garding effective concentration influences. The *in vitro* system for measuring drug release could not distinguish between these two systems due to the large receptor medium volume. The effective concentration parameter was swamped by the excess receptor fluid. However, this result did not occur *in vivo* due to the limited volume (about 7 μl) of precorneal fluid present. This finding also points out problems that can arise when such *in vitro* tests are used to predict *in vivo* performance for systems containing high effective drug concentrations.

The *in vivo* data for these two vehicles (Fig. 1) may not reflect the fivefold difference in effective concentration since the aqueous humor levels produced by the Absorption Base B–25% water vehicle were decreased by less than one-half those values achieved by the Absorption Base B–5% water vehicle. However, these results were quite reasonable if the dilution factor arising from the normal resident tear pool volume (7.5 μl) was considered. The water volume in a 25-mg dose of the Absorption Base B–25% water vehicle was 6.25 μl, with one-fifth this amount (1.25 μl) being present in an equivalent dose of the Absorption Base B–5% water vehicle. If each volume were to be mixed with the tear pool, the calculated decrease in precorneal drug concentration would be no more than 40%. Of course, such mixing would not be instantaneous in either case, but these considerations do explain the observed results.

The data in Fig. 4 show that the Absorption Base A–5% water vehicle was able to release more pilocarpine *in vivo via* shearing than was the Absorption Base A anhydrous vehicle. Inspection of the *in vivo* data presented in Fig. 1 also shows the emulsion system to be superior. The reason for this result may be that as the percent water in the vehicle is reduced to zero, the rate-controlling process is switched from mechanical rupture of the emulsion to dissolution or diffusion control. Further work to determine this mechanism is indicated.

A shearing component is a necessary feature for *in vitro–in vivo* data correlations with ophthalmic ointments. Since some form of shearing action, such as inunction, is common to nearly every semisolid topical dosing system and to many parenteral products, this parameter should have almost universal importance.

These studies show that significant drug bioavailability increases can be achieved by careful design of a system that incorporates shear-facilitated drug release. More importantly, the results also demonstrate that such systems can be used to reduce the total drug amount required for topical ophthalmic dosing. This implies a reduced systemic drug load and decreased side effects, particularly important points for pediatric dosing.

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ACKNOWLEDGMENTS

Supported by Allergan Pharmaceuticals, Irvine, CA 92664, and Grant EY-01332 from the National Institutes of Health.

Enhanced Chartreusin Solubility by Hydroxybenzoate Hydrotropy

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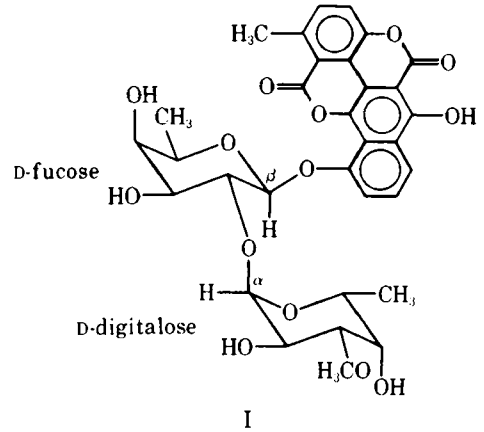
Received October 6, 1978, from the *Analytical and Product Development Section, Pharmaceutical Resources Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20014.* Accepted for publication November 30, 1978.

Abstract □ The apparent aqueous solubility of the water-insoluble cytotoxic agent, chartreusin, was increased at neutral pH in the presence of hydroxybenzoates. Water molecules play an important role in the chartreusin conformation. Studies included solubility and spectral examinations. The weakest and strongest interactants with chartreusin were sodium benzoate and sodium trihydroxybenzoate, respectively, while the effect of mono- and dihydroxybenzoates was intermediate. A plane-to-plane orientation of chartreusin and the ligand molecules brought together by electrostatic and hydrophobic interactions is postulated. The dramatic chartreusin aqueous solubility increase relative to its aglycone, chartarin, under similar conditions was best rationalized by micellization.

Keyphrases □ Chartreusin—aqueous solubility enhanced by hydroxybenzoates, conformation □ Hydroxybenzoates—electrostatic and hydrophobic interactions, stability constants □ Cytotoxic agents—chartreusin, aqueous solubility enhanced by hydroxybenzoates □ Antineoplastic agents, potential—chartreusin, aqueous solubility enhanced by hydroxybenzoates

Chartreusin[1] (I) produced by *Streptomyces chartreusis* was originally reported in 1953 (1), but its chemical structure was not fully elucidated until 1964 (2, 3). The chartreusin aglycone, chartarin (II), possesses essentially a planar ring system. The phenolic group at C-10 is glycosidically (β) bound to a D-fucose, which, in turn, is linked



I

by an α-glycosidic linkage to D-digitalose. Chartreusin has exhibited substantial chemotherapeutic activity in mice against the P-388 and L-1210 leukemias and, to some extent, against B16 melanoma (4). Biochemical studies demonstrated that I binds to DNA and inhibits RNA and DNA syntheses (5).

Low chartreusin solubility (15 μg/ml) inhibits preparation of reasonably concentrated aqueous solutions. Although the solubility may be increased to 2 mg/ml at a high pH (>9), these solutions may be irritating and incompatible with physiological fluids. More importantly, significant irreversible decomposition of these solutions is evi-

[1] Benzo[*h*][*l*]benzopyrano[5,4,3-*cde*][*l*]benzopyran-5,12-dione, 10-[[6-deoxy-2-*O*-(6-deoxy-3-*O*-methyl-α-D-galactopyranosyl)-β-D-galactopyranosyl]oxy]-6-hydroxy-1-methyl.

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dent within a few hours at room temperature. However, at neutral pH, various hydroxybenzoic acids increase chartreusin solubility.

This report describes the preparation of relatively concentrated aqueous chartreusin solutions and the qualitative and quantitative nature of the interactions with various mono-, di-, and trihydroxybenzoic acid sodium salts.

EXPERIMENTAL

Materials—Benzoic acid, salicylic acid, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, and 2,4,6-trihydroxybenzoic acid from the same commercial source[2] were used directly[3]. Chartreusin[4] (NSC-5159), mp 246–249° (benzene–methylene chloride), and chartarin[5], mp 311–314° [lit. (2) mp 315–316°], were used as received. All other chemicals were analytical reagent grade and were used without further purification.

Solubility Studies—Ligand stock solutions of known concentrations were prepared by dissolving the necessary ligand quantities in equimolar amounts of aqueous sodium bicarbonate solution. Excess chartreusin or chartarin was added to 15-ml screw-capped vials containing different aliquots of the proper stock ligand solution, and sufficient water was added to yield 10 ml of aqueous media. The vials were stirred mechanically in a constant-temperature water bath[6] (25 ± 0.1°). To prevent excessive hydroxybenzoate oxidation and to avoid chartreusin or chartarin hydrolysis, no samples remained in solution for more than 48 hr. Chartreusin and chartarin were stable in these solutions (pH ~ 6.0[7]) during the study period, which was sufficient to ensure equilibrium with all ligands tested.

The mixtures were centrifuged for 5 min at 25° and then filtered[8]. An aliquot of the filtrate was diluted quickly with a small amount of water, and the resulting solution was extracted with fixed volumes of chloroform. The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and diluted to the desired volume with additional dry chloroform. The solubilized chartreusin concentration was then measured spectrophotometrically[9]. The extraction procedure was necessary due to the limited chartarin solubility in water.

Following extraction with chloroform, the ligand concentration in the aqueous phase accounted for most (>98%) of the original ligand present as measured spectrophotometrically in the UV region. These same aqueous solutions had no absorption in the visible region, thus indicating that chartreusin and chartarin had partitioned essentially completely into chloroform and that the measured chloroform solution absorbances were due only to the free forms. The I and II concentrations were calculated from appropriate Beer–Lambert plots; I, λ_{max} (chloroform): 424 (log ϵ 4.31) nm; and II, λ_{max} (chloroform): 427 (log ϵ 4.26) nm.

The chartreusin and chartarin solubility diagrams were obtained at 25 ± 0.1° by plotting the observed solubilities against the added benzoate concentrations. The apparent stability constants for both compounds were calculated by the solubility method (7).

Precise solution surface tension[10] measurements were obtained using the Ring method by direct reading (dynes per centimeter).

RESULTS AND DISCUSSION

The solvent effects on the UV–visible spectra of chartreusin (0.025–0.027 × 10^{-3} *M*) in chloroform, ethanol, and water are shown in Fig. 1. The noticeable spectral perturbations in the strong protic solvent, water, may indicate intramolecular associations. These observations, based on the fact that water molecules can influence macromolecular conformation (8, 9) if they change any of the noncovalent bonds that stabilize macromolecular conformation, may explain the observed low chartreusin aqueous solubility. Such information, along with inspection of models, indicated a conformational change in the chartreusin molecule involving an intramolecular association or the folding up of the sugar portion over the aromatic moiety. Furthermore, the D-fucose 3-hydroxyl hydrogen is conformationally capable of hydrogen bonding to the digitalose pyranoside oxygen (10, 11). Such a possibility would create a cage-type chartreusin molecular conformation and diminish its hydration, resulting in the observed low water solubility.

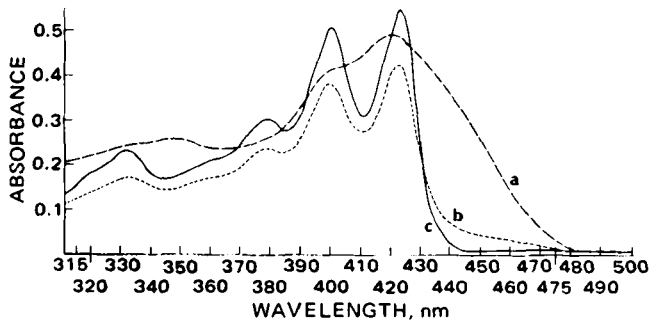

Figure 1—*Chartreusin UV–visible absorption spectra in water (a,– –), 95% ethanol (b,- - -), and chloroform (c,—).*

Hydroxybenzoates have a definite solubilizing action on chartreusin. As shown in Fig. 2, the amount of chartreusin in equilibrium with its solid phase increased progressively with the hydroxybenzoate concentration. The chartreusin solubility increase may be attributed partially to complex formation; however, due to the high reaction product solubilities and instabilities, the exact stoichiometry could not be determined. Nevertheless, the plots indicate that the dependency was nonlinear. The observations strongly support the suggestion that more than one ligand molecule interacts with an individual chartreusin molecule.

The I solubility diagram indicates that, among the ligands studied, the weakest interactant with chartreusin was the unsubstituted benzoate while the strongest was the 2,4,6-trihydroxybenzoate. Between these two were sodium benzoates with different hydroxyl substituents, all increasing I solubility.

The introduction of a hydroxyl group into the ring markedly enhanced the carboxylate "binding" tendencies. With the assumption of 1:1 and 1:2 interactions, the $K_{1:1}$ and $K_{1:2}$ association constants were calculated by the least-squares method (Table I) (7). The *ortho*-substituent had the strongest solubilizing effect on chartreusin. The enhancement effect of *meta*- and *para*-isomers on chartreusin solubility was somewhat less but still appreciable (Fig. 2 and Table I).

The sequential addition of a second or third hydroxyl group at different positions on the aromatic ring intensified chartreusin solubility on a mole to mole basis (Fig. 2). However, due to the limited solubility (0.15 *M*) of sodium 2,4,6-trihydroxybenzoate in water at 25 ± 0.1°, the apparent chartreusin solubility could not be increased more than 120-fold relative to that of water. The additional hydroxyl substitutions may lead to formation of other complexes independent of the original complex or may increase the initial complex strength while maintaining the same structure and, consequently, increase chartreusin solubility. Various ligands may have differently oriented interaction sites with chartreusin, or two complexes of different structures may coexist.

The chartreusin solubility equilibrium in the presence of dihydroxy-

Table I—Apparent 1:1 and 1:2 Stability Constants (M^{-1}) for Chartreusin and Chartarin Interactions with Various Benzoates in Water at 25 ± 0.1° Determined by the Solubility Method

Compound	Chartreusin		Chartarin	
	$K_{1:1}$	$K_{1:2}$	$K_{1:1}$	$K_{1:2}$
Benzoate	3.5	2.1	—[a]	—[a]
p-Hydroxybenzoate	5.9	9.0	—[a]	—[a]
m-Hydroxybenzoate	26.4	2.8	—[a]	—[a]
o-Hydroxybenzoate	5.7	29.1	0.25	10.5
2,4-Dihydroxybenzoate	17.2	48.3	—[a]	—[a]
2,5-Dihydroxybenzoate	30.5	27.5	1.6	28.1
2,6-Dihydroxybenzoate	32.5	38.6	—[a]	—[a]
2,4,6-Trihydroxybenzoate	263.7	17.4	7.9	29.1

[a] Interactions of these benzoates were not studied due to limited chartarin availability.

[2] Aldrich Chemical Co., Milwaukee, WI 53233.

[3] When used at the proper concentrations, the materials received were found to contain no significant impurities that interfered with chartreusin absorption in the region studied.

[4] Chartreusin occurs in several crystalline forms, and its melting point varies according to the degree of hydration and the recrystallization solvent, *i.e.*, mp 180° [acetone (1)], mp 184–186° [methylene chloride–ethanol (6)], and mp 244–246° [benzene–methylene chloride (5)].

[5] Cancer Research Unit, The Upjohn Co., Kalamazoo, MI 49001.

[6] Haake model E52, Haake, Saddle Brook, NJ 07662.

[7] Beckman Zeromatic pH meter, Beckman Instruments, Irvine, CA 92664.

[8] Millex, 0.45-μm Millipore filter, Bedford, MA 01730.

[9] UV–visible Cary 15 recording spectrophotometer, Varian Subsidiary, Monrovia, CA 91016.

[10] Du Nouy tensiometer.

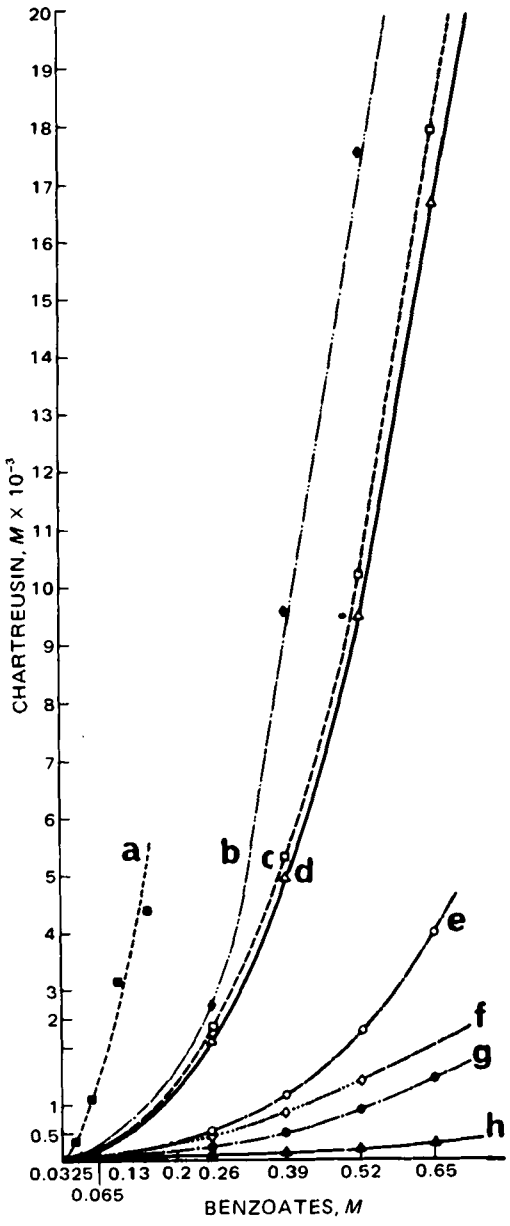

Figure 2—*Chartreusin solubility diagrams in the presence of sodium 2,4,6-trihydroxybenzoate (a, ■), sodium 2,6-dihydroxybenzoate (b, ♦), sodium 2,5-dihydroxybenzoate (c, □), sodium 2,4-dihydroxybenzoate (d, △), sodium salicylate (e, ○), sodium* m*-hydroxybenzoate (f, ◊), sodium* p*-hydroxybenzoate (g, ●), and sodium benzoate (h, ▲) in water at 25 ± 0.1°.*

benzoates, contrary to that with benzoate and monohydroxybenzoates, was achieved quickly (5 min) at concentrations up to 0.5 *M*. Higher ligand concentrations required 30 hr for equilibrium (Fig. 3). Moreover, a difference in the chartreusin solubility equilibration time with benzoate and various monohydroxybenzoates existed, the former being the slowest. Hence, the benzoate hydroxyl group(s) must play a key role in solubility equilibrium and in improving chartreusin solubility.

The increased chartreusin solubility in the presence of monohydroxybenzoates may be rationalized as follows. A hydroxyl group would stabilize the carboxylate anion by delocalization of the negative charge in the following order: *o*-hydroxybenzoate > *m*-hydroxybenzoate > *p*-hydroxybenzoate (12). Such a process would create a π-excessive aromatic system. Furthermore, in *o*-hydroxybenzoate, "ion-dipole interaction" between the phenolic hydrogen and the carboxylate anion (13) tends to keep the carbonyl group in a position coplanar with the benzene ring. Consequently, the carboxylate function π-electrons resonate with the aromatic ring π-electrons and thus increase the area of the π-cloud above and below the plane of the aromatic molecule.

Similarly, the two hydroxyl moieties in 2,6-dihydroxybenzoate are

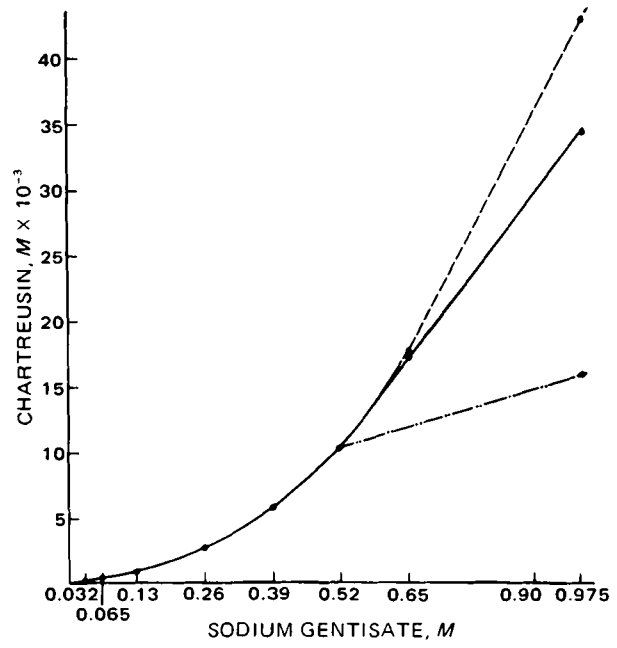

Figure 3—*Chartreusin solubility at 5 min (····), 24 hr (—), and 30 and 48 hr (- -) in the presence of increasing concentrations of sodium gentisate in water at 25 ± 0.1°.*

capable of forming two simultaneous intramolecular hydrogen bonds with the carboxylate anion, resulting in a greater planar π-cloud area relative to the 2,5- and 2,4-dihydroxybenzoates. Such an increase in the benzoate planar surface area may intensify the π–π interaction with the chartreusin aglycone portion, which possesses essentially a planar ring system, and, consequently, explain the observed solubility data: 2,4,6- > 2,6- > 2,5- > 2,4- > *o*- > *m*- > *p*- > sodium benzoate. Thus, charge delocalization on the carboxylate ion coupled with the increase in the ligand molecule π-cloud area would account partially for the difference in apparent chartreusin solubility in the presence of various benzoates. In addition, this relationship parallels the increasing pKa order of their respective acids (14), which reflects the inductive and resonance effects (12, 15).

The area correlation is good evidence for the gross structure of these interactions in solution, which may be described as a plane-to-plane orientation of the substrate and ligand molecules. Such molecular ar-

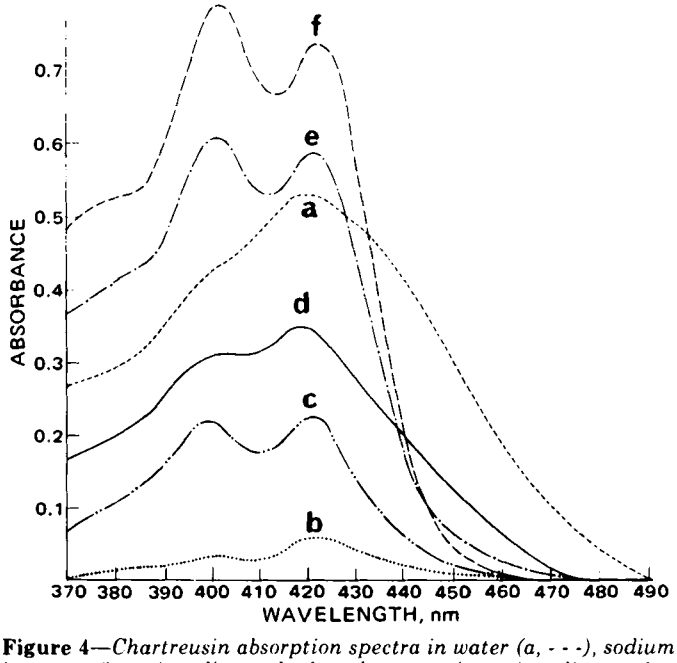

Figure 4—*Chartreusin absorption spectra in water (a, - - -), sodium benzoate (b, . . .), sodium* p*-hydroxybenzoate (c, ·—·), sodium* m*-hydroxybenzoate (d, —), sodium salicylate (e, —·), and sodium dihydroxybenzoate (f, - -) in water at 25 ± 0.1°.*

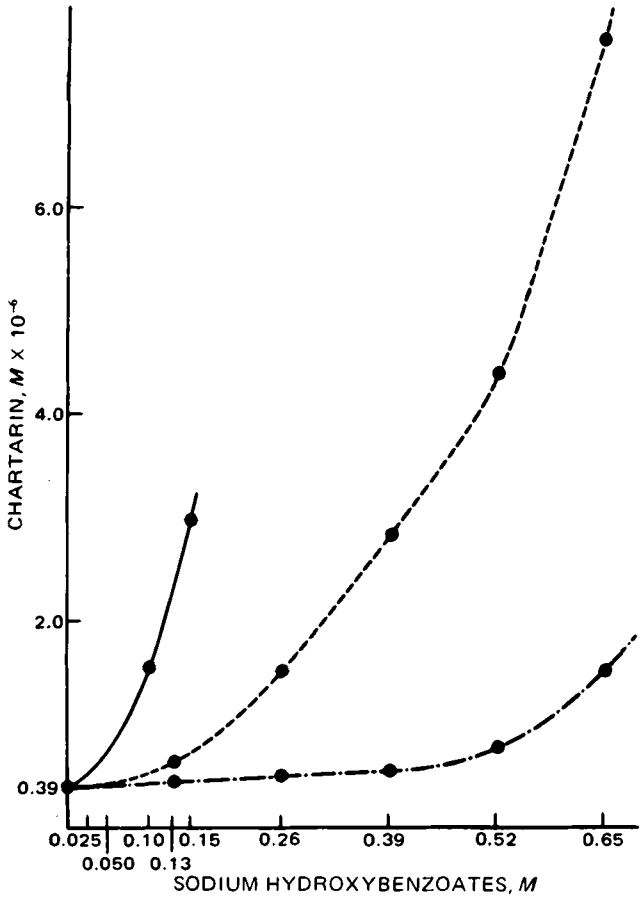

Figure 5—*Chartarin solubility diagrams in the presence of sodium salicylate (·–·), sodium 2,4-dihydroxybenzoate (- - -), and sodium 2,4,6-trihydroxybenzoate (—) in water at 25 ± 0.1°.*

rangements in solution may also indicate electrostatic interactions. Charge transfer forces have orientational properties (16), and their complexes are formed in simple molecular ratios (17). Figure 4 shows the I spectra in the presence of water and various benzoates. The chartreusin spectrum in the presence of benzoate(s) was modified to the extent that it resembled the chartreusin spectrum in chloroform (Fig. 1). This change was most prominent with di- and trihydroxybenzoates. This may be visualized as the chartreusin molecule unfolding due to its interaction with the benzoate, enabling the sugar portion to interact with water as well as with excess benzoate molecules and resulting in enhanced water solubility. Furthermore, a bathochromic shift (2 nm) of the 399- and 421-nm peaks, as well as a marked hyperchromic shift at 399 nm (Fig. 4), was observed.

These ligand-induced spectral changes suggested orientations favoring intermolecular interaction such as local dipole–dipole or dipole-induced–dipole interactions with perhaps some contact-type charge transfer contribution. Interestingly, the hydroxybenzoate sequence 2,4,6 > 2,6 > 2,5 > 2,4 > *o*-hydroxybenzoate > *m*-hydroxybenzoate > *p*-hydroxybenzoate that increased the chartreusin solubility is in the same order as their electron donor strengths, as would be expected from their

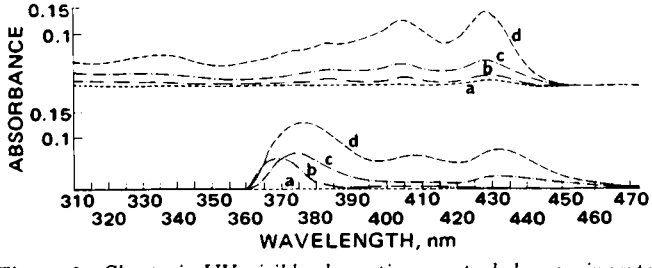

Figure 6—*Chartarin UV-visible absorption spectral changes in water (a, - - -) and in the presence of 0.13* M *(b, ·–·–·), 0.26* M *(c, ·–·), and 0.52* M *(d, – –) sodium gentisate (bottom) and following extraction of these solutions into chloroform (top).*

Table II—Chartarin Spectral Changes in the Presence of Different Benzoate Concentrations

Benzoate Concentration, *M*	Sodium Salicylate λ_{max}, nm	Sodium Gentisate λ_{max}, nm	Sodium 2,4,6-Trihydroxybenzoate λ_{max}, nm
0.025	—	—	328
0.050	—	—	335
0.10	—	—	342
0.13	—	369	—
0.15	—	—	345
0.26	342	373	—
0.39	343	—	—
0.52	344	377	—
0.65	345	—	—

ionization energies (18–20). Conjugation (21, 22) and electron donor groups decrease the π-electron ionization potential (23). Moreover, the polarization model for substituent effects gives qualitatively correct predictions. The addition of two substituent groups having opposite dipole moment directions with respect to the aromatic ring resulted in an ionization potential increasing in the order *o*-hydroxybenzoate > *m*-hydroxybenzoate > *p*-hydroxybenzoate (23). The effects of such substituents are additive but not necessarily linear. However, when studying electron donor–acceptor interactions in aromatic compounds containing heteroatom(s) capable of ρ–π-conjugation, one should recognize that *n*-donor or π-donor properties depend on certain factors (24).

To shed additional light on the interactions that increased chartreusin solubility, the chartreusin aglycone was studied. Chartarin is virtually water insoluble. The effect of various ligands on chartarin solubility is shown in Fig. 5. The shape of the curves corresponds to that of chartreusin. The order of the ligands that increased chartarin solubility was identical to that of chartreusin: trihydroxybenzoate > dihydroxybenzoate > monohydroxybenzoate. With the assumption of 1:1 and 1:2 interactions between chartarin and the ligand, the $K_{1:1}$ and $K_{1:2}$ stability constants were calculated (7) (Table I).

The characteristic chartarin UV-visible maxima were obtained in chloroform[11]. However, due to the low chartarin solubility, the corresponding UV-visible spectrum in water was unobtainable. Significant chartarin spectral changes were induced in the presence of various aqueous benzoates. These changes were observed as the difference spectra of ligand *versus* chartarin plus ligand. A typical difference spectrum is shown in Fig. 6. The $\lambda_{max}^{H_2O}$ at 404 and 427 nm had a 5-nm bathochromic shift, while all other bands[11] present in chloroform disappeared. However, a new absorption band appeared between λ_{325} and λ_{380} nm, depending on the ligand used (Table II). The new peak became the predominant one in the spectrum.

The magnitude of the bathochromic shift was dependent on the ligand concentration (Table II), and the extent of the red shift was more noticeable in the presence of the ligand with greater binding tendencies. On extraction with chloroform, the spectrum of each organic solution was identical to the original spectrum in chloroform (Fig. 6), while the remaining aqueous phase quantitatively accounted (>98%) for the ligand. The increase in chartarin solubility and the significant spectral changes in the presence of sodium hydroxybenzoate(s) support the charge transfer (24) interaction hypothesis.

There also appears to be hydrophobic contribution to the complex stabilization, since there is a direct relationship between the ligand surface area and the stability constants. Such interactions may be important for the association of solute molecules in water, although they may not be the dominant ones. Water, in addition to its role in hydrophobic bonding, may influence the stability of these complexes. Various investigators (25, 26) observed a qualitative correlation between complex stability and solvent surface tension. The solvent surface tension is the "master variable" in determining the complex stability (27). Chartreusin alone and chartreusin plus gentisic acid in chloroform afforded superimposable spectra in the visible region. In water, however, in addition to a significant reduction in the broadness of the shoulder between λ_{435} and λ_{475} nm, red shifts (5 nm) at λ_{421} and λ_{399} nm were observed.

These interactions, although operable in both chartreusin and chartarin, do not account for the remarkable preferential increase in chartreusin solubility. For example, chartarin solubility in the presence of 0.52 *M* sodium gentisate at 25 ± 0.1° was enhanced 10-fold relative to that in water, while chartreusin solubility was intensified more than 300-fold

[11] Chartarin: λ_{max} ($CHCl_3$) 266 (log ϵ 4.53), 274 (4.48), 338 (3.78), 383 (3.95), 404 (4.20), and 427 (4.26) nm.

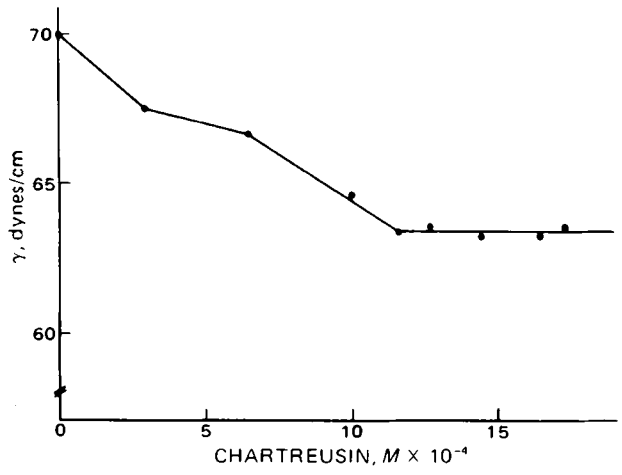

Figure 7—*Surface tension* versus *the chartreusin concentration in 0.26* M *aqueous sodium gentisate solution at 25 ± 0.1°.*

under identical conditions. Such a significant increase may be due to micellization. The commonly accepted picture of micelle structure comes from studies on compounds having a hydrophobic tail joined to a hydrophilic head. The micelles formed by these compounds above the critical micelle concentration (CMC) are nearly spherical aggregates with the heads on the surface and the tails forming a core. Such aggregates are stabilized by hydrophobic interaction between the hydrocarbon tails.

Chartreusin, once unfolded on interaction with the ligand, may be visualized as having a hydrophobic polyaromatic moiety joined to a hydrophilic disaccharide portion. Thus, one can assume that chartreusin molecule aggregates, similar to those of conventional detergent molecules, are built up by hydrophobic interactions between the aromatic surfaces, with the hydrophilic "heads" pointing outward and available to hydrogen bond with the excess ligand as well as with solvent molecules. Where conditions favor the formation of larger secondary micelles, two or more primary micelles could be linked by hydrogen bond donor and acceptor groups. Preliminary surface tension studies of chartreusin and sodium gentisate mixtures indicated possible micelle formation. When the chartreusin concentration was increased ($0–1.72 \times 10^{-5}$ *M*) in the presence of a fixed sodium gentisate concentration (0.26 *M*), a drop in surface tension to a constant minimum value was observed (Fig. 7) (28–30).

Thus, no single mechanism explains hydrotropy, but all of the above-mentioned factors play contributing roles in increasing chartreusin solubility.

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ACKNOWLEDGMENTS

Presented in part at the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1978.

The authors thank Dr. John A. Beisler, Laboratory of Medicinal Chemistry and Biology, National Cancer Institute, for helpful discussions and Dr. J. P. McGovren, The Upjohn Co., for providing the chartarin sample.