Kinetics of Solvolyses of Various *N*-Alkyl-*N*-nitrosoureas in Neutral and Alkaline Solutions

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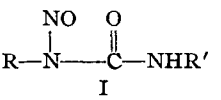
The *N*-alkyl-*N*-nitrosoureas possess antitumor activity but readily decompose at the pH of biological fluids with half-lives about 20 minutes at pH 7 and 37.5°. The effect of substituents, R, on the kinetics of solvolysis of synthesized R—N—(NO)—CO—NH_2 was determined in the phosphate buffer region, pH 6–7.8, as a basis for the proper design of biologically effective compounds. Spectrophotometric techniques were used over a range of several temperatures. The solvolytic rates were first order in substrate and hydroxyl ions and the log *k*-pH profile was linear with a slope of +1.0. No solvent or general acid-base catalysis was observed, and salt effects were negligible. No significant differences in reactivities, entropies, or heats of activation were observed for R = methyl, ethyl, and butyl. The 2-methylpropyl was slightly more reactive. When R = allyl, phenethyl, and benzyl, a significant increase in reactivities was observed. The unsaturated substituents favored hydroxyl ion attack as expected. However, the cyclohexyl compound also had high reactivity to hydroxyl ion attack.

THE ANTILEUKEMIC activities of *N*-nitrosoguanidines (1, 2) and *N*-nitrosoureas (2–4) have been recently observed. Active derivatives of the latter appear to have a higher degree of lipoid solubility, are essentially nonionized, and lack the propensity for plasma protein binding (3, 4).

Degradation studies (5) of the broad spectrum antibiotic streptozotocin (6) indicated that it contained an *N*-methyl-*N*-nitrosourea function, and the maintenance of the biological activity was correlated with the stability of this grouping. It may be postulated that the biological activity of the *N*-nitrosourea group is due to the alkylating action of a diazonium ion intermediate in aqueous solution which most probably goes through a transitory diazoalkane intermediate. This premise intimates that antitumor activity could be by alkylating action.

The correlation of structure with intrinsic activity is confused by the high instability of *N*-nitrosoureas in aqueous solutions at neutral pH values (5, 7). Thus, it is expected that significant degradation of these compounds would be effected in the gastrointestinal tract and in the blood *per se* (5) and may decrease effectively the body retention time of the intact molecule necessary for the exercise of its biological activity.

In general, the anticancer activities of *N*-alkyl-*N*-nitrosoureas (I)



I

were not significantly increased for R′ = H when the substituent R was varied (4). However, since most of the substitutents were more electron-attracting than R = methyl, it is possible that the evaluation of intrinsic biological activity was perturbed by enhanced reactivity to hydroxyl ion attack.

A systematic evaluation of the kinetics of solvolysis of substituted nitrosoureas should provide fundamental information concerning substituent effects on stability and reactivity. The former may permit the design of substitutents which will maintain stability of the compounds at the pH of biological fluids so that *in situ* degradation will not detract from biological activity. The latter may permit insight into the mode of action of these interesting compounds.

In addition, except for streptozotocin, kinetic studies on the solvolysis of this class of compounds in aqueous solutions have not yet been presented.

This paper considers the kinetics of solvolysis of various *N*-alkyl-*N*-nitrosoureas (I), R′ = H, in the phosphate buffer region.

EXPERIMENTAL

Preparation of N-Alkyl-N-nitrosoureas. N-Methyl, N-Ethyl, N-Butyl-N-nitrosoureas.—These ureas were prepared by the method of Werner (8). The substituted ureas (1.0 mole) and sodium nitrite (1.04 moles) were dissolved in water. The solutions were cooled below 0°, slowly siphoned into a mixture of concentrated sulfuric acid and ice, and stirred vigorously. After a few hours, the nitroso deriva-

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TABLE I.—RATE CONSTANTS (10^5k IN SEC.$^{-1}$) FOR APPARENT FIRST-ORDER HYDROLYSIS OF 10^{-4} *M* *N*-ALKYL-*N*-NITROSOUREAS AT VARIOUS pH VALUES OF PHOSPHATE BUFFERS[a] AT 35.0°

	pH				
Alkyl	5.95	6.49	6.82	7.18	7.75
Methyl[b]	4.32[d,e]	15.3	35.8	76.3	240
Ethyl	4.17	16.7	31.5	65.7	238
Butyl	4.85	15.7	32.5[d,e]	67.9	252
Isobutyl	4.90	17.0	38.4	87.2	298
Allyl	8.00	28.0	62.4[d]	116	375
Phenethyl	6.00	21.5	61.0	115	383
Benzyl	10.8	38.4	84.5[e]	182	566
Cyclohexyl[c]	200	867	...	...	...

[a] The pH, $[H_2PO_4^-]$, $[HPO_4^-]$, and ionic strength μ for the various buffer solutions were, respectively: 5.95, 0.0594, 0.0066, 0.08; 6.49, 0.0462, 0.0198, 0.11; 6.82, 0.0330, 0.0330, 0.13; 7.18, 0.0198, 0.0462, 0.16; 7.75, 0.0066, 0.0594, 0.19. [b] Additional 10^5k values for the methyl compound at 30.0° were: pH, 10^5k; 5.96, 1.97; 6.47, 5.90; 6.83, 17.1. At 37.5°, they were: pH, 10^5k; 5.91, 6.19; 6.55, 20.2; 6.91, 46.1; 7.17, 96.5. [c] Additional 10^5k values for the cyclohexyl compound at 25.0° were: pH, 10^5k; 5.80, 52.2; 6.40, 192. At 20.0°, they were: 5.82, 25.5; 6.40, 90.6; 6.76, 203. At 30.0°, they were: 5.90, 120; 6.44, 422. [d] When the concentration of the *N*-alkyl-*N*-nitrosourea was doubled, no significant difference in the apparent rate constants was observed. [e] When the concentration of the phosphate buffer solution was halved, no significant difference in the apparent rate constants was observed. No significant effect was observed in ionic strength ranges 0.10–0.17 for the reaction at the same pH and buffer.

tives, which had separated, were collected, washed, dried in a desiccator, and recrystallized from ether. Based on the substituted urea used, yields were 58% (methyl), 61% (ethyl), and 63% (butyl). Melting points were 121–123° (methyl), 100.5–101° (ethyl), and 82.5–84.0° (butyl). Melting points reported (8) are: methyl, 121°; ethyl, 103–104°; and butyl, 85°.

N-Allyl-N-nitrosourea.—This compound was prepared by the procedure of Marx and Marx-Moll (9). Ten grams of allyl urea was dissolved in 25 ml. of water and cooled. This solution was added to a mixture of 6 ml. of concentrated sulfuric acid and 10 ml. of water. With the temperature maintained below 5°, 8 Gm. of sodium nitrite dissolved in 20 ml. of water was added with stirring. The reaction mixture was filtered, rinsed with cold water, dried, and recrystallized from methanol. The yield was 94%, m.p. 80–81°. The literature value was 79.2° (9).

N-Isobutyl, N-Benzyl, N-Cyclohexyl-N-nitrosoureas.—These compounds were prepared by the method of Kirchner *et al.* (10). The ureas were dissolved in glacial acetic acid and water and cooled. A solution of sodium nitrite (3.1 eq.) was added slowly. After the reactions, the mixture was diluted with ice-cold water, filtered, and recrystallized from water–methanol. Yields were 30% (isobutyl), 26% (benzyl), and 30% (cyclohexyl). Melting points were 104.5°, 100.5°, and 110°, respectively. Literature values were isobutyl, 105° (11); benzyl, 101° (8); and cyclohexyl, 115° (12).

The *N*-phenethyl-*N*-nitrosourea was obtained from the Southern Research Institute of Birmingham, Birmingham, Ala., through the courtesy of Dr. John A. Montgomery. The synthesis of this compound has been reported previously (4).

Kinetic Studies.—The general procedure was to dissolve a specific *N*-alkyl-*N*-nitrosourea in 100 ml. of distilled water maintained at the temperature of the prospective kinetic study. This original solution was subsequently diluted 1:99 with the appropriate buffer solution which was also thermally pre-equilibrated. The difficultly solubilized *N*-benzyl, *N*-phenethyl, and *N*-cyclohexyl-*N*-nitrosoureas were initially dissolved in a small amount of ethanol prior to their dissolution in water. The alcohol concentration of the final reactant solutions was 0.4% by volume. In general, the final concentration of *N*-nitrosourea, about $10^{-4}M$, was optimum for reading spectrophotometric absorbances.

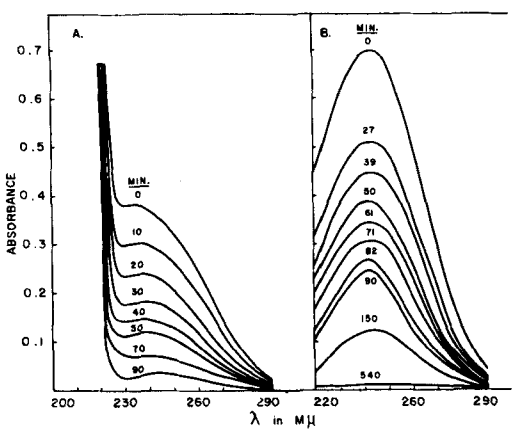

Fig. 1.—Typical curves of the spectral changes during the solution degradations of $10^{-4}M$ *N*-alkyl-*N*-nitrosoureas. The set A is for *N*-benzyl-*N*-nitrosourea (pH 6.45, 35.0°); the set B is for *N*-isobutyl-*N*-nitrosourea (pH 6.45, 35.0°). The time of the readings after the start of the reaction is given in minutes.

For the slow reactions, $t_{1/2}$ > about 30 minutes, the aliquots were cooled rapidly to room temperature before reading at the appropriate wavelengths on the Beckman model DU ultraviolet spectrophotometer. The rates of fast reacting materials, *viz.*, *N*-cyclohexyl-*N*-nitrosourea at pH 6.49, and *N*-allyl, *N*-phenethyl, and *N*-benzyl-*N*-nitrosoureas at pH 7.75, had to be obtained differently. The pertinent reacting solution was permitted to degrade in the thermostatted cell holder of the Beckman DU. The absorbance was plotted continuously on a time-calibrated Sargent SRL recorder with the aid of a Beckman energy recording adaptor (ERA). No photolytically catalyzed reactions were observed.

The conditions for the various studies and the composition of the various phosphate buffers are given in Tables I and II. When necessary, ionic

TABLE II.—RATE CONSTANTS (10^5k IN SEC.$^{-1}$) FOR APPARENT FIRST-ORDER HYDROLYSIS OF 10^{-4} *M* *N*-ALKYL-*N*-NITROSOUREAS AT VARIOUS TEMPERATURES AND AT pH 6.82[a]

Alkyl	30.0°	35.0°	37.5°	40.0°
Methyl	17.1	35.8	46.1	64.0
Ethyl	15.3	31.5	47.4	64.2
Butyl	16.0	32.5	49.6	73.2
Isobutyl	19.2	38.4	55.6	79.4
Allyl	28.8	62.4	87.5	118
Phenethyl	28.9	61.0	80.6	123
Benzyl	42.6	84.5	127	182

[a] The phosphate buffer was $[H_2PO_4^-]$ = $[HPO_4^-]$ = 0.0330, μ = 0.13, and the observed pH at any temperature was not significantly different.

strength was adjusted by the addition of potassium chloride. The wavelengths that were characteristic of the $\lambda_{max.}$ of *N*-alkyl-*N*-nitrosoureas were: methyl, 232 mμ; ethyl, 238 mμ; butyl, 240 mμ; phenethyl, 240 mμ; allyl, 240 mμ; isobutyl, 243 mμ; benzyl, 235 mμ; cyclohexyl, 240 mμ. The absorbance at this $\lambda_{max.}$ was read as a function of time until it approached an asymptotic value. Typical spectrophotometric curves as a function of time are given in Fig. 1 for *N*-benzyl-*N*-nitrosourea and *N*-methyl-*N*-nitrosourea and were recorded on a Cary ultraviolet recording spectrophotometer, model 15.

No significant effects were observed on variation of buffer concentrations, substrate concentrations, or ionic strength.

Verification of the fact that the ultraviolet absorption bands at about 240 mμ characterized the intact *N*-alkyl-*N*-nitrosoureas was obtained by thin-layer chromatographic analysis of aliquots of the *N*-benzyl- and the *N*-methyl-*N*-nitrosourea decompositions in phosphate buffers at pH 6.80 at 35.1°.

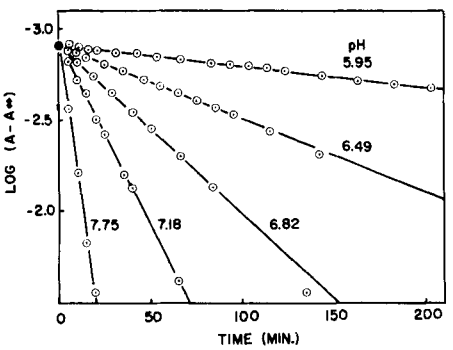

Fig. 2.—Typical apparent first-order plots for the loss of the 232 mμ absorbance at 35° of *N*-methyl-*N*-nitrosourea in various phosphate buffers where A_∞ is the asymptotic absorbance.

Calibration curves for the two compounds in HCl (pH about 3.1) were prepared by applying 10 μl. of known concentrations (10^{-2} to $10^{-3}M$) to 20 × 20 cm. thin-layer plates coated with a 0.4-mm. layer of silica gel G with phosphor indicator (catalog No. 8071, Research Specialties Co.). The plates were developed with chloroform–ethyl acetate (1:2) for the benzyl compound and chloroform–isopropyl alcohol (3:1) for the methyl compound. The solvent front was allowed to advance 13 cm. Detection was under ultraviolet light at 245 mμ. The diameters of the spots were measured in both a horizontal and vertical direction, and the mean value was used to calculate the area of a spot. Plots of spot area *versus* concentration were constructed as calibration curves. The R_f value for the benzyl compound was 0.88 and for the methyl compound was 0.67.

Five-milliliter aliquots of the degradation reactions at pH 6.80 initially 4.65×10^{-3} *M* for the benzyl compound and 8.45×10^{-3} *M* for the methyl compound were added to 1 ml. of HCl solution to obtain a pH of 3.1 where the compounds were stable. Then 10 μl. of the solutions was chromatographed as given above as a function of time for at least 90% of the decomposition as anticipated from

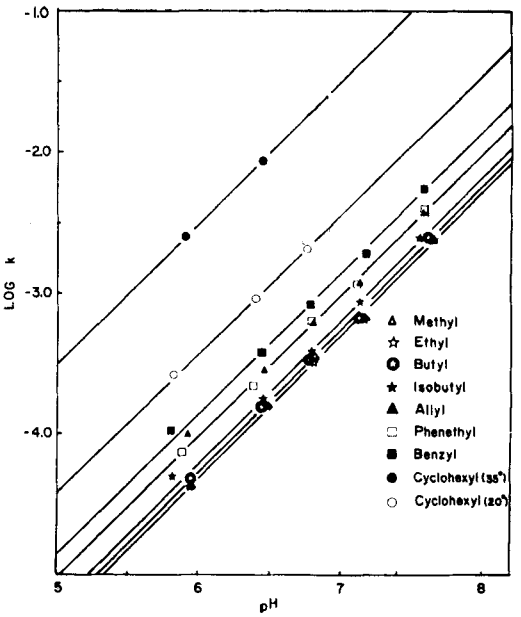

Fig. 3.—The log *k*-pH profiles for the solvolysis of *N*-alkyl-*N*-nitrosoureas at 35° of slope = +1.

spectrophotometric analysis. The areas of the developed spots were determined, and the corresponding concentrations were obtained from the calibration curves. The assays from spectrophotometric analysis and from thin-layer chromatography were coincident. The apparent first-order rate constants by the respective methods were 8.5×10^{-4} and 10×10^{-4} sec.$^{-1}$, respectively, for the benzyl compound; they were 3.6×10^{-4} and 3.3×10^{-4} sec.$^{-1}$, respectively, for the methyl compound.

No other spot was observed for the methyl compound by ultraviolet or sulfuric acid plus heat consistent with the expectation that methyl alcohol is the product of the degradation. The *N*-benzyl-*N*-nitrosourea showed the presence of a spot that had the same R_f value of benzyl alcohol. It was not readily observable under ultraviolet but was readily detected by sulfuric acid plus heat. These facts were also true for standard benzyl alcohol solutions spotted on the plates.

TABLE III.—BIMOLECULAR RATE CONSTANTS, $10^{-3}\,k_{OH^-}$[a] IN L./*M*/SEC. FOR SPECIFIC HYDROXYL ION CATALYZED SOLVOLYSIS OF *N*-ALKYL-*N*-NITROSOUREAS

Alkyl	30.0°	35.0°	37.5°	40.0°
Methyl	1.51	2.38	2.45	3.21
Ethyl	1.57	2.32	2.92	3.29
Butyl	1.68	2.38	3.28	3.76
Isobutyl	2.16	3.16	3.67	4.36
Allyl	3.23	4.30	5.65	6.30
Phenethyl	3.17	4.30	5.32	6.76
Benzyl	4.69	5.74	8.39	10.0
Cyclohexyl[b]	103	152	...	...

[a] The k_{OH^-} values were determined from slopes of the apparent first-order rate constants, *k* in seconds^{-1}, against [OH$^-$], where [OH$^-$] = $10^{-(pK\ -pH}$ where the pK_w appropriate to the temperature was used. In the case of some temperatures the estimate was $k_{OH} = k/[OH^-]$. [b] Additional $10^{-3}k_{OH^-}$ values for the cyclohexyl compound were 79.6 at 25.0° and 53.0 at 20.0°.

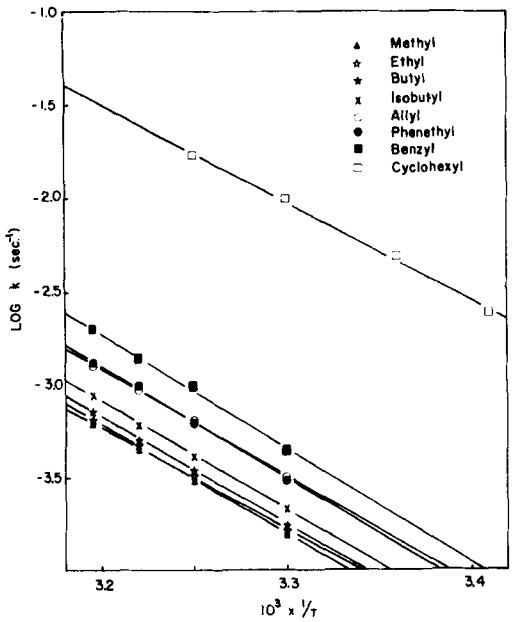

Fig. 4.—Arrhenius plots for the solvolysis of various *N*-alkyl-*N*-nitrosoureas at pH 6.82.

CALCULATIONS AND RESULTS

Rate Constants.—The first-order rate constants, k, were calculated from the slopes of plots of the logarithm of the difference in absorbance, A, at time, t, and the final absorbance, A_∞, against time in accordance with

$$\log (A - A_\infty) = -kt/2.303 + \log A_o \quad \text{(Eq. 1)}$$

Typical first-order plots for the solvolysis of these compounds are given for *N*-methyl-*N*-nitrosourea at various pH values (Fig. 2). The observed rate constants are given in Tables I and II.

Rate Dependency on pH.—In the log k-pH profiles for the solvolyses of the *N*-alkyl-*N*-nitrosoureas at 35° in the phosphate buffer regions studied, the slopes are positive and equal to unity (see Fig. 3). These facts indicated only hydroxyl ion catalyzed reactions in accordance with

$$\log k = \log k_{OH^-} - pK_w + pH \quad \text{(Eq. 2)}$$

where $k_{OH^-} = k/[OH^-]$ is the specific rate constant for the hydroxyl ion catalyzed solvolysis. These values are summarized in Table III.

Rate Dependency on Temperature.—The logarithmic version of the Arrhenius expression is

$$\log k = -[\Delta H_a/(2.303R)]1/T + \log P \quad \text{(Eq. 3)}$$

The Arrhenius plots for the data of the solvolysis of various *N*-alkyl-*N*-nitrosoureas at pH 6.8 are given in Fig. 4 as based on the data of Table II. The pertinent ΔH_a and log P values are listed in Table IV.

DISCUSSION

Prediction of Stability In Vitro and In Vivo.—The studied *N*-alkyl-*N*-nitrosourea solutions are unstable in the normal pharmaceutical pH ranges and temperatures. For example, the half-life of *N*-methyl-*N*-nitrosourea is 32 minutes at 35°, pH 6.8; *N*-cyclohexyl-*N*-nitrosourea has a half-life of 6 minutes at 20°, pH 6.76. Therefore, significant degradation of these compounds can be expected in the blood and the gastrointestinal tract.

Possible Mechanisms and Orders of Reactivities of N-Alkyl-N-nitrosoureas.—There exist two reasonable mechanisms (Fig. 5) for the hydroxyl ion catalyzed solvolysis of *N*-alkyl-*N*-nitrosoureas. The one would implicate direct participation of hydroxyl ion in the transition state (route A), and the other would involve charge transfer (route B). In the case of *N*-methyl-*N*-nitrosourea (7), route B had been postulated since amines have been reacted *in situ* to give high yields of *N*-substituted ureas. It was presumed that the reaction occurs with the formed isocyanic acid (R=H) or an alkyl isocyanate. Unfortunately, the isocyanic acid produced was not quantitative, and route A was just as feasible. The presumption that methyl isocyanate is transiently formed from *N*-nitroso-*N*,*N*′-dimethylurea in boiling water was based on the formation of methylamine and carbon dioxide (7). However, it can be seen readily that these products are available from route A through a classical amide solvolytic mechanism to a hydrolyzable carbamate as well as from route B, Fig. 5.

If the nonnitrosated amine were dialkyl substituted and a gross rate reduction was observed, then route B could be postulated as a primary route. The lack of observed general base-catalyzed solvolysis by acetate ion, however, is not consistent with a charge transfer hypothesis.

Since the reactivity of the transition state is postulated on electronic shifts, it could be predicted that electron accepting groups, R′ and R″, would accelerate the solvolysis of the nitrosoureas.

The sequence of reactivities for hydroxyl ion catalyzed degradations of the *N*-alkyl-*N*-nitrosoureas is cyclohexyl > benzyl > phenethyl = alkyl > isobutyl > butyl = ethyl = methyl, with very minor differences among the last four substituted compounds (Tables I and II). This sequence is almost the same as that for decreasing log P values, *i.e.*, decreasing entropies of activation, except for the cyclohexyl compound, where the heat of activation is significantly lower (Table IV).

This sequence is reasonably consistent with electron-withdrawing properties of the substituents which may abet hydroxyl ion attack by inducing a more positive center, *i.e.*, primary alkyl < allyl < phenethyl < benzyl, where unsaturated carbons are presumed to have greater electronegativity (13).

TABLE IV.—APPARENT THERMODYNAMIC QUANTITIES FOR SOLVOLYTIC DEGRADATION (k IN SEC.$^{-1}$) IN PHOSPHATE BUFFER (pH 6.82) FOR VARIOUS *N*-ALKYL-*N*-NITROUREAS

Alkyl	ΔH_a	log P
Methyl	26.5	15.3
Ethyl	26.6	15.4
Butyl	27.0	15.7
Isobutyl	27.0	15.8
Allyl	27.3	16.2
Phenethyl	27.5	16.3
Benzyl	27.8	16.7
Cyclohexyl	23.8	15.1

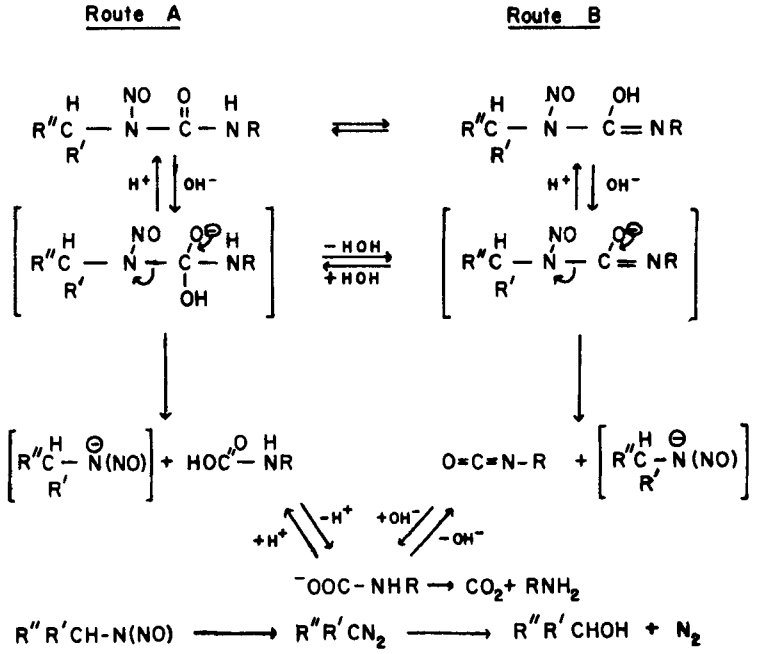

Fig. 5.—Possible routes for hydroxyl ion catalyzed solvolysis of substituted *N*-nitrosoureas.

The concomitant premise is that the electronegative phenyl group transmits its inductive powers throughout the alkyl chain, although its effect is more attenuated by two methylenes than by one (benzyl > phenethyl).

However, the cyclohexyl compound is anomalous in this rationale since its reactivity is the greatest of those studied, although a substituent linked through a secondary carbon should be less electronegative in its properties than the primary alkyl compounds. In addition, the isobutyl compound is not in the proper sequence that could be ascribed to inductive effects. The cyclohexyl and isobutyl compounds have one property in common, a tendency to restrict free rotation about the alkyl-nitrogen bond as per Fisher-Hirschfelder-Taylor models. Steric influences may predispose a configuration where the carbonyl carbon is more susceptible to hydroxyl ion attack. Ordinarily, the C=O and NO oxygens may be anti, but due to the enforced adjacency of these groups to permit rotation at the R'R''C—N bond, the transition states may be H stabilized and thus abet over-all reactivity.

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