Solvent-Dependent Conformational System of Hydroxyureas in Octanol–Water and in Inhibition of Ribonucleotide Reductase

GEORGE R. PARKER ˣ and E. COLLEEN MOORE *

Abstract □ The carbonyl nitrogen IR absorption region of selected hydroxyureas was examined in octanol. The spectra indicated appreciably different absorptions prior to and after equilibration with water. Stability studies on the hydroxyureas indicated that the absorption differences were not due to chemical decomposition in the equilibration process but were due to solvent conformational and/or tautomeric dependency of the system. Preliminary *in vitro* inhibition of the enzyme ribonucleoside diphosphate reductase by selected hydroxyureas also indicated an apparent solvent dependency, which may involve the conformational and/or tautomeric properties of these agents. The implications of the solvent-dependent conformational–tautomeric system observed are discussed in relation to this property in the biological action of these agents.

Keyphrases □ Hydroxyureas, various—solvent-dependent conformation in octanol–water, effect on ribonucleotide reductase activity *in vitro* □ Conformation—various hydroxyureas in octanol–water, effect of solvent equilibration □ Ribonucleotide reductase—activity, effect of various hydroxyureas *in vitro* □ Enzymes—ribonucleotide reductase, effect of various hydroxyureas on activity *in vitro* □ Structure–activity relationships—various hydroxyureas, solvent-dependent conformation in octanol–water, effect on ribonucleotide reductase activity *in vitro*

A solvent-dependent conformational system in substituted hydroxyureas was reported (1) utilizing solvents such as acetone, pyridine, and tetrachloroethane in the presence and absence of water. The conformational aspects of hydroxyureas were investigated, since this property may influence their transport, stability *in vivo*, and mechanism of action. Since there is no accepted explanation for the low anticancer activity of substituted hydroxyurea analogs compared to the parent antileukemia agent hydroxyurea, the conformational properties of hydroxyureas were examined.

In this study, the properties of the carbonyl–nitrogen system of substituted hydroxyureas were examined in the solvent 1-octanol before and after equilibration with water (pH 7.4); this system closely simulates the environment of *in vitro* and *in vivo* systems. The findings further substantiate conformational properties of hydroxyureas as detected by IR spectroscopy in 1-octanol. Initial biological data on the action of hydroxyureas on the enzyme ribonucleoside diphosphate reductase, the proposed site of anticancer action, indicate that the solvent plays a role in the *in vitro* inhibition of the enzyme, which may be due to the solvent-dependent conformation of certain hydroxyureas.

EXPERIMENTAL

Compounds—The 3-substituted analogs were synthesized according to a literature method (2) with modifications (3, 4). Hydroxyurea and the 1-substituted analogs were used as received[1].

IR Spectra—The IR spectra were obtained[2] using liquid cells. The solvent was 1-octanol[3] dried over anhydrous sodium sulfate. Spectra were taken immediately after dissolution, after equilibration with glass-distilled water (adjusted to pH 7.4) for 24 hr, and after 2 weeks.

After equilibration, the solvents were separated; the octanol layer was dried over sodium sulfate prior to recording the IR spectra. All spectra were taken with the following instrument settings: gain, 2; period, 1 sec; balance, 0; chart expansion, 1×; sensitivity, 3; ordinate scale, 100%; scan speed, 600 (8 min); and baseline transmission, 90%. All spectra were taken with dry octanol as the blank.

Stability Studies—The stability and decomposition of hydroxyureas in aqueous solutions were examined *via* the method of Harmon *et al.* (3). The solution of the hydroxyurea in a reaction vessel was connected to a gas dispersion tube immersed in anhydrous ether. Any gas evolved in the hydroxyurea decomposition was observed visually or in the IR spectrum of the ether solution after bubbling through the ether. IR spectra of the ether solutions after standing in water (pH 7.4), in acidic solutions at pH 1.5, and with ferric chloride added (in a ratio of 2 moles of hydroxyurea to 1 mole of ferric chloride) were recorded at intervals (up to 1 week for water at pH 7.4) using a double-beam instrument[4] and liquid cells with ether as the blank. The pH values were determined on a standard pH meter[5]. The pH of the aqueous layers in the equilibration experiments was also checked after 24 hr and 2 weeks and was in all cases 7.4 ± 0.2, indicating that no appreciable pH change occurred in the equilibration process. Water layers were removed by freeze drying[6] (at 20 mm Hg) to afford solids identical to starting materials, as verified by melting points and IR spectra.

Ribonucleotide Reductase Assay—The enzyme ribonucleoside diphosphate reductase was isolated from Novikoff ascites hepatoma of rats and purified according to the method of Moore (5). The assay included 8.3 m*M* phosphate buffer (pH 7), 2.1 m*M* adenosine triphosphate, 4.2 m*M* magnesium acetate, 0.04 m*M* $Fe(NH_4)_2(SO_4)_2$, 0.17 m*M* cytidine ^{32}P-diphosphate, 6.3 m*M* dithiothreitol, 1.0 μl of thioredoxin, and approximately 0.2 mg of partially purified ribonucleotide reductase in a total volume of 0.12 ml. Inhibitors were present in the reaction mixture (in an ice bath) when the enzyme was added; the mixture was then moved to a water bath at 37° and incubated for 30 min. The reaction was stopped with perchloric acid (1 *M*), and the products were hydrolyzed, separated on a cation-exchange resin[7], and analyzed according to the method of Reichard (6).

The hydroxyureas were tested five times over 6 months; each compound was evaluated at four different concentrations with two tests at each concentration. The enzyme activity was plotted *versus* the inhibitor concentration and extrapolated to determine the concentration required to inhibit 50% of the enzyme activity (ID_{50}). The control activities for the enzyme system in the five determinations were 2.28, 0.75, 4.16, 2.20, and 1.50 nmoles/30 min/tube.

RESULTS

The IR spectra of the carbonyl nitrogen region of the hydroxyureas in 1-octanol (with 1-octanol as blank) are shown in Fig. 1. The spectra labeled A were taken immediately after dissolving each compound in dried solvent; those labeled B and C were taken after equilibration with water (pH 7.4) for 24 hr and 2 weeks, respectively. In each case, octanol was separated from the water layer and dried over sodium sulfate, and the IR spectra were taken in the same manner as A.

Since B and C spectra were appreciably different from A spectra in every case except hydroxyurea, the stability of the compounds was examined using the method of Harmon *et al.* (3) to determine if chemical decomposition had occurred in the pH 7.4 water layer compared to so-

[1] Squibb Institute for Medical Research.
[2] Beckman IR 4250 double-beam instrument.
[3] Matheson, Coleman and Bell.
[4] Model 700 Perkin-Elmer IR spectrophotometer.
[5] Model 7, Corning.
[6] Virtis freeze-drying apparatus.
[7] Dowex 50.

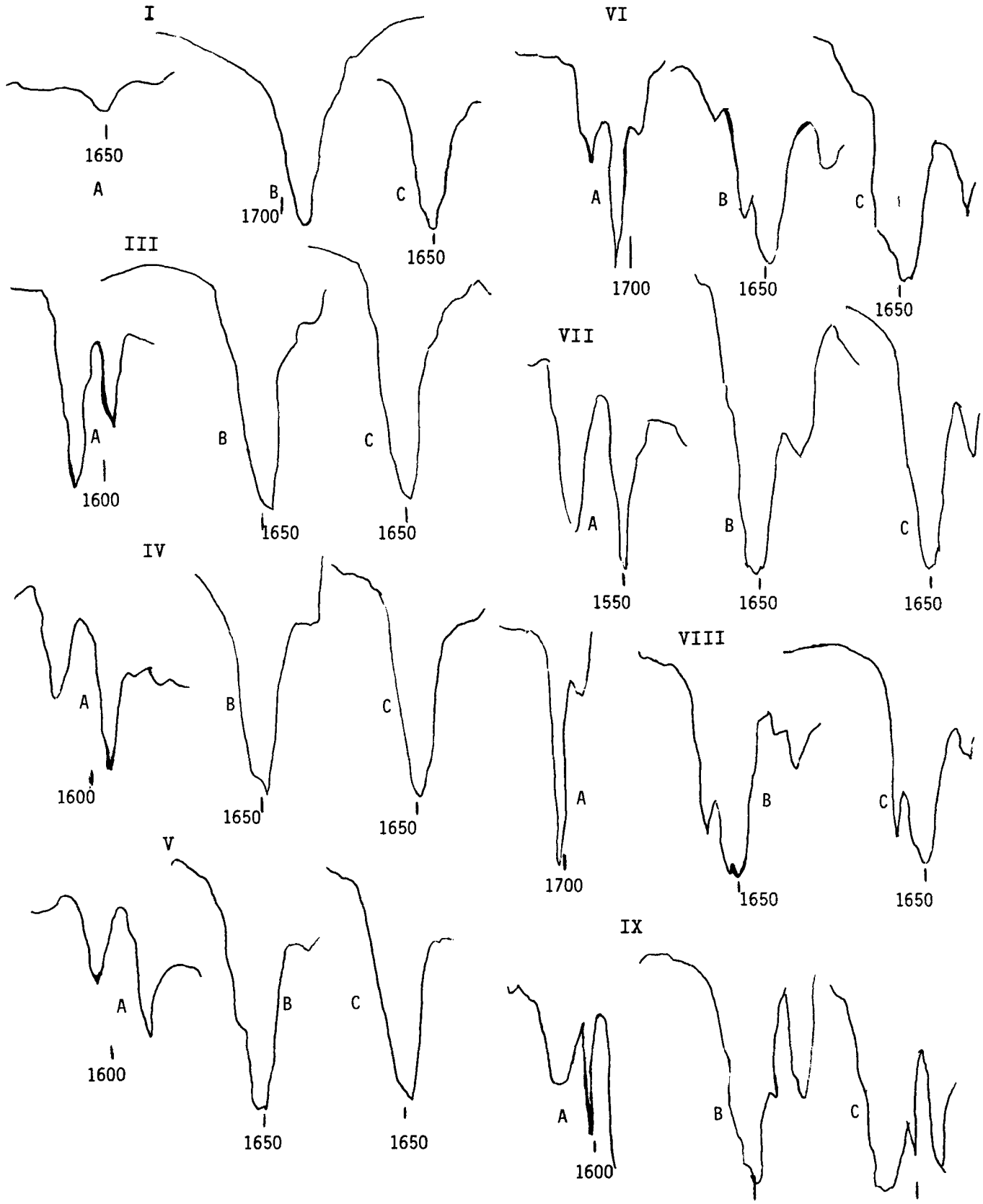


Figure 1—*The C=O and C=N regions in IR spectra of hydroxyureas. Spectra were taken in dry octanol after dissolution (A), after 24 hr of equilibration (B), and after 2 weeks of equilibration (C).*

lutions of pH 1.5 or in solution with ferric chloride (pH 1.5) (Table I). All compounds were stable in pH 7.4 water, whereas only I was stable in pH 1.5 water, with gas evolution observed for the other compounds.

The relative inhibitory concentrations of selected substituted hydroxyureas compared to hydroxyurea are shown in Table II. By using the method of Moore (5) for the purification of the enzyme and the method of Reichard (6) for the evaluation of the inhibitory concentration of the hydroxyureas, the concentration required for 50% enzyme inhibition was

Table I—Stability[a] of Hydroxyureas in Ferric Chloride Solution (pH 1.5)

$$R_1NHC(=O)NOH\text{-}R_2$$

Compound	R_1	R_2	Stability in Ferric Chloride Solution
I	H	H	Gas[b] plus blue color
II	H	CH_3	Gas plus blue color
III	H	C_2H_5	Gas plus blue color
IV	C_2H_5	H	Gas plus blue color
V	n-C_3H_7	H	Gas plus blue color
VI	iso-C_3H_7	H	Gas plus black color
VII	n-C_4H_9	H	Gas plus blue color
VIII	$tert$-C_4H_9	H	Gas plus brown color
IX	p-ClC_6H_4	H	Gas and no color change

[a] All compounds were stable in pH 7.4 solutions; only I was stable in pH 1.5 solutions, with gas evolution observed for the other compounds. Reaction vessels were connected to gas dispersion tube in ether; no signals were detected in IR spectra of ether solutions after 1 week. [b] Gas was detected according to method of Harmon *et al.* (3); signals were observed in the 2350–2200- and 600–700-cm^{-1} regions for all compounds where gas was observed. In most cases, gas was evolved immediately.

determined. Hydroxyurea and the 1-substituted analogs were dissolved in distilled water for all determinations. The 3-*n*-propyl analog (V) was dissolved in water and the 3-*n*-butyl analog (VII) was dissolved in 1 ml of 95% ethanol and diluted with water to 1% for the first determination. For the second, third, and fourth determinations, V and VII were dissolved in 6 and 10% dimethyl sulfoxide, respectively, and diluted to 1% with water. Ethanol and dimethyl sulfoxide were not inhibitory to the enzyme system at 1% concentrations.

A general trend in relative inhibitory activity was observed in all but the first determination (especially for V) where a different solvent was used. A fifth determination was performed with water as the solvent, and again a higher relative concentration was required for V to produce 50% enzyme inhibition compared to the concentration required when the solvent was 1% dimethyl sulfoxide.

DISCUSSION

For a drug molecule to exhibit its biological action at its molecular site of action in an *in vivo* system, it must first be transported from its original entry site to its target site. To simplify the dynamics of drugs in biological fluids (the biophase), various model systems attempt to approximate the biophase (an aqueous system with a nonpolar component present similar to a colloidal suspension) with, for example, the octanol–water system (7–9). The octanol–water system used in determining drug dynamics in complex *in vivo* biophases is a simplification of the actual occurrences but has been accepted as suitable for determining relative membrane *versus* water solubilities. It is the most extensively studied system depicting *in vitro* partitioning between polar and nonpolar biological components (10, 11).

The octanol–water system was selected as a model to evaluate the effect of lipophilicity or hydrophobicity in the biological action of substituted hydroxyureas. Surprisingly, the properties of the substituted hydroxyureas, as observed by their IR carbonyl nitrogen absorption (1720–1550 cm^{-1}), were different in octanol after equilibration with pH 7.4 water compared to spectra taken immediately after dissolving the substituted hydroxyurea in octanol (Fig. 1). This spectral change (C spectra compared to A spectra) after extended equilibration with water (2–4 weeks) occurred after 24 hr of equilibration with water (B spectra), and this process proceeded without a change of pH in the aqueous system. In spectra of all hydroxyureas except hydroxyurea itself, a change occurred in the carbonyl nitrogen region after water equilibration from two distinct peaks at around 1550–1580 and 1650–1690 cm^{-1} [1720 and 1780 cm^{-1} for the isopropyl analog (VI) and 1660 and 1710 cm^{-1} for the *tert*-butyl analog (VIII)] to a broad band centered around 1650 cm^{-1}. The spectra of VI–VIII also had additional shoulders in the broad band at 1650 cm^{-1}.

To determine if a chemical reaction occurred in the aqueous solvents during water equilibration, the compounds were allowed to stand in water (pH 7.4 at room temperature) in sealed flasks. These flasks were attached *via* polyethylene tubing to a gas dispersion apparatus in anhydrous ether. With this method, a gas could be detected immediately after the solution was acidified to pH 1.5 or after ferric chloride was added (with a corresponding color change to blue) for most hydroxyureas (Table I). IR spectra of the ether solutions (using a double-beam instrument with ether as the blank) also indicated the presence of decomposition gases after treatment with acid or ferric chloride. For the hydroxyureas in pH 7.4 water at room temperature, no decomposition gases were observed over 4 weeks, and no absorption could be detected in the ether solution spectra (taken at intervals) in which the gas dispersion tube had been immersed.

Table II—Relative Concentrations Required to Inhibit 50% Enzyme Activity (ID_{50}) of Ribonucleoside Diphosphate Reductase

Compound	Determination 1	2	3	4	5	Inhibitor Concentration Range, m*M*	Average Concentration
I	1.0	1.0	1.0	1.0	1.0	0.055–0.27	0.12
II	2.7	1.7	0.7	0.7	—	0.07–0.15	0.09
III	4.9	2.0	0.9	1.1	1.0	0.11–0.27	0.17
V	15.5	4.2	3.6	3.6	13.3	0.23–0.85	0.48
VII	9.1	6.4	5.8	7.0	7.5	0.35–2.0	0.84
VI	—		—	—	19.0	0.80	—
VIII	—		—	—	9.5	0.40	—

The solutes (hydroxyureas) also were examined for possible decomposition during the equilibration process. Attempts to remove octanol by evaporation under vacuum were unsuccessful (hydroxyurea slowly decomposed) due to the high boiling point of 1-octanol (194–195°), but the water layer could be removed by freeze drying under vacuum. The solids obtained from solutions treated by freeze drying (I and IV–VIII) were identical to starting material (melting points) and showed IR spectra in dry octanol identical to those obtained for the initial solution (*i.e.*, A spectra in Fig. 1). Identical solid materials also could be obtained when the octanol layer was washed with water and the water was removed by freeze drying under vacuum, indicating that chemical decomposition was not the cause of the IR spectral changes in octanol after water equilibration.

Various reactions of the hydroxyureas with water could account for the IR spectra, and the function of water in each reaction is different (Scheme I). Scheme I depicts the conformational system of hydroxyureas (I*e*, I*f*, I*j*, and I*k*) produced by interconversion through canonical forms I*g* and I*h*. Water could: (*a*) catalyze the tautomerization (as could the solvent) of the N-1 or N-3 proton to form tautomers I*a*–I*d* and I*l*–I*o*; (*b*) react with a form similar to canonical form I*h* to produce a diol (I*i*) by addition to the carbonyl function; or (*c*) react with a form that is not internally hydrogen bonded, such as conformer I*k*, *via* hydrogen bonding to form a solvated form of the hydroxyurea (I*p*).

In B and C spectras (Fig. 1) for all substituted hydroxyureas, there is an absence of absorption similar to that observed in A spectra, indicating that the chemical process that caused the new signals had proceeded to 100% completion. The presence of water in the original samples of hydroxyureas could account for different spectra after equilibration and drying due to the removal of all water. This possibility was eliminated when spectra taken after initial solution and those taken after drying over anhydrous sodium sulfate (for 1 and 24 hr) were identical (Fig. 2).

Of the three possible products that can form with water in octanol, the diol (I*i*) is the least likely and would be the most unstable. For diol formation to occur, the carbonyl carbon would have to possess a strong $\delta+$ charge ($\delta+CO^{\delta-}$); therefore, the canonical form I*h* would represent the form of hydroxyurea present. This form would require minimal double bond character between N^1C and N^2C bonds, which is unlikely since any positive charge that forms at the carbonyl carbon causes electron flow

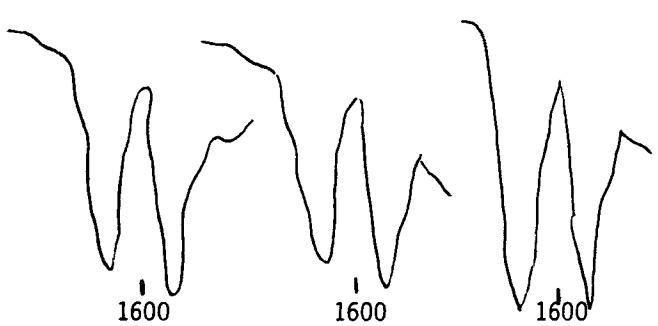
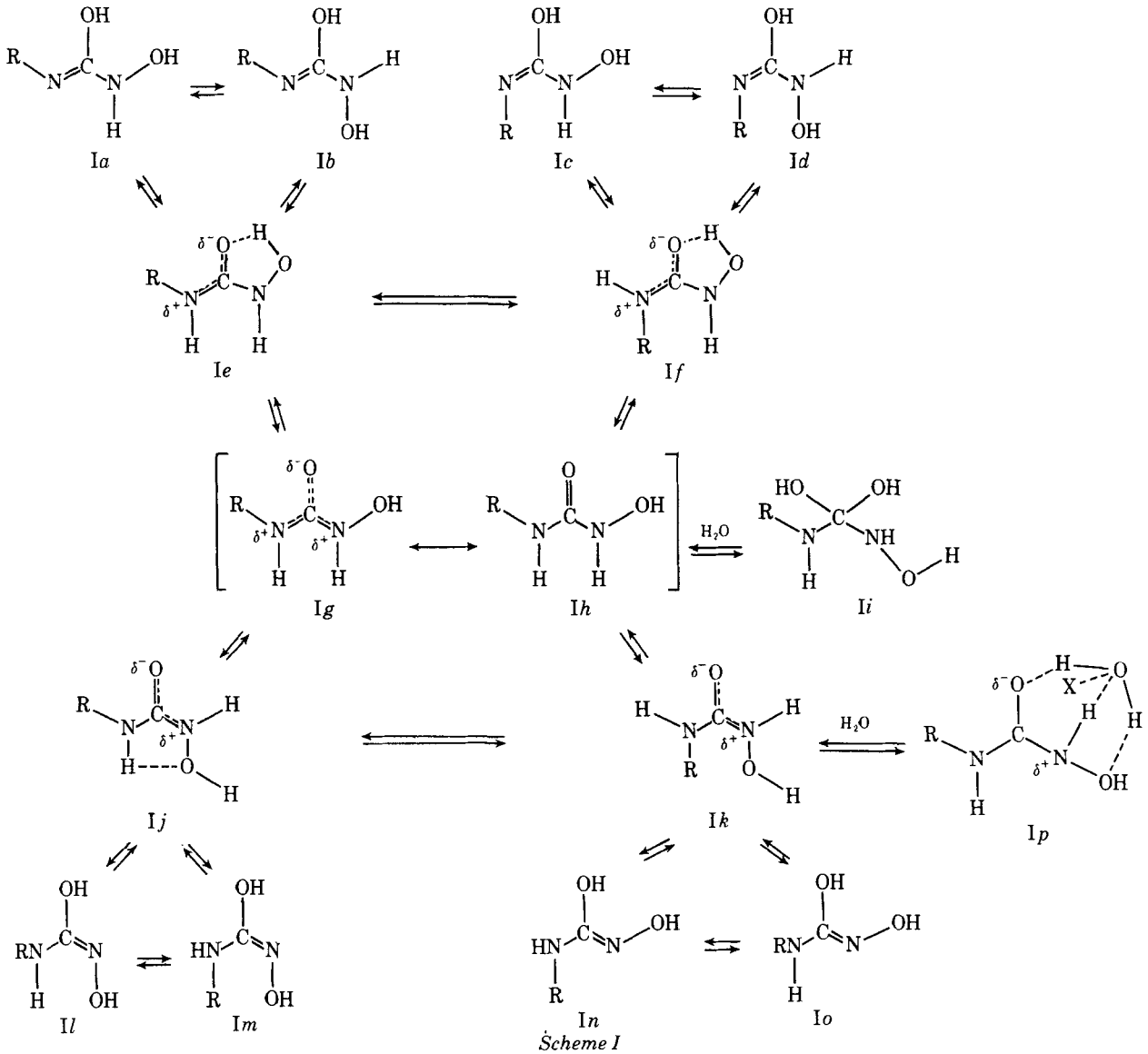


Figure 2—*Carbonyl nitrogen region of IR spectra of VII after dissolution in dry octanol (left) and after drying over anhydrous sodium sulfate for 1 (middle) and 24 (right) hr.*



Scheme I

from the nitrogens toward the δ+C=O to stabilize the carbonyl group. Since the carbonyl absorption completely disappears, it is highly unlikely that it is due to a 100% conversion to diol I*i*, which could not have a C=N system, and it would be hard to rationalize the strong absorption at 1650 cm^{-1}, which is probably due to C=N in the molecules.

The reaction of the hydroxyurea with water in the other two cases involves tautomerization catalyzed by water or the solvent to form tautomers I*a*–I*d* and I*l*–I*o* and the solvate formation with water to form I*p* (Scheme I). In tautomerization, water does not add to the molecule, but rather bond breaking and bond making are promoted by water or by the solvent. The spectral phenomenon observed in octanol also occurred in ether. Upon initial solution, two signals were observed; after water equilibration and drying, a single signal (also around 1650 cm^{-1}) was detected. Attempts to reproduce the spectral changes in a solvent without the addition of water were performed by allowing VII to stand for 3 days in dry solvents with proton affinity closer to water [proton affinities in kilocalories per mole (12) are: water, 164; 1-octanol, 202; and ether and tetrahydrofuran, 187]. Tests with tetrahydrofuran and ether were unsuccessful, indicating that the process observed when water was present did not occur in the dry solvent and that the molecular species in the dry solvents were probably the same.

Therefore, the spectral changes observed in octanol after equilibration with water are due to conformational and/or tautomeric changes similar to those reported in other solvents (1). The conformational and/or tautomeric changes are the result of the stabilization or destabilization of conformational isomers in each solvent system (Scheme I). This type of conformational system would be sensitive to the presence of water or other polar solvents or molecules (allowing hydrogen bonding to water or other polar groups rather than internal hydrogen bonding). The IR spectra of the hydroxyureas taken in dry solvents immediately after solution most likely depict conformers I*e* and I*f*, which are stabilized by OH···O=C hydrogen bonding, and conformer I*j*, which is stabilized by weaker NH···OH hydrogen bonds (Scheme I). All conformers (I*e*, I*f*, I*j*, and I*k*) are formed by interconversion through some intermediate. This intermediate may be similar to canonical form I*g*, where rotation around the NC bonds is restricted due to the C=N character, or the canonical form I*h*, which allows rapid rotation and interconversion.

In dry octanol, the internal hydrogen bonding may exist in preference to hydrogen bonding with the solvent. This effect would be observed in the IR spectra as complex signals due to conformers I*e*, I*f*, and I*j* and the canonical form present during interconversion. Conformer I*k* should not be an important contributor in the IR absorption, since it is not stabilized by internal hydrogen bonding and it would be quickly interconverted to the other conformers through canonical forms I*g* and I*h*. After equilibration with water, a form similar to I*k* is present. This form is stabilized by solvate formation with water, which does not allow it to interconvert back to the other forms due to hydrogen bonding with water. Since I*k* or its solvate form would be stabilized, it would not be able to interconvert into a canonical form, which would have a carbonyl absorption and would primarily possess a C=N absorption observed as strong absorptions at 1650 cm^{-1}.

The implications of these data are relevant to drug action if different forms of the hydroxyurea conformational system are present in preference to others, depending on whether the system is in a polar or a non-

polar environment and whether internal hydrogen bonding is stabilized or whether hydrogen bonding to a solvent or to other biochemical entities has occurred. Hydroxyurea appears to possess the same type of carbonyl nitrogen character before and after equilibration with water. Therefore, no conformational preference may exist in hydroxyurea in solutions where conformational preference may be conferred to substituted hydroxyureas by their bulk or steric hindrance or to some electronic stabilization factor. The crystal structure of hydroxyurea indicates that intermolecular hydrogen bonding exists between the hydroxyl group of one molecule and the carbonyl oxygen of another molecule rather than intramolecular hydrogen bonding (13), and internal hydrogen bonding may not be preferred in solution.

The *in vitro* inhibition of ribonucleotide reductase by selected hydroxyureas (Table II) relative to hydroxyurea seems to indicate a solvent dependency. Although the enzyme system is quite complex and its activity is dependent on many variables (14), its inhibition by hydroxyureas followed a general trend, except for the 3-*n*-propyl analog (V) in the first determination. The solvent for hydroxyurea and the 1-analogs (II and III) was water for all determinations, and water was used for V in the first determination. The solvent for all other determinations for V and VII was 1% dimethyl sulfoxide.

The inhibitory concentration required to obtain 50% enzyme activity with V was much higher in the first determination than in the other determinations (2–4 in Table II), and this result was due to some error in the enzyme determination or to the different solvent. To verify which factor was the determinant in the inhibition study, another analysis was performed (5 in Table II) in which water was the solvent for all compounds. Again the concentration required for 50% enzyme inhibition for V was appreciably higher than in determinations where dimethyl sulfoxide was the solvent. This result implicates the solvent as a potential variable in the ability of hydroxyureas to function in the *in vitro* inhibition of ribonucleoside diphosphate reductase, and this property should be considered in the elucidation of the biodynamics of drugs in the hydroxyurea class.

Although it is not possible to determine whether the solvent-dependent conformational system observed in substituted hydroxyureas is the actual cause of the poor inhibition of the enzyme, with V the conformation and solvent have an interdependence that may affect the biological action of the drug. The transport and partitioning properties of hydroxyureas in octanol–water are currently being investigated to determine whether predicted and actual partition coefficients support the solvent-dependent conformation and the importance of this property in drug transport *in vivo*.

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NOTES

Improved Recovery of Morphine from Biological Tissues Using Siliconized Glassware

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Abstract □ The adsorption of morphine onto glassware during analysis in aqueous solutions and biological tissues is a common problem, resulting in lower recoveries than expected. The problem can be remedied by siliconization of all glassware involved. The same glassware cannot be used repeatedly without incurring a significant loss of morphine.

Keyphrases □ Morphine—adsorption onto glassware during fluorometric analysis, effect of siliconization □ Adsorption—morphine onto glassware during fluorometric analysis, effect of siliconization □ Siliconization—of glassware, effect on adsorption of morphine during fluorometric analysis □ Narcotic analgesics—morphine, adsorption onto glassware during fluorometric analysis, effect of siliconization

Several methods including fluorometric (1, 2), flame-ionization (3) and electron-capture (4) GLC, and radioimmunoassay (5) have been used to measure morphine levels in biological tissues. The fluorometric procedure is still the most widely used method because of its simplicity and rapidity.