Experimental

Fumaramic Acid from **Asparagine**.—Method described in original paper[1]:

Anal. Calcd. for $C_4H_6O_3N$: C, 41.74; H, 4.38; N, 12.18; mol. wt., 115.09. Found: C, 41.45; H, 4.24; N, 12.04; mol. wt. (freezing point depression using glacial acetic acid), 103.2; neut. equiv., 109.3; m.p. (capillary tube 216.5–217.5° (cor.).

Fumaric Acid Monomethyl Ester.—The method of Anschütz and Baeumges[10] as modified by Lutz[8] was employed. A solution of dry methanol (10.7 g.) in 145 ml. of dry benzene was added dropwise to 51.0 g. of fumaryl dichloride with constant stirring. The temperature was kept below 30° and hydrogen chloride was evolved. After standing overnight, under dry conditions, the benzene was boiled off at atmospheric pressure. The product was fractionally distilled through a 12-inch column filled with small porcelain saddles at a pressure of about 18 mm. Four fractions were taken with boiling ranges 62–71°, 71–82°, 82–84° and 84–85°. At 84–85° a product solidified in the column (dimethyl fumarate). The fractions boiling from 71–84° were combined and stirred vigorously with cold water until the emulsion, first formed, solidified and crystallized. After cooling in ice, the suspension was filtered and the crystals washed with ice-water. The yield, after drying at 50° *in vacuo*, was 8.2 g. or 18.9% of crystals melting at 144.0–144.5°.

(10) R. Anschütz and P. Baeumges, *Ann.*, **461**, 188 (1928).

Fumaramic Acid from **Fumaric Acid Monomethyl Ester**.—The fumaric acid monomethyl ester (8.2 g.) was treated with 25 ml. of concentrated ammonium hydroxide at room temperature. Solution occurred immediately. After shaking for several minutes, a product began to crystallize. The suspension of crystals was allowed to stand two hours and then evaporated to dryness in a rotary evaporator. The dry product was taken up in water and redried to remove the last traces of free ammonia. The product was dissolved in water and passed through a column of Dowex 50[11] in the H^+ ion cycle to desalt. After washing with three bed-volumes of water, the effluent and washings were combined, evaporated to a small volume in the rotary still at less than 40° and allowed to crystallize. After recrystallization from water and drying at 50° *in vacuo*, the yield of fumaramic acid was 6.3 g. or 16.4% based upon the fumaryl dichloride. X-Ray powder diagrams and infrared absorption in KBr disks showed the two samples of fumaramic acid to be identical.

Anal. Calcd. for $C_4H_5O_3N$: C, 41.74; H, 4.38; N, 12.18; mol. wt., 115.09. Found: C, 41.85; H, 4.60; N, 11.96; mol. wt. (freezing point depression in glacial acetic acid), 102.2; neut. equiv., 110.4; m.p. (capillary tube), 216.5–217.5°; hydrogenation (g./mole H_2), 118.2.

(11) Mention of commercial products does not imply endorsement by the Department of Agriculture over others of similar nature not mentioned.

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[CONTRIBUTION FROM THE MALLINCKRODT LABORATORIES OF HARVARD UNIVERSITY]

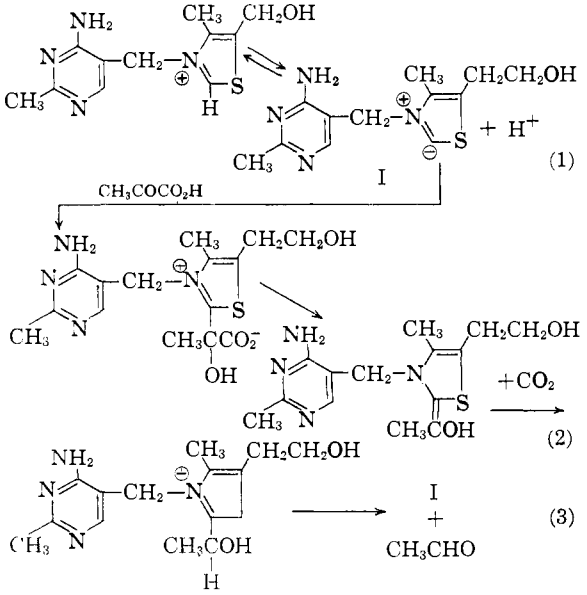
The Role of Thiamin in Carboxylase

BY D. F. DETAR[1] AND F. H. WESTHEIMER

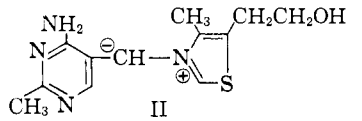
RECEIVED AUGUST 14, 1958

The decarboxylation of pyruvate with yeast carboxylase has been carried out in tritiated water. The coenzyme, thiamin pyrophosphate, was released from the enzyme. Carrier was added, and the coenzyme was cleaved to pyrimidinesulfonic acid. The compound so obtained contained no tritium. This finding for the enzymatic reaction accords with the facts for the known model system which catalyzes the decarboxylation of pyruvate, and with the probable mechanism for the enzymatic and "model" systems.

A mechanism for the action of thiamin has recently been advanced by Breslow.[2] This mechanism involves the ionization of a proton from the 2-position of the thiazolium ring, to form an ylid; this ylid is postulated as the essential intermediate in the decarboxylation of pyruvic acid.



The ionization shown in equation 1 has been demonstrated by deuterium exchange, and the mechanism will account for the reactions observed in model systems.[3] Earlier the ylid II was postulated[4] as an intermediate in the process, but this possibility has been disproved for the model systems.[5]



Although it seems reasonable to assume that the model system and the enzymatic one operate by very similar mechanisms, this point remains for experimental demonstration. The present research is concerned with a study of the mechanism of the enzymatic decarboxylation of pyruvic acid with the

(1) National Science Foundation Senior Post-doctoral Fellow. Professor of Chemistry, University of South Carolina, Columbia, S. C.

(2) R. Breslow, THIS JOURNAL, **80**, 3719 (1958).

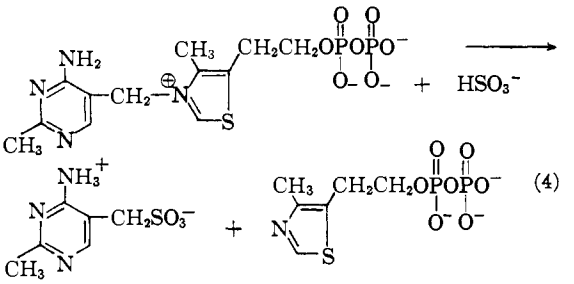
(3) S. Mizuhara and P. Handler, *ibid.*, **76**, 571 (1954).

(4) R. Breslow, *Chemistry & Industry*, R28 (1958); a related species has been suggested by K. Wiesner and Z. Valenta, *Experientia*, **12**, 190 (1956).

(5) L. Ingraham and F. H. Westheimer, *Chemistry & Industry*, 846 (1956); K. Fry, L. Ingraham and F. H. Westheimer, THIS JOURNAL, **79**, 5225 (1957).

enzyme carboxylase, and the coenzyme thiamin pyrophosphate.

Yeast carboxylase was purified and the enzymatic reaction carried out in tritiated water. The enzyme then was precipitated with acid and the coenzyme, thiamin pyrophosphate, liberated from it. Carrier coenzyme was added, and the tritiated water removed in vacuum and replaced with ordinary water. This process was repeated several times, under experimental conditions where control experiments showed that no exchange of hydrogen takes place. Finally the coenzyme was cleaved with bisulfite.[5,6]



(4)

The resulting sulfonic acid was collected and burned to water. The water showed essentially no excess tritium. It then was concluded that the ylid II is not involved in the enzymatic process. Presumably, the enzymatic and model experiments do occur by the same mechanism; at the least, the mechanisms are alike in excluding the ylid II as an intermediate.

Experimental

Carboxylase.—The isolation procedure of Green, Herbert and Subrahmanyan[7] failed in our hands. Although a suspension of Anheuser–Busch yeast showed good activity, the extracts contained only small amounts of enzyme. The following method gave good results. A suspension of Carling ale yeast[8] was centrifuged for 15 minutes at 0–5° at 4000 r.p.m. in an International refrigerated centrifuge. The yeast cells were washed three times with distilled water. A "Vibromischer" was useful in resuspending the cells. Each kilogram of the resulting yeast cake (18% solids) was spooned into a mixture of a liter of ether and 1.5 kg. of Dry Ice. The frozen yeast was separated from the ether–Dry Ice mixture, and most of the ether was blown off. The frozen yeast could be stored at −20° without rapid loss of activity.

In a typical preparation, an 800-g. sample of frozen yeast was melted and transferred, with the aid of 200 cc. of distilled water, to 300-ml. plastic bottles. The mixture was centrifuged at 4000 r.p.m. for 20 minutes at 0°, and 530 cc. of supernatant was obtained. To this solution, 4 g. of protamine sulfate in 50 cc. of water was added, in order to precipitate the nucleic acids. Centrifugation yielded 19 g. of wet residue, which was discarded; the supernatant liquid contained 7500 units of carboxylase or 52 units per g. of (dry) yeast used. The activity ratio, according to Green's definition,[7] was 11–15, where the activity ratio is defined as the absorbance in a 2-cm. cell at 280 mμ, divided by the number of units present. The ratio of optical density at 280 mμ to that at 260 mμ was 0.54–0.56.

The crude enzyme was precipitated by adding 40 g. of ammonium sulfate per 100 cc. of solution. The protein was deposited as a poorly packed thin paste by centrifuging the suspension for 30 minutes at 4200 r.p.m. An upper layer of white scum was removed and the protein layer then transferred to 50-cc. plastic tubes and centrifugated at 10,000 r.p.m. for 30 minutes. The crude carboxylase weighed 30 g. wet and contained about 13% protein and 29% ammonium sulfate; the total activity was 4,000–5,000 units. The protein was dissolved in 0.04 *F* citrate buffer (*p*H 6) to a total volume of 40–50 cc. and fractionated with ammonium sulfate; the fractions collected were soluble in 15% ammonium sulfate (plus the buffer) and insoluble in 30% ammonium sulfate. The various samples of carboxylase were separated by centrifugation at 10,000 r.p.m. for about 40 minutes. They were then dried *in vacuo* over P_2O_5 at room temperature. The best fractions had an enzymatic activity of about 0.37 unit per mg. and contained about 0.60×10^{-9} mole of thiamin pyrophosphate per mg., of which only 2.3% was free; the rest was bound to the enzyme.

Carboxylase Assay.—The assay method, essentially that of Green, Herbert and Subrahmanyan,[7] was conducted at *p*H 6.1 and gave consistent results up to a carbon dioxide yield of about 15 micromoles in 3 minutes.

Thiamin Assay.—The thiamin was oxidized to thiochrome with alkaline ferricyanide, and the thiochrome extracted into isobutyl alcohol and then determined by its fluorescence.[9] The official method gave yields of fluorescing material which varied somewhat with the rate of addition of the oxidant. By reversing the procedure and adding the thiamin solution to the oxidant, maximal amounts of thiochrome were produced, and the analyses were reproducible to ±2%. The fluorescence was measured either with a Coleman or with a Photovolt fluorometer.

Hydrolysis of thiamin pyrophosphate samples to thiamin could be accomplished either with Polidase-S (Schwartz Laboratories) or with Takadiastase (Parke–Davis). To a solution containing 15–75 γ thiamin pyrophosphate in 1–2 cc. of an acetate or citrate buffer of *p*H 3.5–5.5 was added 0.2–1.0 cc. of 10% Polidase-S solution (previously centrifuged to remove insoluble residue). The mixture was then incubated at *p*H 3.1 for an hour. It was possible to obtain considerable hydrolysis at a *p*H of 3.1, but below *p*H 2.7 the hydrolysis is slow. The resulting solution was diluted to give an assay solution containing 0.3–0.6 γ thiamin per cc.

Thiamin.—Thiamin hydrochloride (Merck) was dried over P_2O_5. *Anal.* Calcd. for $C_{12}H_{18}ON_4SCl_2$: C, 42.75; H, 5.38; N, 16.61. Found: C, 43.40; H, 5.88; N, 16.30.

Thiamin Pyrophosphate.—Several samples of thiamin pyrophosphate showed on biochemical analysis only a minor fraction of the coenzyme. Presumably the samples were mixtures of the mono-, di- and triphosphate, so that the analysis is approximately correct, but the actual content of pyrophosphate is low. Sigma thiamin pyrophosphate and Mann lot No. 604 proved excellent, and other samples were compared to them, using the infrared spectrum as a criterion of purity. For the results here discussed, the purity of the thiamin pyrophosphate is not so important, because all the possible compounds cleave with bisulfite to the same sulfonic acid, and even the samples which showed, on infrared analysis, only a small fraction of pyrophosphate gave a good yield of cleavage product. The various impure samples of pyrophosphate therefore served as well as the pure material as "carrier" for radioactive material.

Exchange Experiments.—In a typical experiment, 8.85 g. of tritiated water containing 26×10^{-3} curie of tritium was placed in a 250-cc. round-bottom flask, and a citrate buffer consisting of 87 mg. of sodium citrate (5.5 H_2O) and 53 mg. of citric acid–H_2O was added. In the experiments with pyruvate, 205 mg. of the sodium salt then was added. In runs with ordinary water, the *p*H measured 4.57. A solution of 572 mg. of carboxylase in 1.46 g. of 0.1 *F p*H 6 citrate buffer was made up by weight. The carboxylase solution was added to the tritiated water at about 20°; the resulting mixture had a *p*H of 4.76 (measured using ordinary water). Within 25–30 seconds of the addition of the enzyme, 120–130 microliters (70 if sodium pyruvate was absent) of 36 *N* sulfuric acid was added, so that the *p*H fell to less than 2, and the protein of the enzyme was precipitated. The tritiated water was removed by lyophilization (about 4 hours required) and an aliquot of the distillate was set aside for tritium analysis; the rest was purified with alkaline perman-

(6) R. R. Williams, R. E. Waterman, J. C. Keresztesy and E. R. Buchman, This Journal, **57**, 536 (1935).

(7) D. E. Green, D. Herbert and V. Subrahmanyan, *J. Biol. Chem.*, **138**, 327 (1941); T. P. Singer, in "Methods in Enzymology," Vol. I, Colowick and Kaplan, eds., Academic Press, Inc., New York, N. Y., 1955, p. 460.

(8) We are indebted to the Carling Brewing Co., Natick, Mass., and to Mr. Edward McCann for generous samples of yeast.

(9) Official Methods of Analysis of the Association of Official Agricultural Chemists, 8th ed., 1955, AOAC, Washington, p. 819; *Analyst*, **76**, 127 (1951).

TABLE I

RADIOACTIVITY IN 5-(2-METHYL-4-AMINOPYRIMIDINE)-METHANESULFONIC ACID

Reaction Medium									
Carboxylase, mg.	TPP,[a] moles × 10^9 in the carboxylase	Sodium pyruvate, moles × 10^3	TPP[a], added, moles × 10^6	TOH, 10^3 c. per equiv. of H in reaction	T[c] in TPP, calcd for exchange of 1 H, c. × 10^9	TPP[a,d] carrier, moles × 10^6	Sulfonic acid assayed, moles × 10^6	Calcd.[g] for exchange of 1 H	Found, c × 10^9
..	..	..	..	..	....	..	151	0.0	0.3
..	..	..	..	..	....	..	110	0.0	0.3
355	..	..	272	11.4	3130	..	37[f]	330	1.8
550	..	1.93	289	12.9	3730	..	52[f]	540	4.0
355	605	..	..	28.4	17.2	199	69[e]	2.1	0.2
490	622	..	..	26.8	16.7	128	65[e]	3.4	0.1
538	685	1.89	..	25.4	17.4	199	100[e]	3.0	0.4
521	662	1.86	..	23.0	15.2	284	230[f]	4.8	1.3
448	748	1.91	..	17.1	12.8	196	115[e]	3.0	0.6

[a] TPP in thiamin pyrophosphate. [b] Carrier added to entire solution before removing tritiated water. [c] Tritium. [d] Carrier added to a 40% aliquot of the solution after removing tritiated water. [e] Recrystallized. [f] Not recrystallized. [g] Estimated error about 15%.

ganate for reuse. The residue was extracted with three approximately 4-cc. portions of distilled water and filtered through a sintered glass funnel, with the aid of diatomaceous earth (Celite) directly into a 100-cc. round-bottom flask. The filtrate was lyophilized. Then about 10 cc. more water was added to the residue, and this was removed by lyophilization. The distillate from this lyophilization was analyzed for tritium. It was expected to contain 5×10^{-7} curie if only 50 mg. of water had remained after each lyophilization; in fact it contained 80×10^{-7} curie. In the later runs the dilution and lyophilization was repeated once more.

The residue was diluted to 10 cc. and aliquots taken. A 1-cc. aliquot was assayed for thiamin pyrophosphate. The analysis of a particular sample showed 660×10^{-9} mole, whereas calculation based on the amount of enzyme used gave 625×10^{-9} mole. A 4-cc. aliquot was lyophilized to dryness, 131 mg. of carrier thiamin pyrophosphate added, together with 1 cc. of a 0.56 *F* sodium citrate solution plus 0.40 g. of sodium bisulfite. After the solution had stood for 2 days, 46 mg. (84% of the theoretical) of the pyrimidinesulfonic acid was obtained by filtration.

Tritium Assay.—The samples of tritiated water were generally assayed by scintillation counting by the New England Nuclear Corporation. The minute quantities of tritium (of the order of 10^{-9} curie) which could be anticipated in the pyrimidinesulfonic acid are very difficult to assay with accuracy. The work was carried out by Dr. Kenneth Wilzbach with the coöperation of the Argonne National Laboratories.

Control Experiments.—Our previous work[5] showed that hydrogen is not exchanged in or out of the interannular methylene bridge of thiamin or of the sulfonic acid during the cleavage process, or at mildly acid *p*H. However, the present experiments were conducted at strongly acid *p*H, and it remained to be ascertained whether these experimental conditions were such as to allow for exchange or not. In a typical control experiment, 448 mg. of thiamin pyrophosphate (Sigma) was dissolved in 5 cc. of D_2O plus enough sulfuric acid to lower the *p*H to 0.89. After the solution had stood at room temperature for 24 hours, 5 cc. of a solution of 250 mg. of $NaDCO_3$ in D_2O and 2.65 g. of $NaDSO_3$ were added. After 24 hours, the precipitated sulfonic acid was collected, washed with 1 cc. of D_2O and placed in an evacuated desiccator overnight. The acid then was recrystallized from hot D_2O. The acid was converted to the sodium salt with $NaDCO_3$ in D_2O, and the sodium salt dissolved in cold H_2O, and recovered by vacuum evaporation of the water. This latter process was repeated. Previous experiments[5] had shown that this process involved no exchange at the methylene group of the sulfonic acid. The sodium salt was then burned. Similar experiments were conducted near *p*H 2. The experiment at *p*H 0.89 showed about 0.04 atom of D per molecule; that conducted at *p*H 2, about 0.013 atom of D per molecule. Although these numbers are above the lower limit which can be detected, they are sufficiently small to indicate that, had exchange occurred in the enzymatic reaction, the tritium would have been retained during the work-up, and would have been detected.

Results

The results of a series of experiments are recorded in Table I. The first two experiments consist of samples of ordinary pyrimidinesulfonic acid, and show the level of radioactivity which is obtained in "cold" samples. The next two show samples where the thiamin pyrophosphate carrier was added to the tritiated water (the sulfuric acid was added before the carboxylase in order to inactivate it, and then the carrier was added). The purpose of this experiment was to determine whether non-enzymatic exchange would occur. An experiment using tritiated carrier (labeled in the 2-position of the thiazolium ring) showed that relatively little exchange occurred at this position in the isolation procedure. Furthermore, the controls carried out in D_2O (see Experimental) show that, if tritium had been introduced into the thiamine pyrophosphate, it would not subsequently be lost during the "work-up" in ordinary water. The figures in the next to last column show the amount of radioactivity which would have been found in the samples of pyrimidinesulfonic acid if one tritium atom per molecule had been introduced into the methylene group of the interannular bridge (or elsewhere in the pyrimidine portion of the molecule). The low values found, relative to those projected, show that such introduction of tritium did not take place to an appreciable extent. The next samples were those carried through the experimental procedure already outlined. The enzyme was allowed to react at *p*H 4.6 for about 30 seconds, then denatured with acid, and the tritiated water was removed. Since the sample was in acid almost all the time, and only briefly near *p*H 5, the opportunity for uncatalyzed (*i.e.*, non-enzymatic) exchange of hydrogen was minimized. The sample was assayed for thiamin pyrophosphate (as shown in column 2 of the table), and then carrier thiamin pyrophosphate was added to the solution in ordinary water at *p*H 2. Part of the solution then was used to prepare the sulfonic acid by the method described. The radioactivity which was anticipated for the exchange of one hydrogen atom per molecule, and that found, are shown in the last two columns of Table I. In general, the radioactivity is small and not much greater than that found for "cold" samples. It seems quite

probable, on the basis of these facts, that the enzymatic reaction resembles that in the model system in that the hydrogen atoms on the methylene bridge are inert.

The data, although in support of this statement, are on the edge of experimental precision and therefore leave something to be desired. The excellent analyses for tritium, performed at the Argonne National Laboratory, made it possible to carry through this work, but the total amount of radioactivity (*ca.* 10^{-9} curie) cannot be determined accurately. A further difficulty must be considered; this is the possibility of an adverse isotopic fractionation, which would effectively obscure the results here shown. However, the turnover number of the carboxylase used was about 0.7 per second at the *p*H of 4.6. In 30 seconds, the average enzyme molecule should turn over about 20 pyruvate molecules. If the coenzyme is ionized each time a pyruvate molecule reacts, then the coenzyme should have reached equilibrium with the solvent; this is the postulate on which the values in the next to last column of Table I have been calculated. If the enzyme were to ionize the coenzyme only once, and the ionized coenzyme were then converted to its non-ionic form only on acidification, then an adverse isotope effect could obscure the results. This possibility, although it must be considered, appears remote.

Additional experiments were performed to test the hypothesis that one tritium atom is introduced into the thiazole portion of the coenzyme, in accordance with Breslow's mechanism.[2] The experimental conditions were so chosen that the non-enzymatic exchange of hydrogen and tritium occurs too slowly to obscure the exchange. The results of these experiments, unfortunately, were not clear-cut. However, the crude results indicate that the enzyme probably does induce hydrogen–tritium exchange in the coenzyme molecule.

Acknowledgment.—The authors take pleasure in acknowledging the coöperation of Dr. Kenneth Wilzbach, of the Argonne National Laboratories, who carried out the tritium analyses essential to this work. They wish also to acknowledge the help of Mr. Keelin Fry with the deuterium control experiments, and the generous financial support of the National Institutes of Health.

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[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING DIVISION OF CORNELL UNIVERSITY MEDICAL COLLEGE]

Thiation of Nucleosides. II. Synthesis of 5-Methyl-2′-deoxycytidine and Related Pyrimidine Nucleosides[1]

BY JACK J. FOX, DINA VAN PRAAG, IRIS WEMPEN, IRIS L. DOERR, LORETTA CHEONG, JOSEPH E. KNOLL, MAXWELL L. EIDINOFF, AARON BENDICH AND GEORGE BOSWORTH BROWN

RECEIVED AUGUST 1, 1958

Uracil or thymine nucleosides were converted into a series of analogs in which the oxygen atom at position 4 has been replaced by other functional groups. This procedure involves thiation of suitably-protected nucleosides and has led to a facile synthesis of the hitherto-rare, naturally-occurring nucleoside, 5-methyl-2′-deoxycytidine, from thymidine. Similarly, 5-methylcytidine and cytidine were prepared from 1-β-D-ribofuranosylthymine and uridine, respectively. The 4-thio intermediates of blocked nucleosides served as excellent chemical precursors for the synthesis of a host of 4-substituted derivatives of 1-β-D-ribofuranosyl-, 2′-deoxyribofuranosyl-, -xylofuranosyl-, and -glucopyranosyl-pyrimidine nucleosides such as the 4-alkylamino, 4-hydrazino, 4-hydroxylamino, 4-thio, 4-azido (or tetrazolo) and other analogs. A comparison of the spectrally-determined pK_a values of nucleosides with and without a 5-methyl substituent is given and the ultraviolet absorption spectra of the more important nucleosides at different *p*H values are described. 1-Methyl-4-thiouracil was synthesized from 1-methyluracil and converted to 1-methylcytosine. 4-Hydrazino-2(1H)-pyrimidinone was prepared from 4-ethoxy-2(1H)-pyrimidinone and 1,5-dimethylcytosine was synthesized from 1,5-dimethyl-4-ethoxy-2(1H)-pyrimidinone.

On the basis of metabolic studies[2] it was demonstrated that in the mammal the naturally-occurring pyrimidine nucleosides (cytidine, and to a lesser extent uridine and thymidine) are extensively incorporated into the nucleic acids. These studies pointed to the desirability of developing methods for the synthesis of pyrimidine nucleosides adaptable for the incorporation of radio-isotopes and for the synthesis of pyrimidine nucleoside analogs for screening as potential chemotherapeutic agents. As a result, relatively facile synthetic routes (*via* the mercuri procedure) to cytidine, 5-methyluridine and uridine[3,4] were developed, and, indeed, doubly-labeled cytidine has since been prepared by these procedures.[5]

Chemical conversion of uracil or thymine moieties of furanosyl nucleosides to other derivatives have been reported.[6] These alterations, however, were effected at position 5 (*i.e.*, 5-halogeno, 5-amino, 5-hydroxy, *etc.*) and at position 3 (*i.e.*, 3-methyluridine) of the pyrimidine residues. Chemical alteration of cytosine moieties of nucleosides to their

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(2) See chapter on the biosynthesis of nucleic acids, G. B. Brown and P. M. Roll, in "The Nucleic Acids," Vol. II (Chargaff and Davidson, eds.), Academic Press, Inc., New York, N. Y., 1955, p. 350.

(3) J. J. Fox, N. Yung, J. Davoll and G. B. Brown, THIS JOURNAL, **78**, 2117 (1956).

(4) J. J. Fox, N. Yung, I. Wempen and I. L. Doerr, *ibid.*, **79**, 5060 (1957).

(5) J. F. Codington, R. Fecher, R. Y. Thomson, M. H. Maguire and G. B. Brown, *ibid.*, **80**, 5164 (1958).

(6) (a) P. A. Levene and F. B. LaForge, *Ber.*, **45**, 615, 616 (1912); (b) M. Roberts and D. W. Visser, THIS JOURNAL, **74**, 668 (1952); (c) D. W. Visser, G. Barron and R. Beltz, *ibid.*, **75**, 2017 (1953); (d) T. K. Fukuhara and D. W. Visser, *ibid.*, **77**, 2393 (1955); (e) T. K. Fukuhara and D. W. Visser, *J. Biol. Chem.*, **190**, 95 (1951); (f) P. A. Levene and W. A. Jacobs, *Ber.*, **43**, 3150 (1910).