Reactions were carried out in sealed degassed tubes placed in thermostats and irradiated to complete reaction as previously. Very dilute solutions at 0° were irradiated for 6 hr. by a 140-watt ultraviolet lamp at a distance of 6 in. Shorter times and weaker light were used in other systems. Reaction times were somewhat arbitrary since the disappearance of hypochlorite color could not be seen in dilute solutions. However, all tubes were checked for complete reaction by reaction with iodide on opening. Gas phase reactions were carried out by attaching a small tube containing a 9:1 mole ratio of cyclohexane and *t*-butyl hypochlorite and a 265-ml. bulb equipped with a side-arm to a vacuum manifold, degassing the system, and allowing it to equilibrate at room temperature. The bulb was then isolated by closing an intervening stopcock and sealed off after freezing out the contents in the side-arm. After irradiation in a thermostat, the contents were again frozen out, sealed off, and held for analysis.

Experiments with CuCl-*t*-butyl perbenzoate and di-*t*-butyl peroxylate were carried out in the same manner as the hypochlorite runs except that no irradiation was required.

Analyses were carried out by g.l.c. using an Aerograph Model 600 Hy-Fi equipped with a flame ionization detector. Columns were 10-ft. lengths of $^1/_8$ in. tubing packed with 60/80 firebrick containing 15% Carbowax 20M, 20% diethylene glycol succinate (DEGS), 20% neopentyl glycol succinate (NPGS), or 20% Ucon polar, depending on the system to be analyzed. Peak areas were determined from the product of heights and width at half-height, and were related to compositions by calibration against known mixtures of acetone–alcohol (or cyclohexyl chloride) in the same solvents.

In the isomer distribution experiments, peak areas were taken as proportional to concentration. Material balances were determined in some systems by using a small amount of benzene as internal standard and again calibrating against known mixtures. Analyses were in duplicate or triplicate and agreement was within 3%, usually within 2%. Ratios of rate constants and Arrhenius parameters were calculated in the usual manner and standard deviations are indicated in the tables. No correction was made in calculations for the small change in cyclohexane concentration during reaction since measured k_a/k_d ratios were shown to be independent of cyclohexane:hypochlorite ratios from 2:1 to 20:1.

Kosower *Z*-values were not available for a number of our solvents, and were determined from the visible absorption spectra of N-ethyl-4-carbomethoxypyridinium iodide solutions in them.[20] In solvents for which no value is given, the salt was too insoluble for measurement.

Thermal expansion of solvents was determined in a sealed 1-ml. pipet graduated in hundredths, the volume of each solvent being measured two or three times at each experimental temperature. Actual volume changes over the 100° range amounted to 8–15%. Since k_a/k_d ratios obtained from plots such as Fig. 1 have the dimensions of l. mole^{-1} they were corrected accordingly in Table II and Fig. 2.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND LAWRENCE RADIATION LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIF.]

The Biosynthesis of Nicotine in *Nicotiana glutinosa* from Carbon-14 Dioxide. Formation of the Pyrrolidine Ring[1]

BY WILLIAM L. ALWORTH,[2] ARNOLD A. LIEBMAN,[3] AND HENRY RAPOPORT

RECEIVED MARCH 28, 1964

Radioactive nicotine, isolated from the root and aerial portions of *Nicotiana glutinosa* after a 6 hr. exposure to $^{14}CO_2$, was degraded and the activity determined in the pyridine ring, the N-methyl group, and C-2′ and C-5′ of the pyrrolidine ring. The results support previous conclusions reached from $^{14}CO_2$ feedings. In addition, equal activity has been found at C-2′ and C-5′, consistent with the glutamate-symmetrical intermediate hypothesis. However, the labeling pattern required is not derivable from any of the known glutamate biosyntheses. An attempt has been made to reconcile the various data by suggesting a new glutamate biosynthesis.

Introduction

In a previous paper,[4] we presented our initial data on the biosynthesis of nicotine obtained by short-term growth of *N. glutinosa* in the presence of $^{14}CO_2$. Among a number of interesting results was the observation that C-2′ consistently contained a very low fraction of the total nicotine activity, a fact difficult to reconcile with the current hypothesis for biosynthesis of the pyrrolidine ring. In this paper, we present further data on the labeling pattern in the pyrrolidine ring and suggestions as to how these data and those obtained by other precursor feedings might be made compatible.

The current concept for formation of the pyrrolidine ring of nicotine is based on feeding experiments with ornithine-2-^{14}C,[5,6] putrescine-1,4-^{14}C,[7] and glutamic acid-2-^{14}C.[8] Activity at C-2′ in the resulting nicotine was established by oxidation to, and decarboxylation of, nicotinic acid. To determine the activity at C-5′, use was made[7,9] of the small yield of 3-nitro-5-(3′-pyridyl)pyrazole[10] isolated as a by-product from the nitric acid oxidation of nicotine or, more directly,[8,11] by oxidation of the nicotine to cotinine[12] and hydrolysis of the latter to 4-methylamino-4-(3′-pyridyl)-butyric acid, whose N-benzoyl derivative was decarboxylated by the Hofmann amide degradation. In each case, it was established that half of the incorporated activity was at C-2′ and the remainder at C-5′.

On the basis of these expeerimnts, Leete[7,13] proposed the glutamate-symmetrical intermediate hypothesis (Scheme I) for the biosynthesis of the pyrrolidine ring of nicotine. Cyclization of the δ-semialdehyde I, obtainable from glutamic acid and ornithine, gives Δ^1-pyrroline-5-carboxylic acid (II)[14] which, on decarboxyla-

(1) Sponsored in part by the United States Atomic Energy Commission.

(2) Public Health Service Predoctoral Research Fellow of the National Institute of General Medical Sciences.

(3) Public Health Service Postdoctoral Research Fellow of the National Institute of General Medical Sciences.

(4) W. L. Alworth, R. C. De Selms, and H. Rapoport, *J. Am. Chem. Soc.*, **86**, 1608 (1964).

(5) E. Leete, *Chem. Ind.* (London), 537 (1955).

(6) L. J. Dewey, R. U. Byerrum, and C. D. Ball, *Biochim. Biophys. Acta*, **18**, 141 (1955).

(7) E. Leete, *J. Am. Chem. Soc.*, **80**, 2162 (1958).

(8) B. L. Lamberts and R. U. Byerrum, *J. Biol. Chem.*, **233**, 939 (1958).

(9) E. Leete and K. J. Siegfried, *J. Am. Chem. Soc.*, **79**, 4529 (1957).

(10) The formation of this compound was first reported by G. A. C. Gough and H. King [*J. Chem. Soc.*, 2968 (1931)] who assigned to it the structure 4-nitro-5-(3′-pyridyl)pyrazole. The correct structure was suggested by H. Lund [*ibid.*, 686 (1933)] and established by synthesis [G. R. Clemo and T. Holmes, *ibid.*, 1739 (1934)].

(11) B. L. Lamberts, L. J. Dewey, and R. U. Byerrum, *Biochim. Biophys. Acta*, **33**, 22 (1959).

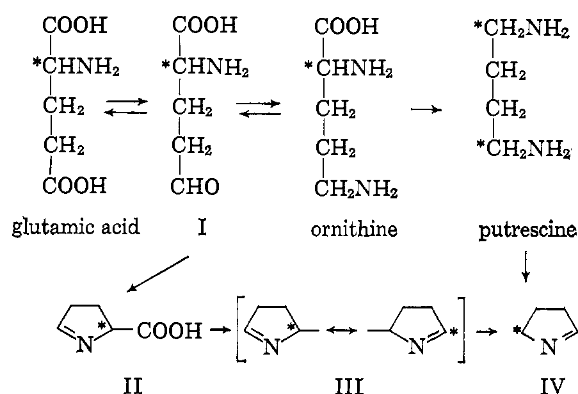
(12) A. Pinner, *Ber.*, **26**, 292 (1893).

(13) E. Leete, *J. Am. Chem. Soc.*, **78**, 3520 (1956); recently modified by E. Leete, E. G. Gros, and T. J. Gilbertson, *Tetrahedron Letters*, 587(1964).

(14) Evidence from feeding experiments with Δ^1-pyrroline-5-carboxylic acid-5-^{14}C also has been sighted as support for this hypothesis [V. Krampl and C. A. Hoppert, *Federation Proc.*, **20**, 375 (1961)]; however, 29% of the activity was found in the N–CH_3. This, plus the highly unstable nature of II [H. J. Strecker, *J. Biol. Chem.*, **235**, 2045 (1960)], weaken this evidence.

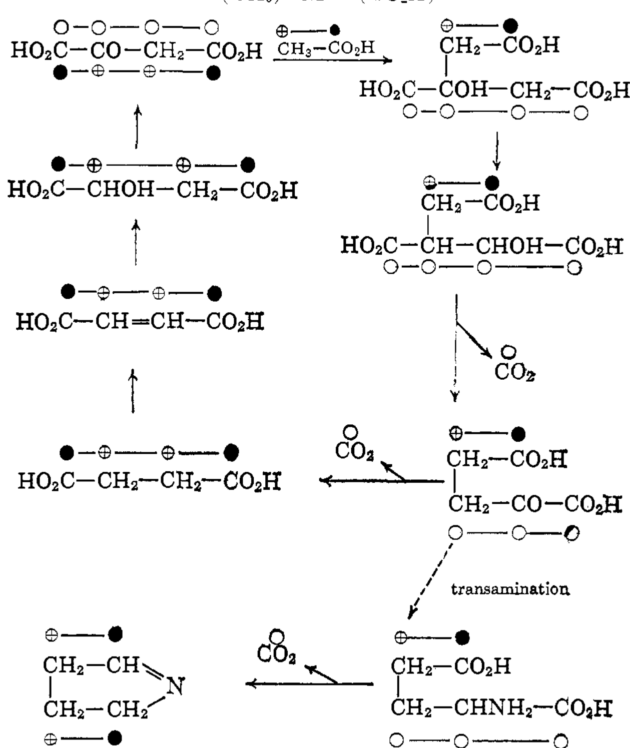
Scheme I

Glutamate Symmetrical Intermediate Pathway for Pyrrolidine Ring Biosynthesis



Scheme II

Formation of Glutamic Acid and Δ^1-Pyrroline *via* Tricarboxylic Acid Cycle. Acetate Carbons Are Indicated by ⊕ (CH_3) and ● (CO_2H)



tion, forms the symmetrical anion III. Incorporation of this anion or the resulting Δ^1-pyrroline (IV) as the pyrrolidine ring of nicotine leads to equal labeling at C-2′ and C-5′. An alternative path[7] results from decarboxylation of ornithine to the symmetrical putrescine and conversion of the latter to IV; however, under similar conditions, incorporation of putrescine was only 25% that of ornithine.

Support for pyrrolidine ring formation *via* Scheme II has been obtained by the feeding of simple 2-, 3-, and 4-carbon precursors such as acetate, glycerol, propionate, and aspartate. The labeling pattern present in the pyrrolidine ring after feeding such molecules specifically labeled with ^{14}C has been consistent with the glutamate-symmetrical intermediate hypothesis. Furthermore, the results have been interpreted to indicate formation of the glutamic acid by a transamination of α-ketoglutaric acid formed by operation of the tricarboxylic acid cycle as indicated in Scheme II. The formation of glutamic acid through such a pathway has been established in animals and certain bacteria.[15]

From the diagram, it can be seen that acetate-1-^{14}C would result in activity only at positions 2 and 5 of the pyrrolidine ring. Even continued recycling of the acetate-1-^{14}C within the tricarboxylic acid cycle would not lead to randomization of the label, since the ^{14}C incorporated into the carboxyl groups of oxaloacetic acid during one cycle is lost as $^{14}CO_2$ in the subsequent cycles leading to Δ^1-pyrroline. When acetate-1-^{14}C was incorporated by intact *N. rustica* over a 168-hr. period, C-2′ and C-5′ were found[16] to contain 50% each of the activity in the pyrrolidine ring. Feeding[17] acetate-1-^{14}C to excised *N. tabacum* root cultures over 4 weeks led to 51% of the activity of the pyrrolidine ring at C-2′. In addition, those precursors which would be expected to be metabolized to acetate-1-^{14}C, such as glycerol-2-^{14}C, pyruvate-2-^{14}C, and propionate-3-^{14}C, also formed active nicotine in which 40–50% of the activity of the pyrrolidine ring was present at C-2′,[16,17] consistent with prediction.

The labeling pattern predicted by the hypothesis of Scheme II for acetate-2-^{14}C incorporation is more complex. Glutamic acid formed from acetate-2-^{14}C during the first cycle should be labeled only in C-4, resulting in a Δ^1-pyrroline intermediate with no activity at C-2. During subsequent cycles, however, activity from acetate-2-^{14}C enters other positions of glutamic acid *via* oxaloacetic acid-2,3-^{14}C.[18] The Δ^1-pyrroline formed from α-ketoglutarate during the second cycle should have 12.5% of the activity at C-2. After *n* cycles, 16.5% of the label within the pyrroline ring from acetate-2-^{14}C should be found at C-2. When incorporation of acetate-2-^{14}C into nicotine was examined[16] as a function of time, 18, 20, 22, 10, and 14% of the pyrrolidine label was found at C-2′, after 1, 2, 3, 6, and 168 hr., respectively. Propionate-2-^{14}C and glycerol-1,3-^{14}C, which would be expected to be metabolized to acetate-2-^{14}C, also were found[16] to result in 14% of the pyrrolidine label being located at C-2′ after 168 hr. The incorporation of acetate-2-^{14}C into C-2′ of nicotine as a function of time does not appear to be consistent with the hypothesis of Scheme II, and no explanation was offered for this discrepancy.[19]

Incorporation of acetate-2-^{14}C by root cultures gave[17] nicotine with 11% of the pyrrolidine activity at C-2′. Precursors of acetate-2-^{14}C such as glycerol-1-^{14}C and alanine-3-^{14}C gave similar results. The 4-carbon intermediates such as fumarate-2-^{14}C, succinate-2-^{14}C, and aspartate-2,3-^{14}C were incorporated into nicotine by root cultures with about 25% of the pyrrolidine activity at C-2′, as would be expected if these compounds were incorporated into glutamic acid through the tricarboxylic acid cycle.

For the precursors discussed above, except for acetate-2-^{14}C, all incorporations have been consistent with

(15) M. R. Stetten in W. D. McElroy and H. B. Glass, "Amino Acid Metabolism," Johns Hopkins University Press, Baltimore, Md., 1955, p. 277; H. J. Vogel, *J. Biol. Chem.*, p. 335.

(16) P. L. Wu, T. Griffith, and R. U. Byerrum, *ibid.*, **237**, 887 (1962).

(17) D. R. Christman and R. F. Dawson, *Biochemistry*, **2**, 182 (1963).

(18) S. Aronoff, "Techniques of Radiobiochemistry," Iowa State University Press, Ames, Iowa, 1956, p. 91.

(19) If a sufficient experimental error is invoked, so that the values were actually 16 ± 6%, the pattern would become consistent with the predicted i ncorporation after several turns of the cycle.

the formation of a symmetrical pyrrolidine precursor arising from glutamic acid derived from an intermediate of the tricarboxylic acid cycle. However, certain precursors[17] gave labeling patterns that cannot be explained by this scheme. From Scheme 2, it can be seen that succinate-1-^{14}C and citrate-1-^{14}C should give labeling patterns identical with acetate-1-^{14}C; that is, 50% at C-2′. Actually, from succinate-1-^{14}C, only 15.1% of the pyrrolidine ring activity was located at C-2′, and in the case of citrate-1-^{14}C, only 3% of the activity was located at C-2′. Glucose-6-^{14}C was found to give active nicotine with 19% of the pyrrolidine ring activity located at C-2′, but glucose-1-^{14}C gave active nicotine with only 4% of the pyrrolidine activity at C-2′. Since, *via* the Embden–Meyerhof glycolytic pathway, both glucose-1-^{14}C and -6-^{14}C should form acetate-2-^{14}C, the variation in the labeling pattern from these hexoses also is not clear.

Speculation as to the mode of incorporation of the four compounds described above would be meaningless without a full knowledge of the labeling patterns of the nicotine produced. However, it is important to note that in the case of one of the more fundamental substrates, glucose, and in the only case in which the incorporation has been examined carefully as a function of time, acetate-2-^{14}C, the reported labeling patterns are not consistent with the formation of a pyrrolidine ring from glutamate that had been derived from an intermediate of the tricarboxylic acid cycle.

Labeling Patterns from $^{14}CO_2$.—Previous[4] $^{14}CO_2$ biosynthesis experiments with *N. glutinosa* revealed a consistently low order of activity at C-2′ of nicotine, *viz.*, 0.5–2.5% of the total, the variation being dependent on the time of exposure and the portion, root or aerial, of the plant. If the glutamate-symmetrical intermediate hypothesis is invoked, an equal percentage of the total nicotine activity would be assigned to C-5′. The balance of the pyrrolidine ring activity must then residue equally in C-3′ and C-4′, conferring significantly greater activity on this pair than on C-2′ and C-5′. This scheme requires glutamate with higher specific activity in the methylene carbons, a requirement contrary to current ideas of glutamate biosynthesis. Various explanations for this dilemma are possible; *e.g.*, there is more than one biosynthetic pathway for the pyrrolidine ring of nicotine, an as yet undiscovered biosynthesis of glutamate is involved, or the glutamate-symmetrical intermediate hypothesis does not apply to $^{14}CO_2$ biosynthesis. We have sought to test the last alternative by a direct determination of the specific activity of C-2′ and C-5′ of nicotine isolated from a $^{14}CO_2$ biosynthesis.

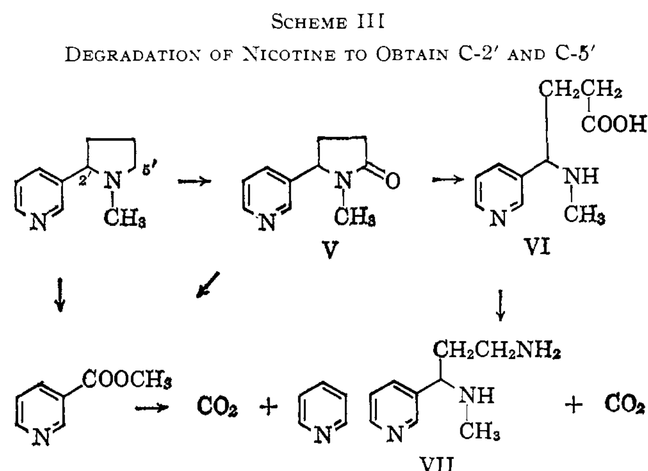
Since a 6-hr. exposure had produced the maximum incorporation of ^{14}C into C-2′, this experiment was selected for repetition. The initial $^{14}CO_2$ feedings had been carried out either in the absence of additional $^{12}CO_2$, or $^{12}CO_2$ was added to reconstitute normal concentration (0.03%) after 1.5 hr. of exposure, during which period 95% of the $^{14}CO_2$ had been absorbed. In the present experiment, in order to maintain conditions as close as possible to normal, the CO_2 concentration was kept at 0.03% throughout the entire run.

The degradation paths are shown in Scheme III.

The procedure for determining the specific activity at C-2′ is essentially the same as previously reported.[4]

SCHEME III

DEGRADATION OF NICOTINE TO OBTAIN C-2′ AND C-5′



However, since low activities and small differences were anticipated, a number of improvements were made (see Experimental) which increased the accuracy and reproducibility of the procedure. In particular, it was necessary to avoid any source of CO_2 other than the carboxyl carbon of methyl nicotinate. Atmospheric carbon dioxide or calcium carbonate in the calcium oxide would lead to dilution with inactive CO_2 and a lowered specific activity for C-2′. This was avoided by preheating the calcium oxide to 1,000° and by using a thorough prior nitrogen sweep of the apparatus and scrupulously CO_2-free reagents. In this way, the blank was kept to 0.5 mg. of barium carbonate, in comparision to an actual decarboxylation which yielded about 25 mg. Another extraneous source of CO_2 is decomposition of the pyridine ring. In several trial runs, when pyridine was heated with calcium oxide, about 7% of the pyridine carbon was recovered as barium carbonate. This is not a direct correlation with the actual decarboxylation process, but does indicate a small, unavoidable contamination. Since the pyridine nucleus in these decarboxylations is quite active, it probably contributes CO_2 of higher specific activity than the low-activity carboxyl group. Thus, the C-2′ specific activity reported probably represents a maximum value.

The degradation sequence to obtain C-5′ began with the oxidation of nicotine to cotinine (V)[12] and hydrolysis of cotinine to 4-methylamino-4-(3′-pyridyl)-butyric acid (VI).[20] Previously decarboxylation of the butyric acid was effected by Hofmann amide degradation of the N-benzoyl derivative. In an attempt to simplify this step, we subjected the N-tosyl derivative to the Schmidt reaction, but the yield of carbon dioxide was only 15%. However, when the amino acid itself was directly treated with hydrazoic acid, a 60% yield of carbon dioxide was obtained. That the reaction was taking its normal course was established by isolation of the decarboxylated product, 3-amino-1-methylamino-1-(3′-pyridyl)propane (VII), as its bisphenylthiourea derivative.

The N–CH_3 group was obtained by the usual Herzig–Meyer analysis of nicotine and conversion of the liberated methyl iodide to methyltriethylammonium iodide.

The final dilution of the aerial nicotine represented a somewhat low order of radioactivity. Therefore, to

(20) H. McKennis, Jr., L. B. Turnbull, E. R. Bowman, and E. Wada, *J. Am. Chem. Soc.*, **81**, 3951 (1959); H. McKennis, Jr., L. B. Turnbull, H. N. Wingfield, Jr., and L. J. Dewey, *ibid.*, **80**, 1634 (1958).

Table I
Percentage of Total Activity in Various Portions of Nicotine

Nicotine sample	Methyl nicotinate	Pyridine	C-2′	C-5′	N–CH_3
Root, sample 1	79.3	79.8	2.3	1.7	2.0
sample 2	80.6	80.8	1.7	2.0	2.2
Aerial	75.5	72.5	2.0	1.3	1.8

Table II
Labeling Patterns in Nicotine from a 6-Hr. $^{14}CO_2$-Biosynthesis[a]

Plant portion	Pyridine ring[c]	Pyrrolidine ring[d]	C-2′ + C-5′	C-3′ + C-4′	(C-3′ + C-4′)/(C-2′ + C-5′)
Root[b]	79.1	18.8	3.8	15.0	4.0
Aerial	73.0	25.2	3.3	21.9	6.6

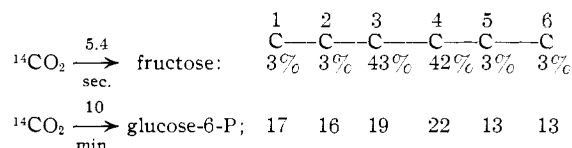
[a] Values are given as percentages of total nicotine activity. [b] Average of root samples 1 and 2. [c] Average of the pyridine value and the methyl nicotinate minus C-2′. [d] Determined by subtracting pyridine and N–CH_3 values from 100%.

ensure that accurate specific activity analyses of the 2′- and 5′-carbon atoms were being obtained, an additional purification step was inserted by using cotinine (V) for the reference compound. The cotinine was prepared as described above and purified by gas–liquid chromatography. Cotinine was found to react with potassium permanganate in a manner similar to nicotine and to give a comparable yield of methyl nicotinate. The specific activities found for the various portions of the nicotine molecule are presented in Tables I and II as percentages of the total nicotine activity.

A number of conclusions may be drawn from the data of the present experiment. First, the method of feeding carbon dioxide appears to have no influence on the labeling pattern in nicotine. Feedings have now been carried out by maintaining normal carbon dioxide concentration, and previously[4] by allowing the carbon dioxide concentration to fall to one-tenth its normal value, with and without replenishment. In each case, the labeling pattern was essentially the same. Second, the previous conclusion of independent nicotine synthesis in the root and aerial portions, based in part on different labeling patterns, is supported by the present results, although a biosynthesis as long as 6 hr. is far from optimum for observing this difference.

The most significant conclusion concerns the activities at the various pyrrolidine ring carbons. Although there are slight differences between C-2′ and C-5′, these differences are within experimental error, and the data are consistent with, and in fact confirm, the equal label found at C-2′ and C-5′ by other precursor feedings. However, we also see that the activity of C-3′ and C-4′ is much greater[21] than that at C-2′ and C-5′. Thus, the glutamate-symmetrical intermediate hypothesis is substantiated only if a glutamate biosynthetic pathway can be found leading to such a labeling pattern. Therefore, we have evaluated known glutamate biosyntheses to determine if any exists by which $^{14}CO_2$-fixation forms a glutamate in which carbons 3 and 4 would contain significantly more activity than carbons 2 and 5.

Glutamate Biosyntheses. Reconciliation with $^{14}CO_2$ Labeling Patterns.—In our experiments, $^{14}CO_2$ represents the original source of radioactive carbon. From the carbon reduction cycle, we can expect a hexose with the label:[22,23]



By Embden–Meyerhof glycolysis, carbons 3 and 4 become carbon 1 (carboxyl) of pyruvate which is subsequently lost during decarboxylation to form acetate. Carbons 1 and 6 of the hexose become carbon 2 of acetate and carbons 2 and 5 become carbon 1 (carboxyl) of acetate. Therefore, the acetate formed under the conditions of our experiment should be equally labeled. From operation of the tricarboxylic acid cycle (Scheme 2), it is seen that acetate equally labeled in the 1- and 2-positions would form a Δ^1-pyrroline having equal labeling in positions 2, 3, 4, and 5.

If oxaloacetic acid is formed by the combination of $^{14}CO_2$ and a three-carbon unit related to pyruvate,[24] the carboxyl carbons should contain the majority of the label. This is the case, since the pyruvate would have the labeling pattern indicated above. Scheme 2 shows that when such a four-carbon intermediate enters the tricarboxylic acid cycle, both the carboxyl groups are lost in the formation of Δ^1-pyrroline. This possibility, therefore, degenerates to the previous case where the four pyrroline carbons are derived from acetate.

Recently,[25] a glutamate biosynthesis beginning with the condensation of glyoxylic acid and oxaloacetic acid has been reported in *Acetobacter suboxydans*. Carbons 4 and 5 of the resulting glutamic acid are represented by carbons 2 and 1, respectively, of glyoxylic acid. Also, glycolic acid formed in *Nicotiana* leaves after 15 min. exposure to $^{14}CO_2$ has been found to be equally labeled, and a high concentration of glycolic acid oxidase is present in *Nicotiana* leaves.[26] Therefore, the glyoxylic acid formed in *Nicotiana* after exposure to $^{14}CO_2$ should be equally labeled. Applying this information to the *A. suboxydans* glutamate biosynthesis, we see that carbons 3 and 4 of the resulting glutamic acid will contain more activity than carbons 2 and 5 only if carbon 3 is more active than carbon 2. However, carbons 2 and 3 are derived from carbons 2 and 3 of oxaloacetic acid and are, therefore, equally labeled (Scheme II).

Another possible glutamate biosynthesis would be the reversal of glutamate fermentation by *Clostridium tetunomorphum*[27] or the so-called citramalic pathway.[25] Here, acetate condenses with pyruvic acid to form citramalic acid. This, in turn, is converted to β-methylaspartic acid which rearranges to give glutamic acid. The specificity of this enzymatic rearrangement is such that carbon 1 (carboxyl) of pyruvate becomes carbon 5 of glutamate. The remaining carbons, being equivalent to acetate carbons, should be equally labeled, each having activity less than carbon 5. Therefore, this pathway would result in glutamic acid

(21) Equality of C-3′ and C-4′ has been assumed. However, this equality is the ultimate test for a symmetrical intermediate, and a degradation of the pyrrolidine ring which will establish those carbons directly is now in progress. Since a higher activity will be present at these sites, greater radiochemical accuracy will be possible.

(22) J. A. Bassham and M. Calvin, "The Path of Carbon in Photosynthesis," Prentice-Hall, Inc., New York, N. Y., 1957, p. 39.

(23) E. A. Havir and M. Gibbs, *J. Biol. Chem.*, **238**, 3183 (1963).

(24) C. R. Benedict and R. W. Rinne, *Biochem. Biophys. Res. Commun.*, **14**, 474 (1964).

(25) Y. Sekizawa, *et al.*, *Chem. Eng. News*, **41**, No. 27, 46 (1963).

(26) I. Zelitch, *J. Biol. Chem.*, **234**, 3077 (1959).

(27) H. A. Barker, "Bacterial Fermentations," John Wiley and Sons, Inc., New York, N. Y., 1956.

TABLE III[a]
DERIVATION OF GLUTAMATE CARBONS

Pathway	Carbon atoms C-1	C-2	C-3	C-4	C-5
Tricarboxylic acid cycle	CO_2[b]	m[c]	c[d]	m	c
Glyoxylate condensation	CO_2	m	c	gly 2	gly 1
Citramalic pathway	c	m	m	c	CO_2
C. kluyveri	c	m	c	m	CO_2
R. rubrum	CO_2	c	m	m	c

[a] Data presented in this table taken from footnotes 28, 29, and that presented by S. R. Elsden, *Federation Proc.*, **21**, 1047 (1962). [b] CO_2 = carbon readily derived from CO_2 fixation such as pyruvate C-1 or oxaloacetate C-1 and C-4. [c] *m* = acetate C-2. [d] *c* = acetate C-1.

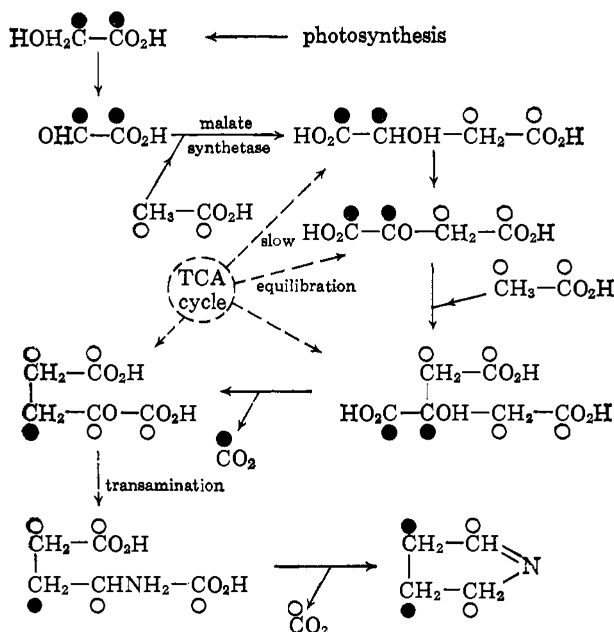
with greater activity at carbon atoms 2 and 5 than at 3 and 4, a pattern opposite to that found.

Two other glutamate biosyntheses are indicated by the labeling patterns of glutamic acid formed by *Rhodospirillum rubrum*[28] and *Clostridium kluyveri*.[29] The pathways to glutamate in these microorganisms are as yet unknown; therefore, we have simply reproduced the indicated labeling patterns (Table III).

Assuming that the glyoxylic acid formed in *Nicotiana glutinosa* as a result of $^{14}CO_2$ fixation is equally labeled and that the acetic acid formed in *Nicotiana glutinosa* as a result of $^{14}CO_2$ fixation is equally labeled, the above table clearly shows that none of the five known glutamate biosyntheses will give a labeling pattern such that carbon atoms 3 and 4 will contain more activity than carbon atoms 2 and 5. It can only be concluded that if glutamate is the precursor of the pyrrolidine ring of nicotine, this glutamate must be formed by an as yet undetermined biosynthetic pathway.

A hypothetical but plausible glutamate pathway consistent with our data and involving known enzyme systems is proposed in Scheme IV.

SCHEME IV
HYPOTHETICAL GLUTAMIC ACID BIOSYNTHETIC PATHWAY OUTSIDE THE TCA CYCLE



One of the initial products of photosynthesis, glycolic acid, has been shown[26] to contain up to 50% of the early photosynthetically fixed carbon in tobacco leaves (determined in the presence of a glycolic acid oxidase inhibitor). The glycolic acid was equally labeled and the large amount of glycolic acid oxidase, present in the green leaves, would normally result in formation of glyoxylic acid. If highly labeled glyoxylic acid thus derived from photosynthetic fixation of $^{14}CO_2$ enters the glyoxalate cycle,[30] the malic acid formed by the action of malate synthetase has the labeling pattern indicated in Scheme IV. If this malic acid were oxidized to oxaloacetic acid, condensed with acetic acid, and subsequently converted to α-ketoglutaric acid by enzymatic reactions analogous to, but isolated from, the tricarboxylic acid cycle, the α-ketoglutarate would be labeled only in position 3. Subsequent conversion to glutamic acid and Δ^1-pyrroline provides a pyrrolidine ring precursor having all of the label in the 3- and 4-positions. Since $^{14}CO_2$ feeding should label acetate, but to a lesser extent than glyoxylate, some activity would appear at positions 2 and 5, giving a pattern consistent with our experimental observations for short-time exposure.[31]

It is readily seen that acetate-1-^{14}C entering such a pathway would show 50% of the incorporated label at C-2′ of nicotine. Acetate-2-^{14}C entering this pathway would lead to a pyrrolidine ring in which 25% of the label is located in C-2′. Previous experiments in which acetate-1-^{14}C was fed[16] showed that 50% of the incorporated label was located in C-2′. Also, incorporation of acetate-2-^{14}C after 1-, 2-, and 3-hr. exposures were 18, 20, and 22%, respectively, into C-2′. The labeling pattern resulting from this early incorporation of acetate-2-^{14}C is quite consistent with the above pathway and inconsistent with incorporation through the tricarboxylic acid cycle. As exposure time increases, it is reasonable to assume that intermediates of the tricarboxylic acid cycle would equilibrate with intermediates of the above pathway. This would account for the fact that acetate-2-^{14}C after 168 hr. of exposure was found to provide only 14% of the incorporated label at C-2′, and for the fact that intermediates of the tricarboxylic acid cycle have been shown to function as precursors of the pyrrolidine ring.[16,17]

Experimental

Counting Technique.—All scintillation counting was performed with a Nuclear–Chicago 720 series liquid scintillation instrument. The scintillator solutions used were toluene solutions of 2,5-diphenyloxazole and 1,4-bis-2-(5-phenyloxazoyl)benzene (both Arapahoe scintillation grade). Samples to be counted were dissolved in 95% ethanol, *p*-dioxane was added to ensure a clear scintillation solution (about 1 ml.), and finally 10 ml. of the scintillator solution was added. Background under these conditions was found to be 26 to 28 c.p.m. Counting efficiency was determined in each case by addition of internal toluene-^{14}C standard and varied from 73 to 75%, except in the case of the CO_2 determination according to the method of Woeller,[32] where it varied from 65 to 69%. In all cases, samples were counted for a sufficient time such that the 0.9 statistical error was within ±2% of the average counting rate.[33] Determinations were made on weighed samples and on aliquots of solutions whose concentrations were ascertained by ultraviolet absorption. All values are given in d.p.m./mmole unless otherwise stated.

(28) D. S. Hoare, *Biochem. J.*, **87**, 284 (1963).
(29) N. Tomlinson, *J. Biol. Chem.*, **209**, 605 (1954).
(30) H. L. Kornberg and H. A. Krebs, *Nature*, **179**, 988 (1957).
(31) One 7-day feeding experiment with glycolic acid-2-^{14}C has been reported in which 95% of the nicotine activity resided in the N–CH_3. However, because of the long time involved, this experiment may not be pertinent to the present suggestion and probably reflects a totally different mode of glycolic acid incorporation [R. U. Byerrum, L. J. Dewey, R. L. Hamill, and C. D. Ball, *J. Biol. Chem.*, **219**, 345 (1956)].
(32) F. H. Woeller, *Anal. Biochem.*, **2**, 508 (1961).
(33) Footnote 18, p. 208.

Biosynthesis.—*Nicotiana glutinosa* plants were grown hydroponically, exposed to $^{14}CO_2$, and the nicotine was extracted and purified as previously described in detail.[4] For these experiments, XI in the series, 8 plants were exposed to 76 mc. of $^{14}CO_2$ for a total of 6 hr. The plants were 2 months old and had been growing in nutrient solution for 5 weeks. They were 28–30 cm. high and had 10–11 large leaves. As determined by sacrificing two other plants of the same batch, the aerial portion of each plant weighed 45–50 g. and the roots weighed 8–10 g. In contrast to previous runs I–X, $^{12}CO_2$ was added as soon as the infrared analyzer indicated the CO_2 level in the biosynthesis chamber was falling below the normal air concentration. This occurred 5 min. after the chamber had been sealed and the generated $^{14}CO_2$ fed into the chamber. A flow of $^{12}CO_2$ of 4–5 ml./min. was necessary to maintain the normal concentration. Under these conditions, uptake of $^{14}CO_2$ was rapid, about 90% being taken up during the first 2 hr. After 5 hr., ^{14}C could no longer be detected in the atmosphere of the chamber by the vibrating reed electrometer. The total plant material was frozen in liquid N_2 within 20 min. of the end of the indicated 6-hr. exposure period.

Determination of Nicotine Specific Activity.—The specific activity of the g.l.c. purified nicotine was determined by g.l.c.-proportional counting as described previously.[4] Nicotine isolated from the root portions had a specific activity of 7.43 μc./mmole and that from the aerial portions, 0.42 μc./mmole. As determined by g.l.c., the aerial portions yielded 3.1 times as much nicotine as did the root portions; therefore, the root portions contained 5.8 times as much total nicotine-^{14}C activity as did the aerial portions. These values resemble closely those found previously for $^{14}CO_2$ incorporation into nicotine under slightly different conditions, lying between those obtained in run VIII (4-hr. exposure to $^{14}CO_2$, $^{12}CO_2$ added after 1.5 hr.) and run IX (6-hr. exposure to $^{14}CO_2$, $^{12}CO_2$ added after 1.5 hr.).[4]

Dilution of Nicotine for Degradation and Establishment of Radiochemical Purity.—The purified nicotine was diluted with inactive nicotine and this diluted radioactive nicotine (1.5–3 g.) utilized for all the degradations. Paper chromatography[4] of the diluted nicotine revealed only trace amounts of impurities. Owing to the low specific activities involved, it was impractical to determine the radiochemical purity of this nicotine by radioautography. Therefore, a sample of the diluted aerial nicotine was subjected to further g.l.c. (10 ft. × 3/8 in. column, 10% polybutylene glycol on KOH-treated 60/80 firebrick, 238°, and 200 ml. He/min., R_t 10.5 min.), the nicotine collected, and subjected to yet another g.l.c. purification (10 ft. × 3/8 in. column, 15% SF-96 on hexamethyldisilazane-treated 60/80 Chromosorb W, 220°, and 200 ml. He/min., R_t 6.75 min.). The specific activity of the nicotine was not changed by either of these additional purification steps. Furthermore, the specific activity of cotinine (see below) formed from the nicotine isolated after this final g.l.c. purification was equal to that of the nicotine. This sequence established the fact that nicotine purified by two g.l.c. fractionations as described previously[4] is radiochemically pure.

Determination of Activity in C-2′. A. Conversion of Nicotine to Methyl Nicotinate.—This conversion was carried out as previously described,[4] except for the following improvements. (a) After filtration of the manganese dioxide, the filtrate was adjusted to pH 10.5 and extracted continuously with methylene chloride until no more ultraviolet-absorbing material (~5%) was removed. (b) After reflux, the esterification solution was cooled, made alkaline (pH 8.5) with sodium bicarbonate, concentrated *in vacuo*, and extracted continuously with methylene chloride after adding 100 ml. of water to the residue; g.l.c. of the methyl nicotinate (5 ft. × 0.25 in. column, 15% SF-96 on hexamethyldisilazane-treated 60/80 Chromosorb W, 165°, and 80 ml. He/min., R_t 1 min. 27 sec.) showed only one peak.

In the case of the aerial nicotine, oxidation was first carried out to cotinine (see below) and the cotinine was converted to methyl nicotinate in 60% yield exactly as described above for nicotine; specific activity of root methyl nicotinate: sample 1, 13,598; sample 2, 13,834; 79.3 and 80.6%, respectively, of the activity of original root nicotine (17,160); specific activity of aerial methyl nicotinate: 2276, 75.5% of the activity of original aerial cotinine (3010).

B. **Conversion of Methyl Nicotinate to Pyridine and Barium Carbonate.**—Methyl nicotinate was hydrolyzed and decarboxylated as previously described[4] except for the following improvements; (a) 0.6 ml. of water was used per g. of calcium oxide, and (b) heating to effect decarboxylation was carried out for 20 min. at 400°. The pyridine was swept into 7 ml. of isopropyl alcohol and 0.5 ml. of 60% perchloric acid. This solution was concentrated to 3 ml., isopropyl alcohol was added, and the concentration repeated until the water was removed and the **pyridine perchlorate** crystallized. Recrystallization to constant specific activity gave material of m.p. 288–294° (reported[34] m.p. 288°).

Anal. Calcd. for $C_5H_6ClNO_4$: C, 33.4; H, 3.4; N, 7.8. Found: C, 33.2; H, 3.5; N, 7.8.

Carbon 2′, remaining as calcium carbonate, was recovered and counted exactly as previously described.[4]

Root portion specific activities: pyridine, sample 1, 13,699; sample 2, 13,866 (79.8 and 80.8% of nicotine); C-2′, sample 1, 284; sample 2, 395 (1.7 and 2.3% of nicotine); aerial portion specific activities: pyridine, 2179 (72.5% of cotinine); C-2′, 59.5 (2% of cotinine).

Determination of Activity in C-5′. A. Oxidation of Nicotine to Cotinine (V).—Nicotine (1 ml.) was converted *via* dibromocotinine perbromide[12] into 600 mg. of crude cotinine.[20] This material was purified by preparative g.l.c. (10 ft. × 3/8 in. column, 15% SF-96 on hexamethyldisilazane-treated 60/80 Chromosorb W, 240°, and 200 ml. He/min., R_t 15 min.), passing the emerging gas stream through a U-tube cooled in liquid N_2. The collected cotinine, which crystallized in the collecting tube, was removed with chloroform and the chloroform evaporated to leave 400 mg. of pure cotinine, m.p. 44–49° (reported[12] m.p. 50°); ultraviolet absorption, λ_{max}^{EtOH} 263 mμ (ε 3090); thin layer chromatography on silica gel using methanol for development gave a single spot (gold), R_f 0.59, when sprayed with 5% ethanolic *p*-aminobenzoic acid and then exposed to BrCN vapor.

Anal. Calcd. for $C_{10}H_{12}N_2O$: C, 68.2; H, 6.9; N, 15.9. Found: C, 68.1; H, 6.8; N, 15.8.

B. **Hydrolysis of Cotinine to 4-Methylamino-4-(3′-pyridyl)-butyric Acid (VI).**—Cotinine (purified by g.l.c.) was hydrolyzed and the products isolated according to the procedure of McKennis, *et al.*[20] About 25% of the cotinine was recovered (pure by t.l.c. and g.l.c.) and the 4-methylamino-4-(3′-pyridyl)butyric acid (~50% yield) was crystallized from methanol and air-dried; m.p. 127° (reported[20] m.p. 133°); ultraviolet absorption, λ_{max}^{EtOH} 259 mμ, $\lambda_{max}^{KOH-EtOH}$ 261 mμ (ε 2740); t.l.c. on silica gel (1:1:4, 0.5 *N* NH_4OH-EtOH-*n*-BuOH) gave a single spot (orange) with *p*-aminobenzoic acid–BrCN, R_f 0.1.

Anal. Calcd. for $C_{10}H_{14}N_2O_2 \cdot H_2O$: C, 56.6; H, 7.6; N, 13.2. Found: C, 56.9; H, 7.2; N, 13.1.

The N-*p*-toluenesulfonyl derivative was prepared by shaking an ethereal solution of *p*-toluenesulfonyl chloride with an aq. sodium carbonate solution of 4-methylamino-4-(3′-pyridyl)butyric acid. Adjusting the aqueous phase to pH 6 with hydrochloric acid precipitated the N-*p*-toluenesulfonyl derivative which was crystallized from ethanol; m.p. 214–216°.

Anal. Calcd. for $C_{17}H_{20}N_2O_4S$: C, 58.6; H, 5.6; N, 8.0; S, 9.2. Found: C, 58.7; H, 5.6; N, 7.7; S, 8.7.

C. **Decarboxylation of 4-Methylamino-4-(3′-pyridyl)butyric Acid (VI).**—4-Methylamino-4-(3′-pyridyl)butyric acid (65–100 mg.) was added to 5 ml. of concentrated H_2SO_4 at room temperature and a slow N_2 stream was bubbled through the stirred solution, a U-tube trap, and two traps containing 0.1 *N* NaOH; 1 ml. of 1.4 *N* HN_3 in chloroform was added and the temperature raised immediately to 55° and then to 70° over a 1-hr. period, an additional 1 ml. of NH_3 solution added, and the temperature held at 70° for 2 hr. A final 1 ml. of HN_3 solution was added and the reaction mixture heated at 70° for 1 additional hr. At the end of this period, saturated $BaCl_2$ solution was added to the NaOH traps and the precipitated $BaCO_3$ was centrifuged and washed with two portions of freshly boiled water and one portion of ethanol. After being dried 12 hr. *in vacuo* at 80°, the $BaCO_3$ activity was determined. The yield of $BaCO_3$ was from 31–36 mg. Using the precautions described above to exclude extraneous CO_2, less than 0.5 mg. of $BaCO_3$ was obtained in a blank experiment. Scintillation counting gave the following activities: root, sample 1, 296; sample 2, 336; aerial, 40.1. Based on root nicotine (17,160), 1.7–2.0% of the activity of the nicotine from the root is located at C-5′. Based upon the aerial cotinine (3010), which was used as starting compound for both C-2′, (see above) and C-5′ determinations, 1.3% of the activity of the nicotine from the aerial portion is located at C-5′.

From the sulfuric acid solution, the **3-amino-1-methylamino-1-(3′-pyridyl)propane (VII)** was recovered by continuous extraction with ether after dilution and alkalization with sodium hydroxide.

(34) F. Arndt and P. Nachtwey, *Ber.*, **59**, 448 (1926).

Evaporation of the ether and treatment of the residue in methanol with phenyl isothiocyanate gave the bisphenylthiourea derivative, m.p. 108–111° after crystallization from benzene.

Anal. Calcd. for $C_{23}H_{25}N_5S_2$: C, 63.5; H, 5.8; N, 16.1; S, 14.7. Found: C, 63.7; H, 6.2; N, 15.9; S, 14.4.

N-CH_3 Determination.—Nicotine (50 µl.) was subjected to a standard Herzig–Meyer determination and the CH_3I generated was collected in 500 µl. of dry toluene cooled in a Dry Ice–acetone trap. Triethylamine (2 ml. of a 5% solution in absolute ethanol) was added and the mixture was allowed to stand at room temperature for 24 hr. Evaporation of the solvent *in vacuo*, crystallization of the residue two times from 1–1.5 ml. of isopropyl alcohol, and drying at 50° (10 µ) for 12 hr. yielded 28 mg. of pure methyltriethylammonium iodide, m.p. 297° dec.

Anal. Calcd. for $C_7H_{18}NI$: C, 34.5; H, 7.5; N, 5.8. Found: C, 34.7; H, 7.4; N, 5.8.

From scintillation counting of weighed samples of the methyltriethylammonium iodide, the following activities were obtained: root, sample 1, 336; sample 2, 370; aerial, 54. The 95% ethanol and *p*-dioxane were allowed to stand over sodium bisulfite for 24 hr. before being used to dissolve these samples. Under these conditions, the scintillation solutions remained colorless during the entire counting period and gave counting efficiencies of 72–74% with samples as large as 14 mg. Since the root nicotine had an activity of 17,160 and the aerial nicotine had an activity of 3010, the methyltriethylammonium iodide activities show that 2.0–2.2% of the activity of the root nicotine and 1.8% of the activity of the aerial nicotine was present in the N-CH_3.

[CONTRIBUTION FROM INSTITUTE OF GENETICS, UNIVERSITY OF COLOGNE, GERMANY]

Ultraviolet-Induced Dimerization of Free and Metal-Complexed Orotic Acid

BY A. HAUG

RECEIVED FEBRUARY 21, 1964

A red shift of the π–π^* absorption band, arising from the interaction of orotic acid with paramagnetic and diamagnetic ions, is studied. With all ions used 1:1 complexes are formed. The behavior against ultraviolet radiation, as measured by dimerization of orotic acid, is somewhat different with the paramagnetic Ni^{++} compared to the diamagnetic Zn^{++}. The orotic acid–Ni^{++} (1:1) complex is stable toward intense irradiation with ultraviolet light in contrast to the corresponding Zn^{++} complex. The quantum yield (measured in nitrogen) of the photochemical decomposition of the Zn^{++} complex is comparable with the quantum yield when irradiating uncomplexed orotic acid in oxygen atmosphere

Introduction

The extent to which orotic acid (6-carboxy-2,4-dioxypyrimidine) is photochemically decomposed by irradiation with ultraviolet light depends distinctly upon the environmental medium. Beukers, *et al.*,[1] reported that the percentage of orotic acid, sensitive to the light of a germicidal lamp, increases from 14 to 37% when bubbling oxygen or nitrogen, respectively, through the solution. Further, orotic acid, first irradiated in a nitrogen atmosphere, is partially reconstituted on subsequent irradiation in oxygen. Replacing oxygen by paramagnetic ions, 10^{-3} *M* solution of Ni^{++} or Cu^{++} in 0.2 *M* HCl, brought about the same partial recovery of orotic acid. The authors concluded that paramagnetic molecules or ions partially inhibit the dimerization of orotic acid in solution. They further believed that the paramagnetic substances change the photochemical equilibrium between orotic acid and its dimer.

However, recent work of Johns, *et al.*,[2] studying the dimerization of thymine upon irradiation with monochromatic ultraviolet light, indicates that the photochemical equilibrium between thymine and its dimer is strongly dependent on the wave length. At 2350 Å. the equilibrium mixture is over 90% thymine, at 2750 Å. it is over 80% thymine dimer.

Tsubomura and Mulliken[3] investigated spectrophotometrically the extra absorption caused by oxygen bubbling into organic solvents like ethanol, dioxane, or benzene. They found that new absorption bands occur as a result of charge-transfer interaction between oxygen as an electron acceptor and the organic solvent as an electron donor. Theoretically they demonstrated that this electron donor–acceptor interaction may also produce the enhancement of the singlet–triplet absorption bands by oxygen.

Having in mind these two effects mentioned above (wave length dependence of the photochemical equilibrium and the enhancement of transition probabilities of the singlet–triplet transitions) the present research was carried out in the hope of explaining more clearly the mechanism of dimerization of orotic acid.

Experimental

Materials.—Orotic acid was purchased from the Aldrich Chemical Co., Milwaukee, Wis. It was purified by recrystallizing it from water. All other chemicals used ($NiSO_4 \cdot 6H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $Ni(CHO_2)_2 \cdot 2H_2O$, $Hg(C_2H_3O_2)_2$ were obtained from E. Merck, Darmstadt, or Th. Schuchardt, Munich, respectively. Compressed nitrogen and oxygen gas was purchased from the Linde Co.

Doubly distilled water from a quartz column was stored in polyethylene bottles. Solutions of orotic acid were freshly prepared immediately prior to use.

Apparatus.—Irradiation experiments were carried out with a high flux ultraviolet monochromator, first constructed at the California Institute of Technology, copied and further improved at our Institute. The samples were irradiated at room temperature in a Teflon stoppered Beckman cuvette (1 cm. depth, 3 ml. vol.). Nitrogen or oxygen, respectively, was passed in through a small glass tube fixed in an aperture of the stopper and out through a second small gap. Before flushing the filled Beckman cell with nitrogen, the gas was bubbled through a stock solution of orotic acid for several hours. The nitrogen was deoxygenated in an alkaline pyrogallol solution and subsequently passed through a column of distilled water to prevent evaporation loss in the Beckman cell. During irradiation, stirring of the solution was achieved by a constant flow of nitrogen or oxygen, respectively, into the upper part of the cuvette, which was not exposed to the impinging ultraviolet beam.

Absorption spectra were measured with a Cary Model 15 or Zeiss PMQ 2 spectrophotometer; pH was measured using a pH meter 22 from Radiometer Inc., Copenhagen. Descending paper chromatography was performed on Whatman 3 mm. paper in 2-propanol–NH_3–H_2O, 7:1:2 (v./v.). The molar extinction coefficients were taken from Chargaff and Davidson.[4]

(1) R. Beukers and W. Berends, *Biochim. Biophys. Acta*, **38**, 573 (1960); R. Beukers, J. Yjlstra, and W. Berends, *Rec. trav. chim.*, **78**, 879, 883 (1959).

(2) H. E. Johns, S. A. Rapaport, and M. Delbrück, *J. Mol. Biol.*, **4**, 104 (1962).

(3) H. Tsubomura and R. S. Mulliken, *J. Am. Chem. Soc.*, **82**, 5968 (1960).

(4) E. Chargaff and J. N. Davidson, "The Nucleic Acids I," Academic Press, Inc., New York, N. Y., 1955, p. 113.