Hydrotropic Solubilization—Mechanistic Studies

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Purpose. This study examines the mechanism of hydrotropic solubilization using the riboflavin-nicotinamide system. The most commonly proposed mechanism for hydrotropic solubilization is complexation, and therefore, is investigated. Additionally, since nicotinamide and several other hydrotropic agents self-associate in aqueous solution, the possibility that self-association of the hydrotropic agent is important mechanistically is examined by studying the effect of temperature on hydrotropic ability. Researchers have shown that the degree of self-association decreases with increasing temperature. Therefore, if temperature affects the solubilizing capacity of nicotinamide, self-association must be mechanistically significant.

Methods. The complexation hypothesis is tested by looking at nicotinamide's ability to quench riboflavin fluorescence and by examining changes in the UV/Vis spectrum of riboflavin upon addition of nicotinamide. The solubility of riboflavin in nicotinamide solutions as a function of temperature is determined to assess the impact of self-association on hydrotropicity.

Results. Nicotinamide does not alter the intrinsic fluorescence of riboflavin nor are changes indicative of complexation observed in the UV/Vis spectrum. Temperature does have an effect on the hydrotropic ability of nicotinamide. Specifically, as temperature increases, the solubilizing capacity of nicotinamide decreases.

Conclusions. Because nicotinamide is unable to quench riboflavin fluorescence, and does not produce significant spectral changes, complexation of nicotinamide and riboflavin does not occur. However, since increasing temperature causes a decrease in the hydrotropic ability of nicotinamide and in its degree of self-association, it is proposed here that the self-association of nicotinamide impacts the hydrotropic mechanism.

KEY WORDS: solubilization; complexation; fluorescence; self-association; hydrotropic agent; nicotinamide.

INTRODUCTION

Increasing the water solubility of insoluble and slightly soluble compounds is of major concern. For parenteral formulations, the aqueous solubility of a drug is often the limiting factor for product feasibility. Development of viable solubilization methods is important, especially since many new therapeutic entities are large, sometimes marginally soluble proteins and nucleosides. Some solubilization techniques such as micellization and the use of co-solvent systems are commonly employed but may not always be desirable. For example, surfactants such as Cremaphor® and the Tweens used in micellized formulations have demonstrated various types of toxicities including liver damage (1). Additionally, pharmaceuticals formulated in propylene glycol-water co-solvent systems have been associated with precipitation of drug microcrystals upon intravenous injection and with myotoxicity upon intramuscular injection (2,3). Therefore, while solubilization of therapeutic agents is a key formulation goal, the development of safe, non-toxic methods of solubilization cannot be overlooked.

Aqueous solubilization can also be achieved via addition of hydrotropic agents. Hydrotropy refers to an increase in water solubility caused by the addition of large amounts of a second solute. This is in contrast to "normal" solution behavior since addition of a second compound, especially in large concentrations, generally causes precipitation of the less-soluble solute. Several hydrotropic agents such as urea, caffeine and other xanthine derivatives, tryptophan, certain antihistamines, sodium benzoate, sodium salicylate, and nicotinamide have been identified (4–9). Use of the term, hydrotropy, does not imply a mechanism, and for most hydrotropic systems, the solubilization mechanism is unknown. Suggested mechanisms include complexation, "salting-in", and changes in the nature of the solvent (10,11). The most commonly proposed mechanism is complexation and in some cases, complexation is indeed, the solubilization mechanism (most notably, caffeine). However, complexation does not explain all hydrotropic systems. A characteristic that many hydrotropic agents share is the ability to self-associate in aqueous solution, particularly at hydrotropic concentrations (i.e., >1M) (12–18). This correlation between hydrotropicity and self-association and the mechanistic implications have not been explored. To that end, a more comprehensive investigation into the hydrotropic mechanism was undertaken.

Nicotinamide, vitamin B_3, was chosen as the model hydrotropic agent because of its ability to solubilize a wide variety of compounds (9,19–24). Like many other hydrotropic agents, nicotinamide self-associates in aqueous solution, primarily as dimers and trimers and Kopecky' and co-workers found that the extent of self-association decreases with increasing temperature (16–18,25). Nicotinamide has the added attraction of being a potentially feasible formulation additive for two reasons: its very low toxicity, and its FDA approval status. In one of the earliest studies on hydrotropicity, nicotinamide demonstrated the ability to dramatically increase the solubility of riboflavin (9). These results were confirmed in our laboratories and showed a 36-fold increase in riboflavin solubility at 2.0 M concentrations of nicotinamide (25). Riboflavin, vitamin B_2, was chosen as a model of slightly soluble compounds for that reason and because it is easily assayed, is fluorescent, and consists of a relatively complex multi-aromatic ring system. This final attribute makes it an ideal representative of slightly soluble, aromatic compounds such as nucleosides and small peptides that may be formulated as aqueous solutions.

The primary goal of this study was to determine the mechanism of hydrotropic solubilization using the riboflavin-nicotinamide system. The possibility of complex-formation between nicotinamide and riboflavin was studied by determining the effect of nicotinamide on both the fluorescence and the UV/Visible spectrum of riboflavin. Additionally, the effect of hydrotrope self-association on hydrotropic ability was examined by studying the effect of temperature on solubilizing power.

MATERIALS AND METHODS

Chemicals

Riboflavin USP (RFN) and nicotinamide USP (NA) were used as supplied by Pharmavite (Los Angeles, CA). Caffeine,

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and cytosine were purchased from Sigma Chemicals (St. Louis, MO).

Fluorescence Studies

The ability of NA to quench the fluorescence of RFN was determined using an SLM Aminco 8000C® spectrofluorimeter (SLM Instruments, Urbana, IL). The excitation wavelength was set at 447 nm with the emission being measured at 530 nm. The excitation bandpass was set at 16 nm and the emission bandpass was fixed at 0.5 nm. In all experiments, solutions of RFN were prepared by adding the required amount of RFN solid either to deionized, distilled water or to NA solutions of the stated concentration. NA is not fluorescent. Three sets of experiments were performed. Each set of experiments used different concentrations of RFN and NA and are outlined in Table I. For all experiments, fluorescence of solutions containing RFN alone in the specified concentrations was measured. For each set of experiments, the sample containing only RFN at the highest concentration was used to autorange the fluorimeter output to show approximately 8–9 $\times$ 10^4 fluorescence units as recommended by the manufacturer, and all subsequent measurements made relative to the autoranged values. As positive controls, 0.01 M solutions of caffeine and cytosine (known RFN complexers) were used to dissolve enough RFN to produce a final RFN concentration of 7.0×10^{-6} M. Fluorescence of these solutions was measured as described above.

Effect of Nicotinamide on the UV and Visible Spectra of Riboflavin

The effect of NA on the UV and visible spectra of RFN was determined using a Beckman DU-7 Spectrophotometer® (Fullerton, CA) in the wavelength range of 300–500 nm. Sample solutions contained RFN 50 μg/ml alone or with NA in concentrations ranging from 0.1 to 2.0 M. Peak position and absorbance was monitored. Additionally, the spectra of solutions of NA (0.1 and 2.0 M) in the specified wavelength range were determined to insure NA did not absorb in this range.

Effect of Temperature on the Hydrotropic Nature of Nicotinamide

The effect of temperature on the hydrotropic ability of NA to solubilize RFN was studied by performing solubility studies at four different temperatures: 30°, 37.5°, 45°, and 50°C (±0.1°). The solubility of RFN in aqueous solutions was determined using the phase solubility method. A quantity of RFN far in excess of the intrinsic solubility (approximately 100 mg) was placed in a 25 ml scintillation vial. The vial was covered with aluminum foil in order to prevent photodegradation of RFN. To each vial, 20 ml of either deionized, distilled water (DDI-H_2O) or aqueous NA solution was added. The NA solutions ranged in concentration from 0.1 to 2.0 M. A small magnetic stir-bar was added and the vial sealed first with parafilm, then the vial screw-top cap, and finally with an additional exterior layer of parafilm. This was done in order to prevent leakage from the water bath into the vials. The vials were then placed in a Haake-D1® (Haake, Germany) magnetic-stirring, circulating water bath at the desired temperature and allowed to equilibrate, with stirring, for 18 hours. Determination of the time to achieve equilibrium is described below. The equilibrated mixture was filtered and diluted, then analyzed spectrophotometrically at 444.5 nm for concentration. Although NA does not absorb at this wavelength, a standard curve was generated using RFN in aqueous solutions of NA (10 and 20 μg/ml) to ensure that NA would not interfere with the RFN spectrophotometric assay.

Preparation of Aqueous Nicotinamide Solutions

Aqueous solutions of NA (0.1 to 2.0 M) were prepared using DDI-H_2O. pH was determined using a Fischer Accumet pH Meter® (Fairlawn, NJ) and found to remain constant over the concentration range studied at 7.20 ± 0.02.

Determination of Equilibration Time

The time required for aqueous RFN solutions to reach equilibrium was determined by analyzing for concentration at 8 hours, 16 hours, 18 hours, 24 hours, and 48 hours. Time to equilibrium was defined as the time at which RFN concentration in the filtrate did not change more than 4% and occurred between 16 and 18 hours.

RESULTS AND DISCUSSION

Fluorescence Studies

When complexes form between fluorescent and non-fluorescent molecules, the result is a marked decrease in fluorescence, a phenomenon known as fluorescence quenching. If complexation is the mechanism of hydrotropic solubilization, RFN fluorescence should be quenched upon its dissolution in aqueous NA solutions. It must also be noted that fluorescent molecules also exhibit the ability to self-quench at higher concentrations. For RFN, self-quenching is noted above concentrations of 7.0×10^{-6} M, which means that above 7.0×10^{-6} M, fluorescence of RFN will not be a linear function of concentration. Since the goal of these experiments is merely to qualitatively assess whether or not complexation occurs, a linear relationship between RFN concentration and fluorescence is not necessary. The only concern is an evaluation of fluorescence in response to the addition of NA at a single RFN concentration. In this study, three sets of experiments were performed. The results are shown in Table I.

The objective of experiment I was to study the effect of NA in hydrotropic (i.e., solubilizing) concentrations on RFN fluorescence. The sample containing only RFN 7.0×10^{-6} M was used to autorange the fluorimeter to 8.592×10^4 Fluorescence Units. There is approximately a 10-fold difference in fluorescence of the RFN 7.0×10^{-7} M sample, confirming that RFN concentrations are below self-quenching levels. No significant changes in RFN fluorescence at either concentration were seen upon addition of NA. This suggests complexation does not occur.

NA can increase RFN solubility even at very low concentrations (0.1 mM) (25). Since Experiment I showed no evidence of complexation at higher NA concentrations, the hypothesis that the mechanism could be composed of two or more distinct processes was proposed. Complexes have a definite stoichiometry, and generally the mole ratio of the two compounds does not exceed 1:10. Therefore, complexation would be more likely to predominate mechanistically at lower NA concentrations (i.e., concentrations where the NA concentration is approxi-

Table I. Effect of Nicotinamide on Riboflavin Fluorescence

Experiment	[RFN] (M)	[NA] (M)	Fluorescence Units × 10^4
	7.0×10^{-7}	—	0.9758[a] (0.015)
	7.0×10^{-7}	0.1	1.0090 (0.018)
	7.0×10^{-7}	0.5	0.9670 (0.014)
	7.0×10^{-7}	1.0	0.9957 (0.003)
I[b]	7.0×10^{-7}	2.0	1.1920 (0.036)
	7.0×10^{-6}	—	8.592 (0.087)
	7.0×10^{-6}	0.1	8.534 (0.170)
	7.0×10^{-6}	0.5	7.710 (0.503)
	7.0×10^{-6}	1.0	8.900 (0.036)
	7.0×10^{-6}	2.0	9.390 (0.335)
	7.0×10^{-7}	—	0.8081 (0.015)
	7.0×10^{-7}	7.0×10^{-8}	0.8739 (0.011)
	7.0×10^{-7}	7.0×10^{-7}	0.8597 (0.018)
II[c]	7.0×10^{-7}	7.0×10^{-6}	0.8670 (0.016)
	7.0×10^{-6}	—	7.760 (0.091)
	7.0×10^{-6}	7.0×10^{-8}	7.790 (0.110)
	7.0×10^{-6}	7.0×10^{-7}	7.953 (0.432)
	7.0×10^{-6}	7.0×10^{-6}	7.693 (0.086)
	0.05×10^{-5}	—	5.860 (0.073)
	0.05×10^{-5}	1.0×10^{-6}	6.224 (0.110)
	0.05×10^{-5}	1.0×10^{-5}	5.578 (0.036)
	0.05×10^{-5}	1.0×10^{-4}	6.263 (0.086)
	1.0×10^{-4}	—	7.650 (0.094)
	1.0×10^{-4}	1.0×10^{-6}	7.170 (0.087)
III[d]	1.0×10^{-4}	1.0×10^{-5}	7.360 (0.115)
	1.0×10^{-4}	1.0×10^{-4}	7.217 (0.120)
	1.0×10^{-4}	—	8.1190 (0.100)[e]
	1.0×10^{-4}	0.01	8.9060 (0.096)
	1.0×10^{-4}	1.0	8.689 (0.122)
	1.0×10^{-4}	2.0	9.357 (0.105)

[a] Average of 3 readings. Standard deviation in parentheses.
[b] Riboflavin below self-quenching levels; nicotinamide at hydrotropic concentrations.
[c] Riboflavin below self-quenching levels; nicotinamide at equitable mole:mole ratio.
[d] Riboflavin at solubility levels; nicotinamide at equitable mole:mole ratio and at hydrotropic concentrations.
[e] The last four experiments shown were performed separately. The fluorimeter was autoranged with the sample containing riboflavin 1.0×10^{-4} M alone.

mately equimolar with RFN). At higher NA concentrations, another mechanism would take over. This hypothesis was tested in Experiment II. In this set of experiments, RFN concentrations were below self-quenching levels, and NA concentrations were chosen to be approximately equimolar. Like Experiment I, no significant changes in RFN fluorescence were observed.

Experiment III was designed to parallel the solubility experiments as closely as possible. In order to avoid precipitation of RFN at room temperature, RFN concentrations were 25% and 50% of the intrinsic solubility at 30°C. These concentrations are above self-quenching levels, and therefore, the two-fold difference in RFN concentration does not produce a two-fold difference in fluorescence. However, a qualitative assessment of NA's effect on RFN fluorescence at a given concentration is valid. NA concentrations ranged from 1.0×10^{-6} to 2.0 M. As with the previous two experiments, NA was unable to quench RFN fluorescence.

These experiments were repeated with cytosine and caffeine. Other researchers have found evidence of complex-formation between RFN and these compounds using methods other than fluorescence detection (4,26). In this experiment, RFN concentration was 7.0×10^{-6} M and gave an intrinsic fluorescence of 9.943×10^4 fluorescence units. The addition of 0.01 M cytosine resulted in a 52% reduction in fluorescence while addition of 0.01 M caffeine resulted in an 80% reduction. Thus, the complexation that occurs between riboflavin and caffeine or cytosine is evidenced by a dramatic decrease in RFN fluorescence. From these fluorescence experiments, it appears that NA does not complex with RFN; hence, complexation cannot be responsible for the solubilization of RFN by NA.

Effect of Nicotinamide on the UV/Visible Spectrum of Riboflavin

In order to confirm that RFN and NA do not form complexes, the effect of NA on the UV/Visible spectrum of RFN was studied. RFN has two characteristic peaks at 374 and 444.5 nm. As NA was added to the solution in increasing concentration, a gradual bathochromic peak shift along with a decrease in absorbance was observed. At 2.0 M NA, the peaks had shifted 5 nm and absorbance of the major peak had decreased by 0.35 absorbance units from an intrinsic absorbance of 1.62. Spectral changes like those seen here have been associated with π-π complexation; however, π-π complexation requires a π-electron acceptor and a π-electron donor. Both NA and RFN are π-electron acceptors and would not be expected to enter into π-π complexes. Further, the spectrum was also scanned above 500 nm for the appearance of a new peak corresponding to a RFN-NA complex. No new peaks were observed. The spectral changes seen are not consistent with the formation of long-lived complexes. In fact, changes in peak position and absorbance commonly are seen when a compound is spectrophotometrically analyzed in different solvent media (27). Thus, the data presented in this section and the fluorescence data presented in the previous section support the hypothesis that NA exerts its hydrotropic action not via direct interaction with RFN, but rather by altering the nature of water as a solvent.

Effect of Temperature on the Hydrotropic Solubilization of Riboflavin by Nicotinamide

A study by Kopecky' and co-workers has documented that the extent of NA self-association decreases with increasing temperature. Comparing the degree of NA self-association that occurs at 40°C versus the freezing point, the results of this study indicated that more NA exists as aggregates, and that higher order aggregates are formed at the freezing point (18). Therefore, if self-association plays a role in hydrotropy, increasing temperatures should result in a decrease in the hydrotropic ability of NA. Figure 1 shows RFN solubility as a function of NA concentration at different temperatures. Solubility is plotted in terms of the solubility factor (the solubility of RFN at a given NA concentration divided by the intrinsic solubility at a specific temperature). The 30°C solubility curve lies far above the other three curves, indicating that the hydrotropicity of NA

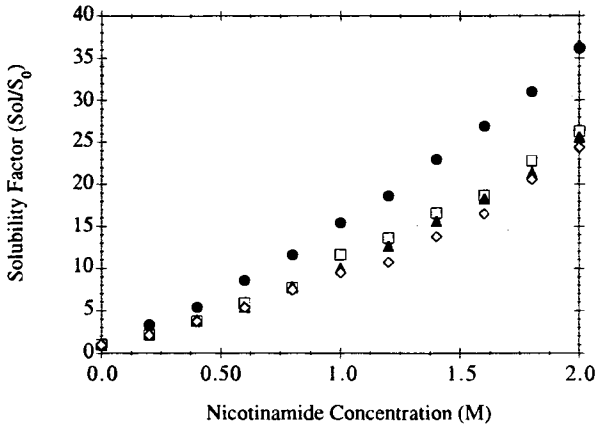

Fig. 1. Solubility factor as a function of temperature. Closed circles = 30°C; Open squares = 37°C; Closed triangles = 45°C; and Open diamonds = 50°C.

is most pronounced at that temperature. As the temperature increases, each solubility curve is sequentially lower. From this data, it is evident that temperature does affect the hydrotropicity of NA. Specifically, the solubilizing capability of NA is diminished as temperature increases. Since NA self-associates to a lesser extent at higher temperatures, it is proposed that hydrotrope self-association plays a role in the solubilization mechanism; however, further work is needed to fully characterize the self-association of NA at the temperatures studied here. It may be that the self-association in conjunction with very high concentrations of hydrotropes in aqueous solution actually changes the nature of water as a solvent. That is, hydrotropic systems may be thought of as co-solvent systems in which one component is a solid in solution rather than a liquid. This hypothesis would also be consistent with the spectral observations discussed in the previous section.

In conclusion, it is evident that for the RFN-NA hydrotropic system, the solubilization of RFN cannot be attributed to complexation. It was found that the hydrotropic ability of NA is inversely related to temperature. Because temperature also affects the extent and degree of NA self-association, the hypothesis that self-association of the hydrotrope is mechanistically important is proposed. Additionally, evidence is suggestive of hydrotropic systems behaving as solid-liquid co-solvent systems. Understanding the hydrotropic mechanism will enable researchers to identify potential hydrotropic agents for difficult-to-solubilize compounds and will aid in the formulation of compounds for which traditional solubilization techniques are not desirable.

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