[17] H. OZEKI, *Genetic Studies with Bacteria*, Carnegie Inst. Wash. Publ., 612 (1956) 97.
[18] B. O. D. STOCKER, *J. Gen. Microbiol.*, 15 (1956) 575.
[19] H. EPHRUSSI-TAYLOR, *Recent Progress in Microbiology, Symposia held at VII Intern. Congr. for Microbiology, 1958*, p. 51.
[20] R. D. HOTCHKISS, in W. D. MCELROY AND B. GLASS, *The Chemical Basis of Heredity*, The Johns Hopkins Press, Baltimore, 1957, p. 321.
[21] J. MONOD, A. M. PAPPENHEIMER AND G. COHEN-BAZIRE, *Biochim. Biophys. Acta*, 9 (1952) 648.
[22] L. GORINI AND W. K. MAAS, *Biochim. Biophys. Acta*, 25 (1957) 208.
[23] A. B. PARDEE, F. JACOB AND J. MONOD, *J. Mol. Biol.*, 1 (1959) 165.
[24] J. G. FLAKS, J. LICHTENSTEIN AND S. S. COHEN, *J. Biol. Chem.*, 234 (1959) 1507.

Biochim. Biophys. Acta, 45 (1960) 155–163

STUDIES ON THE BIOSYNTHESIS OF 5-RIBOSYLURACIL PHOSPHATE IN *NEUROSPORA CRASSA* 36601

JOHN B. HALL AND FRANK WORTHINGTON ALLEN
Department of Biochemistry, University of California, School of Medicine, San Francisco, Calif. (U.S.A.)

(Received June 27th, 1960)

SUMMARY

(1) While the pyrimidine portion of 5-ribosyluracil phosphate has been found to be derived from orotic acid in yeast, it has been found to be derived largely from cytidine of the medium in the pyrimidine requiring mutant Neurospora, *Neurospora crassa* 36601.

(2) Experiments with [2-^{14}C]uracil indicate that uracil is not an intermediate in the conversion of cytidine into 5-ribosyluracil phosphate.

(3) Reasons are presented for the belief that a uridine phosphate, such as uridine 5′-phosphate, is an intermediate in the biosynthesis of 5-ribosyluracil phosphate.

(4) The possibility is discussed that 5-ribosyluracil phosphate is normally derived from a pathway in which orotic acid is first incorporated into uridine 5′-phosphate, which then forms an unknown intermediate and is finally converted to 5-ribosyluracil phosphate.

INTRODUCTION

Studies by HALL AND ALLEN[1] have shown that orotic acid is an effective precursor of 5-ribosyluracil phosphate in yeast. When yeast was grown in the presence of [6-^{14}C]orotic acid it was found that, within the limits of experimental error, 5-ribosyluracil phosphate and uridylic acid were equally labeled by ^{14}C and that cytidylic acid was less highly labeled than the two uracil-containing nucleotides. There seem to be two pathways that could explain these results (Fig. 1). Possibly there is yet another orotic acid pathway, similar to these, in which orotic acid reacts with 5-phosphoribose 1-pyrophosphate, or with some other activated ribose, to give 5-ribosylorotidyl

phosphate, which is an isomer of orotidine 5′-phosphate, in which the ribose is linked to the carbon atom of position 5 of the orotate molecule, rather than with the nitrogen atom of position 3. This compound could then be directly decarboxylated to give 5-ribosyluracil phosphate, just as orotidine 5′-phosphate yields uridine 5′-phosphate upon decarboxylation. The other possibility is that 5-ribosyluracil phosphate is derived from some nucleotide intermediate further along the orotic acid pathway. Cytidylic acid is ruled out by the pattern of labeling in the experiment on yeast,

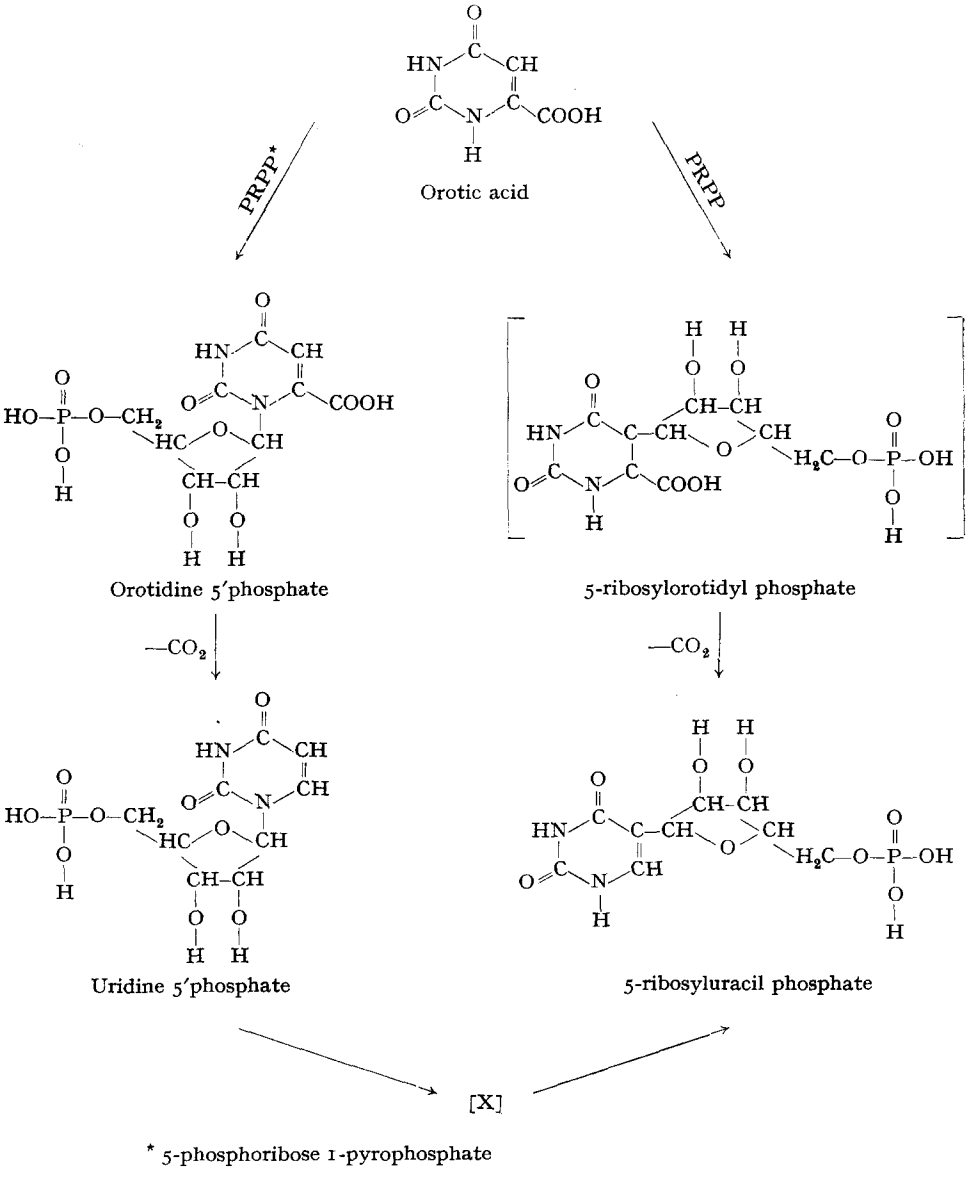

Fig. 1. Possible pathways for the biosynthesis of 5-ribosyluracil phosphate from orotic acid.

because it is unlikely that a compound could be derived from another compound in the metabolic sequence that is less highly labeled. The data, however, indicate that 5-ribosyluracil phosphate could be derived from uridylic acid, and that perhaps it is derived exclusively from this compound.

To examine these two possibilities it was decided to determine the origin of the pyrimidine of 5-ribosyluracil phosphate in a mutant organism which can not utilize orotate for the biosynthesis of its pyrimidine nucleotides. *Neurospora crassa* 36601 was selected for this purpose. This fungus, which was first described by MITCHELL, HOULAHAN AND NYC[2], requires pyrimidines for growth and grows best on nucleosides, such as cytidine. Orotidine and orotic acid accumulate in the culture medium, so that the fungus appears to lack the decarboxylase which converts orotidine 5'-phosphate to uridine 5'-phosphate. This mutant does not to any extent incorporate orotic acid into the pyrimidine nucleotides uridylic acid and cytidylic acid. If orotic acid were to be incorporated into 5-ribosyluracil phosphate, the existence of an orotic acid pathway parallel to the one utilized for the synthesis of the principal pyrimidine nucleotides would be demonstrated. If, on the other hand, orotic acid were not to be incorporated into 5-ribosyluracil phosphate, then the problem of its origin from other precursors would arise. This latter, is in fact, what has been observed in this study, so that it seems probable that the pyrimidine of 5-ribosyluracil phosphate is derived from the cytidine furnished in the medium. The pathway by which this conversion is accomplished is the subject of this research.

EXPERIMENTAL

Materials

Neurospora crassa 36601 was obtained as a dry pellet of lyophilized mycelia in a sealed vial from the American Type Culture Collection, 2112 M Street, N.W., Washington 7, D.C. The pellet was put into a sterile test tube which contained about 20 ml of culture medium and was allowed to grow at room temperature. Growth was apparent in about 3 days and portions of the mycelia were than transferred to agar slants and maintained as stock cultures. The purity of the strain was periodically checked by transferring a portion of the mold to a test tube containing the same medium on which it had been grown, with the exception that cytidine was omitted. The mutant requires pyrimidines for growth, and in no case did growth occur on a medium deficient in these. This procedure guarded against the possibility of back-mutation and of contamination of the culture by organisms that did not require cytidine.

Culture medium for Neurospora crassa *36601*

The culture medium for this mutant Neurospora is the one described by BEADLE AND TATUM[3] and is based on a salt mixture developed by FRIES[4] designated No. 3. To this medium 20 units penicillin/ml and 40 μg streptomycin/ml[5] are added to suppress the growth of any bacteria that might gain entrance after autoclaving.

The medium was made up to 5.5 l and placed in a 20-l pyrex bottle. The mouth of the bottle was stoppered with a cotton plug and the bottle was autoclaved at 100° for 1 h. This low-pressure autoclaving was sufficient to sterilize the medium and did not induce the formation of sediment or cause caramelization of the sugar.

The medium for the seed cultures was of the same composition as that for the batch cultures. This medium was poured into test tubes stoppered with cotton plugs and autoclaved. The agar slants were prepared from the same medium with agar added to a concentration of 1.5 % (w/v).

Radioactive compounds

[2-^{14}C]Uracil and [6-^{14}C]orotic acid were purchased from the Research Specialties Company of Richmond, Calif.

[2-^{14}C]Cytidine was obtained from the Schwarz Laboratories of Mount Vernon, N.Y.

A small amount of contaminating uracil was removed from the orotic acid by chromatography on a Dowex-1-acetate column. The purity of the cytidine and of the uracil was examined by paper chromatography in butanol–water. The uracil appeared to be pure, but the cytidine was contaminated by a trace of material which was believed to be uridine. It was thought that the amount of this contaminant was too small to interfere with the experiments.

Methods

11 mg [6-^{14}C] orotic acid containing $27 \cdot 10^6$ counts/min was added to 5.5 l of the culture medium. The medium was inoculated with a seed culture containing 50–100 mg *Neurospora crassa* 36601 and the bottle of medium, of approx. 20-l capacity, was placed on its side on a rotator. This device caused the bottle to rotate about its long axis and stirred and aerated the medium. After 2 days the mold was harvested by pouring the medium containing it through a layer of cheese cloth. The fungus was then squeezed dry and weighed. About 10 g was usually obtained. This was about one-third of the maximum weight obtainable when longer growth periods were used, a fact which indicated that the mold was still growing vigorously at the time of harvest. The fungus was placed in a chilled Waring blendor, which contained 100 ml of a cold solution of 8 parts ethanol to 2 parts of a 10-% solution of perchloric acid, and it was blended for 10 min. The resulting homogenate was centrifuged and the precipitate was washed with 10 % perchloric acid to remove additional acid-soluble material. The homogenized residue was neutralized with solid pellets of potassium hydroxide and 100 ml 1 *N* potassium hydroxide was added. The mixture was thoroughly stirred and allowed to stand at room temperature for 24 h to hydrolyze the nucleic acids to mononucleotides. This procedure, which is a modification of that of SCHMIDT AND THANNHAUSER[6], was adopted after two attempts to extract the nucleic acid of this organism by the phenol method of LITTAUER AND EISENBERG[7] had failed to give satisfactory yields. Treatment of a commercial sample of cytidylic acid in a similar manner showed that hydrolysis with potassium hydroxide under these conditions does not produce significant deamination of cytidylic acid. The potassium hydroxide of the Neurospora homogenate was neutralized by the addition of concentrated acetic acid and centrifuged. The residue was washed twice with 67 % ethanol. A cloudy supernatant was obtained from the original centrifugation, and this was poured into two volumes absolute alcohol, which precipitated a pinkish-white, curdy material. This step was introduced when it was found that the cloudy material interfered with further purification and was not soluble in lipid solvents or precipitated by extremes of pH. The precipitate was removed by centrifugation and washed with 67 % ethanol. The

alcoholic solution containing the nucleotides was reduced in volume on a rotary evaporator and desalted on a charcoal column[8].

After adsorption on charcoal and elution, the solution containing the nucleotide was dried on a flash evaporator to remove ammonia and alcohol. A drop of acetic acid was added to reduce the pH to approx. 6, and the material was washed on to the surface of a Dowex-1-acetate column. The system of column chromatography used to fractionate the nucleotides was that devised by COHEN[9] and described by LANE AND BUTLER[10]. This system resulted in excellent separation of 5-ribosyluracil phosphate from uridylic acid. The elution of fractions from this column was recorded by the recording unit of a GME automatic fraction collector. The fractions containing the nucleotides were collected and desalted on charcoal. The solution of desalted nucleotides was reduced in volume and placed on Whatman No. 1 chromatography paper to be purified by ascending chromatography in either isopropanol–hydrochloric acid–water (170:20:39)[11] or in isopropanol–acetic acid–water (60:30:10)[12]. Approx. 200 μg/cm was applied to the paper and the chromatograms were developed overnight. The first solvent required but one submission, but, when the second was used, two submissions were necessary. The chromatograms were photographed in u.v. light and the areas that absorbed were cut out and eluted. Spectral ratios of the purified nucleotide solutions were calculated.

Aliquots of the nucleotide solutions were now plated in duplicate or triplicate, dried in a vacuum desiccator under an infra-red lamp, and counted three times on the automatic gas flow counter. The counts/min were averaged and background subtracted, and the specific activities in counts/min/mmole were calculated.

Procedures for the subsequent experiments were essentially the same as that described for the incorporation of orotic acid into the nucleotides of the mutant Neurospora. Different substances containing the ^{14}C label were added to the medium, but the conditions of growth and the isolation of the nucleotides were largely the same in other respects. When labeled cytidine was used, it was added to the medium as 0.37 mmoles cytidine with an activity of $7.0 \cdot 10^7$ counts/min/mmole. In the experiment designed to determine the role of uracil in the biosynthesis of 5-ribosyluracil phosphate, 0.17 mmoles uracil containing $2.1 \cdot 10^8$ counts/min/mmole, was added to the cytidine supplemented medium.

In order to determine the dilution of uracil before its incorporation into the nucleotides in this last experiment, the free uracil of the cell was isolated from the Neurospora homogenate by the following method. The combined alcohol–perchloric acid supernatant solution from the Neurospora homogenate and the 10 % perchloric acid wash were neutralized with potassium hydroxide. 1.5 g of barium acetate was added to this solution, which was about 300 ml in volume, and the solution was then adjusted to pH 9 with barium hydroxide. The precipitate of potassium perchlorate and the barium salts of the nucleotides was centrifuged and washed with 100 ml water. The supernatant solution was evaporated to a volume of approx. 100 ml on the rotary evaporator and desalted on charcoal. An aliquot of the resulting solution containing approx. 3 mg material was placed across a 1-cm wide strip of Whatman No. 1 paper and twice submitted to ascending chromatography in butanol saturated with water[13]. The material separated into several bands. Scanning with a radioactivity counter showed that one of these, which had moved in about the position expected of uracil in this solvent, was highly radioactive. This band was cut out,

eluted, and submitted to chromatography in isopropanol–hydrochloric acid–water, to remove any traces of contaminating material. The material moved at a rate identical with that of a reference spot of uracil on the same sheet of paper. The area containing the radioactive-uracil was cut out and eluted and the spectral ratios were determined, and the pyrimidine was plated and counted as described earlier.

TABLE I

SPECIFIC ACTIVITIES OF NUCLEOTIDES DERIVED FROM THE NUCLEIC ACIDS OF *Neurospora crassa* 36601 GROWN ON VARIOUS ^{14}C LABELED PRECURSORS

	Specific activity, counts/min/mmole			
	Precursor			
	Orotic Acid	*Cytidine 1*	*Cytidine 2*	*Uracil*
Precursor	$3.9 \cdot 10^8$	$7.0 \cdot 10^7$	$7.0 \cdot 10^7$	$2.1 \cdot 10^8$
Cytidylic acid	$1.8 \cdot 10^6$	$7.0 \cdot 10^7$	$6.4 \cdot 10^7$	$1.2 \cdot 10^7$
Uridylic acid	$2.3 \cdot 10^6$	$6.8 \cdot 10^7$	$6.2 \cdot 10^7$	$2.0 \cdot 10^7$
5-Ribosyluracil phosphate	$2.3 \cdot 10^6$	$5.8 \cdot 10^7$	$5.1 \cdot 10^7$	$1.7 \cdot 10^7$
Adenylic	$3.5 \cdot 10^4$	$1.1 \cdot 10^6$	$1.0 \cdot 10^6$	$1.0 \cdot 10^5$
Guanylic acid	$2.8 \cdot 10^4$	—	$6.3 \cdot 10^5$	$8.0 \cdot 10^4$
Cellular uracil	—	—	—	$5.1 \cdot 10^7$

TABLE II

THE UTILIZATION OF VARIOUS ^{14}C LABELED COMPOUNDS AS PRECURSORS FOR NUCLEIC ACID NUCLEOTIDES IN *Neurospora crassa* 36601

	Per cent nucleotide derived from precursor			
	Precursor			
	Orotic acid	*Cytidine 1*	*Cytidine 2*	*Uracil*
Cytidylic acid	0.5	100	91	5.7
Uridylic acid	0.6	97	89	9.5
5-Ribosyluracil phosphate	0.6	83	73	8.1
Adenylic acid	0.009	1.6	1.4	0.05
Guanylic acid	0.007	—	0.9	0.04
Cellular uracil	—	—	—	24

RESULTS AND DISCUSSION

The specific activities of the nucleotides isolated from *Neurospora crassa* 36601, after this fungus had been grown in the presence of various labeled pyrimidine precursors, are given in Table I. The efficiency of each precursor for the formation of the pyrimidine nucleotides is more readily seen in Table II, in which the data are presented as the % nucleotide derived from each precursor, obtained by dividing the specific activity of the isolated nucleotide by that of its precursor and multiplying by 100. Data for the purine nucleotides are included as a measure of the degradation and non-specific incorporation of the labeled compound into nucleic acids.

It can be seen from Table II that orotic acid produces very little labeling of any of the pyrimidine nucleotides. Because the orotic acid had been purified, it was unlikely

that any pyrimidine precursor would be present at levels that would produce the labeling observed. It was therefore decided that the slight incorporation of ^{14}C into the pyrimidine nucleotides represented a leak in the block of the orotic acid pathway. It will be seen that the pattern of incorporation produced by this precursor is identical with that reported earlier for yeast[1]. The possibility that 5-ribosyluracil phosphate, unlike the major pyrimidine nucleotides, could be derived from orotate in this organism is excluded by this experiment. The most probable source, therefore of the pyrimidine of this compound is the cytidine furnished in the medium. When the Neurospora was grown in the presence of ^{14}C-labeled cytidine, it was found that this nucleoside was an effective precursor for the pyrimidine of all three pyrimidine nucleotides. This was the expected result, but it raised the question of what is the sequence of metabolic transformations that are utilized by the organism to convert a nucleoside, with the ribose moiety attached to a nitrogen atom in the 3 position of the ring, into a nucleotide, in which the ribose is attached to a carbon atom in the 5 position. It seems unlikely that 5-ribosyluracil phosphate is derived directly from cytidine, through some intermediate such as 3,5-diribosylcytosine, because the specific

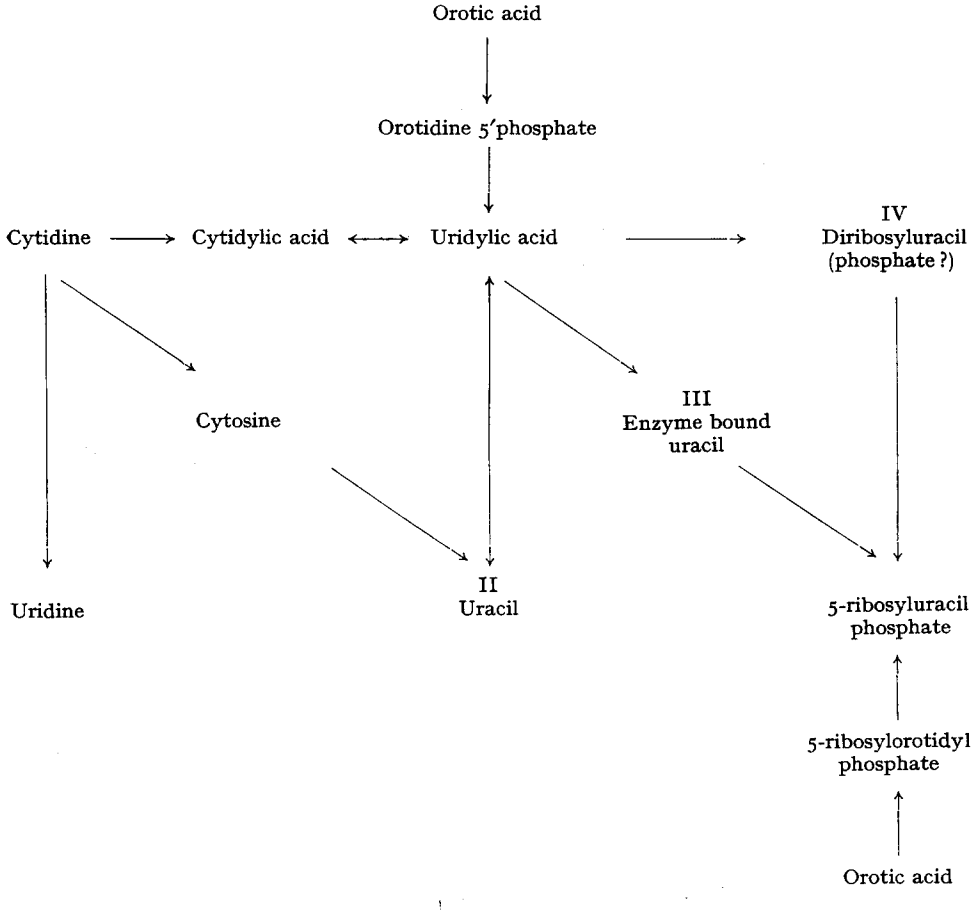

Fig. 2. Some possible intermediates in the biosynthesis of 5-ribosyluracil phosphate.

activity of 5-ribosyluracil phosphate is even less than that of uridylic acid, instead of being comparable to that of cytidylic acid, as would be expected if radioactive-cytidine were the precursor. A second possibility is that cytidine is first converted to a free pyrimidine, which is then conjugated with ribose to form 5-ribosyluracil phosphate (Fig. 2). To examine this possibility, the Neurospora was grown on a cytidine supplemented medium to which [2-^{14}C]uracil had been added. It was again found that 5-ribosyluracil phosphate was less highly labeled than uridylic acid, although its specific acitivity was higher than that of cytidylic acid, as it was in the orotic acid experiments. If uracil were an intermediate in the biosynthesis of 5-ribosyl-uracil phosphate, we should expect this nucleotide to be much more, rather than less highly labeled than uridylic acid. The fact that the free uracil isolated from the Neurospora homogenate was more than two and a half times as radioactive as 5-ribosyluracil phosphate showed that this nucleotide was not derived principally from uracil. It is possible that cytosine, and not uracil, is the free pyrimidine intermediate. This seems unlikely, however. If cytosine is derived from cytidylic acid, it is clear that it cannot be the precursor of 5-ribosyluracil phosphate in the uracil or orotic acid experiments in which cytidylic acid was less highly labeled than the uracil nucleotides were. If, on the other hand, the cytosine were formed from uracil, and the 5-ribosyluracil phosphate were derived entirely from cytosine, we should expect this nucleotide, to be more highly labeled than uridylic acid when radioactive uracil was present in the medium, because the major portion of the cytidylic acid and the uridylic acid was derived from the cytidine in the medium.

The remaining possibility, which the data seem to favor, is that 5-ribosyluracil phosphate is derived from the labeled pyrimidines via some derivative of uridylic acid, probably uridine 5'-phosphate. This would explain the nearly equivalent labeling of uridylic acid and 5-ribosyluracil phosphate in the orotic acid and uracil experiments, and also the decrease in specific activity from cytidylic acid to uridylic acid to 5-ribosyluracil phosphate when labeled cytidine is the precursor. This pathway requires the existence of an additional intermediate between uridylic acid and 5-ribosyluracil phosphate, such as 3,5-diribosyluracil, or an enzyme-bound uracil which is not in equilibrium with the free uracil of the cell.

The relationship of this pathway to the orotic acid pathway must now be examined. It may be supposed that wild strains of Neurospora can form 5-ribosyluracil phosphate directly from orotate, via 5-ribosylorotidyl phosphate. If this is so, however, it must be assumed that this organism possesses two pathways for the biosynthesis of 5-ribosyluracil phosphate, the alternative explanation being the rather improbable one that the pathway just described for the mutant Neurospora arose *de novo* during the mutation that deleted the decarboxylase. Thus it seems probable that either the uridylic acid pathway of 5-ribosyluracil phosphate synthesis is the sole pathway in Neurospora, or that two routes for the synthesis exist. In non-mutant organisms, the pathway here described would lead from orotic acid to uridylic acid, then to the unknown intermediate, and finally to 5-ribosyluracil phosphate. At first glance this appears to be a rather indirect and inefficient pathway (see Fig. 1), and its existence in this organism gives rise to the suspicion that the direct pathway from orotic acid may not be available for this synthesis. Possibly the nitrogen atom of position 3 of the uracil ring must be substituted, or the carboxyl group on position 4 removed, before the carbon atom at position 5 can react with a ribosyl compound

to form a 5-ribosyluracil derivative. This is highly speculative, however. All that can be said with confidence is that the data indicate that this mutant of neurospora is able to utilize cytidine and uracil for the synthesis of 5-ribosyluracil phosphate by a pathway which probably involves uridylic acid as an intermediate.

ACKNOWLEDGEMENTS

This work was supported in part by Grant-in-aid RG 2496, U.S. Public Health Service, Grant G 8971 National Sciences Foundation and Cancer Research Funds of the University of California.

REFERENCES

1 J. B. HALL AND F. W. ALLEN, *Biochim. Biophys. Acta*, 39 (1960) 557.
2 H. K. MITCHELL, M. B. HOULAHAN AND J. F. NYC, *J. Biol. Chem.*, 172 (1948) 525.
3 G. W. BEADLE AND E. L. TATUM, *Proc. Natl. Acad. Sci.*, 27 (1941) 499.
4 N. FRIES, *Symbolae Bot. Upsaliensis*, Vol. 3, No. 2, (1938) p. 1.
5 *Difco Manual*, 9th ed. (1953) p. 238.
6 G. SCHMIDT AND S. J. THANNHAUSER, *J. Biol. Chem.*, 161 (1945) 83.
7 U. Z. LITTAUER AND H. EISENBERG, *Biochim. Biophys. Acta*, 32 (1959) 320.
8 I. G. WALKER AND G. C. BUTLER, *Can. J. Chem.*, 34 (1956) 1168.
9 L. COHEN, *Ph. D. Thesis*, University of Toronto, 1954.
10 B. G. LANE AND G. C. BUTLER, *Can. J. Biochem. Physiol.*, 37 (1959) 1329.
11 G. R. WYATT, *Biochem. J.*, 48 (1951) 584.
12 F. F. DAVIS AND F. W. ALLEN, *J. Biol. Chem.*, 227 (1957) 907.
13 R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 45 (1949) 294.