, the synthesis of which was carried out as follows. One-tenth mole (13.6 g.) of potassium dihydrogen phosphate and 0.1 mole (8.1 g.) of potassium cyanate were dissolved in 100 ml. of water and heated to 30°C for 30 minutes. The mixture was then cooled on ice and 0.2 moles of perchloric acid containing 0.3 moles of lithium hydroxide was added. The white precipitate of potassium perchlorate and lithium phosphate was removed by filtration, and the solution of

lithium carbamyl phosphate was precipitated by slow addition of an equal volume of ethanol. The crystals were filtered off, redissolved, reprecipitated by ethanol, refiltered, and dried in a dessicator over calcium chloride. The yield was 7.1 g., 48 per cent of the theoretical value. The effect of this compound on the rate of growth of the mutant on aminobutyric acid was studied in the event that carbamyl phosphate might donate a carbamyl group to this acid in the formation of the pyrimidine ring. Furthermore, Fairley (25) had demonstrated the powerful inhibitory effect of arginine on the growth of this mutant on aminobutyric acid, and it was of interest to see if added carbamyl phosphate overcame this inhibition. The work of Grisolia and Wallach (31) also implicated carbamyl phosphate as the donor of a carbamyl group to beta-alanine to form beta-ureidopropionic acid, a pyrimidine precursor, and the mutant was therefore grown with various combinations of these substances. The synthesis of beta-ureidopropionic acid was carried out by the reaction of potassium cyanate with beta-alanine, as described by Lengfeld and Stieglitz (61). Two g. of beta-alanine and 1.85 g. of potassium cyanate were simply placed in an evaporating dish and evaporated on the steam bath to a syrup. This residue was transferred to a small beaker and placed in the refrigerator. The resulting crystals were separated, redissolved in hot water, filtered, and the solution again

placed in the regrigerator. The yield of beta-ureidopropionic acid was 1.6 g., a 62 per cent yield. The melting point was 169-170°C.

A series of vitamins, including folic acid, pyridoxine, thiamine, and vitamin B_{12} were also studied as to their effect on the growth of the mutant on uracil, aminobutyric acid, and uridine. Folic acid and thiamine were of particular interest because of the possession of a pyrimidine structure in the molecule, and vitamin B_{12} was implicated by the work of Jukes et al. (62).

The results of the growth studies are presented in Table VII. It can be seen that alpha-hydroxybutyric acid does not replace the pyrimidine requirement of Neurospora 1298. Furthermore, carbamyl phosphate does not stimulate growth on aminobutyric acid nor does it overcome the inhibitory effect of arginine. The combination of beta-alanine and carbamyl phosphate was also ineffective in replacing the pyrimidine requirement. The vitamin studies suggest that thiamine and vitamin B_{12} have no effect on the utilization of aminobutyric acid. However, folic acid has a marked stimulatory effect on growth with both aminobutyric acid and uracil, and not with uridine, though the effect is noted only at low levels of either precursor. Pyridoxine has a surprising inhibitory activity in the case of growth on aminobutyric acid.

TABLE VII

THE EFFECT OF VARIOUS COMPOUNDS ON GROWTH OF NEUROSPORA 1298

Expt. No.	Supplement to 12.5 ml. of Basal Medium	Growth as mg. dry mycelium
1	None	0
	5 mg. hydroxybutyric acid	0
2	None	0
	5 mg. aminobutyric acid	30
	" " " -2 mg. carbamyl phosphate	31
	2 mg. carbamyl phosphate	0
3	None	0
	5 mg. aminobutyric acid	28
	" " " - 5 µg arginine	0
	" " " " " " - 2 mg. carbamyl phosphate	0
	" aminobutyric acid - 5 µg. arginine - 20 mg. carbamyl phosphate	0
4	None	0
	5 mg. aminobutyric acid	32
	" " " - 2 mg. carbamyl phosphate	34
	" beta-alanine	0
	" " - 2 mg. carbamyl phosphate	0
	2 " carbamyl phosphate	0
	5 " beta-ureidopropionic acid	0
5	None	0
	1 mg. aminobutyric acid	8
	" " " - 1 µg Vitamin B_{12}	6
	" " " - 2 µg " "	12
	3 " " "	27
	" " " " - 1 " " "	31
	" " " " - 2 " " "	34

THE EFFECT OF VARIOUS COMPOUNDS ON GROWTH OF NEUROSPORA 1298 (CONT.)

Expt. No.	Supplement to 12.5 ml. of Basal Medium	Growth as mg. dry mycelium
6	None	0
	5 mg. aminobutyric acid	22
	" " " - 0.1 mg. folic acid	31
	" " " - .02 " " "	26
	0.1 mg. folic acid	0
	0.02 " " "	0
7	None	0
	5 mg. aminobutyric acid	28
	" " " - 0.1 mg. folic acid	28
	" " " - 0.5 " thiamine	27
	" " " - 0.5 " pyridoxine	11
	2 mg. " "	13
	" " " - 0.1 " folic acid	24
	" " " - 0.5 " thiamine	13
	" " " - 0.5 " pyridoxine	4
	" uracil	37
	" " - 0.1 " folic acid	61
	" " - 0.5 " thiamine	38
	" " - 0.5 " pyridoxine	41
	0.2 mg. uridine	16
	" " - 0.1 " folic acid	14
	" " - 0.5 " thiamine	13
	" " - 0.5 " pyridoxine	13

It should be noted that negative results such as those obtained with carbamyl phosphate and ureidopropionic acid are difficult to interpret, since the possibility always exists, especially with charged compounds, that a permeability barrier is responsible for the non-utilization of the compound tested.

The Utilization of Aminobutyric Acid for Pyrimidine Biosynthesis in the Rat

Materials

One male albino rat weighing 200 g. was injected intraperitoneally with 1 ml. of a water solution containing 0.1 mc. (0.1 mM) of 3-C^{14} alpha-aminobutyric acid which was obtained commercially*.

Isolation of Deoxyribonucleic Acid

The method of isolation was the same as that used in the earlier methionine study (see page 11).

Isolation of Purine and Pyrimidine Bases

The hydrolysis of the deoxyribonucleic acid was carried out as before (see page 13) but the resulting perchloric acid solution of the purine and pyrimidine bases was treated by the method outlined on page 30 since this had been found to be a more useful procedure.

Purification of the Purine and Pyrimidine Bases

The fractions from the column separations were banded on 7-inch sheets of Whatman No. 1 filter paper and chromatographed in isopropanol - water as outlined earlier (see page 33).

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Concentration and Radioactivity Measurements

The bases were treated exactly as outlined previously (see page 35) for the determination of specific activity.

Results

The activities of the purine and pyrimidine bases are shown in Table VIII. It is seen that alpha-aminobutyric acid is not a precursor in the rat under these experimental conditions. The relatively higher labeling in the cytosine might suggest that the routes of synthesis for thymine and cytosine are different, but the order of activity is so low that the validity of any such interpretation of the data is highly questionable.

TABLE VIII

INCORPORATION OF AMINOBUTYRIC ACID-3-C^{14} INTO RAT DNA COMPONENTS

Compound Isolated	Specific Activity (c.p.m./μM)
Adenine	42
Thymine	24
Cytosine	180
Aminobutyric acid supplied	1.73×10^6

DISCUSSION

DISCUSSION

The Utilization of Methionine for Thymine Biosynthesis

The results of the study of the utilization of methyl-labeled methionine for the biosynthesis of the purines and pyrimidines of deoxyribonucleic acid indicate that the methionine methyl carbon is indeed a precursor of the ureide carbons of the purines and of the methyl group of thymine. This conclusion is based on the observation of significant labeling of these compounds after methionine administration. It has been assumed that the distribution of activity is the same for methionine-methyl incorporation as that found for formic acid incorporation into uric acid by Sonne, Buchanan, and Delluva (63) and into nucleic acid purines and thymine by Totter, Volkin, and Carter (26). Support for the validity of this assumption is supplied by the experimental finding of extremely low amounts of isotope in cytosine. The labeling of thymine and the purines must, therefore, have been accomplished by means of a specific process, rather than by random labeling which would have been accompanied by labeling of the cytosine as well. In addition, that fact that both methionine and formic acid gave rise to thymine which on degradation gave highly labeled iodoform substantiates the view that the activity was located in the methyl carbon in each case.

The metabolic interrelationship of formic acid and methionine has also been demonstrated by the work of Berg (28), who has shown the conversion of formic acid to methionine in pigeon liver, and by Weinhouse and Friedmann (64), who, by a formate-trapping procedure, have found that methyl-labeled methionine rapidly gives rise to labeled formate in the urine of rats. These studies clarified earlier work by du Vigneaud *et al.* (65, 66) wherein it was demonstrated that formate and formaldehyde could be converted to a variety of "labile" methyl-group donors including creatine, choline, and methionine. The latter is therefore clearly involved in the pool of "active one-carbon units" described by Tarver (67).

However, it should be noted from the dilution values that formic acid was used for purine synthesis to about ten times the extent to which the methyl group of methionine was used. On the other hand, a comparison of incorporation of the two precursors into thymine indicates only a two-fold greater utilization of formic acid. It must therefore be concluded that methionine was utilized for thymine synthesis by some route not involving free formic acid. Further evidence for an alternate pathway is supplied by the work of Elwyn and Sprinson (27) wherein it was demonstrated that the beta-carbon atom of serine is converted readily to the methyl group of thymine by a mechanism allowing the retention of both hydrogen atoms

originally bound to this carbon atom, an impossibility if the beta-carbon atom had been oxidized to formic acid. These results suggest conversion of both serine and methionine to some active one-carbon unit at the oxidation state of formaldehyde which may be readily converted to the methyl group of thymine. Berg (28), on the basis of the increase of formate incorporation into both methionine and serine caused by homocysteine, postulated an S-hydroxymethyl derivative of this compound as the active intermediate. However, a considerable amount of work points to a role for a hydroxymethyl derivative of tetrahydrofolic acid. By the single addition of tetrahydrofolic acid, Kisliuk and Sakami (68) have been able to restore the ability of Dowex 1-treated dialyzed pigeon liver extracts, to combine labeled formaldehyde and glycine to form labeled serine. Formic acid could replace formaldehyde in this reaction by the addition of adenosine triphosphate, diphosphopyridine nucleotide, glucose-6-phosphate, magnesium ion and tetrahydrofolic acid. These results suggest that formaldehyde reacts readily with tetrahydrofolic acid to give a hydroxymethyl derivative, presumably N^5-hydroxymethyl-tetrahydrofolic acid, the active one-carbon unit at this oxidation state, but that formate must first react in the presence of adenosine triphosphate to form a formyl derivative of tetrahydrofolic acid which is then reduced by a diphosphopyridine nucleotide enzyme system to the

hydroxymethyl derivative. Hamill (69) has supplied further evidence for the involvement of this intermediate since labeled formaldehyde was found to be at least as good a precursor of thymine as is formate in the rat. Such a route would appear unlikely if the sole route of incorporation of formaldehyde required preliminary oxidation to formic acid. The finding, by Lowry, Brown, and Rachele (70), that one deuterium atom is lost on utilization of dideutero-C^{14}-labeled formaldehyde for synthesis of the thymine methyl group in the rat was explained as an isotope effect. The direct interaction of formaldehyde and tetrahydrofolic acid has in fact recently been demonstrated by Kisliuk (71). The product has been found to function as the active one-carbon donor in the conversion of glycine to serine, and appears, therefore, to be the intermediate through which both serine and methionine are utilized for the synthesis of the thymine methyl group.

It is interesting to note that hydroxymethyl derivatives of uracil (72) cytosine (73) and cytidine (74), which may represent the products of reaction of these substances with the tetrahydrofolic acid derivative, have been isolated from natural sources.

The other possible mechanism of involvement of methionine in thymine biosynthesis, involving direct transfer of the methyl group, can not be a quantitatively important route for thymine synthesis since formic acid, formaldehyde and serine are all utilized to a greater extent than

is the methyl group of methionine. These compounds are, therefore, not utilized by prior conversion to methionine. However, the reservation must be made that it is difficult to determine the extent to which each of these metabolites is utilized under normal conditions. The sizes of the metabolic pools of the various precursors may differ widely, in which case a comparison of the incorporation of isotopic carbon contained in these compounds is a questionable measure of the relative value of these substances as precursors of the compound studied.

Keeping this reservation in mind, it may be concluded that methionine is a minor source of carbon atoms 2 and 8 of the purines of deoxyribonucleic acid. The data for thymine, however, suggests that methionine may play a significant role in the biosynthesis of the methyl group of this pyrimidine.

The Utilization of Aminobutyric Acid for Pyrimidine Biosynthesis in Neurospora

The study of the labeling of the purines and pyrimidines of the ribonucleic acid of Neurospora crassa strain 1298 after growth on alpha-aminobutyric acid-3-C^{14} indicates a specific utilization of this precursor for biosynthesis of the pyrimidine ring in this organism. It may be seen in Table V that after the four-day growth period the isolated pyrimidines, uracil and cytosine, had specific activities in the region of 5000 c.p.m./μM.

whereas the purines, adenine and guanine, had specific activities of 1000 c.p.m./μM. The same labeling pattern was also found in the nucleotides of the acid-soluble fraction, the pyrimidine nucleotide, uridine-5'-phosphate, being labeled to the extent of 4000 c.p.m./μM and the purine nucleotide, adenosine-5'-phosphate, having a specific activity of 800 c.p.m./μM. The values for the purines are about what would be expected if the labeled aminobutyric acid was being utilized as a carbon source at the same relative rate as the other carbon sources of the medium - sucrose and tartrate. This low order of activity was likewise found for aspartic acid, glutamic acid, and threonine isolated from the soluble proteins of this organism.

The high order of labeling in protein isoleucine, isolated from the wild strain after four days growth and from the mutant after a six-day growth period, is what would be expected since Abelson and Vogel (75) and Adelberg et al. (57)had demonstrated that aminobutyric acid is a normal intermediate in isoleucine biosynthesis in Neurospora. The mechanism of its utilization, as proposed by the latter authors and also by Strassman, Thomas, and Weinhouse (76), involves a ketone condensation between alpha-ketobutyric acid (obtained from the amino acid by deamination) and some activated form of acetaldehyde followed by a pinacol-type rearrangement and then amination to give isoleucine.

Another result of this study is the corroboration of the postulate of Adelberg *et al.* (57) that isoleucine and valine are formed by different routes in *Neurospora*. The low labeling found in valine from the soluble proteins indicates that aminobutyric acid is not a valine precursor, whereas, as mentioned previously, the high labeling of isoleucine established the involvement of aminobutyric acid in isoleucine biosynthesis.

However, as may be seen in Table V, the labeling of pyrimidines and isoleucine was only one-tenth the value of the precursor, aminobutyric acid. But this dilution of the isotope in the course of its utilization is explained by the finding that the specific activity of aminobutyric acid in the acid-soluble fraction is also greatly decreased, the value being 2300 c.p.m./μM at the end of the six-day growth period. This specific activity compares favorably with the labeling observed in both uracil and isoleucine obtained in the same experiment. The dilution of the isotope is therefore explained by a corresponding dilution of the precursor in the course of growth, apparently by endogenous aminobutyric acid.

In regard to the mutation in *N. crassa* 1298 which brings about its pyrimidine requirement, it may be noted that Mitchell and Houlahan (3) have described a number of "partial block" mutants of *Neurospora*. The organisms are suggested as being deficient in a single reaction step, perhaps through a decreased affinity of enzyme for substrate.

This barrier in the metabolic pathway is surmountable by means of a large excess of the substrate or by incubation at a lower temperature, the latter being explained on the basis of the heat-sensitivity of the enzyme. In the present study it seems plausible that the large excess of aminobutyric acid at the start of the incubation period enables sufficient accumulation of the precursor to bring about reaction at the partially-blocked step. Once growth begins the organism can presumably synthesize its own aminobutyric acid, and therefore the pool of this precursor is subsequently diluted, with a consequent dilution in the labeling of uracil and cytosine. This phenomenon of an organism having the requirement of a substance for growth and yet the ability to synthesize the substance in the course of growth has also been demonstrated by Bonner, Yanofsky and Partridge (77) in tryptophaneless mutants of Neurospora. The authors describe this occurrence as "leakage", and Haldane (78) suggests that such a phenomenon proves the over-similicity of the "metabolic block" idea.

This combination of a "partial block" of some reaction step on the route to pyrimidines with the "leakage" phenomenon as an explanation for the behavior of this Neurospora mutant is not, however, without its uncertainties. It has been noted that the labeling of the aminobutyric acid recovered from the mycelium after six days growth was of the

same order of magnitude as the labeling of the pyrimidines. It might be expected that the formation of pyrimidines and of aminobutyric acid would be processes occurring in relatively constant proportions throughout the growth period. Under these conditions it would be expected that the labeling of the pyrimidines would be higher than the labeling of the aminobutyric acid, since some of the pyrimidines would be formed at earlier stages of growth, when the aminobutyric acid pool had not been diluted to the final extent. The fact that this result is not obtained suggests, therefore, the greater part of the dilution occurred at a stage in growth before much pyrimidine biosynthesis had occurred.

The suggestion might be made that aminobutyric acid or some closely related derivative possesses an additional role as a catalytic factor. The greater-than-additive effect of combined uracil and aminobutyric acid on the growth of the mutant, noted in earlier work (24) suggests a dual role for this amino acid. In addition, the much greater growth response of the mutant to uridine suggests that the latter, or some closely related derivative, may be the catalytic factor to which aminobutyric acid is converted. The importance of uridine-containing coenzymes in metabolism has been suggested by the demonstration of a whole series of naturally-occurring uridine diphosphate derivatives where the latter is combined with glucose (79), galactose (80), acetylglucosamine (81), and an amino sugar

which is in turn joined to a series of amino acids (82). A group of compounds similar to the last have also been demonstrated by Binkley (83) to result by the digestion of hog kidney with a proteolytic enzyme. The fact that these substances also had enzymatic activity suggests that uridine derivatives may form the core of some enzymes, and that therefore the involvement of such substances in enzyme synthesis may not be due solely to their function as nucleic acid constituents. A catalytic role of aminobutyric acid may thus result by virtue of its initial conversion to a uridine-containing compound which may either act as a cofactor or become a part of an enzyme involved either directly or indirectly in aminobutyric acid synthesis. This additional effect might therefore explain the synergism noted in growth of the mutant on combined uracil and aminobutyric acid. It also satisfies the requirement of early dilution of the precursor, mentioned previously as the probable explanation of the similar specific activities of aminobutyric acid and pyrimidines. If aminobutyric acid was also acting in an autocatalytic manner to form more aminobutyric acid, early dilution of the precursor would certainly occur.

Granted that aminobutyric acid is a precursor of pyrimidines in Neurospora, it then becomes of interest to consider the possible reactions for conversion of the amino acid to the pyrimidine ring.

The low labeling found for protein aspartic acid in

all experiments precludes the possibility of conversion of aminobutyric acid to aspartic acid as the route of its utilization. If this amino acid or a closely related derivative were involved, the labeling of the aspartic acid pool should be of the same order of magnitude as the aminobutyric acid - shown to have a specific activity of 2500 c.p.m./uM. The aspartic acid pool should also be in equilibrium with the Neurospora proteins, and the aspartic acid of the latter should therefore also be highly labeled. Since this is not found to be the case, a specific activity of 300 c.p.m./uM being found instead, free aspartic acid is apparently not an intermediate in the conversion of aminobutyric acid to pyrimidines.

However, the selective labeling of the pyrimidine bases and nucleotides is in agreement with the possibility that the carbon chain of aminobutyric acid is utilized as a source of the pyrimidine ring. If aminobutyric acid may be considered to be utilized in a manner similar to that demonstrated for aspartic acid in Z. oroticum by Lieberman and Kornberg (84), the labeled beta carbon atom should appear as carbon 5, the middle carbon atom of the three-carbon chain of the pyrimidines. However, degradation of the isolated uracil indicates that the label, though concentrated in the carbon chain, is distributed about equally between two carbon atoms of the chain. This result does not appear reasonable, though Lagerkvist et al.

(12) have reported results which suggest a much greater utilization of the methylene carbon than the carboxyl carbon of aspartic acid-3-C^{13}-4-C^{14} for pyrimidine biosynthesis in the rat. It is possible that cleavage of the carbon chain of either four-carbon precursor occurs to give two two-carbon fragments, and that the part consisting of carbon atoms 3 and 4 then combines with a like fragment to give the doubly-labeled chain. In the case of aminobutyric acid, cleavage might be preceded by conversion to threonine by means of dehydrogenation and subsequent hydration of the resulting beta-gamma unsaturated acid. Threonine might then give rise to acetaldehyde, a reaction demonstrated in Neurospora by Wagner and Bergquist (85). However, the selective recombination of fragments to give a doubly-labeled compound must be regarded with considerable doubt. On the contrary, the results of considerable metabolic study point to an interrelationship between amino acid metabolism and pyrimidine biosynthesis such that aminobutyric acid would be expected to give rise to the 5-labeled pyrimidine. The degradation results might thus be explained on bases such as the presence of an impurity in the pyrimidine or the unreliability of the degradation procedure. The former possibility seems unlikely in view of the conformity of the absorption ratios of the isolated compounds to those of known compounds, because of the close agreement of radioactivity and concentration of the column eluates, and by

virtue of the constancy of specific activity after repeated paper chromatography in several different solvent systems. The results therefore seem to case some doubt on the reliability of the degradation procedure, at least when carried out on this small scale.

The work of Mitchell and Houlahan (7) implicated oxalacetic acid and aminofumaric acid as pyrimidine precursors in Neurospora, and subsequent studies by Lagerkvist et al. (12) and by Woods, Ravel, and Shive (13) demonstrated the involvement of aspartic acid in pyrimidine biosynthesis. The mutant under investigation in the present work, N. crassa strain 1298, has been shown to utilize, in addition to aminobutyric acid, either threonine or homoserine but not aspartic acid for growth (24, 25). The central role of homoserine in the biosynthesis of methionine and threonine was suggested by the work of Teas, Horowitz, and Fling (86) wherein homoserine was found to replace the joint threonine-methionine requirement of a Neurospora mutant. More recently Abelson and Vogel (75), by the use of an isotope-competition technique, have demonstrated a biosynthetic sequence in Neurospora consisting of aspartic acid, homoserine, threonine, alpha-ketobutyric acid, alpha-keto-beta-methylvaleric acid, and isoleucine. Homoserine was also found to be converted to methionine. In addition, the metabolic formation of homoserine and aminobutyric acid from methionine has been demonstrated by Matsuo and Greenberg (87).

The relationship between aminobutyric acid, homoserine and threonine explains the ability of N. crassa 1298 to utilize any of these three amino acids for pyrimidine biosynthesis. In addition, since the magnitude of the growth response was similar for all three compounds, and since aminobutyric acid was shown in the present work to be a pyrimidine precursor, the possibility exists that all three amino acids may be converted to a common intermediate in the course of pyrimidine biosynthesis. Teas et al. (86) have suggested that threonine may be converted to homoserine by dehydration to the beta-gamma unsaturated acid and subsequent hydration of the double bond. A similar dehydration reaction has been suggested by Chargaff and Sprinson (88) as involved in the deamination of serine. The utilization of aminobutyric acid might proceed by dehydrogenation to the beta-gamma unsaturated acid followed by hydration to homoserine. Furthermore, the reversible conversion of homoserine to aspartic acid has recently been demonstrated in yeast extracts by Black and Wright (89). The primary alcohol group of homoserine is oxidized to an aldehyde group, giving aspartic acid beta-semialdehyde. This compound is then oxidized in the presence of inorganic phosphate to the corresponding acyl phosphate, beta aspartyl phosphate, and the latter is subsequently dephosphorylated to give aspartic acid. The final step in this sequence of reac-

tions can not, however, occur reversibly in Neurospora. The fact that in the present study aspartic acid from the soluble proteins is only randomly labeled while the aminobutyric acid pool has a specific activity of 2300 c.p.m./uM. is not compatible with the idea of a rapid interconversion of the two metabolites. The conclusion therefore seems reasonable that the enzyme for one of the steps in the reaction sequence from homoserine to aspartic acid is not present in Neurospora.

The route of utilization of aminobutyric acid for pyrimidine biosynthesis in Neurospora may therefore involve homoserine and also one or more of the previously-mentioned aspartic acid derivatives. A reactive compound such as beta-aspartyl phosphate may readily react with carbon dioxide and ammonia by cleavage of the phosphate bond to give ureidosuccinic acid. The latter might then undergo cyclisation to dihydroorotic acid and then oxidation to orotic acid, as demonstrated in Z. orotieum by Lieberman and Kornberg (84).

The low labeling found for threonine from the soluble proteins suggests that aminobutyric acid is not readily convertible to threonine under these experimental conditions, but that hydration of the beta-gamma unsaturated intermediate may occur in such a manner as to give only homoserine. The possibility also exists that the unsaturated intermediate may not undergo hydration to either

homoserine or threonine in this organism, but that this reactive compound is instead in some way directly utilized for the formation of the pyrimidine ring. However, in lieu of the known role of homoserine as a methionine precursor in Neurospora, the tentative identification of highly labeled methionine sulfone in the acid-soluble fraction of N. crassa 1298 in the present work suggests that conversion of aminobutyric acid to homoserine must actually occur in this organism.

Another possible pathway whereby aminobutyric acid may be utilized for pyrimidine biosynthesis is suggested by the studies of Fink et al. (30, 90) wherein it was demonstrated that dihydropyrimidines, beta-ureido acids and beta-amino acids result as reduction products of thymine and uracil in rat liver. These authors suggested that this may represent not only a degradative pathway for pyrimidines but also a method for their synthesis. This suggestion was strengthened by the recent work of Grisolia and Wallach (31) in which they demonstrate the reversible conversion of dihydrouracil and beta-ureidopropionic acid in beef liver extracts. Furthermore, crude extracts were shown also to convert beta-ureidopropionic acid to beta-alanine, and these authors were also able to demonstrate the reverse reaction in rat liver mitochondrial preparations and in bacterial extracts (91) upon the addition of carbamyl phosphate. It would therefore appear

that beta-alanine may be a precursor of the pyrimidine ring.

Since beta-alanine has been shown to arise by the decarboxylation of aspartic acid in Cl. welchii (92), the mediation of the previously-mentioned beta-aspartyl phosphate in aminobutyric acid utilization seems quite possible. Aminobutyric acid might be converted to homoserine and then to beta-aspartyl phosphate, and the latter might then undergo decarboxylation of the carboxyl group representing carbon 1 to give a phosphorylated derivative of beta-alanine. Addition of carbon dioxide and ammonia, perhaps as carbamyl phosphate, if necessary, would yield beta-ureidopropionic acid, which could then be cyclisized and oxidized to form the pyrimidine ring. In the present study, the attempt to replace the pyrimidine requirement of N. crassa 1298 with a mixture of carbamyl phosphate and beta-alanine was unsuccessful. Woods, Ravel, and Shive (13) noted a similar inability on the part of the aspartic acid-requiring L. arabinosus 17-5 to utilize either beta-alanine or ureidosuccinic acid. However, these negative results are of little significance since they quite possibly may have been due to the inability of the compounds to pass through the cell wall of the organism. Such permeability barriers are known to be significant where highly charged and polar substances are concerned.

The conclusion therefore seems reasonable that aminobutyric acid is utilized for pyrimidine biosynthesis by conversion to homoserine followed by oxidation and perhaps phosphorylation of the gamma carbon atom to give a reactive intermediate such as beta-aspartyl phosphate. The latter may then proceed to pyrimidines directly by reaction with carbon dioxide and ammonia with subsequent cyclisation and oxidation. Alternatively, beta-aspartyl phosphate might undergo decarboxylation to form a derivative of beta-alanine which might then react with carbon dioxide and ammonia to form beta-ureidopropionic acid followed by cyclisation and oxidation to give the pyrimidine ring.

In connection with this suggestion of a different route for pyrimidine biosynthesis in Neurospora, it might be noted that Davis (93) has observed that lysine biosynthesis in Neurospora involves an entirely different pathway from that which exists in E. coli. He has demonstrated that diaminopimelic acid is an intermediate in lysine biosynthesis in E. coli, whereas Good, Heilbronner and Mitchell (94) have shown alpha-aminoadipic acid to be involved in lysine biosynthesis in Neurospora. The same difference has also been noted between T. utilus and N. crassa by Abelson and Vogel (75). Furthermore, the observation by Woods, Ravel, and Shive (13) that lysine - in addition to threonine and pyrimidines - spares the aspartic

acid requirement of L. arabinosus 17-5 suggested to those authors that lysine was involved in pyrimidine biosynthesis. Mitchell and Houlahan (95) had earlier demonstrated the accumulation of pyrimidines by lysineless mutants of Neurospora, and Doermann reported an inhibitory effect of arginine in Neurospora similar to that observed by Fairley (25) with the pyrimidineless mutant used in the present work.

The fact that a definite difference exists between Neurospora and other organisms in regard to lysine biosynthesis, when coupled with the observed pyrimidine-lysine relationship, suggests that the unusual biosynthetic pathway for both pyrimidines and lysine in Neurospora may be attributable to some alteration in an intermediate common to both pathways. The studies of Abelson et al. (96), wherein lysine was shown by isotopic competition experiments to be an aspartic acid metabolite, indicates that aspartic acid or some derivative may be this common intermediate. The involvement of such an intermediate in lysine biosynthesis remains to be clearly demonstrated, but the lysine-pyrimidine relationship at least serves to demonstrate the existence of real differences in biosynthetic pathways in different types of living things.

As may be seen in Table V, the labeling of the purines and pyrimidines of the wild strain, while not as markedly

different as in the case of the mutant, nevertheless demonstrates that aminobutyric acid is a normal precursor of pyrimidines in this organism. The finding of aminobutyric acid as a component of the free amino acid pool of the wild strain - grown without aminobutyric acid - also supports this suggestion.

However, regardless of the pathway of utilization of aminobutyric acid for pyrimidine synthesis, it seems reasonable to conclude that this amino acid must be a significant precursor of these nucleic acid constituents in Neurospora. The fact that the labeling of isoleucine - for which aminobutyric acid is a known precursor - is similar in magnitude to that of the pyrimidines must be regarded as strong support for a prominent position for aminobutyric acid in pyrimidine biosynthesis in Neurospora.

The Utilization of Aminobutyric Acid for Pyrimidine Biosynthesis in the Rat

The finding that aminobutyric acid is not readily utilized for pyrimidine biosynthesis in the rat is not unexpected since the previous study strongly suggests a special situation for pyrimidine biosynthesis in Neurospora.

The pathway involving aspartic acid, demonstrated by Lieberman and Kornberg (17, 84) in Z. oroticum, is undoubtedly also the major route for pyrimidine biosynthesis in

the rat. Weed and Wilson were able to demonstrate utilization of orotic acid (7) and of ureidosuccinic acid (11) for the synthesis of polynucleotide pyrimidines in rat spleen, and Reichard (14) recently reported that rat liver mitochrondria are capable of converting aspartic acid, carbon dioxide, and ammonia to ureidosuccinic acid.

The dilution of isotopic aminobutyric acid on conversion to rat deoxyribonucleic acid cytosine in the present study is approximately ten times as great a s that found in earlier studies of methionine utilization for thymine biosynthesis. This represents a dilution by about 5000 times in the course of utilization, whereas methionine was diluted over 25,000 times in the course of conversion of the labeled methyl group to the ring carbons of cytosine. There thus appears to be some slight selectivity in the use of aminobutyric acid in the rat, particularly since cytosine was labeled to four times the extent of adenine. The lower specific activity of isolated thymine suggests a different route of pyrimidine biosynthesis for the two major deoxyribonucleic pyrimidines, thymine and cytosine.

However, the specific activities of the isolated components are so low that the above interpretation must be regarded as extremely tentative. A more definite conclusion must await further study of aminobutyric acid and pyrimidine biosynthesis in the rat.

SUMMARY

SUMMARY

1. Methionine-methyl-C^{14} has been shown to give rise to significant labeling of the purines and thymine of deoxyribonucleic acid in the rat. The data suggests that utilization of the methionine methyl carbon occurs by way of the "1-carbon pool", but that formic acid is not an intermediate in this process. Rather, an intermediate at the oxidation state of formaldehyde appears more likely. By comparison with formic acid, the methionine methyl group appears to be of negligible importance in purine biosynthesis, but the data for the pyrimidines suggests that the methyl group of methionine serves as a significant precursor of the thymine methyl carbon.

2. A pyrimidineless mutant of Neurospora crassa has been shown to utilize alpha-aminobutyric acid-3-C^{14} for pyrimidine biosynthesis. The data suggests that free aspartic acid is not an intermediate in this conversion, but an aspartic acid derivative, derived from aminobutyric acid by way of homoserine, appears as a reasonable choice for one of the intermediates involved. The importance of aminobutyric acid as a pyrimidine precursor in Neurospora is indicated by the similar magnitude of the labeling of isoleucine and the pyrimidines. The

biochemical nature of the mutation possessed by N. crassa 1298 and possible reasons for the ten-fold dilution noted on conversion of the precursor to the pyrimidine are discussed.

3. Alpha-aminobutyric acid-3-C^{14} was also studied as a possible pyrimidine precursor in the rat. Some selectivity in utilization of the isotope for cytosine synthesis was found, though the specific activities of the compounds isolated were of such a low order of magnitude that any interpretation of the data must be qualified as highly tentative.

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APPENDIX

APPENDIX

A. The molar extinction coefficients for thymine and the purines in the eluting solvents were determined as follows.

In the case of thymine a 15 mg. sample of the recrystallized base* was dissolved in 25 ml. of 0.1 N hydrochloric acid. One 5 ml. aliquot was diluted to 100 ml. with 0.1 N hydrochloric acid and the optical density determined at 260 mμ. A second 5 ml. aliquot was diluted to 100 ml. in 0.015 M ammonium formate buffer of pH 8.25, and the optical density at 260 mμ also determined. The molar extinction coefficient was then calculable by the following relationship.

$$k_u = k_s \times \frac{D_u}{D_s}$$

where k_u = molar extinction coefficient in eluting solvent buffer of pH 8.25

k_s = molar extinction coefficient in 0.1 N hydrochloric acid

D_s = optical density at 260 mμ in 0.1 N hydrochloric acid

D_u = optical density at 260 mμ in eluting solvent

The procedure for adenine was similar except that

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*Nutritional Biochemicals Corp., Cleveland, Ohio

10 mg. of adenine sulphate* was dissolved in 25 ml. of 0.1 N hydrochloric acid and dilutions were made to 100 ml. with 0.1 N hydrochloric acid and 4 N hydrochloric acid. The optical densities were again determined at 260 mμ. For guanine, 10 mg. of guanine hydrochloride** was dissolved in 25 ml. of 0.1 N hydrochloric acid, and dilution was made with 0.1 N and 3 N hydrochloric acid. In this case the optical densities were measured at 249 mμ, the wavelength of maximum absorption for guanine in acid solution.

B. The formula used to convert the observed counts of the purines and pyrimidines to specific activity.

$$S = \frac{Co \times k \times 1000 \text{ ml.}}{D \times 10^6}$$

where S = specific activity (counts/minute/micromole)

Co = observed counts (counts/minute/ml.of sample)

k = molar extinction coefficient

D = optical density of sample counted

10^6 = factor to convert moles to micromoles

Sample calculation: thymine

Co = 104 c.p.m./ml. of sample $k = 7 \times 10^3$ (260mμ,pH 8.25)

D = 0.693

$$S = \frac{104 \times 7 \times 10^3 \times 1000}{0.693 \times 10^6} = 1050 \text{ c.p.m./}\mu\text{M}$$

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*Bios Laboratories, New York

**Eastman Kodak Co., Rochester, New York