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Oxidation of Pyruvic Acid by Flavins in the Presence of Amines[1]

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Pyruvic acid or other enolizable α-keto acids in the presence of primary or cyclic secondary amines in aprotic polar solvents efficiently reduce flavins and isoalloxazines to their 1,5-dihydro derivatives. The other product of this reaction is the diamide of citraconic acid (**1**). In the absence of flavin, the diamide of methylsuccinic acid (**2**) is obtained as the major product. The specificity for α-keto acid and amine, stoichiometric requirements, rates and quantities of CO_2 evolution, and the kinetics of the reaction were studied. On the basis of these data a mechanism is proposed that involves an enamine condensation followed by decarboxylation and then either reaction with flavin to give ultimately (**1**) and dihydroflavin, or reaction with an additional amine to give (**2**). The possible import of this kind of mechanism in biochemical systems is briefly discussed.

INTRODUCTION

During a survey of organic functional groups that might be potentially redox active with flavins (or isoalloxazines) and as possible models for flavoenzyme reactions, we observed that mixtures of pyruvic acid and amines (but neither component alone) efficiently reduce flavins in polar aprotic solvents. The reaction was of an unknown nature and represented one of the few (but gradually increasing in number) non-photochemical oxidations of organic molecules by isoalloxazines or flavins (*1–3*)[4]. All the components of the reaction are potential natural reactants.

Pyruvic acid itself is not very readily oxidized with air alone, but it is oxidized by hydrogen peroxide and a variety of other oxidizing agents, including redox active metal ions. It is quite susceptible to condensation reactions, both with itself and with

[1] Paper V in the series *Flavin Redox Reactions*. Part IV is M. J. Gibian and A. L. Baumstark, *J. Org. Chem.* **36**, 1389 (1971), and Part III is Ref. *1*.

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[3] Taken in part from the Ph.D. dissertation of J. A. R., University of California, Riverside, 1971.

[4] It should be pointed out that we had reported (*1*) some peculiar behavior of 10-phenylisoalloxazine when treated with strong base as in Hamilton's experiment (*3a*). The isoalloxazine spectrum with anaerobic *t*-BuO^- alone is shifted to a λ_{max} at approximately 400 nm, as also reported in *3a*, but this solution shows 50% spins by esr. The visible spectral change is reversed back to 437 nm by air or by HCl. We picture this as a substitution and disproportionation. At any rate, the starting material when one adds methyl phenylglycinate or mandelate to the reaction mixture is not simply 10-phenylisoalloxazine.

aldehydes. Being activated by the α-carboxyl function, the carbonyl is quite reactive. There is thus a wide range of chemistry possible in our system.

In an earlier publication we described some of the studies involving flavin oxidation of compounds related to pyruvic acid (*1*), and we here report an examination of the scope and pathway of this new reaction from an organic viewpoint and explore the possible biochemical import of mechanisms such as are found here.

RESULTS

General Properties of the Reactions

In anaerobic Schlenk tubes with 10^{-4} *M* flavin or isoalloxazine in DMF, 10^{-2} *M* pyruvic acid causes an irreversible loss of the visible isoalloxazine spectrum in several days. Amines alone with isoalloxazines have virtually no effect. When ammonia or a primary or cyclic secondary amine and pyruvic acid are added together, complete flavin reduction occurs in a matter of minutes. Addition of oxygen causes immediate and quantitative return of flavin spectrum (a property of 1,5-dihydroflavin).

Preliminary experiments showed that DMF, DMSO, and acetonitrile are good solvents for the reaction while protic solvents, such as water or methanol, completely suppress it. Small amounts of water added to DMF significantly slow the rate.

TABLE 1

EFFECT OF PYRUVIC ACID DERIVATIVES AND RELATIVES[a]

Compound	Flavin reduction
CH_3COCO_2H	Rapid (<4 min)
$CH_3COCO_2C_2H_5$	No reaction
$CH_3CH(OH)CO_2H$	No reaction
$C_6H_5COCO_2H$	No reaction
$C_6H_5CH_2COCO_2H$	Reduction
$HCOCO_2H$[b]	Slow reduction
$HCOCHO$[b]	Very slow
$CH_3COCOCH_3$	No reaction
$HOCH_2CO_2H$	No reaction
HO_2CCO_2H	No reaction
CH_3CHO	No reaction
$CH_3CH(N{=}CHC_6H_5)CO_2^-Na^+$	Slow reduction
$CH_3CH_2COCO_2H$	Reduction

[a] All reactions contained 10^{-2}–10^{-1} *M* carbonyl compound, 2-fold excess benzyl or isopropyl amine (both effective with pyruvic acid), and 10^{-4} *M* 3-benzyl or 3-methyllumiflavin in DMF. Reaction was judged by a change in flavin absorption spectrum. All changes in fact were oxygen reversible flavin reduction.

[b] Glyoxylic acid and glyoxal behave differently from the other compounds in that their reactions are base catalyzed (not specifically amine). They belong in the group of flavin reductants discussed in Ref. *1*.

A survey of pyruvic acid derivatives and relatives is given in Table 1. The salient points are: (1) free carboxylate is required, (2) hydrogens α to the carbonyl are required, (3) oxalic acid and α-diketones do not react, (4) α-hydroxy acids do not react, (5) substitution of one of the hydrogens on the methyl of pyruvic acid by alkyl or phenyl is deleterious, and (6) the tautomer of the imine from pyruvate and benzylamine, that formed from alanine and benzaldehyde, reduces flavin at a significantly slower rate than a mixture of pyruvate and benzylamine.

Neither hydroxide nor cyanide causes any reduction of flavin by pyruvate. Tables 2 and 3 give approximate times for half-complete flavin reduction using various amines. In the acyclic series primary and secondary amines are effective, but the latter much less

TABLE 2

EFFECTIVENESS OF VARIOUS AMINES (3×10^{-2} *M*) WITH PYRUVIC ACID (10^{-2} *M*) FOR REDUCTION OF 3-BENZYLLUMIFLAVIN (10^{-4} *M*) IN DMF

Amine	pK_a (amine)	$t_{1/2}$ (min)
Pyrrolidine	11.3	4
n-$C_4H_9NH_2$	10.8	13
s-$C_4H_9NH_2$	10.8	20
t-$C_4H_9NH_2$	10.8	17×10^3

TABLE 3

EFFECTIVENESS OF VARIOUS AMINES (2×10^{-2} *M*) WITH PYRUVIC ACID (2.5×10^{-3} *M*) FOR REDUCTION OF 3-METHYLLUMIFLAVIN (10^{-4} *M*) IN DMF

Amine	pK_a (amine)	$t_{1/2}$(min)
Pyrrolidine	11.3	23
$(CH_3)_2CHNH_2$	10.7	300
$C_6H_5CH_2NH_2$	9.3	138
Morpholine	8.3	approx. 10^3
$(n\text{-}C_4H_9)_2NH$	10.9	$>10^4$
$C_6H_5NH_2$	4.6	$>10^5$
$(C_2H_5)_3N$	11.0	$>10^5$
Quinuclidine	11.8	$>10^5$
NH_3[a]	9.3	(approx. 3×10^3)

[a] Estimated from more dilute solution because of precipitation at these concentrations.

so. Pyrrolidine was the most reactive amine of those tried, and all tertiary amines failed to effect flavin reduction. There is no correlation with basicity of the amine but rather a parallel with nucleophilicity toward carbonyl carbon, and, for the primary amines, with the equilibrium constants for imine formation (*4*).

Some of the experiments to determine the stoichiometry of the reaction are depicted in Fig. 1. Two equivalents of pyruvic acid are required to reduce one equivalent of

flavin. At least a 2-fold molar ratio of amine to pyruvic acid was necessary (or one equivalent of amine with sodium pyruvate). One mole of amine is obviously being consumed in salt formation with the acid and may thus be replaced with any base, but the second mole must be reacting. These stoichiometry experiments were carried out at high flavin concentrations for reasons to be apparent later.

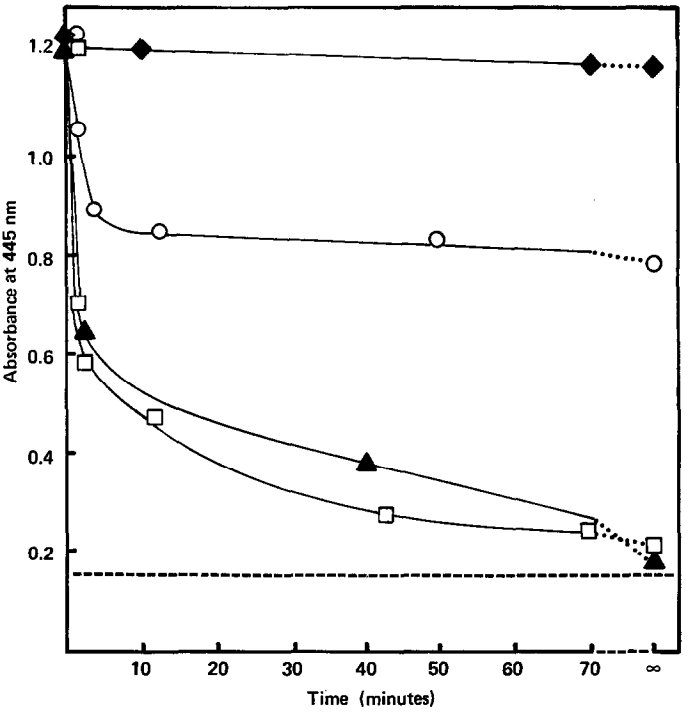


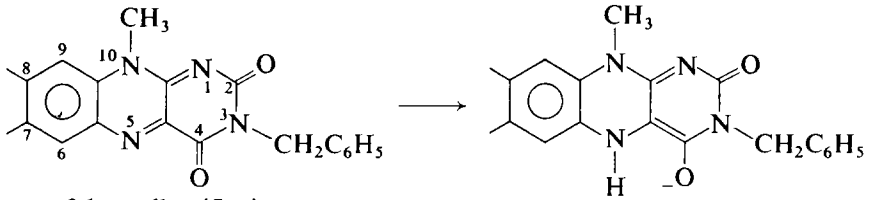
FIG. 1. Effect of pyruvate and pyrrolidine concentrations upon the degree of flavin reduction. Dashed line indicates absorbance of aliquot at complete reduction. 3-Benzyl-10-methylisoalloxazine (0.050 *M*) in DMF. Pyruvic acid and pyrrolidine, respectively: both 0.050 *M* (♦), 0.050 *M* and 0.15 *M* (○), 0.10 *M* and 0.20 *M* (▲), 0.10 *M* and 0.60 *M* (□).

Nuclear magnetic resonance experiments revealed that the methyl protons of pyruvate are rapidly lost in the presence of amine. At 0.1 *M* pyruvate and pyrrolidine or benzyl amine, all the methyl protons disappear within 5 min, but with phenylhydrazine (which forms the stable hydrazone, analogous to the imine form from an amine) the methyl protons are retained.

Products

Flavin. The visible spectrum of a solution after reaction with pyruvate and amine was virtually identical to that of a flavin solution reduced by dithionite. From other work we knew that 1,5-dihydro-3-benzyllumiflavin is insoluble in acetonitrile, and in fact when the pyruvate–amine reaction was run in this solvent a dull-orange solid precipitated. When isolated in an oxygen-free dry box and spotted on tlc plates, this material migrated (in butanol/water/acetic acid; 8/1/1) with the same R_f as authentic

1,5-dihydro derivative. When dissolved in methanol, removed from the dry box, and shaken with air for a few seconds, it then migrated with authentic 3-benzyllumiflavin. No other spots were observed. The R_f values are quite sensitive, and we judge the flavin product to be typical dihydroflavin, most likely precipitated as the ammonium salt.



3-benzyllumiflavin

F **FH⁻**

Pyruvic acid–amine. Solubility problems and the necessity of using large quantities of flavins for product studies led us to examine a range of isoalloxazines. 3-Benzyllumiflavin is quite soluble in DMF, but it is tedious to prepare. The corresponding isoalloxazine (devoid of the 7- and 8-methyl groups) retains essentially the same spectral, electrochemical, and general chemical properties, and is easily prepared in two steps. Product studies and many of the kinetic studies were often performed with isoalloxazines rather than flavins.

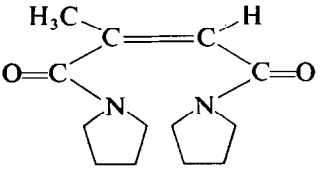
Pyruvic acid undergoes a range of fairly rapid chemical reactions with amines, so that a reaction mixture of 5×10^{-3} *M* flavin and 2-fold excess pyruvate and amine shows 6–8 separated spots on tlc. The mixture without flavin is also complex, but one distinct strong spot in the former is now missing. With benzylamine and pyruvic acid no *N*-benzylacetamide is formed, nor was there any detectable amount of acetic acid in any of the reactions.

Chromatography on silica gel of a worked-up (see Experimental) concentrated reaction mixture gave two major fractions. One contained a nonredox product (30% here), and the other an oxidation product (57% based on flavin, after purification).

The oxidation product was unique to the flavin runs, and was identical on tlc to the spot previously observed. On the basis of microanalysis, mass spectrum, nmr, ir, and hydrolysis to citraconic acid, the structure assigned was the pyrrolidinodiamide of citraconic acid (**1**). Details of spectra and chemical and physical properties are given in the Experimental section. Rational synthesis from citraconic acid and pyrrolidine produced a material identical with **1**.

The other fraction from chromatography was identical to the major product of the pyruvic acid–pyrrolidine nonflavin reaction. Analogous products from pyruvic acid and morpholine and benzylamine were also obtained in high yield by allowing acid and amine to react anaerobically in DMF or acetonitrile for several days. None of these compounds would reduce flavin with or without additional amine. Because the pyrrolidine compound was difficult to purify (mp 50–55°C, tending to oil) most of the analytical work (given in the Experimental section) was performed on the other two analogs, although confirmatory evidence for the pyrrolidine structure was also obtained. On the

basis of microanalysis, mass spec, nmr, ir, and hydrolysis to methylsuccinic acid, the structures **2** were assigned to these products.



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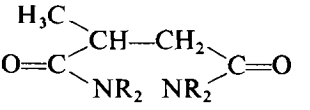
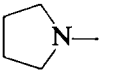
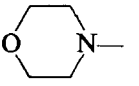


2

a: $NR_2 =$ 

b: $NR_2 =$ 

c: $NR_2 = C_6H_5CH_2NH$—

The structures of both products imply a decarboxylation of one pyruvate, and each contains two pyruvates and two amines. The oxidation product **1** is simply dehydrogenated **2**, oxidized by two electrons from starting material. The stoichiometry is thus

$$2CH_3\overset{O}{\overset{\|}{C}}CO_2^- + 2R_2NH + F \longrightarrow \mathbf{1} + CO_2 + FH_2 + 2H_2O,$$

and in the absence of flavin (and as a side-reaction in its presence)

$$2CH_3\overset{O}{\overset{\|}{C}}CO_2^- + 2R_2NH \longrightarrow \mathbf{2} + CO_2 + 2H_2O.$$

Because **2** does not reduce flavin under the reaction conditions, the actual reducing agent must precede its formation. Carbon dioxide was identified as a product in both the presence and absence of flavin when the reactions were run on a vacuum line connected to a gas infrared cell (strong 2320 cm^{-1} absorption). Subsequent gravimetric determinations of CO_2 (in conjunction with kinetic experiments described below) on a gas train using ascarite traps showed that one CO_2 is evolved per flavin reduced (or per two pyruvates reacted).

Rates of CO_2 evolution. Analysis for CO_2 was complicated by carbamate formation with excess amine (Eq. 1)

$$R_2NH + CO_2 \rightleftharpoons R_2N{-}CO_2H \overset{R_2NH}{\rightleftharpoons} R_2N{-}CO_2^- + R_2NH_2^+ \qquad (1)$$

By exhaustive sweeping with argon it was possible eventually to trap all the CO_2 on ascarite, but control experiments using known amounts of CO_2 in a DMF solution of amine showed that the liberation of CO_2 was rather slow. It was thus not possible to compare directly the rate of flavin reduction with CO_2 evolution. Experiments using concentrated solutions of reactants revealed that CO_2 could be recovered quantitatively from standard mixtures. Figure 2 shows a typical result of this kind of experiment, in

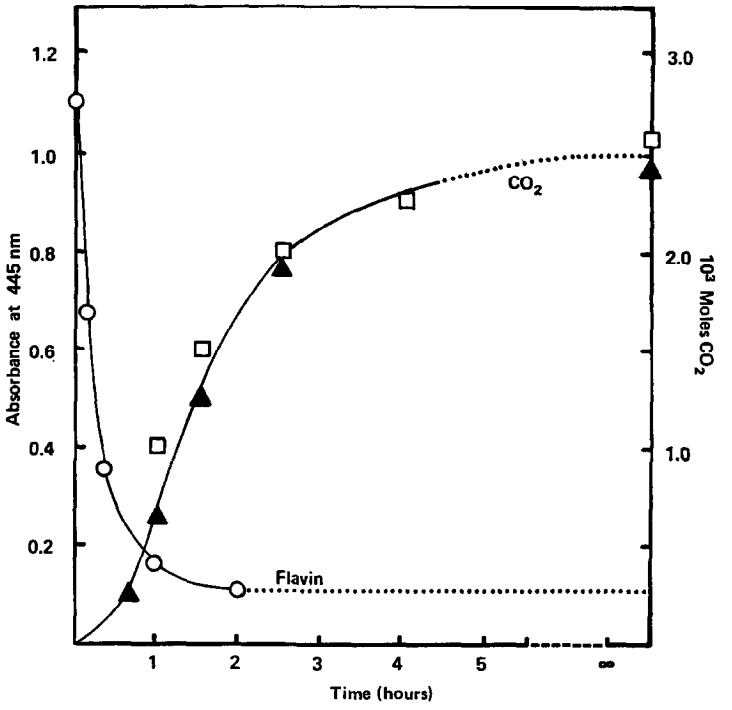


FIG. 2. CO_2 absorption from 5 mmoles pyruvic acid (0.1 *M*) and 10 mmoles pyrrolidine (0.2 *M*) in 50 ml DMF in the presence (□) and absence (▲) of 0.05 *M* 3-benzyllumiflavin and the rate of reduction of flavin as shown by absorbance of anaerobic aliquots of reaction mixture (○).

which small aliquots of reaction mixture were removed and anaerobically diluted to monitor flavin reduction while argon was still sweeping through the reaction flask and ascarite train. It is clear that the CO_2 trapping rate is the same in the presence and absence of flavin, but that it lags behind the actual production due to carbamate formation. In both cases, the ultimate stoichiometry is one CO_2 per two pyruvates. It is unlikely that CO_2 evolution is slower than flavin reduction, because with standard CO_2 solutions the trapping rate is not much faster than that in our experiments, and if CO_2 evolution were slow in the reaction we would have found a much slower absorption of it on the ascarite. We conclude that since decarboxylation is on the reaction path, it occurs in the flavin reduction step or before it.

Rate of flavin reduction. Figure 3 shows the rates of flavin reduction for various pyruvic acid and amine concentrations, and Fig. 4 those for various flavin concentrations. In this series the ratio of pyruvic acid to pyrrolidine was constant (1:2), and their

concentrations were always in significant excess of flavin. All these runs were performed by successively adding small aliquots of amine and pyruvic acid to a DMF solution of flavin. They always showed induction periods, which were shorter at higher concentrations of pyruvic acid and pyrrolidine and persist if sodium pyruvate is used rather than the free acid.

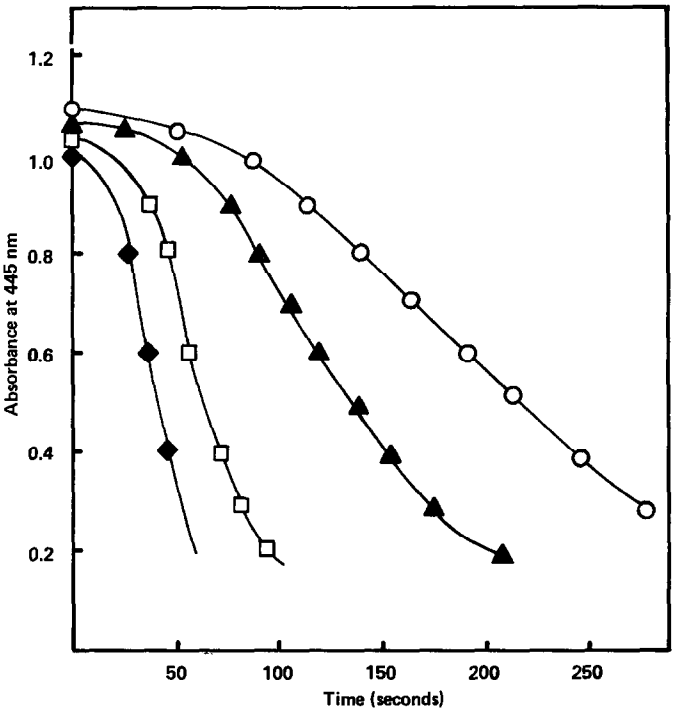


FIG. 3. Reduction of 3-benzyl-10-methylisoalloxazine (1.0×10^{-3} *M*) with pyruvic acid and pyrrolidine, respectively: 0.01 *M* and 0.02 *M* (○), 0.02 *M* and 0.04 *M* (▲), 0.04 *M* and 0.08 *M* (□), 0.08 *M* and 0.16 *M* (♦) in DMF. Each component added separately in 1-mm path cell.

Figure 5 shows the result when high concentrations of pyruvic acid and pyrrolidine were mixed externally to the reaction cell, and then added to flavin. The induction period is eliminated, and the course of reaction appears to be zero order in flavin and pseudozero-order in the other components (they were present in very large excess here).

When the flavin concentration was similar to that of the other reactants and in the ratios demanded by the stoichiometry in these premix experiments, the results were as shown in Figs. 6 and 7 for two different sets of concentrations. All data fit overall first-order plots. For the more concentrated runs (Fig. 6) with 10^{-3} *M* flavin (1-mm cell) the apparent first-order rate constant is 4.0×10^{-3} sec^{-1}. At 10^{-4} *M* flavin and a concommitant decrease in the other components (Fig. 7) the apparent rate constant is 5.1×10^{-3} sec. At 5×10^{-2} *M* flavin, 0.1 *M* pyruvic acid, and 0.2 *M* pyrrolidine, approximate half-lives of 150–200 sec can be estimated from Fig. 2, giving a first-order rate

constant of 4.6×10^{-3} sec^{-1} to 3.5×10^{-3} sec^{-1}. Over a 500-fold change in flavin concentration, the observed rate constant is thus unchanged, and we conclude that the reaction is zero order in flavin under these conditions.

The induction period in the nonpremixed runs may be attributed to chemical reaction between pyruvic acid and the amine to produce an intermediate that is the actual flavin reductant. Apparently this species is short-lived. Figures 6 and 7 show that as the premixed pyruvic acid–amine solution is allowed to incubate, reducing capability is

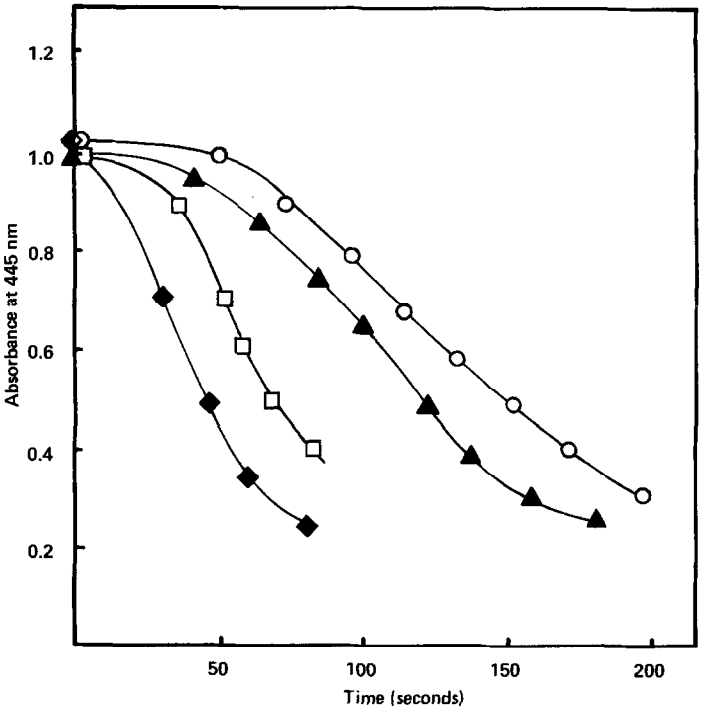


FIG. 4. Reduction of 3-benzyl-10-methylisoalloxazine by pyruvic acid (0.02 *M*) and pyrrolidine (0.04 *M*), components added separately. 10^{-3} *M*, 1-mm path cell (○); 5×10^{-4} *M*, 2-mm path cell (▲); 10^{-4} *M*, 1-cm path cell (□); 2×10^{-5} *M*, 5-cm path cell (♦) in DMF.

lost and the flavin reaction does not go to completion (recall that these solutions have exactly the concentrations demanded by the stoichiometry). At incubation times of less than 60 sec, the induction period is still evident. The runs in each of these figures are all at the same total flavin concentration, and the decreasing intercepts with time reflect the fact that flavin reduction stops before it is all used up, thus reflecting a mixture of oxidized and reduced flavin. In both sets of data the highest intercept represents runs in which 100% flavin reduction occurs.

The fact that all these runs are overall first order, and reproducibly so over a wide range of concentrations, implies that an intermediate formed from pyruvate and pyrrolidine undergoes a unimolecular reaction in the rate-determining step. An attractive possibility is a decarboxylation.

When the other two reactants are added successively to a flavin solution and if the pyruvate is not in high concentration, a complicated kinetic situation results. The entire reaction is very much slower than runs with the similar concentrations of premixed reagents in the reaction cell, and the overall kinetics are not of any simple order. Figure 8 shows the effect on the approximate half-life in this sort of experiment when the

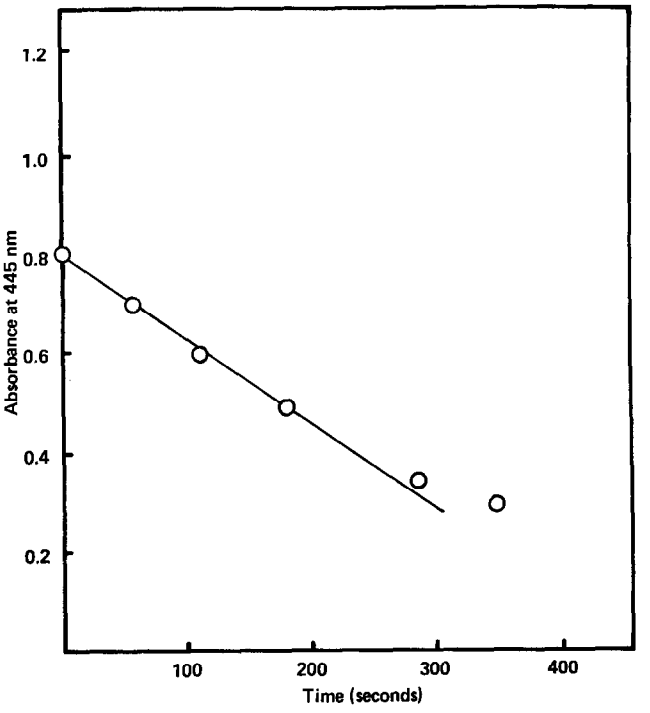


FIG. 5. Reduction of 3-benzyl-10-methylisoalloxazine (6×10^{-5} *M*) in DMF by an aliquot of a 120-sec incubated mixture of pyruvic acid (0.05 *M*) and pyrrolidine (0.10 *M*). Final concentrations of the latter two were 2×10^{-3} *M* and 4×10^{-3} *M*, respectively.

pyrrolidine concentration is regularly increased while the other components are constant (and at lower concentrations). Up to a certain level, the rates are approximately first-order in amine, and above that amine has no further effect. A similar result, but with somewhere between first- and second-order dependence, was obtained when pyruvate was varied at three different concentrations in excess of flavin and with large excess of pyrrolidine.

DISCUSSION

The following summarizes the major observations. (a) The oxidation product **1**, a decarboxylated pyruvate dimer, is formally two electrons oxidized from **2**, the major product in the absence of flavin. These products account for the stoichiometric requirements for flavin reduction and carbon dioxide formation. (b) An α-keto acid with

β hydrogens is necessary for oxidation. (c) Various amines show various degrees of effectiveness in the reaction, generally paralleling the ease of imine or enamine formation. (d) CO_2 evolution parallels flavin reduction. (e) There is an induction period involving pyruvate and amine. (f) The reaction rate is flavin independent. (g) When the pyruvate and amine are allowed to react independently so that the induction period is eliminated, the reaction is overall first order (if the reactants are present in the stoichiometric ratios).

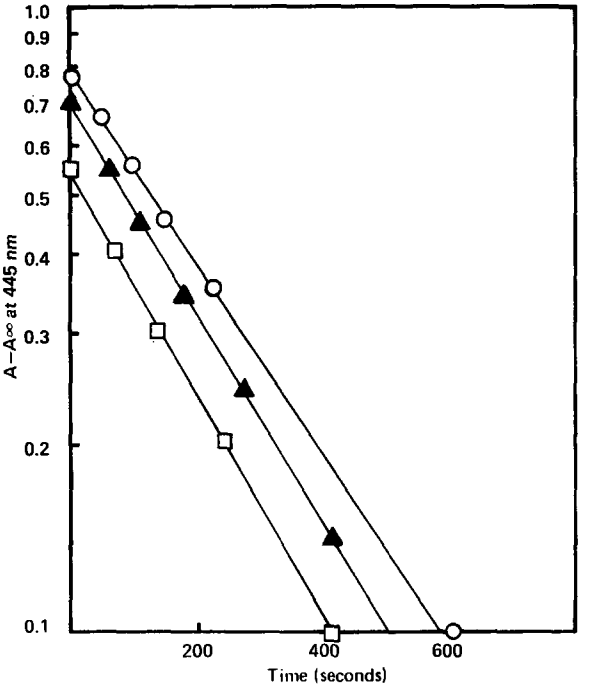


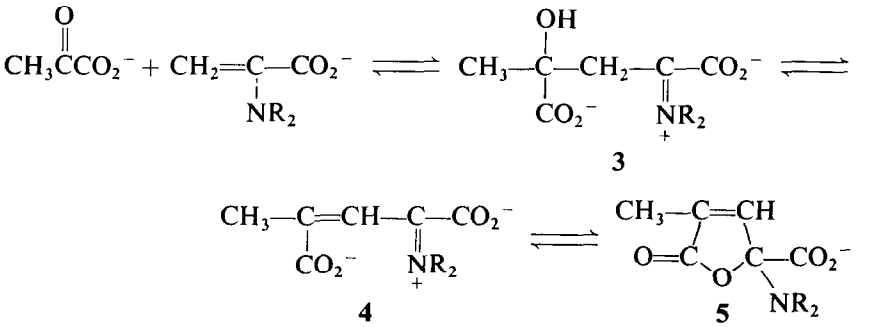
FIG. 6. Reduction of 3-benzyllumiflavin (10^{-3} *M* in 1.0 ml DMF) by 0.040-ml DMF aliquots of pyruvic acid (0.05 *M*) and pyrrolidine (0.10 *M*) after 120 sec (○), 180 sec (▲), and 300 sec (□). The resulting solution is 1:2:4 in the respective components.

The non-redox reaction. Pyruvic acid and amine certainly first undergo a very rapid proton transfer reasonably followed by amine attack at the very reactive keto function and loss of water in the usual manner to form an enamine (Eq. 2).

$$CH_3{-}\overset{\overset{O}{\|}}{C}{-}CO_2^- + R_2NH \rightleftharpoons CH_3{-}\overset{\overset{{}^+NR_2}{|}}{\underset{\|}{C}}{-}CO_2^- \rightleftharpoons CH_2{=}\underset{\underset{NR_2}{|}}{C}{-}CO_2^- \quad (2)$$

Protic solvents would reverse this reaction, explaining their strong inhibitory effect.

The next reaction, well-precedented, is condensation between enamine and pyruvate (Eq. 3), possibly also largely reversible.



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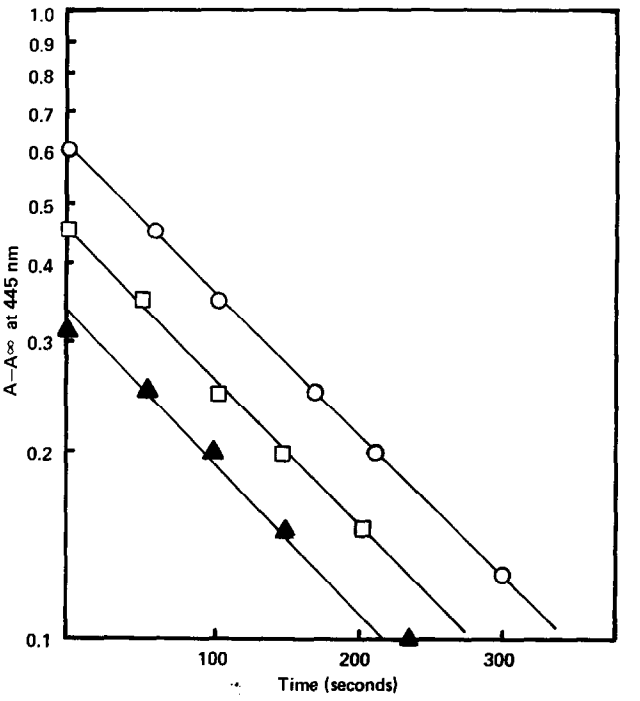


FIG. 7. Reduction of 3-benzyllumiflavin (10^{-4} *M* in 3.0 ml DMF) by DMF aliquots of incubated pyruvic acid and pyrrolidine. Respectively, 0.003-ml aliquot of 0.20 *M* and 0.40 *M* after 60 sec (○), same after 120 sec (□), 0.012-ml aliquot of 0.05 *M* and 0.10 *M* after 120 sec (▲).

The dehydration step (**3** to **4**) could be DMF catalyzed. Intermediates **4** and **5**, β,γ-unsaturated carboxylates, could undergo decarboxylation at this stage.

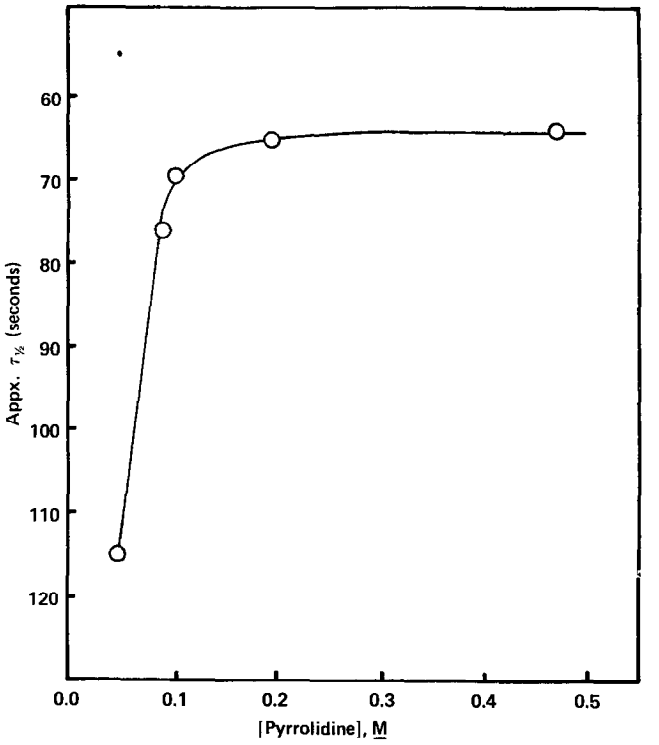
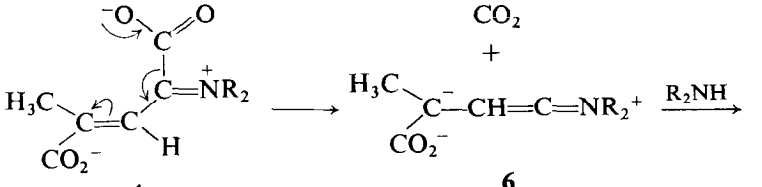
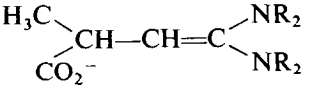


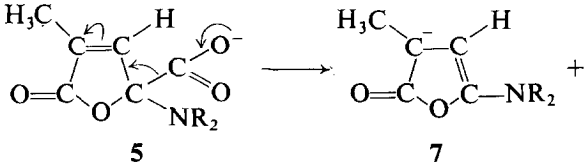
FIG. 8. Effect of pyrrolidine concentration on the approximate half-life of 3-benzyllumiflavin (10^{-3} *M*) with pyruvic acid (2×10^{-2} *M*) in DMF, components added separately.



4 **6**

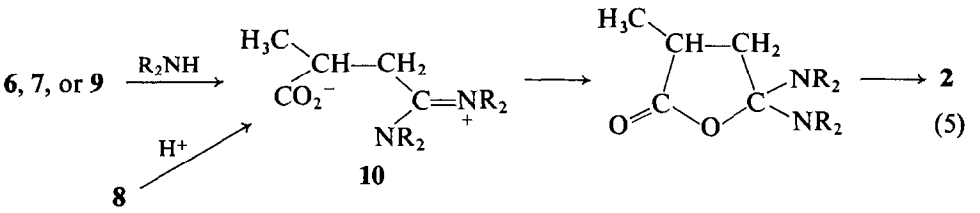


8 (4a)



5 **7** (4b)

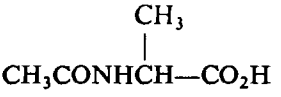
Another possibility is protonation of **4** and cyclic decarboxylation of the resultant species to give **9**, a keteniminium ion, which can react with pyrrolidine to give **8**. The reaction of **6**, **7**, or **9** with amine would yield **10**. Protonation of **8** gives **10**. Intramolecular attack by the carboxylate on the amidinium carbon yields an intermediate that in the presence of pyrrolidine should produce **2**, the nonredox product.



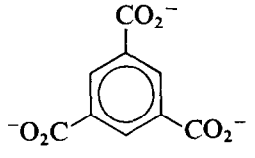
An alternative route from **6**, **7**, or **9** is attack by water (from the initial condensation) to form the amine salt of the monoamide of succinate and then dehydration of the carboxylate ammonium salt in DMF during work-up.

Analogies to this process exist. Pyruvic acid itself when heated forms methylsuccinic acid, and does so very effectively at 100°C with HCl (*5*, *6*), likely via an enol mechanism similar to that given here. In aqueous base, pyruvate dimerizes reversibly to an aldol product, with an equilibrium constant of 6.0 *M* (*7*). That the methyl of pyruvate readily condenses with aldehydes, and that the enolate is involved, were shown by Wermuth (*8*).

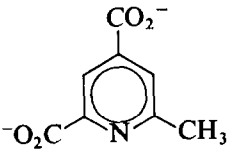
Amines accelerate these types of condensations (*5*). Ammonium pyruvate in alkaline solution produces a mixture of **11** and **12**, while in acid *N*-acetyl alanine



is the major product. Recently Pojir and Roe (*9*) found that anilines react with pyruvate esters to give aminated dimers akin to **1**.



11



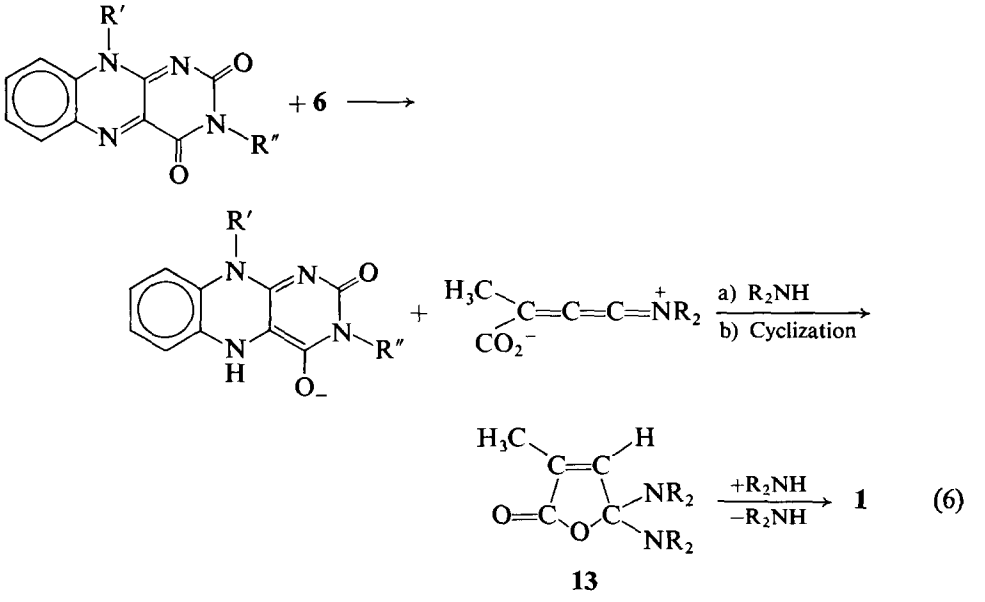
12

The usual decarboxylations of pyruvic acid by amines (*10*, *11*) are very different from the reaction reported here. These processes are acid catalyzed, involve tautomerism of α, β imines to β,γ imines with decarboxylation at this stage, and reveal very different specificities for the α-keto acid substrate and the amine than those seen here. Pyrrolidine, our most effective catalyst, does not catalyze this type of decarboxylation reaction. Phenylglyoxylic acid does not give flavin reduction, but rather the competing monomer decarboxylation (*11b*, see Table 1). For these same reasons, pyridoxal (*12*) and acetoin (*13*) type mechanisms may be discounted. The amine-catalyzed decarboxylations of pyruvate are much less facile than the process we observe in DMF, usually requiring hours of reaction time at elevated temperatures.

Oxidation-reduction. The kinetic results, along with the fact that 100% flavin reduction can be achieved with stoichiometric amounts of reactants, indicate that the non-redox and redox paths are identical up to some intermediate that is produced in or

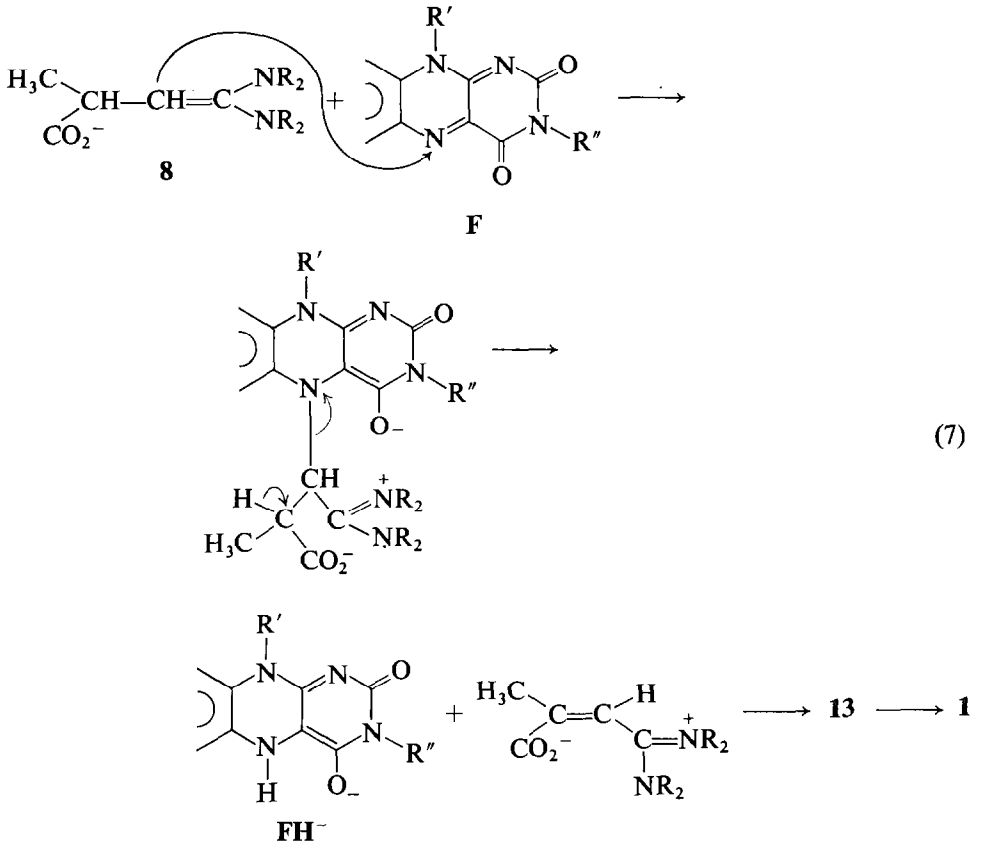
after the rate-limiting step. If no flavin is present to intercept it, product **2** results. The reaction of the intermediate with flavin must be fast, since the rate of flavin disappearance depends only on the rate of formation of this intermediate.

Since CO_2 evolution is at least as rapid as flavin reduction, and probably proceeds via one of the cyclic processes of Eq. 4 (all of which are unimolecular), we conclude that decarboxylation is the rate-limiting step. The product **6**, for example, could be either oxidized by flavin (Eq. 6) or undergo reaction with pyrrolidine via Eq. 5.



Similarly **7**, upon two-electron oxidation by flavin and then reaction with amine, gives **13** directly. Analogous paths for **8** may be envisaged. At this stage, there is no way to choose among these various species (**6–9**) which are all related by proton shifts, ionizations, or additions of amine. With sufficient flavin all of the intermediate is trapped.

We are left with the question of the mode of the flavin reaction. Because of the complexity of the early steps in this reaction and the fact that the flavin step is extremely rapid, little direct information was gained about it. Various possibilities were tested (Table 1), and though we may rule out some possibilities, we are still left only with hypotheses. For **6**, we have drawn Eq. 6 as a hydride transfer, and implied a similar path for **7**. For **8**, however, we do not have anions, so that an electron transfer or hydride path is difficult to envision. All these species should be alkylating agents toward electrophilic centers. For anions **6** and **7** this is obvious, and **8**, a dienamine, should be quite reactive as a nucleophile. There is some evidence that a mechanism (discussed by Hamilton (*3b*)) involving substitution by nucleophiles on the isollaoxazine nucleus, and leading formally to redox products, operates with various nucleophiles, such as mercaptide ion and enolates (*1*, *14*, *15*). One such scheme for **8** is given in Eq. 7.



(7)

We have chosen to write these substitutions as occurring at N-5 of the isoalloxazine somewhat arbitrarily, awaiting results of more definitive investigations. Experiments were performed with repeated visible and esr spectral scanning during the course of the pyruvate–amine–flavin reaction at high concentrations, and no spectral intermediates or radical signals were observed. We thus cannot, at this stage, define the nature of the oxidation step with any certainty.

CONCLUSIONS

While this reaction does not precisely duplicate any known enzymatic reaction, it resembles a good number of them, and may help furnish a clue to the understanding of others. It has been shown (*16*) that acetoacetate decarboxylase functions through formation of an imine, and then enamine, from substrate and an ϵ-amino group of a lysine unit, and that several aldolase reactions (*17*) also proceed *via* enamines. There are distinct advantages, manifest in the current study also, for condensation and decarboxylation to proceed through an enamine stage (*18*). The process we have studied bears much resemblance to these, but differs in that oxidation with flavin occurs. There are bacterial pathways for oxidation of pyruvate that involve the flavoenzyme pyruvate

oxidase. In two cases (*19*) the reaction involves thiamine pyrophosphate and FAD and results in oxidative decarboxylation, probably by flavin oxidation of the hydroxyalkyl TPP intermediate, very much like our process. In several organisms pyruvate is dimerized and either mono- or didecarboxylated as a major path.

EXPERIMENTAL

Materials

Lumiflavin (*20*), 3-methyllumiflavin (*21*), 3-benzyllumiflavin *via* 3-benzylbarbituric acid (*22*), 10-phenylisoalloxazine (*23*), and 3-benzyl-10-phenylisoalloxazine (*24*) were synthesized by published routes. Pyruvic acid (MCB) was regularly freshly distilled at 70–72°C/20 mm. All reagents in Tables 1, 2, and 3, with the exception of the next to last entry in Table 1, were from commercial sources and of reagent grade. They were distilled when used in kinetic experiments. *N*-Benzylidine alanine sodium salt was prepared from alanine and benzaldehyde (*25*). DMF, acetonitrile, DMSO, and chloroform were of commercial reagent grade, and DMF was distilled from calcium hydride.

Products

Oxidation reaction. 3-Benzyllumiflavin (0.025 *M*, 0.75 mmole), pyruvic acid (0.12 *M*, 3.75 mmole), and pyrrolidine (0.38 *M*, 11.2 mmole) were allowed to react in DMF (30 ml) under argon at room temperature until all the flavin was reduced (several hours). DMF was removed *in vacuo*, and water (50 ml) was added, dissolving essentially all products other than flavin. The resulting aqueous solution was thrice extracted with chloroform, and the combined chloroform extracts dried and evaporated to leave a yellow oil. The oil was chromatographed on silica gel using CCl_4, THF, CH_3OH, and finally H_2O as eluants. Upon evaporation of the solvent from each fraction two major products had been separated. The first was a barely crystalline material melting from 30 to 50°C, and the second over a range of 50–70°C. The largest spot from the second fraction on tlc (several solvent systems) was identical to the spot that was unique to the flavin reactions, while the first fraction was identical to material that had been obtained from pyruvic acid and pyrrolidine alone.

The oxidation product was sublimed (110°C/0.1 mm) to give a still impure material (mp 79–90°C) which was then recrystallized from ether–hexane to yield 100 mg of white crystals of mp 99–101°C (based on structure **1** and the amount of flavin, this is a 57% yield).

Analytical data. Calcd for $C_{13}H_{20}N_2O_2$: C, 66.0; H, 8.48; N, 11.85. Found: C, 65.78; H, 8.88; N, 11.95. Mass spectrum 236 (M+); 166 (M-C_4H_8N); 139 (M-COC_4H_8N); ir (CCl_4) 1640 (s); nmr (CCl_4, δ) 6.00 (1H; q, vinyl); 2.03 (3H; d, CH_3); 3.45 (8H; t, pyrrolidine, α to N); 1.90 (8H; m, pyrrolidine β to N).

Synthesis of **1** was *via* citraconic acid and thionyl chloride followed by the addition of pyrrolidine in a straightforward manner. The resultant material was identical spectrally and on mixed m.p. with the oxidation product.

Nonoxidation. The nonoxidative product (crude mp 30–50°C) was with difficulty recrystallized from cyclohexane (mp 50–55°C), but was identical to material (mp 52–55°C) obtained in approx. 30% yield after purification from an anaerobic mixture of

pyrrolidine (0.2 *M*) and pyruvic acid (0.1 *M*) in DMF. This material was still impure by tlc, and although microanalysis best fit **2** plus one H_2O, spectral data supported structure **2** (see below). Analogous products were isolated in high yield (>70%) from morpholine and benzylamine with pyruvic acid by allowing amine and pyruvic acid to stand in DMF or acetonitrile for several days. Evaporation of the solvent left oils which slowly crystallized. The morpholine derivative readily sublimed, and was recrystallized from cyclohexane (mp 115–117°C). The benzylamine derivative melted at 200°C.

Analytical data. Pyrrolidine cpd (**2**). Calcd for $C_{13}H_{22}N_2O_2 \cdot H_2O$: C, 61.0; H, 9.38; N, 10.9. Found: C, 61.32; H, 9.61; N, 10.6. Mass spectrum 238 (M+), 168 (M-C_4H_8N); nmr (CCl_4) 1.08 (3H; d, CH_3); 2.0–3.0 (3H; m, CH and CH_2 α to amide); 1.90 (8H; m) and 3.35 (8H; m), pyrrolidine protons; ir (CCl_4) 1640 (m).

Morpholine cpd (**2**). Calcd for $C_{13}H_{22}N_2O_4$: C, 57.7; H, 8.15; N, 10.33. Found: C, 57.69; H, 8.42; N, 10.24. Mass spec: 270 (M+); 184(M-C_4H_8NO); 156(M-COC_4H_8NO); nmr (CCl_4) 1.05 (3H; d, CH_3); 2.5–3.0 (3H; m, CH and $CH_2\alpha$ to amide); 3.60 (16H; s, all morpholine protons; ir (CCl_4) 1640 (s).

Benzylamine cpd (**2**). Mass spectrum 310 (M^+), 204 (M-$NHCH_2C_6H_5$); nmr (CCl_4); 1.05 (3H; d, CH_3); 2.2–3.0 (2H; m, CH_2); 3.32 (1H; s, CH); 4.30 (4H; d, NCH_2); 7.3 (10H; s, C_6H_5); ir (Nujol): 1660 (s), 1640 (m).

The microanalyses of the first two, and mass spectra of all derivatives, agree with the assignment. The ir spectra of the first two contain bands at 1640 cm^{-1} as the only carbonyl region absorption, indicative of amides of cyclic secondary amines. The nmr spectra are all consistent with the structures, especially for the morpholine compound. Morpholine itself has resonances at 2.90 and 3.70 ppm, each a triplet; all amides of morpholine show one singlet at 3.50–3.64 ppm. Our compound has a 16-proton singlet at 3.60 ppm. The long-range splitting by the vinyl proton of **1** of the CH_3 (2.03δ, J = 2.5 cps) is replaced in all three derivatives of **2** by a resonance at 1.05–1.08 and J = 7 cps (d).

Carbon dioxide determination. The method used here was to attach a 250-ml three-necked round-bottomed flask using all $\bar{s}$ and ball joints through a reflux condenser to (in sequence) a trap kept at –20°C, two U-tubes with stopcocks containing first ascarite (approx. two-thirds) and then drierite (one-third), and finally an oil bubbler. The flask had one neck stoppered; the other had a septum through which DMF-saturated argon was continuously, but slowly, bubbling. The concentrated reaction mixtures (usually 50–100 ml) were stirred, and spectral monitoring was accomplished by diluting 0.005-ml aliquots into anaerobic DMF solutions in Schlenk tubes. The ascarite tubes were weighed periodically after closing them and the reaction mixture off.

Kinetic procedures. The Schlenk tube method for following the visible absorptions has been previously described (*24* and references therein). For studies at 5×10^{-4} and 10^{-3} *M* flavin, 2-mm and 1-mm cells were adapted onto Schlenk tubes, as was a 5-cm cell for a 2×10^{-5} *M* flavin reaction.

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