melting nor decomposition were observed on heating to 350°; ir (in KBr, ν_{max} in cm^{-1}) 3060, 2910, 1630, 1602, 1488, 1240, 1190, 1120, 1013, 798, 782, 728, 750; MS (rel intensity) 648 (M^+) (100), 324 (M^{2+}) (45%). Anal. Calcd for $C_{44}H_{28}N_2S_2$: C, 81.48; H, 4.32; N, 4.32; S, 9.88. Found: C, 80.44; H, 4.43; N, 4.23; S, 9.82.

(Tetraphenyl-21,23-dithiaporphyrin)iron(III) Triperchlorate. Porphyrin (32.4 mg, 5×10^{-5} mol) was dissolved in 100 ml of dry, pure chloroform (Fluka). $Fe(ClO_4)_3$ (3.54 g, 10^{-2} mol) (Non-Yellow, The G. Frederick Smith Chemical Co.) was dissolved in 4 ml of absolute ethanol, 1 ml of triethyl orthoformate was added, and the solution was stirred for 5 min. Then 0.1 ml (excess) of the $Fe(ClO_4)_3$ solution was added to the porphyrin solution, and the solution turned deep green immediately. The solvent was evaporated to dryness under reduced pressure and the residue was dissolved in 250 ml of dry, pure methylene chloride. The green solution thus obtained was concentrated under reduced pressure to 20–25 ml and put in a desiccator containing pentane. Green crystals (31 mg, 60% yield) were obtained.

1-(Phenylmethylene)-5,10,15-triphenyl-20,22-diselenabilatriene *abc*. 2,5-Bis(phenylhydroxymethyl)selenophene (113 mg, 3.38×10^{-4} mol) and 22.6 mg of pyrrole were dissolved in 100 ml of dry pure benzene containing 2% (w/w) chloroacetic acid. The mixture was refluxed for 30 min, and, after cooling to room temperature, was washed twice with 100 ml of 5% ammonia solution and once with water and dried over sodium sulfate. This solution was concentrated to ~5 ml under reduced pressure and separation was performed on a Varian liquid chromatograph, Model 8500. The column was Micropak S.I.-10, *l* 25 cm, i.d. 2.2 mm. The effluent was 40% benzene in hexane, 100 ml/hr. The detector was a Varian spectrophotometer, Model 635, which was also used to run the visible spectra during elution.

Acknowledgment. We would like to acknowledge the many inspiring conversations we had with the late Dr. A. Wolberg before his death in the war of October 1973. This work is a continuation of our cooperative efforts. We would like to thank F. Frolow and D. Rabinovitz for their X-ray analysis studies on the material.

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Pyruvamide Semicarbazone Formation. Kinetics, Mechanism, and Pertinence to Pyruvamide-Dependent Histidine Decarboxylase[1]

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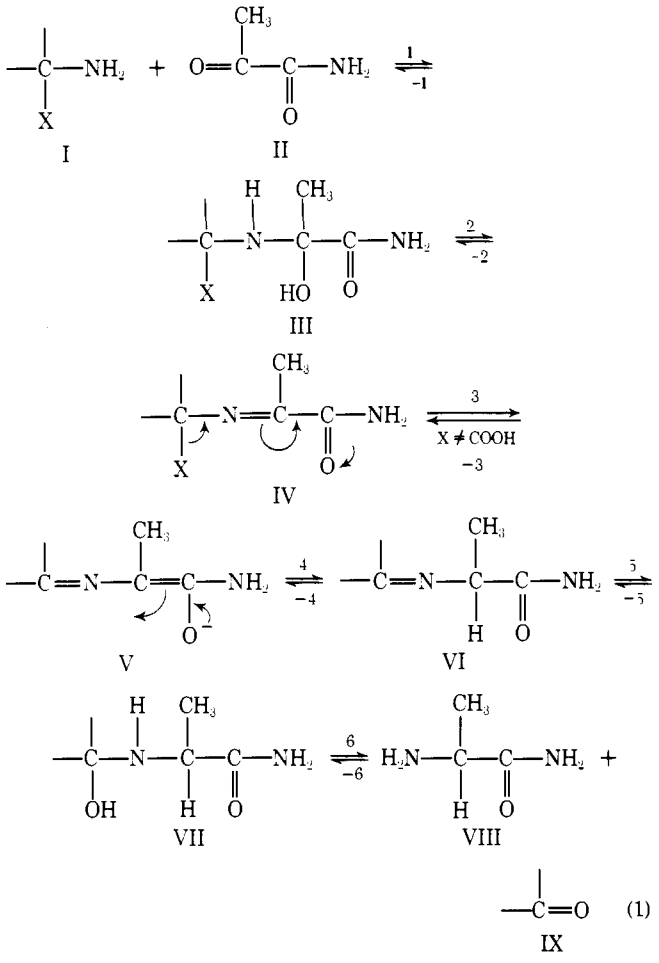
Abstract: Pyruvamide semicarbazone formation proceeds by way of readily recognizable carbinolamine intermediates. Rate and equilibrium constants for uncatalyzed, acid catalyzed, and phosphate buffer catalyzed modes of carbinolamine formation and dehydration, and equilibrium constants for pyruvamide hydration, are reported for pyruvamide itself, and for *N*-propylpyruvamide, pyruvanilide, pyruvoylphenylglycine, and *N,N*-dimethylpyruvamide. The fast uncatalyzed and phosphate catalyzed first step for *N*-protopyruvamides appears to proceed in a structurally unique manner via a zwitterionic intermediate stabilized by duple intramolecular hydrogen bonding and/or sigmatropic rearrangement in a bridged eight-centered system which is tantamount to simultaneous concerted intramolecular general acid and general base catalysis of the coordination process. An unusually low-lying transition state and a metastable isoamide intermediate likely are involved. The reaction is strongly general acid catalyzed by $H_2PO_4^-$ and, apparently, general base catalyzed by PO_4^{2-}! The second step, carbinolamine dehydration, is quite slow. Comparison of pertinent rate constants with known kinetic parameters for histidine decarboxylase reveals that reaction of the pyruvamide prosthetic groups with histidine to give the enzyme histidine carbinolamine needs little or no catalysis by the apoenzyme protein, but that dehydration of the carbinolamine must needs be accelerated 10^6-fold.

Pyruvamide residues and related functions appear to be involved in a variety of enzyme catalyzed reactions.[2,3] The most completely authenticated case is the involvement of N-terminal pyruvoylphenylalanine residues of histidine decarboxylase (histidine carboxy-lyase, E.C.4.1.1.22) as prosthetic groups for the decarboxylation of histidine, as demonstrated by Snell et al.[2] Recently[4] we have shown that pyruvamide itself promotes the deamination of amines and the decarboxylation of phenylglycine under very mild conditions and, consequently, have undertaken a study of what promises to be a revealing organic model of an enzyme catalyzed reaction, the transamination and decarboxylation of amines and α-amino acids by pyruvamide and N-substituted pyruvamides.

A likely sequence of reactions[2,4] involving the pyruvamide moiety is shown in general form in eq 1.



(1)

The Schiff's base formation (steps 1, 2), tautomerization or decarboxylation (steps 3, −4) via the enolate V, and hydrolysis (steps 5, −6) are entirely analogous to the corresponding reactions of pyridoxal in related enzymic and nonenzymic processes.[5] Our observation that Schiff's bases IV derived even from aliphatic amines are stable enough to be isolated and characterized, a property attributable to intramolecular hydrogen bonding of the pyruvamide $-NH_2$ group to the imine nitrogen atom,[4] leads us to anticipate the possibility of dissecting the multistep sequence into its constituents and studying the kinetics and mechanism not only from the starting materials I, II, VIII, and IX but, also, and in both directions, from intermediates IV and VI.

The present paper reports a study of reactions 1 and 2, specifically of pyruvamide and a selection of N-substituted pyruvamides, with semicarbazide. This amine was chosen so that further reactions (3, etc.) would not complicate matters and to permit comparison and contrast with its well-documented behavior with other carbonyl compounds.

Experimental Section

Materials. Pyruvamide and *N*-propylpyruvamide were prepared by permanganate oxidation of the corresponding lactamides. Pyruvanilide and pyruvoylphenylglycine resulted from acid hydrolysis of the appropriate α,α-dimethoxypropionamides. Details of these and other pyruvamide syntheses will appear elsewhere. *N,N*-Dimethyl- and -diethylpyruvamides were prepared from the appropriate amines by reaction with the pyridine salt of hydroxymaleic anhydride[6] (75–80% yield). These materials and stock solutions (10–50 m*M*) of them in purified[7] dioxane were kept in the dark since they are adversely affected by light. Semicarbazide hydrochloride (Aldrich 99%+) was recrystallized from aqueous ethanol. Other materials were reagent grade.

Kinetics. 1. General Considerations. A consistent model for the reactions studied, reactions 1 and 2 of eq 1 and hydration (hyd) of the ketone, is shown as eq 2; reaction 2 is essentially irreversible

(2)

under our conditions. I is semicarbazide, II is the free (unhydrated) ketone, II H_2O the ketone hydrate (geminal diol), III the carbinolamine, and IV the semicarbazone. Reaction 1 is quite fast ($t_{1/2} \sim$ 30–300 msec at pH 5–8, depending on buffer and semicarbazide concentration), hydration is relatively slow $t_{1/2} \sim$ 5–60 sec at pH 7), and reaction 2 is slower still (acid dependent; $t_{1/2} \sim$ 5–8 min at pH 6). Pyruvamide and the *N*-alkylpyruvamides (propylpyruvamide, dimethylpyruvamide, and pyruvoylphenylglycine) absorb light measurably at 240 nm, whereas the corresponding hydrates and carbinolamines are essentially transparent. Thus, the initial decrease in optical density to an equilibrium value upon dilution of the pyruvamide into water, the further very rapid decrease upon addition of semicarbazide, and the eventual increase to a value well above that initially exhibited, all are readily resolved and interpreted. The hydrate and the carbinolamine from pyruvanilide, interestingly, exhibit extinctions greater than that of the parent ketone (although less than that of the semicarbazone), a phenomenon attributable to greater involvement of the unshared electron pair of the amide nitrogen with the aromatic ring when the carboxamide carbonyl group is not itself flanked by a further carbonyl group. Nevertheless, the optical density changes observed, three successive increases, again are resolvable.

2. The Fast Reaction (Reaction 1). Ketone solutions, prepared by diluting 10–40 μl. portions of stock solutions in dioxane to 10 ml with water 30 min before use to permit equilibrium hydrate formation, were mixed with solutions of semicarbazide hydrochloride in phosphate buffer in a Durrum D-110 stopped-flow spectrophotometer. Semicarbazide (10–200 m*M*) was always in large excess over ketone. The pH was ascertained to be unchanged after each reaction. Observations were made at 240 nm and 25 ± 0.1° using a 1-mm slit. Oscilloscope traces of absorbance changes vs. time (3–50 to 3–1000 msec) were recorded photographically. At pH >5, steady A_∞ values were obtained, reactions 2 and -hyd being imperceptible on the stopped-flow time scales; linear log $(A_t - A_\infty)$ vs. t plots were invariably obtained and gave apparent pseudo-first-order rate constants (k_{obsd}) in the usual way. At pH 4–5, good A_∞ values did not obtain; here, Guggenheim's method was used to corroborate the approximate k_{obsd} values indicated by the biphasic semilogarithmic plots.

3. The Slow Reaction (Reaction 2). Portions of ketone in dioxane–water mixture were quickly diluted to 3.0 ml with buffered semicarbazide. Changes in absorbance (240 nm, 25 ± 0.1°) with time, after a mixing time of 6 sec, were observed with a Cary Model 14 recording spectrophotometer. Values of k_{obsd} for semicarbazone formation were deduced as usual from semilogarithmic plots of $A_\infty - A_t$ vs. t.

4. Hydration. Optical densities of ketone solutions diluted into water or buffer (no semicarbazide) were followed in the Cary spectrophotometer in the usual way. Equilibrium optical densities (A_{H_2O}) resulted directly; extrapolation to $t = 0$ gave initial values (A_0) for unhydrated ketone and the initial slopes gave values of k_{hyd} sufficiently accurate to establish their magnitude relative to k_1 and k_2. Separately, *initial* optical densities of appropriate ketone–semicarbazide mixtures determined under 3 above were plotted reciprocally (1/OD vs. 1/SC), thus to obtain optical densities (A_{sc}) at SC = ∞. On the assumption that the extinctions exhibited by the various carbinolamines accurately simulate those of the hydrates, the equilibrium constants given in Table I for hydrate formation, $K_{hyd} = (A_0 - A_{H_2O})/(A_{H_2} - A_{sc})$, were obtained.

Results

1. Carbinolamine Formation (Reaction 1). The k_{obsd} (extrapolated to zero buffer concentration) vs. pH profiles shown in Figure 1 for the reaction of pyruvamide and *N*-propylpyruvamide with 50 m*M* semicarbazide are typical and demonstrate that the rates of carbinolamine formation are specific acid independent in the pH range $5 < pH < 8$ but increase rapidly at lower pH. Accordingly, we have

Table I. Rate and Equilibrium Constants for Pyruvamide–Semicarbazide Carbinolamine Formation and Dehydration and Pyruvamide Hydrate Formation[a]

	K_1, M^{-1}	$k_1, M^{-1}\ min^{-1}$	k_{-1}, min^{-1}	$k_1^{H_2PO_4^-}, M^{-2}\ min^{-1}$	$k_1^{HPO_4^{2-}}, M^{-2}\ min^{-1}$	K_{hyd}	$k_2^H, M^{-1}\ min^{-1}$
Pyruvamide	39.2 ± 2.2 (46 ± 2.5)	490 ± 10	12.5 ± 0.3	3.5×10^4	1.6×10^4	0.8 ± 0.04	7900 ± 800
N-Propylpyruvamide	38.3 ± 2.3 (36 ± 0.5)	240 ± 80	6.1 ± 2.0	3.3×10^4	1.6×10^4	0.27 ± 0.03	3700 ± 100
N,N-Dimethylpyruvamide	1.9 ± 0.2	19 ± 2	10.4 ± 0.5	($k_1^{PO_4}$ at pH 7.5 is 2.2×10^3)			
Pyruvanilide	29.9 ± 4.4 (55 ± 1.5)	430 ± 10	14.4 ± 4.4	2.7×10^4	1.5×10^4	0.32 ± 0.01	2500 ± 100
Pyruvoylphenylglycine	98 ± 22 (81 ± 1)	630 ± 100	6.5 ± 1.0	2.8×10^4	0.56×10^4	0.77 ± 0.01	3800 ± 100

[a] $K_1 = k_1/k_{-1}$ = [carbinolamine]/[unhydrated ketone][semicarbazide]; K_{hyd} = [ketone hydrate]/[unhydrated ketone]; other constants are defined by eq 3, 3a, 4, 4a, 5, and 5a.

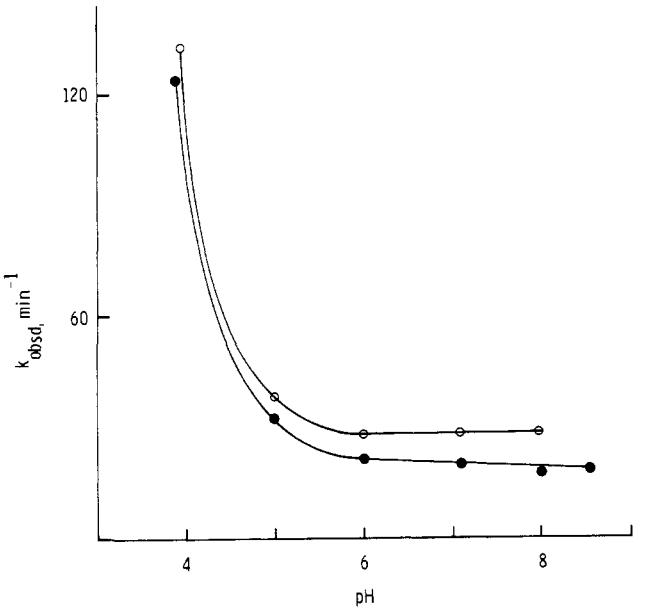


Figure 1. Dependence of rate of carbinolamine formation from semicarbazide and pyruvamide (○) or *N*-propylpyruvamide (●) upon pH. Values of k_{obsd} are at 0.05 *M* semicarbazide, are extrapolated to zero buffer (phosphate) concentration, and at pH 3.9 are corrected for protonation of semicarbazide.

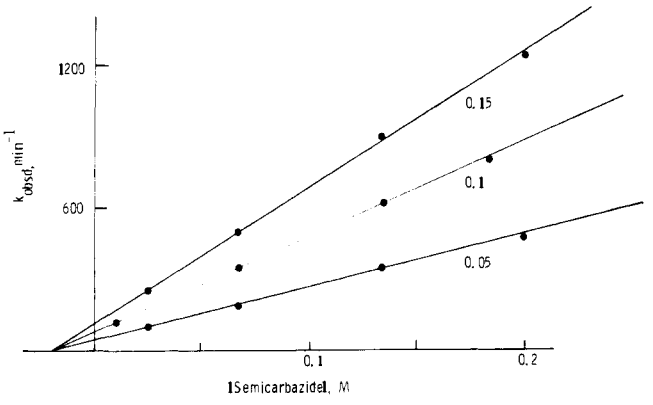


Figure 2. Dependence of rate of carbinolamine formation from semicarbazide and pyruvamide at pH 6 upon semicarbazide concentration at three concentrations of phosphate buffer (0.05, 0.1, and 0.15 *M*). Slopes are $k_1^{apparent}$; intercepts are $k_{-1}^{apparent}$ at the indicated phosphate concentrations.

studied the reaction in this pH region; pertinently, perhaps, the action of histidine decarboxylase is almost pH independent in this same region.[2a] Figure 2 shows the dependence of k_{obsd} upon semicarbazide at pH 6 and at three concentrations of phosphate buffer. Clearly, although the reaction rate increases linearly with semicarbazide and is pH independent at pH 6, it is strongly catalyzed by phosphate. Figure 3, a secondary plot of the (least-squares) slopes of the

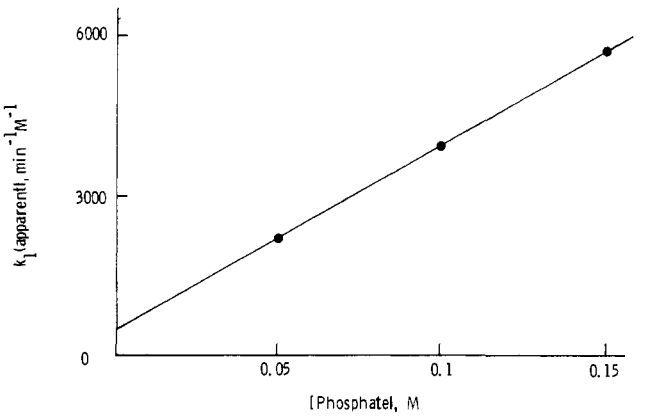


Figure 3. Dependence of $k_1^{apparent}$ (slopes of lines in Figure 2) upon phosphate buffer concentration. Intercept is k_1; slope is $k_1^{PO_4}$ for reaction of pyruvamide with semicarbazide at pH 6.

lines in Figure 2 *vs.* total phosphate reveals this dependence to be linear. Similar results obtain throughout the pH range 5–8 with all four pyruvamides examined. Since reactions 2 and -hyd may be neglected at these pH's, the pertinent model and the related integrated rate expression are 3 and 3a:[8]

$$\text{I} + \text{II} \underset{-k_1}{\overset{k_1 + k_1^{PO_4}[PO_4]}{\rightleftharpoons}} \text{III} \qquad k_{-1} + k_{-1}^{PO_4}[PO_4] \tag{3}$$

$$k_{obsd} = (k_1[\text{I}] + k_{-1})(1 + k_1^{PO_4}[PO_4]/k_1) \tag{3a}$$

Interpretation of the experimental data in terms of eq 3a gives rise to the values given in Table I for k_1, k_{-1}, and K_1. Not surprisingly, $k_1^{PO_4}$ is not pH independent. Figure 4, a plot of k_{obsd} at constant [SC] vs. fraction of buffer present as $H_2PO_4^-$ (as opposed to HPO_4^{2-}), reveals the reaction to be catalyzed by both of these anions and gives the values given in Table I for the third-order constants $k_1^{H_2PO_4^-}$ and $k_1^{HPO_4^{2-}}$ as defined by the further modified model and integrated rate expression (eq 4 and 4a):

$$k_1^{HPO_4^{2-}}[HPO_4^{2-}]$$

$$k_1^{H_2PO_4^-}[H_2PO_4^-]$$

$$\text{I} + \text{II} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{III} \qquad k_{-1}^{H_2PO_4^-}[H_2PO_4^-] \quad k_1^{HPO_4^{2-}}[HPO_4^{2-}] \tag{4}$$

$$k_{obsd} = (k_1[\text{I}] + k_{-1})(1 + (k_1^{H_2PO_4^-}[H_2PO_4^-] + k_1^{HPO_4^{2-}}[HPO_4^{2-}])/k_1) \tag{4a}$$

2. Semicarbazone Formation (Reaction 2). Initial slopes of Cary absorbance vs. time traces at various values of pH and semicarbazide concentration sufficed to demonstrate that, in this case as in a wealth of others,[9] the rate of dehydration of carbinolamines is linearly dependent on hydrogen ion concentration in the pH region 4–7. At pH 5, semilogarithmic plots of $A_\infty - A_t$ vs. t were linear through at least 5 half-lives except in the case of pyruvoylphenylglycine for which they were biphasic at low semicarbazide concentrations. Modest buffer catalysis was evident. Since both carbinolamine formation and hydrate dehydration are fast on the Cary time scale under these conditions, the pertinent model and related integrated rate expression are eq 5 and 5a:

$$\mathrm{I} + \mathrm{II} \underset{}{\overset{K_1 = k_1/k_{-1}}{\rightleftharpoons}} \mathrm{III} \xrightarrow{\substack{k_2^{PO_4}[PO_4] \\ k_2^{H}[H]}} \mathrm{IV} \qquad (5)$$

$$K_{hyd} \Big\Downarrow$$

$$\mathrm{II}\ \ H_2O$$

$$k_{obsd} = k_1(k_2^{H}[H] + k_2^{PO_4}[PO_4])[I]/(k_{-1}(1 + K_{hyd}) + k_1[I]) \qquad (5a)$$

Inversion of eq 5a gives the linear double reciprocal relationship, eq 5b:

$$1/k_{obsd} = k_{-1}(1 + K_{hyd})/k_1[I](k_2^{H}[H] + k_2^{PO_4}[PO_4]) + 1/(k_2^{H}[H] + k_2^{PO_4}[PO_4]) \qquad (5b)$$

A typical plot of $1/k_{obsd}$ vs. $1/[I]$, for *N*-propylpyruvamide at two phosphate concentrations, is shown in Figure 5. Secondary plots of reciprocals of the ordinal intercepts of such lines (i.e., $k_2^{H}[H] + k_2^{PO_4}[PO_4]$) vs. total phosphate were straight lines. Their ordinal intercepts, $k_2^{H}[H]$, gave the values for k_2^{H} listed in Table I. One observes, moreover, that the form of eq 5b affords an interesting check of the self-consistency of the models proposed and the data obtained. The common abscissal roots of double reciprocal plots such as those in Figure 5 should be $-K_1/(1 + K_{hyd})$. Values of K_{hyd} being known, values of K_1 were computed and are included (in parenthesis) in Table I for comparison with the values obtained in the stopped-flow studies. The agreement, considering the magnitudes of the extrapolations involved and similar uncertainties, is reassuring.

Discussion

Reactions of amines with ketones and aldehydes have been the subject of considerable study for over half a century.[9] Since the classic paper by Jencks some 15 years ago,[10] it has been clearly recognized that imine (semicarbazone, oxime, etc.) formation is a two-step reaction, a carbinolamine being an obligatory intermediate, that either or both steps commonly may be acid or general acid catalyzed or uncatalyzed, and that the magnitudes of the various rate constants for the two steps often are such that a change in rate determining from the first to the second occurs with increasing pH so that a complete set of rate constants can be extracted from a simple study of the dependence of the rate of imine formation upon pH. When supported by other criteria such as breaks in linear free energy relationships and differential effects of catalysts, "breaks" and maxima in pH vs. rate profiles are rather reliably diagnostic of changes of rate-determining step. Direct examination of the first step is an obvious and penetrating additional criterion. Sander and Jencks have used stopped-flow methods for equilibrium studies[11] and Diebler and Thorneley have studied the very fast reaction of secondary amines by temperature-jump procedures[12] but, in general, despite the wide availability of

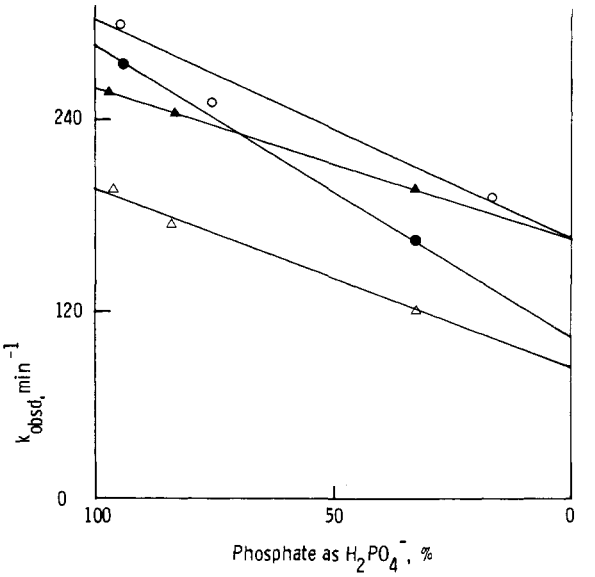


Figure 4. Dependence of k_{obsd} for reaction of semicarbazide with four pyruvamides at constant semicarbazide (0.05 *M*) and total phosphate (0.1 *M*) concentration upon fraction of phosphate present as $H_2PO_4^-$ (as opposed to HPO_4^{2-}). (○) pyruvamide; (●) *N*-propylpyruvamide; (△) pyruvoylphenylglycine; (▲) pyruvanilide.

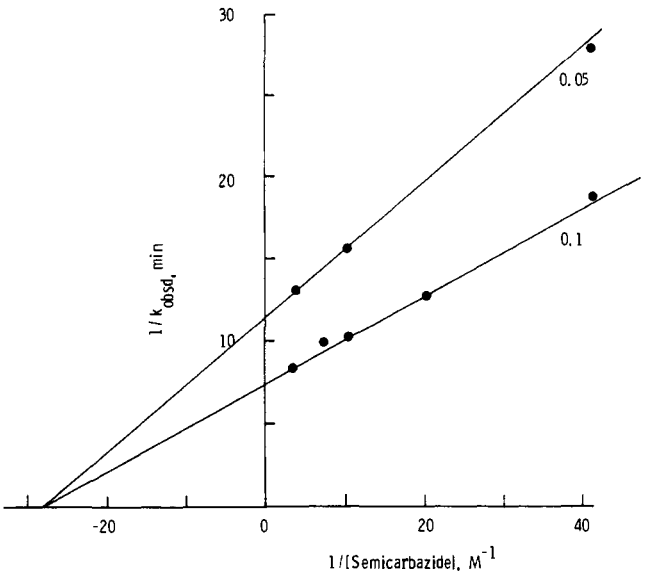


Figure 5. Double reciprocal plot of k_{obsd} vs. [semicarbazide] for semicarbazone formation from *N*-propylpyruvamide at pH 5 and two phosphate buffer concentrations (0.05 and 0.1 *M*). Ordinal intercepts are reciprocal of $k_2^{H}[H] + k_2^{PO_4}[PO_4]$; abscissal root is $K_1/(1 + K_{hyd})$.

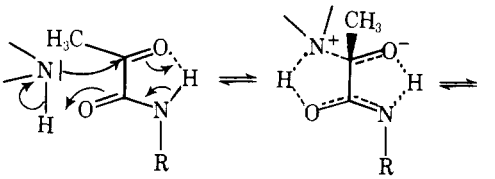
stopped-flow equipment for observing fairly fast reactions, this criterion has been little used. We would point out that simple initial slope values, which are easily read off from oscilloscope traces even when these are nonhyperbolic owing to intervention of subsequent steps, equilibria, side reactions, and like complications, give rate constants sufficiently accurate for this purpose. Indeed, while the constants reported in Table I derive rigorously from linear semilogarithmic plots, numbers required merely for comparison purposes (such as the rate constant for reaction of ethyl pyruvate) were obtained from such initial slope data.

With pyruvamides (and methyl pyruvate[13]), the second step being rather slow, the first fast, and acid catalysis intervening early, no change in rate-determining step could occur at any reasonable pH. Breaks, if any, in extended

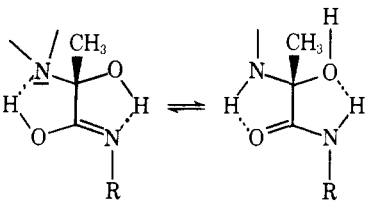
pH-rate profiles for the overall reaction might well be ambiguous and subject to misinterpretation.

The values of the acid-independent rate constants k_1, and the equilibrium constants for carbinolamine formation from N-monosubstituted pyruvamides are large for the reaction of ketones with the rather poor nucleophile semicarbazide. This is not surprising of itself; the carboxamide is a poweful electron-withdrawing group attached directly to the site of nucleophilic attack, and the effects of such groups in promoting both the rates[14,15] and equilibria[15] of formation of carbonyl addition compounds are well documented and entirely reasonable. Such groups simultaneously enhance the electrophilic character of the carbonyl carbon atom and electrostatically destabilize the starting material relative to the product. That *N*-propylpyruvamide is moderately (twofold) less reactive than pyruvamide itself is reasonably in keeping with the inductive effect of the alkyl group. However, a variety of considerations suggest that the inductive effect alone is insufficient to account for the magnitudes of the constants. Thus: (1) The carboxyalkyl group is far more strongly −I than the carboxamide group and yet k_1 for the reaction of ethyl pyruvate with semicarbazide is at most no greater than k_1 for propylpyruvamide and probably is significantly less.[16] (2) *N,N*-Dimethylpyruvamide is 10 times less reactive than propylpyruvamide and 20 times less reactive than pyruvamide itself; similarly, ethyl pyruvate is at least 20 times less reactive than pyruvic acid.[13,16] (3) The equilibrium constant for carbinolamine formation from the dialkylpyruvamide is less than one-tenth of that for any of the N-monosubstituted compounds. (4) The reaction is pH independent over a wide range. (5) Powerful catalysis by both phosphate monoanion and phosphate dianion is evidenced.

Hydrogen bonding of amide NH to carbonyl oxygen, tantamount to intramolecular general acid catalysis of the addition reaction, immediately springs to mind as a possible root of the first two at least, of these properties. The amide proton is five centers away from the ketone oxygen, a situation clearly conducive to intramolecular hydrogen bonding. The NMR spectrum of pyruvamide in aprotic solvents shows this proton shifted downfield approximately 1 ppm from the other NH absorption. Pyruvamide imines[4] and amides of phenylpyruvic acid (R. G. Lawton, personal communication) show hydrogen bonded spectra and ultraviolet spectral data[18] are also consistent. A modest enhancement of the electrophilicity of the carbonyl carbon might well result, as might also a fair modicum of general acid catalysis by the amide group even though this is a very weak acid and a poor general catalyst.[17] However, a much greater catalytic potential can be recognized. According to the detailed mechanism for carbinolamine formation recently advanced by Sayer, Jencks, et al.,[19] the key intermediate stage at pH >2-4 is the carbinolamine zwitterion ($T^{\pm}$) which is trapped in a rate-determining step subsequent to its formation by a water-mediated proton switch, by protonation at the expense of a general acid or, at lower pH, via protonation by the hydronium ion (eq 6). The reaction of semicarbazide (a moderate nucleophile) with a pyruvamide (a good electrophile) fits the criteria for such a pathway very well indeed, and there is no reason to doubt that the reactions of N,N-disubstituted pyruvamides (and pyruvate esters) at near-neutral pH proceed in this manner. A necessary consequence of the rate-determining nature of the second (proton switch or protonation) step is that $T^{\pm}$ must be in some sort of equilibrium, albeit one to which it contributes very little, with the reactants, and that the overall reaction rate will be governed by its equilibrium concentration. Thus, stabilization of $T^{\pm}$ will greatly accelerate the overall reaction. Now, in the case of the zwitterionic intermediate (XI) from an *N*-protopyruvamide (X), not only is the developing carbinoxide anion hydrogen bonded to a potential proton donor but also, in a manner which is almost unique to the pyruvamide structure, the carboxamide oxygen, to which any anionic charge and concomitant basicity will be mesomerically transferred upon partial or complete deprotonation of the amide NH, finds itself in the proximity of the protons attached to the attacking nucleophilic center upon which a cationic charge and concomitant acidity are fast developing.



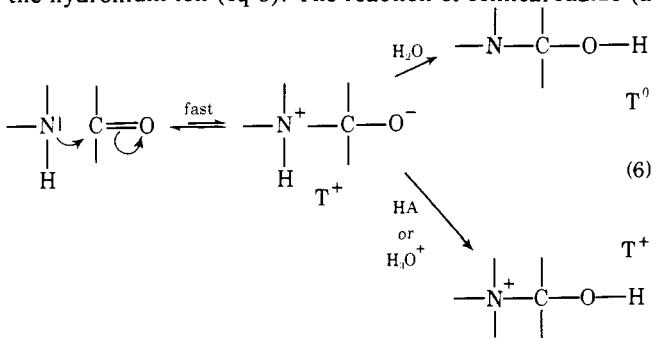
X XI



XII XIII

Taken to the extreme of complete duple proton transfer, the intermediate would be represented as the carbinolamine *isoamide* XII in which all needs for zwitterionic charge separation and the development of strongly basic and acidic centers disappear. Since duple hydrogen bonding is likely in both, any structural (as opposed to energetic) distinction between XI and XII begins to be rather moot as does also the question of whether the acceleration is attributable to stabilization of the intermediate or to more-or-less-concerted bifunctional acid-base catalysis within the reactants themselves by sigmatropic rearrangement in a bridged eight-center bicycle. Certainly, considerable stabilization seems very likely; and the "intramolecular" proton transfer is in many senses akin to that from hydroxylamino OH or unusually acidic hydrazino β-NH proposed[19] to account for rate accelerations amounting to two orders of magnitude or more.

We have direct but as yet tentative evidence that carbinolamine isoamides XII are capable of independent existence. Carbinolamines precipitate instantly, in good yield, analytically pure, when solutions of pyruvamide and various amines (propylamine, benzylamine; piperazine gives a bis adduct!), in aprotic solvents such as tetrahydrofuran, are mixed. Under these conditions, proton switch must surely be extremely slow. The infrared spectra of these products in the solid state lack completely the amide NH bending absorption at 1550 cm^{-1}, as would be expected for isoamides. Pyruvamides and pyruvamide imines show this absorption very clearly. The carbinolamines dissociate completely upon dissolution in water and other solvents (pyridine) in which they are soluble. Their existence undoubtedly depends upon



(6)

association energy in the solid state and their generation in solvents in which they are poorly soluble.

In water, *N*-protopyruvamides react to a considerably greater equilibrium extent with semicarbazide than do dialkylpyruvamides; the respective free energies of carbinolamine formation, calculated from the equilibrium constants in Table I, are between −2.02 and −2.62 as opposed to −0.38 kcal/mol. However, the rate constants for the back-reactions are little different from each other so that the activation energies for reversion of carbinolamine to transition state are similar. Thus, *both* the carbinolamines *and* the transition states from *N*-protopyruvamides are stabilized relative to the starting materials by some factor worth about 1.6–2.2 kcal, which stabilization is not enjoyed by *either* the transition state *or* the carbinolamine from dimethylpyruvamide. A reasonable suggestion is carbinolamine stabilization by intramolecular hydrogen bonding (XIII) entirely akin to the proposed (XI, XII) transition-state stabilization. Amides, of course, are much more stable than isoamides. In water, proton switch is inevitable and the end-products (prior to dehydration) must be carbinolamine amides. Instabilities of carbinolamine zwitterions ($T^{\pm}$) and neutral carbinolamine isoamides relative to these can be estimated. Fersht[20] estimates a typical amide–isoamide equilibrium constant to be about 10^{-8} which corresponds to a free energy difference of about 11.1 kcal/mol. The $T^0 \rightleftarrows T^{\pm}$ equilibrium constants (K_z) for carbinolamines from methoxyamine and *p*-chloro-, *p*-methoxy-, and *p*-nitrobenzaldehyde are estimated by Sayer[19] all to be 3×10^{-7}. A ΔpK_a of 1.2 (i.e., 4.73–3.65) between a methoxyammonium ion and a comparably substituted semicarbazidium ion leads to a K_z of 2×10^{-8} for a semicarbazide–pyruvamide carbinolamine. It is assumed that replacement of H by CH_3 and Ar by CONH will be without effect since their effects on the hydroxyl and amino groups will cancel each other, a supposition supported by the noneffect of change of Ar in Sayer's examples. Estimates based on known and estimated pK_a values for alaninamide and lactamide suggest a similar or slightly smaller K_z, about 10^{-8}. These equilibrium constants again correspond to a free energy difference of 10.8–11.1 kcal/mol. Thus, the energy contents relative to the common product of two possible metastable intermediates, the zwitterion and the isoamide, arrived at by totally independent estimation procedures, are remarkably similar. Given the considerable uncertainties in both estimations, either or other might truly be the more stable. Hydrogen bonding as in XI or XII would stabilize either by about 2 kcal, and the energy barrier between them would be quite small.

These considerations permit the construction of rather detailed energy profiles such as that shown in Figure 6 for pyruvamide and dimethylpyruvamide. Setting the starting systems, A′ and A respectively, arbitrarily at zero, products E′ and E are at −2.2 and −0.4 kcal. Zwitterion C is about 11 kcal above E and transition state D for water-mediated proton switch is about 7 kcal higher ($\Delta F^{\ddagger}$ = 17,400 − 1360 log *k* at 25°). This correlates roughly but reassuringly with D being about 10 kcal above C, a value estimated from Sayer's energy barrier for protonation of $T^{\pm}$ in 1 *M* H_3O^+ (~3 kcal) linearly extrapolated to pH 5 since our pH-independent region sets in at that pH. The transition state for proton switch of the hydrogen-bonded intermediate, D′, is set 1.9 kcal below D, this being |E − E′| (1.8 kcal) plus a correction of 0.1 for the small difference between the k_{-1} values. Exactly the same ΔE_{act} may be deduced from the ratio of the forward rate constants (490/19). The double dip (XI ⇋ C′ ⇋ XII) in the pyruvamide profile arbitrarily assumes that XII is somewhat more stable than XI and truly lies on the reaction pathway. If the converse should be

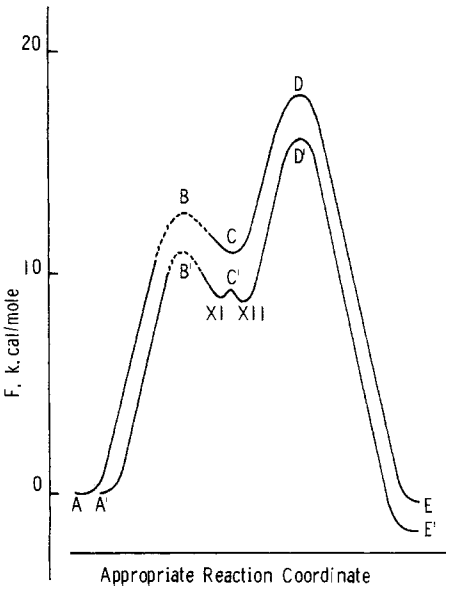
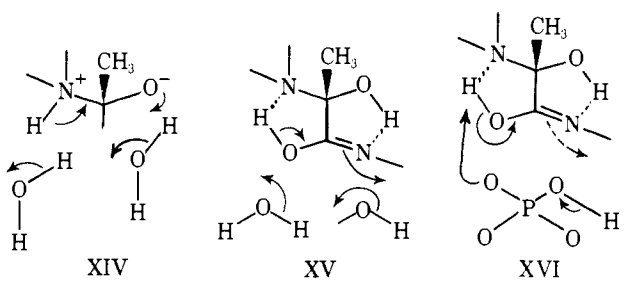


Figure 6. Estimated energy profiles for water catalyzed reaction of *N,N*-dimethylpyruvamide (A) and pyruvamide (A′) with semicarbazide. E,E′ are product carbinolamines; C,C′ are metastable carbinolamine zwitterion and/or carbinolamine–isoamide intermediates.

true, a single dip (XI = C′) would suffice. Equal stabilities would require both pathways (XI → D′ and XI ⇌ XII) → D′) to operate. C′ is set 1.8–1.9 kcal below C by analogy with D′–D and E′–E. Barriers B′ and B are rough estimates. The profile shows clearly that the 25-fold acceleration of carbinolamine formation is due not to an intrinsic lowering of the energy barrier for the rate-determining step (D–D′ ≈ C–C′) but to the enhanced accessibility and greater equilibrium concentration of the metastable intermediate.

Whereas there exists but one pathway (XIV) for water-mediated proton switch for a simple ($T^{\pm}$) carbinolamine zwitterion, several additional possibilities, involving the amide group as intermediary, may be envisaged for the hydrogen-bonded zwitterion XI and the isoamide XII. Many



XIV XV XVI

of these may be disfavored on the principle of minimization of the numbers of bonds to be made and broken in the transition state, but one possibility, XV, for the isoamide is particularly attractive in that it involves no more such steps than does the normal switch. Indeed, inasmuch as the isoamide group, like amide, undoubtedly is a hybrid in which C–O has much double-bond and C=N much single bond character, XV may be even more accessible than XIV. That D–D′ is a little larger than E–E′ may well be ascribed to the existence of two independent pathways for water-mediated proton switch. Now phosphate catalysis is substantial. This is not remarkable of itself; general acid catalysis of carbinolamine formation is commonplace and, indeed, our third-order rate constants ($k_1^{H_2PO_4^-}$ and $k_1^{HPO_4^{2-}}$, Table I) are very similar to those published for a wide variety of acids in similar reactions.[19] The catalytic act for $H_2PO_4^-$ and the other acids is undoubtedly the diffusion-controlled protonation of the oxygen atom of the carbinolamine zwitterion (XI

or $T^{\pm}$) or possibly, at the expense of additional bond shifts restoring isoamide to amide, of XII. What is remarkable, however, is catalysis by HPO_4^{2-}. It is not reasonable to suggest that this very weak acid could compare with $H_2PO_4^-$ as a general catalyst, yet the alternative, acid catalysis by $H_2PO_4^-$ and base catalysis of the same reaction by its conjugate base, is equally unattractive. However, general base catalyzed stabilization (XVI) by *de*protonation of the *isoamide* OH of the carbinolamine isoamide is entirely plausible. Isoamide is likely a moderately strong acid (pK_a ~ 7-8), and arguments for the diffusion-controlled rate-determining nature of its reaction with HPO_4^{2-}, a relatively strong base, exactly parallel those for rate-determining protonation of $T^{\pm}$ alkoxide (a strong base) by weak buffer acids in the normal way. As is suggested by the broken arrows in XVI, the interaction may be to some extent concerted but, in the transition state, one would expect O-H bond breaking to be further advanced than N-H bond formation, giving a somewhat anionic character to the amide, just as in XIV O-H joining is reasoned[19] to lead N-H separation, the carbinolamine being slightly cationic.

It is pertinent to note that the rate constant for uncatalyzed addition of semicarbazide to undissociated pyruvic acid ($6.3 \times 10^3\ M^{-1}\ min^{-1}$)[13] is at least 20 times greater than that for its addition to ethyl pyruvate[16] and that intramolecular general acid catalysis was suggested as a possible reason.[13] One observes that the acid is just as well suited as a pyruvamide for the bicyclic sigmatropic rearrangement, with the additional feature that amide-isoamide tautomerization is not involved.

The second step in semicarbazone formation, dehydration of the carbinolamine, is slow for pyruvamide carbinolamines, just as the first step is fast. Second-order rate constants of $2\text{-}8 \times 10^3\ M^{-1}\ min^{-1}$ (Table I) for the acid catalyzed dehydration may be compared with $2.4\ (4.8) \times 10^6$, 2.0×10^6, and 6×10^4 for the semicarbazide carbinolamines of pyruvate anion,[21] furfural,[10] and methyl pyruvate,[22] respectively. Most of the deceleration undoubtedly is attributable to the inductive effect of the carboxamide group, but again it is noteworthy that the rates are even lower (x10) than that for the ester carbinolamine. This is nicely accounted for by the requirement that half the hydrogen-bonding stabilization of XIII (about 1 kcal) must be lost upon conversion to the singly hydrogen-bonded[4] product, a correlation which reciprocally confirms the supposition of duple rather than single hydrogen bonding in XIII, etc. Dehydration, like initial attack, is catalyzed by phosphate buffer but to a less dramatic extent; in 0.1 phosphate at pH 5, it is three times as fast as the uncatalyzed reaction.

A major purpose in studying prosthetic group reactions in the absence of the apoenzyme protein is to recognize the steps in the reaction pathway at which promotion of reaction rate by the protein must be involved and to clarify the nature of such involvement. The turnover number of the holoenzyme reflects the first-order rate constant for the slowest transformation of the enzyme-substrate complex, and this number divided by the substrate concentration required for half-maximum velocity (the apparent Michaelis constant) reflects the minimum possible second-order constant for the formation of the complex. The turnover number for histidine decarboxylase is 2600 min^{-1} per pyruvamide residue, and the $S_{0.5}$ is 0.31 mM[2a] so that the second-order constant for enzyme-carbinolamine formation must not be less than $8 \times 10^6\ M^{-1}\ min^{-1}$. The corresponding constants for pyruvamide-semicarbazide carbinolamine formation, $\sim 5 \times 10^2$, although fast, do not approach such a value. However, semicarbazide is a weak base ($pK_a = 3.65$) and arelatively poor nucleophile while the (unprotonated) α-amino group of histidine is a strong one ($pK_a = 9.17$). There is considerable evidence that the nucleophilicities of amines toward carbonyl compounds parallel their basicities rather closely; the most convincing example of this, perhaps, arises from the recent temperature-jump studies of Diebler and Thorneley[12a] which show the rate constant for reaction of piperazine ($pK_a = 9.97$) with pyridine 4-aldehyde to be $2\text{-}3 \times 10^5\ M^{-1}\ sec^{-1}$, while the constant for piperazine monocation ($pK_a = 5.80$) is a mere $65\ M^{-1}\ sec^{-1}$. Using this as justification for a large and admittedly tentative extrapolation, the rate constant for the pyruvamide-histidine reaction might well be about $10^8\ M^{-1}$. Allowance for rather less than 1% of the α-amino groups in question being in the free base form at an intracellular pH ~7 leaves us no more than tenfold short of the requisite minimum, a factor readily accessible by enhancement of the free base contribution by a hydrophobic environment at the active site, combined, perhaps with a modicum of general catalysis to which pyruvamide carbinolamine formation is so susceptible. Thus, as Jencks observed many years ago, "rate acceleration by enzymes .... of the reactions of .... carbonyl compounds may be due to an acceleration of the decomposition of addition compounds rather than to acceleration of the addition of a nucleophilic reagent to the carbonyl group".[10]

The second step presents a very different picture. The turnover number of 2600 min^{-1} is equivalent to a second-order rate constant of $2.6 \times 10^8\ M^{-1}\ min^{-1}$ for acid catalyzed dehydration at pH 5, the pH optimum for the enzyme. At neutrality, the effective constant would be over 10^{10}. The second-order rate constants exhibited for acid catalyzed dehydration of pyruvamide carbinolamines, $2.5\text{-}8 \times 10^3\ M^{-1}\ min^{-1}$, fall short by a factor of $10^5\text{-}10^7$. Carbinolamine dehydration seems to be little affected by the basicity or nucleophilicity of the amine component per se; k_2 for acid-catalyzed dehydration of furfural-semicarbazide carbinolamine ($2.0 \times 10^6\ M^{-1}\ min^{-1}$) is almost identical with k_2 for the furfural-hydroxylamine adduct ($2.2 \times 10^6\ M^{-1}\ min^{-1}$)[10]. However, aniline catalysis of semicarbazone formation[23] and available data on the formation and hydrolysis of Schiff bases[24] indicate that rates of dehydration of carbinolamines from simple amines are, at minimum, considerably greater than those involving "α-effect" amines such as semicarbazide and hydroxylamine. Thus, there is no basis for a simple extrapolation such as that applied to the first step, although a remarkably fast spontaneous dehydration would seem necessary if no assistance were rendered by the enzyme protein. Fortunately, Schiff bases of pyruvamides with aliphatic amines are unusually stable and readily prepared.[4] Studies of them, currently in hand, should help to clarify the situation.

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cating the step in the reaction sequence and superscripts (H, PO_4, $H_2PO_4^-$, HPO_4^{2-}) denoting the catalyst, if any. PO_4 is total phosphate. K_{hyd}(unitless) is $[\text{II } H_2O]/[\text{II}]$ in water.
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Intramolecular Effects of Radioiodine Decay in *o*-Iodophenol, a Model for Radioiodinated Proteins[1]

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Abstract: As a model for the chemical effects of the decay of radioiodine in iodinated proteins, [^{131}I-^{14}C]-*o*-iodophenol has been synthesized and the iodine-131 allowed to decay in aqueous solutions containing various phenolic additives. Reference samples were also studied in which the radioiodine was added as $^{131}I^-$ to [^{127}I-^{14}C]-*o*-iodophenol. Similar products were observed from hydrolytic deiodination of the *o*-iodophenol, radiolysis of the molecule by external radiation, and as a result of decay of organically bound iodine-131. A mechanism is proposed in which decay of the iodine in [^{131}I-^{14}C]-*o*-iodophenol leaves a carbonium ion [$C_6H_4(OH)^+$] which in solution reacts to form catechol. It is postulated that the other products formed are oxidation products of catechol.

Although proteins have been radiolabeled with tritium[4,5] and carbon-14,[6] radioiodinated proteins have also been extensively used for both in vivo[7] and in vitro tracer studies.[8] In these studies an iodine atom is substituted for a hydrogen atom so that it is important to know that the molecule retains its biological activity. It has been suggested that one iodine atom on a protein molecule does not alter biological activity, but overiodination correlates with loss of activity.[9] In a protein iodinated at a high specific activity, there is a high probability that one protein molecule will contain more than one atom of radioiodine. Oncley has proposed a binomial formulation for the distribution of iodine atoms attached to a population of molecules.[10] Rosa et al. applied this mathematical treatment to human serum albumin with the prediction that at radioiodine levels of 0.5–1 atom per protein molecule, about 39 and 60%, respectively, of the iodine atoms are bound to molecules containing more than one iodine atom.[11] This theory presents a lower limit to the degree of multiple iodination in that anything less than perfect mixing would lead to regions with even more molecules containing more than one iodine atom. Therefore, at high levels of iodination there is a high probability that one radioiodine atom in a molecule will decay and leave the molecule still labeled. This nuclear decay process results in the release of very large amounts of energy to be distributed between the leaving nuclear particles and the residual heavy nucleus. In order to determine the molecular alterations caused by such a decay and to assess the significance of this local damage on the properties of a molecule, we have initiated a program to assess the chemical effects of iodine decay in model compounds.

The major site of iodination in proteins is at the tyrosine residue[12] to form 3-iodotyrosine which is a substituted *o*-iodophenol. Our initial studies have centered on the intramolecular effects of radioiodine decay in *o*-iodophenol. The method used to study this effect was to prepare doubly labeled [^{131}I-^{14}C]iodophenol and to measure the ^{14}C-labeled residues after β^- decay of the ^{131}I. In a comparison study high specific activity [^{127}I-^{14}C]iodophenol was prepared and the products of the decomposition of this molecule were measured. Any decomposition observed will be a product of hydrolytic deiodination caused by the weak carbon-iodine bond strength and radiolysis due to the effect of extramolecular radiation.

Methods. High specific activity (35 mCi/mmol) uniformly labeled [^{14}C]phenol (0.55 ^{14}C atoms/phenol molecule) (Amersham Searle Corp., Arlington Heights, Ill.) was iodinated with "carrier-free" protein iodination grade iodine-131 (Industrial Nuclear Corp., St. Louis, Mo.) using the chloramine-T method of iodination.[13] Phenol (10 μCi of carbon-14) was allowed to react with 50 mCi of iodine-131 at pH 8.5 using 100 μg of chloramine-T. The iodinated phenols were separated by liquid chromatography [Sephadex G-10-120, bed volume 15 ml, eluted with ammonium hydroxide–ammonium chloride (buffer 0.0025 *M*, pH 9.4) at 40 ml/hr]. In the labeling experiment only the *o*- and *p*-iodophenols were observed, and a good separation of the doubly labeled iodophenol was obtained. Labeling yields based on ^{131}I incorporation of >60% were obtained, of which ~70% was *o*-iodophenol. The *o*-iodophenol was stored at 2–4° at an initial concentration of 0.5 mCi/ml (0.5 mCi of ^{131}I/ml of $\sim 10^{-8}$ *M* iodophenol) containing 0.1 *M* ethanol and dissolved nitrous oxide as scavenger to reduce the amount of radiolysis. The pH was maintained at 7.0 using a