# Natural Cyclodextrins as Efficient Boosters of the Chemiluminescence of Luminol and Isoluminol: Exploration of Potential Applications

**Raquel Maeztu,[†] Gloria Tardajos,[‡] and Gustavo González-Gaitano*[,†]**

*Departamento de Química y Edafología, Universidad de Navarra, 31080 Pamplona, Navarra, Spain, and Departamento de Química-Física I, Facultad de Ciencias Químicas, Universidad Complutense, 28040 Madrid, Spain*

*Received: October 10, 2009; Revised Manuscript Received: December 17, 2009*

The chemiluminescent oxidation of luminol (LUM) and isoluminol (ISOL) is notably enhanced, both in intensity and duration, in the presence of natural cyclodextrins (α-, β-, γ-CD). The experiments have considered some of the most widespread applications of these compounds: the determination of metal cations and the revealing of bloodstains by oxidation with hydrogen peroxide in alkaline solution in the presence of Co(II), Fe(III), human hemoglobin, and blood, in order to explore potential applications. The largest enhancement in the emitted intensity occurs for the reaction of LUM with Co(II) in the presence of β-CD. The use of the more soluble γ-CD permits to expand the range of concentration and obtain more intense emission, although soluble derivatives of the β-CD (methyl, hydroxypropyl-β-CD, and a soluble cross-linked epichlorhydrin polymer) do not improve the chemiluminescence (CL) yield. In the case of hemoglobin and diluted human blood, the CDs aid in producing more light but only at high concentration of CDs, with a more lasting luminescence, up to three times longer. The changes in CL when glucose is used instead, much lower than with any of the CDs, imply that the cyclic structure of these oligosaccharides plays a key factor in the boosting of the emission. The results are explained in terms of the binding between the luminescent intermediate of the reaction, 3-aminophthalate (3-AP) and the CD, rather than to the luminescent reactant itself. The association constants obtained by steady-state fluorescence by assuming 1:1 stoichiometries reveal that the most stable association occurs between β-CD and the intermediate, in accordance with the trend in the chemiluminescence. The topology of the complex deduced via ROESY experiments confirms a shallow inclusion of the double-charged intermediate by the primary rim of the CD, which accounts for the low stability of the complexes.

## 1. Introduction

Chemical methods based upon the phenomenon of chemiluminescence (CL), i.e., production of light during the course of a chemical reaction, offer as advantages a high sensitivity and no requirement for sophisticated equipment, since the excitation source for irradiating the sample is not necessary. This leads to applications in quite diverse fields such as biotechnology, molecular biology, chemical analysis, and environmental chemistry, among others.[1−5]

One of the most efficient compounds in terms of emitted intensity and probably the most studied is luminol (3-aminophthalhydrazide), whose emission of blue light upon oxidation in alkaline solution was discovered by Albrecht in 1928.[6] The mechanism of this reaction was not elucidated until 40 years later, when E. H. White and co-workers[7,8] proposed the different chemical transformations undergone by luminol during its oxidation (Scheme 1). Since then, many studies have focused on the mechanism of this chemiluminescent reaction,[9−12] as well as the reactivity of other derivatives of luminol.[13,14] Although there is still some controversy about certain details of the mechanism, it is admitted that the cause of the light emission is the intermediate, 3-aminophthalate (3-AP, structure **V** in scheme 1), that comes out in highly basic media (pH > 11).

The interest in luminol lies mainly in the fact that different transition metals can catalyze the reaction and in its comparatively high quantum yield of luminescence (0.01 in water and 0.05 in DMSO[15]). This makes the reaction useful for the quantification of metal cations,[16,17] but probably the most well-known application is to reveal the presence of bloodstains, thanks to the catalytic properties of hemoglobin.[18] It is also employed as a luminescent probe in protein or DNA labeling in immunoassays to identify antibodies.[19,20] Substitution in some positions of the heterocycle can be chosen according to the needs, although the derivatives are usually less emissive than the parent luminol[21−23] and other approaches are necessary for enhancing or, at least, maintaining its luminescent power.

One way of modifying the emission of luminescent molecules, mostly their fluorescence, is by formation of supramolecular associations with cyclodextrins (CDs).[24] These are torus shaped, cyclic oligosaccharides, formed by 6, 7, or 8 glucose units (α-, β-, and γ-CD, respectively) which configure a hydrophobic cage that may lodge a guest molecule of appropriate size. The included molecule can gain in stability against photodissociation, undergo changes in its absorption and emission spectra, modify its aggregation properties, etc.[25] The possibilities expand if the CD is chemically modified by substitution of the hydroxyl groups or polymerized with an appropriate cross-linker.[26]

In spite of the overwhelming number of studies with CDs, there are quite a few investigations on CDs and CL. Some chemiluminescent guests used are, for example, peroxioxalates,[27,28] imidazoles,[29,30] or acridinium salts.[31,32] In the case of luminol derivatives the results are sometimes contradictory. Thus, in certain studies, β-CD increases the CL of luminol[33] and an

* To whom correspondence should be addressed. E-mail: gaitano@unav.es.
[†] Universidad de Navarra.
[‡] Universidad Complutense.

10.1021/jp909707x 
Published on Web 02/04/2010

**SCHEME 1: Mechanism of the CL Reaction of Luminol (Ref 7)**

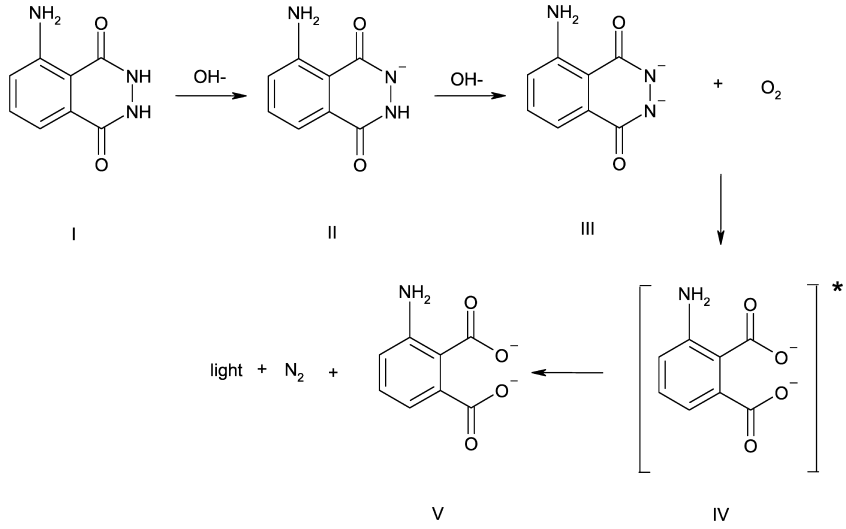
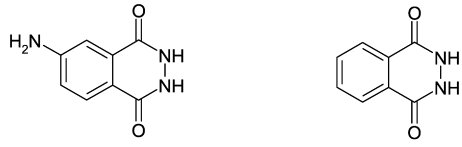



**CHART 1: Molecular Structures of Isoluminol (ISOL) and Phthalhydrazide (PHY)**




isoluminol derivative,[34] whereas in other investigations the emission diminishes in the presence of CDs.[35,36]

The present work is based on the hypothesis that the CD may offer a protective environment that can either stabilize the luminescent intermediate of the CL reaction or form a complex with the reactant itself, producing a more lasting and/or intense emission with all the potential applications that this may offer. To prove this hypothesis we have (i) investigated how the CL of luminol is modified in the presence of natural and some substituted CDs, either in the intensity emitted or its duration, with the catalysts that produce the highest CL (Fe(III), Co(II), hemoglobin, and human blood); (ii) studied by fluorescence spectroscopy and NMR the stability and structure of the supramolecular complexes of the parent luminol, and its luminescent intermediate 3-aminophthalate, and other analogues such as isoluminol (4-aminophthalhydrazide) and phthalhydrazide (Chart 1); and (iii) discussed the results in terms of the role that the complexes have in the mechanism of the reaction, exploring possible applications.

## 2. Materials and Methods

**2.1. Chemicals.** Luminol (LUM) and its derivatives isoluminol (ISOL), phthalhydrazide (PHY) and 3-aminophthalic acid, were supplied by Sigma Aldrich and used without further purification (98, 98, 99, and 90% purities, respectively). Human hemoglobin and D-(+)-glucose (99.5% purity) were supplied by the same company. Panreac provided $Co(NO_3)_2 \cdot 6H_2O$, $K_3Fe(CN)_6$, NaOH, and $H_2O_2$ 30% v/v.

Natural CDs (α-, $\beta$-, and $\gamma$-CD, 98% purity) were obtained from Wacker, with water content of 11, 14, and 11%, respectively, as determined by thermal analysis. Hydroxypropyl $\beta$-CD (HP-$\beta$-CD) and methyl-$\beta$-CD (Me-$\beta$-CD) were supplied by Cyclolab with substitution degrees of 4 and 12 OH groups. A soluble $\beta$-CD polymer (reference CYL-265, $M_w$ = 5600 g mol$^{-1}$, 50−55% in CD) was acquired from Cyclolab. The polymer was fractionated prior to its use with an ultrafiltration membrane of 5000 MWCO (Millipore) under $N_2$ (60−70 psi), in order to exclude residues of nonreacted $\beta$-CD or epichlorhydrin.

For the CL measurements with blood, this was extracted and frozen in the presence of heparin to prevent coagulation and preserve its features (0.2 cm$^3$ heparin v/v). Two luminol-based commercial kits used for the revealing of bloodstains in crime scene were bought to Novakit Biotech (Bluestar Forensic and Bluestar Forensic "Training").[37]

**2.2. Chemiluminescence Assays.** The CL measurements were performed in a Perkin-Elmer LS50B spectrofluorimeter in the bioluminescence mode. Two types of experiments were carried out: (a) scanning of the CL emission over a wavelength range, and (b) measurement of the CL at a fixed wavelength along time. In the first case the scan rate was set to the maximum allowed (1500 nm/min). For the time drive measurements, the $\lambda_{max}$ in the emission spectrum has been chosen, varying between 410 and 420 nm in each case. The emission slit width was also set according to the luminescent yield of each reaction (12−15 nm), and a 1% transmittance filter was employed in the emission monochromator in the cases the CL emitted was too intense. All the measurements were reproducible after duplicate assays.

The concentrations of the luminescent compounds were fixed at 1.0−1.2 mM, and prepared in 0.5 M NaOH. The oxidant agent was $H_2O_2$ 30% v/v in a final concentration in the cuvette of 0.02−0.1 M. Aqueous solutions of Co(II), Fe(III), human hemoglobin ($9 \times 10^{-5}$, $9 \times 10^{-4}$, and $2 \times 10^{-6}$ M), and human blood (1:100 dilution) were used as catalysts. The addition of oxidant is not required for the assays with the Bluestar kits since the peroxide is included in the formulation.

For the bloodstain tests, pieces of cotton fabric and a plate for tests were impregnated with 25 $\mu$L of diluted blood (1:1000). After drying, a solution of luminol (200 $\mu$L) in the absence or presence of CD was added, followed by 25 $\mu$L of $H_2O_2$ 30% and the emission recorded in the darkness with a digital camera at 30 s intervals. A Nikon digital camera mounted on a tripod was used to take the photographs with a time exposure of 8 s, aperture *f*-4.6, and a 400 ISO setting.

**2.3. Steady-State Fluorescence.** Fluorescence measurements were performed with an Edinburgh FLS920 spectrofluorimeter. The excitation wavelengths corresponded to maxima in the absorption spectra of each compound (a HP-8452A spectrophotometer was used), and the emission spectra were recorded

at 120 nm/min with different slit widths, according to the fluorescence of each compound (1.5−3.0 nm in excitation and 4.5−9.0 nm for the emission). 1.000 cm path length quartz cells were used both in fluorescence and in absorption, and the temperature was controlled at 25 ± 0.1 °C with an external heating bath (Lauda Ecoline E100).

Concentrations of LUM, ISOL, PHY, and 3-AP were fixed at $2.1 \times 10^{-5}$, $1.8 \times 10^{-5}$, $4.4 \times 10^{-5}$, and $8.0 \times 10^{-5}$ M, respectively. These solutions were prepared in 0.5 M NaOH in order to reproduce the conditions in which the CL reaction takes place. The measurements of the fluorescent spectra upon increasing the CD concentration were carried out by direct addition of a concentrated CD solution to the measuring cell. This was prepared by dissolving the CD in the same stock solution as that of the luminescent agent (0.1 M for α- and γ-CD, and $1.4 \times 10^{-2}$ M for β-CD).

**2.4. 1D and 2D $^1$H-RMN.** 1D proton NMR spectra were recorded at 298 K in a Bruker Avance 500 Ultrashield, by averaging 32 scans. The samples were prepared in $D_2O$ (99.9% in deuterium Sigma Aldrich) and NaOH until reaching alkaline pH (12.1−13.2, measured in the NMR tubes with a SPINTRODE pH electrode, dia. 3 mm). The HDO signal was used as the reference with presaturation of the solvent.[38] ROESY experiments were carried out in the same spectrometer by using the pulse sequence described in the literature.[39] The optimal mixing time for these systems was 600 ms. Molar ratios CD: substrate were 1:4 for 3-AP with β-CD and PHY with α- and γ-CD, and 1:2 for PHY with β-CD.

## 3. Results and Discussion

**3.1. Chemiluminescence Assays.** ***3.1.1. Effects of Natural CDs in the CL of LUM and ISOL with Different Catalysts.*** *(a) Metal Cations.* There are a number of metal cations that catalyze the CL of LUM.[40] Co(II) and Fe(III) have been chosen in this work due to their high catalytic activity. The color of the emitted light is slightly different, depending on the catalyst employed: blue with Co(II) and blue-green with Fe(III). The catalytic role of these cations is also different as the reactions thus catalyzed occur via different mechanisms: in the case of Fe(III) $OH^{\bullet}$ radicals are formed,[41] whereas in the case of Co(II) the formation of a complex cobalt−hydrogen peroxide is necessary for the chemiluminescent emission.[42] Previous studies have reported that the CL emission of LUM with iron can appear in the absence of hydrogen peroxide.[16] In order to study the CL yield with both catalysts in similar conditions, all the reactions have been carried out in the presence of $H_2O_2$, varying the order of addition of the oxidant to the mixture of reaction with the metal employed in each case. Thus, for Co(II) the highest intensity was obtained by adding first the cation and then $H_2O_2$. In the case of Fe(III), if the metal was added before $H_2O_2$, practically all the CL occurred and the recording of the emission after addition of the oxidant was unsuccessful. Hence in this case the oxidant was added first and then the Fe(III).

Figure 1 shows the emission of LUM 1.2 mM with cobalt (Figure 1, a and b) and iron (Figure 1, c and d) as the catalysts. The different catalytic capacities of both metal cations are shown both in the intensity and in the persistence of CL. In the absence of CDs, for reaching a similar intensity (500 with Fe and 300 with Co) the iron concentration must be 10-fold higher than the cobalt concentration. Regarding the extent of the emission, this is shorter with Fe (III) (about 10 s) than with Co(II) (3 times more, approximately).

Co(II). The CL of both LUM and ISOL is strongly enhanced in the presence of any of the three natural CDs. In spite of the different quantum yield of CL (for example, in the reaction with $H_2O_2$ as the oxidant and hemoglobin,[22] ISOL is about 10 times less luminescent that LUM), the relative variation in the intensity of CL due to the CDs ($I_{CD}/I_{without\ CD}$) is similar for both compounds (Figure 2a). At low CD concentration (14 mM), the highest enhancement is achieved with β-CD, increasing the

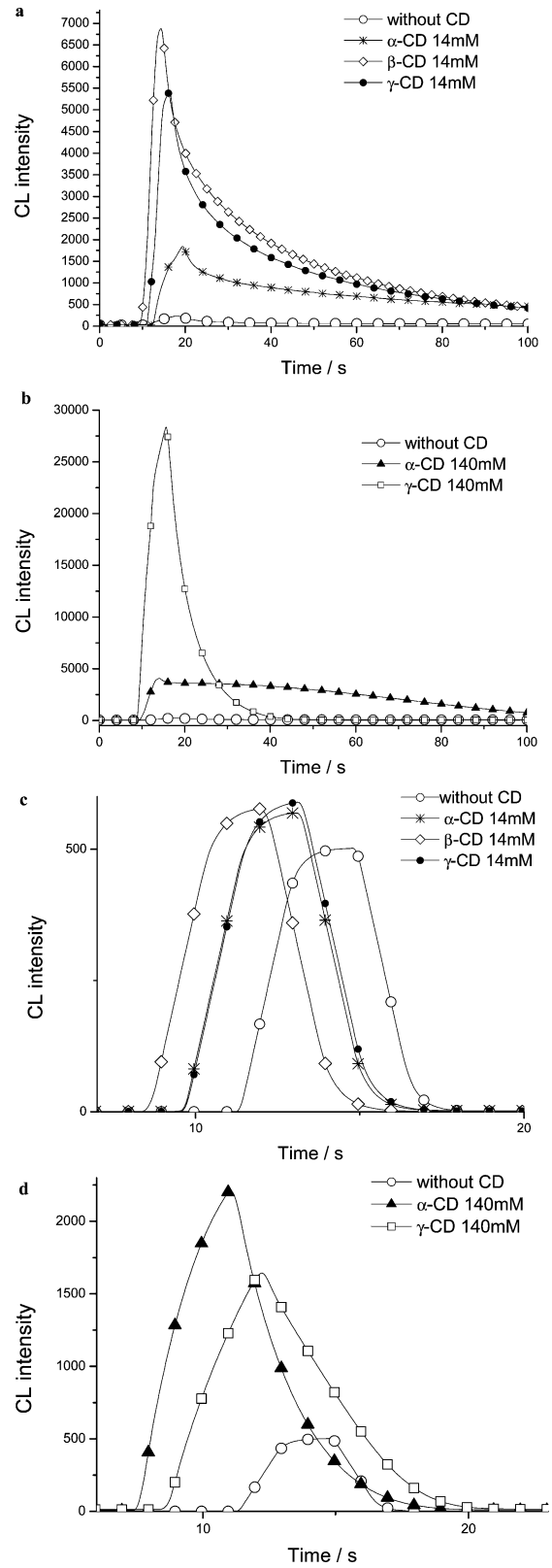



**Figure 1.** CL kinetics at 420 nm of 1.2 mM LUM with Co(II) $9 \times 10^{-5}$ M (a,b); and Fe(III) $9 \times 10^{-4}$ M (c,d) in the absence and presence of different concentrations of the natural CDs.

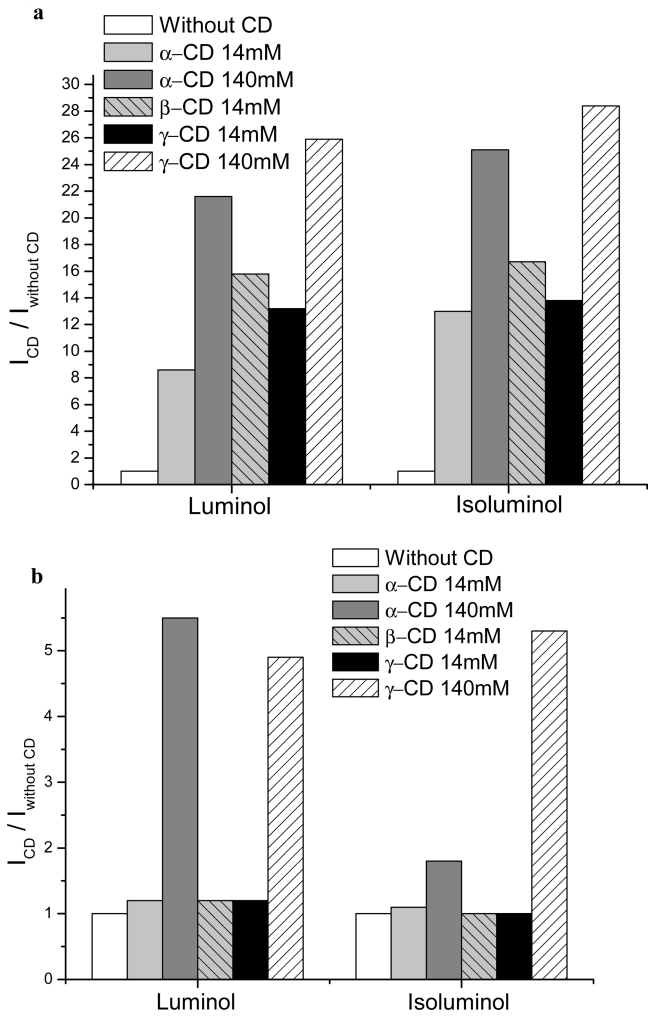



**Figure 2.** Plots of the relative changes of CL intensity ($I_{CD}/I_{without\ CD}$ ratios) of LUM and ISOL 1.2 mM in the presence of (a) $9 \times 10^{-5}$ M $Co^{2+}$ and (b) $9 \times 10^{-4}$ M $Fe^{3+}$. The intensities have been integrated during 180 s for $Co^{2+}$ and 60 s for $Fe^{3+}$.

CL of LUM 16-fold and that of ISOL 17-fold. The other macrocycles, α- and γ-CD, produce a gain in the CL emission by factors of 9 and 13 for LUM and ISOL, respectively, and 13 and 14 in the case of the γ-CD. Addition of higher CD concentrations is only possible with α- and γ-CD, since the solubility of β-CD in water is limited to 1.85 g/100 mL at 25 °C.[43] In these conditions, a stronger catalytic effect in the CL is observed, resulting in amplification factors of 22 (LUM) and 26 (ISOL) with the α-CD, and 25 (LUM) and 28 (ISOL) with the γ-CD. These results provide evidence that the γ-CD is the best enhancer of CL at high CD concentration (140 mM). As an example of the enhanced sensibility in the quantification of Co(II), the CL was measured with increasing concentrations of the catalyst at the same concentration of LUM (Figure 3). The CL could detect cobalt concentration up to $10^{-4}$ M in the absence of CD, while the addition of γ-CD 140 mM increased the detection up to $10^{-7}$ M Co(II). This represents an enhancement by a 1000 factor.

Fe(III). Figure 2b shows that low concentrations of CD barely increase the CL of either LUM or ISOL, whereas at higher [CD] the emission is enhanced 5-fold for LUM and 2- and 5-fold for ISOL with α- and γ-CD, respectively. The lesser catalytic capability of iron implies that more CD is required to perceive the effect of the addition of these macrocycles, so it has not been considered in the subsequent essays.

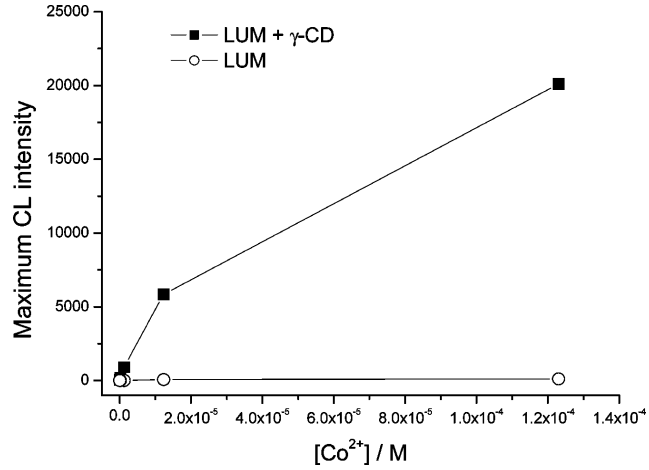



**Figure 3.** CL of 1.2 mM LUM in presence of γ-CD 140 mM at different Co(II) concentrations (range $1.23 \times 10^{-7}$ to $1.23 \times 10^{-4}$ M).

**TABLE 1: Absolute Integrated CL Intensities ($\times 10^{-4}$) during 60 s for LUM and ISOL 1.2 mM with Hemoglobin ($2 \times 10^{-6}$ M) and Diluted Blood (1:100) in the Presence and Absence of CDs**

| | | α-CD | | β-CD | γ-CD | |
|---|---|---|---|---|---|---|
| | without CD | 14 mM | 140 mM | 14 mM | 14 mM | 140 mM |
| LUM + Hb | 1.64 | 1.85 | 4.10 | 1.85 | 1.89 | 5.94 |
| LUM + blood | 2.10 | 2.24 | 4.03 | 2.36 | 2.33 | 4.61 |
| ISOL + Hb | 0.92 | 0.90 | 1.84 | 0.99 | 0.81 | 2.35 |
| ISOL + blood | 0.87 | 1.08 | 1.89 | 1.04 | 1.07 | 2.17 |

In the case of cobalt, there is a clear correlation between the size of the CD cavity and the CL yield, β-CD being the most suitable, followed by γ-CD and α-CD. In principle, the cation could coordinate with the CDs, although the small size of the metal makes its inclusion unlikely in any of the three CDs' cavities. Some authors have reported about the formation of complexes between metal cations and CDs and their catalytic capacity as metalloenzyme model.[44−46] These complexes are formed under the alkaline conditions in which the CL reactions take place, as a high pH involves the ionization of the hydroxyl groups of the CD that can coordinate the central atom by its d orbitals. However, these studies refer to complexes in solid form in the absence of any solvent. Such associations are much less likely to occur in solution at the low metal concentrations used in our CL experiments ($10^{-4}-10^{-5}$ M). That is why the changes in the CL of LUM and ISOL must be ascribed to interactions of the reactants or some of their intermediates with the CDs, ruling out the binding of the transition metal.

*(b) Human Hemoglobin and Blood.* The catalytic capacity of hemoglobin (Hb) with luminol compounds is due to its heme group, whose peroxidase activity catalyzes the peroxide decomposition and the oxidation of LUM.[18] In general, the CL yield of LUM and ISOL is much higher with blood and Hb than with any of the aqueous solutions of both metals (an attenuator was necessary prior to detection in all the measurements). Table 1 shows the effect produced by the presence of CDs in the CL, at the same concentration of luminescent reactant. The intensity emitted is nearly similar without CD and with 14 mM CD. If the concentration of oligosaccharide is increased 10 times, the CL intensity is only doubled or, as much, trebled. The interpretation of the results obtained with blood and Hb is more intricate than with Co(II) and Fe(III). The number of compounds present in blood (membranes, proteins,

**TABLE 2: Absolute Integrated CL Intensities ($\times 10^{-4}$) during 300 s for LUM and Co(II) and Ratios $I_{CD}/I_{LUM}$ in the Absence and Presence of Natural and Modified CDs**

| | α- CD | | β-CD | γ-CD | | Me-β-CD | | HP-β-CD | |
|---|---|---|---|---|---|---|---|---|---|
| LUM | 14 mM | 140 mM | 14 mM | 14 mM | 140 mM | 14 mM | 140 mM | 14 mM | 140 mM |
| 1.66 | 13.37 | 27.42 | 19.02 | 16.44 | 32.04 | 1.92 | 3.11 | 7.61 | 32.51 |
| 1 | 8 | 17 | 12 | 10 | 19 | 1 | 2 | 5 | 20 |

lipids, etc.) may produce uncontrolled effects in the luminescent process upon addition of CDs. For instance, the formation of inclusion complexes between CDs and Hb has been reported,[47] which involves a competitive reaction for the luminescent compound. It is also well-known the hemolytic capacity of CDs,[48] which produce the rupture of the membranes of the erythrocytes, thus modifying the total free Hb available. Phospholipids, which make up the cellular membranes, also form complexes with CDs,[49] as an additional factor of competition for the LUM. All these processes make that the changes in CL cannot only be explained in terms of the interactions between the luminescent species and the CDs, although the results show that the addition of CD at high concentration enhances the intensity emitted.

One of the most important uses of luminol is as presumptive test of blood in forensic investigations. The blood exposed to the atmosphere suffers a number of degradation processes, one of them the oxidation of Fe(II) of hemoglobin to Fe(III), which is reflected as a change from the red color of the blood to brown. When the bloodstains are sprayed with the LUM solution and hydrogen peroxide, the oxidation takes place accompanied by light emission. Hence it is valuable if the luminescence may be enhanced by the addition of CDs in an appropriate solution sprayed over bloodstains on a solid surface.

When a cotton fabric was employed as a test surface, the diffusion gradient produced a lack of reproducibility in the results, so we chose a ceramic plate for the subsequent experiments. The photos taken in these conditions were reproducible, obtaining brighter emission when the blood was left dried for some time, in agreement with literature results.[50] Thus, as in the case of blood diluted in water, the addition of 14 mM of any of the three natural CDs does not increase the amount of light emitted with respect to the LUM without CDs. However, in the presence of α- or γ-CD 140 mM, CL is extended in time, up to 3 times longer than luminol, in spite of the fact that the initial intensity is similar to that of LUM. Although not much more intense, a longer time of emission may be of interest when recording a photograph under poor lighting conditions (the current ones in this kind of test in a crime scene), as the photograph does not need so much exposition time.

According to the previous results, we have explored the addition of CDs to increase the CL yield of optimized commercial kits used for the detection of traces of blood (Bluestar Training and Bluestar Forensic, from Novakit Biotech). When this assay is carried out in solution and also with bloodstains in a solid surface, only the addition of β-CD enhances slightly the intensity emitted. It is worth noting that, when the measurements are carried out after 1 h of the preparation of the solutions, all the solutions that contain CD maintain their initial luminescent power, whereas the Bluestar solution decreased its emission by 22%. Thus, and in contrast to the results obtained with LUM and CDs, the incorporation of natural CDs as an additive in this kit, although scarcely helps to improve its CL, is useful for stabilizing the solution.

***3.1.2. Effects of Chemically Modified CDs.*** The previous measurements of the CL of LUM have demonstrated that CDs enhance the emission of CL, β-CD being the the most efficient one, and that increasing the CD concentration involves a more intense emission. However, its limited solubility compared to α- and γ-CDs prevents the use of larger amounts of this oligosaccharide. Two derivatives of the β-CD have been chosen due to their higher solubility in water, Me-β-CD and HP-β-CD (40 and 45 g/100 mL, respectively at 25 °C).

**TABLE 3: Absolute Integrated CL Intensities ($\times 10^{-4}$) during 300 s for LUM and Co(II) and Ratios $I_{CD}/I_{LUM}$ in the Absence and Presence of Natural CDs and of a Soluble Polymer of β-CD**

| LUM | α-CD | β-CD | γ-CD | poly-β-CD |
|---|---|---|---|---|
| 1.46 | 13.78 | 20.23 | 16.99 | 12.23 |
| 1 | 9 | 14 | 12 | 8 |

Table 2 shows that at low concentration of any of these two derivatives Me-β-CD does not improve the CL yield of LUM whereas HP-β-CD does, although the enhancement is lower than those produced by any of the three natural CDs in the same conditions. However, when the CD concentration is augmented in a factor of 10 (140 mM) HP-β-CD causes an enhancement of CL comparable to that of γ-CD. The width of the β-CD cavity is in principle the same for both derivatives but the presence of hydroxypropyl or methyl groups replacing some of the hydroxyl groups of the rings of the CD necessarily modifies the interactions with the guest. The substitution by methyl groups implies a stronger hydrophobic character of the cavity, whereas the hydroxypropyl substituents are slightly hydrophilic due to the secondary −OH. The ionized state of LUM (forms II and III, Scheme 1) produced by the alkalinity of the solution makes the interaction with Me-β-CD unlikely. In addition, this CD derivative has a higher substitution degree than HP-β-CD so it seems clear that the presence of OH groups in the CDs is an important factor in the CL. In the case of HP-β-CD, the presence of hydroxypropyl groups as substituents is reflected in a lesser enhancement of the CL compared to that of the natural CDs. The weaker interaction with the HP-β-CD compared to natural CDs is reflected in the need of a higher amount of this derivative to produce a larger enhancement of CL.

The effect of the addition of a highly soluble polymer of β-CD made up by CD units cross-linked with chains of epichlorhydrin has been also tested. A similar concentration in CD (14 mM) was chosen for comparison. Table 3 shows that, for the same moles of CD, the highest increase in CL is produced again by β-CD, followed by γ-CD and α-CD, and the less effective is the polymer. Most likely, the tridimensional structure of the polymer makes that all the oligosaccharide units are not accessible to contact with LUM. Besides, the epichlorhydrin network may entrap some guest molecules, avoiding the contact with the oxidant and the catalyst and, therefore, decreasing the CL quantum yield. In conclusion, of the three studied derivatives of β-CD, only HP-β-CD at high concentrations can produce a slight higher rise in CL compared to α- and γ-CDs.

**3.2. Justification of the CL Results.** ***3.2.1. Comparison of the Effect of Glucose vs Natural Cyclodextrins on the CL.*** At this point it seems clear that the CL enhancement is due to the interaction of the luminescent reactant or any of the intermedi-

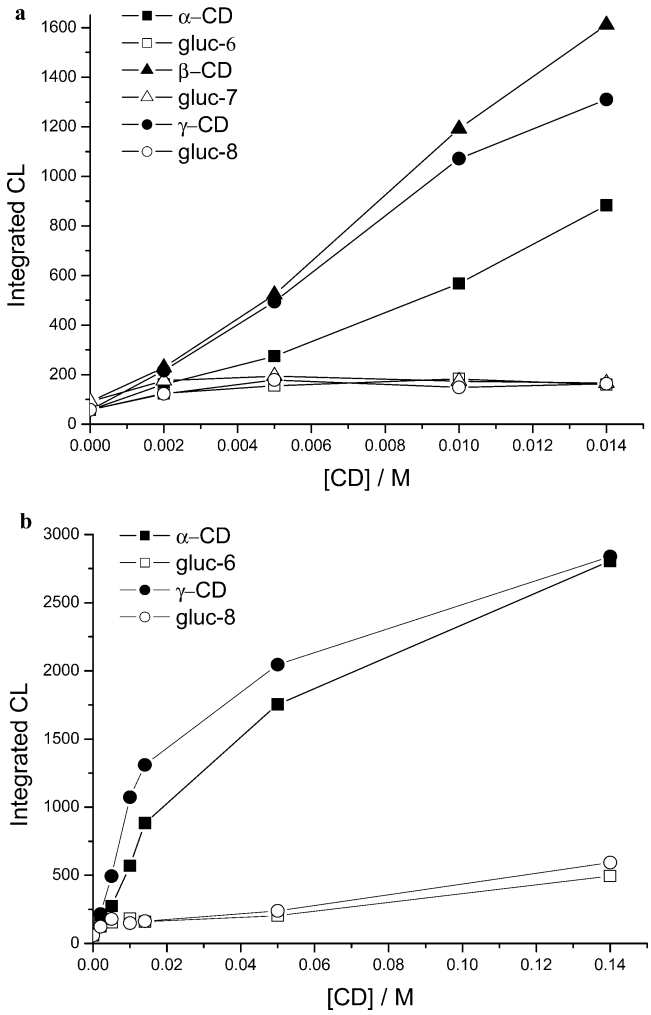



**Figure 4.** Integrated CL intensities during 100 s of LUM and Co(II) in the presence of natural CDs and glucose in equivalent concentration: (a) from 0 to 14 mM; and (b) from 0 to 140 mM.

ates with the CD, and not with the catalyst. The different yield obtained with CDs having variable width suggests an inclusional interaction. In order to ensure whether this is the case, the reaction of LUM with Co(II) was carried out in the presence of increasing concentrations (Figure 4a,b) of the three natural CDs and with glucose solutions in equivalent concentration (1 mol of α-, $\beta$-, and $\gamma$-CD are equivalent to 6, 7, or 8 mol of glucose). The addition of glucose in free or cyclic form increases the CL of LUM but in a quite different manner, being considerably larger as in the CD form. With any of the three natural CDs at low concentration (0−0.014 M) the increase in CL is practically linear, whereas when the same amount of free glucose is added the changes in CL do not depend on the saccharide concentration and remains constant (Figure 4a). At higher concentrations of CD (above the $\beta$-CD solubility), α- and $\gamma$-CDs increase the intensity until reaching a plateau (Figure 4b).

The lack of correlation between the CL intensity and glucose concentration in contrast to the increasing CL emission at larger CD amounts has to be related with a chemical equilibrium. It is known that the addition of polyhydroxyl compounds such as glucose or glycerol raises the CL of LUM, as a result of changes in the viscosity of the solution.[35] However, the fact that the increase in CL is much larger with CDs seems to be related with an association between a chemiluminescent species and the CD, and the presence of an equilibrium which is shifted when increasing the CD concentration favoring the emission of light. The CD would act as a cocatalyst of the reaction. Once more, $\beta$-CD is the macrocycle that produces the highest enhancement of CL yield of LUM what implies that the size of the $\beta$-CD cavity lets the establishment of stronger interactions.

***3.2.2. Stability of the Complexes.*** The stability of the associations between the luminescent derivatives and the CDs has been probed with molecular spectroscopies, namely, fluorescence and NMR.

*Steady-State Fluorescence Spectroscopy.* The addition of increasing concentrations of $\beta$-CD (0−10 mM) to solutions of LUM or ISOL scarcely modifies the absorption spectra, precluding the use of this spectroscopy to study the interactions between the LUM and its derivatives with CDs. Thus, steady-state fluorescence emission has been chosen instead to measure the changes produced in the spectra of LUM, ISOL, 3-AP, and PHY with the addition of increasing concentrations of the three natural CDs. The fluorescence of 3-AP has been studied because it is the chemiluminescent intermediate of LUM and the changes in CL may also be related with interactions of this molecule and the CDs. On the other hand, PHY, although not chemiluminescent by itself, has a molecular structure similar to that of LUM or ISOL except by the absence of the amino group. This is important in order to determine the role of this group in the interaction with the CDs.

At pH = 13.5, the fluorescence spectrum of LUM shows two emission bands at 410 and 505 nm due to the coexistence of the mono and dianion of this molecule, respectively (Scheme 1, structures II and III). The addition of any of the CDs produces an increase of the intensity of the band at 410 nm and a decrease of the other one (Figure 5a). These changes are accompanied by the presence of isoemissive points at 460, 470, and 490 nm for α-, $\beta$-, and $\gamma$-CD, respectively, which suggests the presence of an equilibrium. In the case of ISOL, PHY, and 3-AP, the addition of CDs has different effects on the fluorescence spectra. Thus, upon addition of CD, PHY suffers quenching of their emission (Figure 5b); ISOL also undergoes quenching at low CD concentration but its fluorescence rises upon increasing the concentration of oligosaccharide (Figure 5c); finally, 3-AP increases its fluorescence all over the range of CD concentration (Figure 5d).

Changes in the fluorescence spectra are widely used to determine the binding constants between CDs and guest molecules.[51,52] From the steric point of view, the small size of these derivatives suggests a 1:1 interaction with the CD, in which the emission, $F$, depends on the concentration of the fluorescent species:

$$F = k_S[S] + k_{S:CD}[S:CD] \quad (1)$$

where S and S:CD correspond to the free and associated substrate, and $k_S$ and $k_{S:CD}$ are constants related to properties of the substrate in its free or complex form such as the fluorescence quantum yield and molar absorptivity. The recording of the emission at a certain wavelength (typically at $\lambda_{max}$) versus the CD concentration is usually employed to obtain the binding constant by the Benesi−Hildebrand approach or similar. Nevertheless, in our case the changes in the fluorescence intensity are small and the solubility limitations make it impossible to record the binding isotherm in a great extent. The approach has been to carry out a multivariable nonlinear least-squares fitting procedure that makes use of the whole emission spectra and described elsewhere.[24] In this method, the fit is more robust due to the increased number of data, including a weight factor

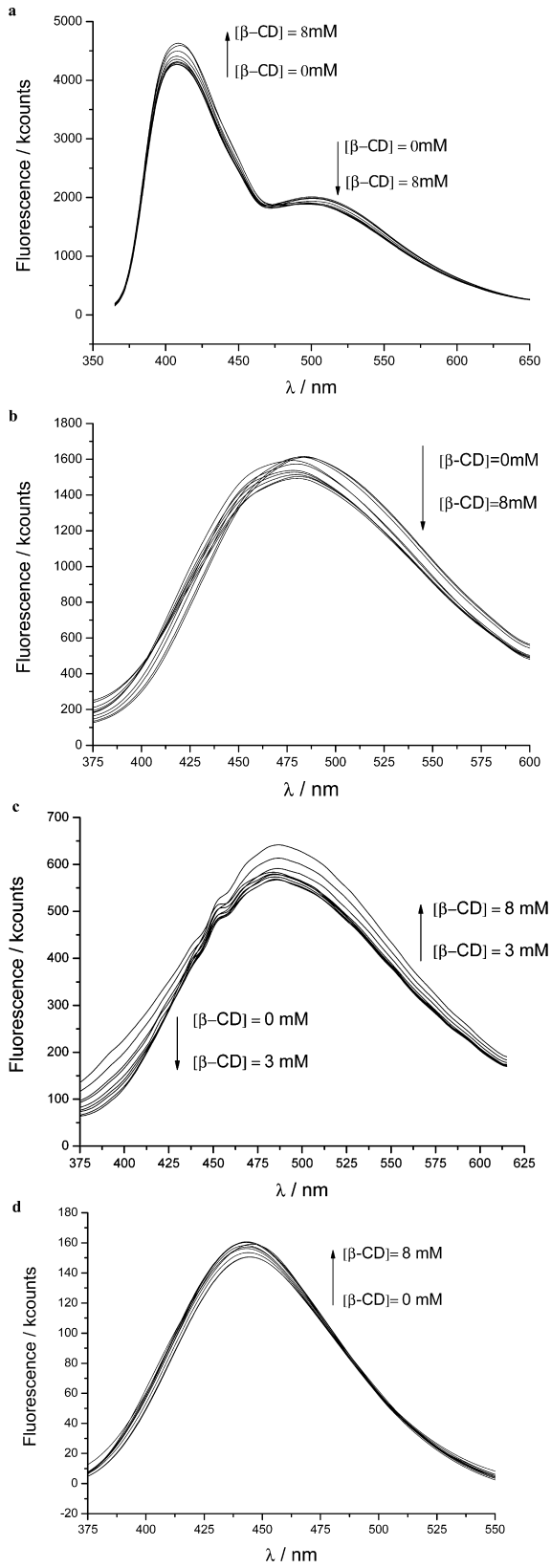



**Figure 5.** Fluorescence spectra in NaOH 0.5 M with increasing concentrations of $\beta$-CD (0−8 mM) solutions of (a) $2.1 \times 10^{-5}$ M LUM; (b) $4.4 \times 10^{-5}$ M PHY; (c) $1.8 \times 10^{-5}$ M ISOL; and (d) $8.0 \times 10^{-5}$ M 3-AP.

to give a higher statistical weight to the wavelengths whose changes in intensity are more significant.

Table 4 compiles the binding constants calculated with the routine described above. Although the presence of isoemissive points in the spectra of LUM and the tiny changes in the fluorescence of ISOL imply some sort of association between these molecules and the CDs, the complexes are not very stable. The binding constants have high standard deviations, and it is not possible to provide a reliable value for this parameter in some cases. For 3-AP, the constants could be calculated and are shown in Table 4. The $\beta$-CD yields the more stable association, following the same trend as the CL of LUM. However, when the fluorescence of this molecule is measured with the same increasing number of moles of free glucose, no changes in its spectra are perceived. According to these results, it seems straightforward to conclude that the improvement in the CL quantum yield of LUM is related to the inclusion of the luminescent intermediate, 3-AP, within the cavity of the $\beta$-CD. The most likely mechanism for such improvement may be connected to a protection of the excited intermediate from collisional quenching by the CD. This could be tested by measuring the CL at different temperatures in the presence and absence of CD. Nevertheless, the constants obtained with α- and $\gamma$-CDs, although 1 order of magnitude lower than those for $\beta$-CD, are nearly similar to each other, which does not seem to match the CL trend. A possible explanation may be in the formation of complexes of higher stoichiometry in the case of the $\gamma$-CD (2 CDs) along with 1:1 complexes, something not improbable due to the larger size of this oligosaccharide. In this case, the intermediate would be sandwiched between two CDs and more protected from quenching, what would produce an augmented or more lasting CL. Due to the tiny changes in fluorescence, it was not possible to apply a 2:1 model with consistent results.

**TABLE 4: Association Constants ($L \cdot mol^{-1}$) at 25 °C for the Phthalhydrazide Derivatives with Natural CDs**

| | α-CD | $\beta$-CD | $\gamma$-CD |
|---|---|---|---|
| LUM | n.a.[a] | n.a. | n.a. |
| ISOL | n.a. | n.a. | n.a. |
| 3-AP | 3.37 ± 0.03 | 51 ± 2 | 3.11 ± 0.03 |
| PHY | 0.76 ± 0.12 | 15 ± 2 | 5.09 ± 0.04 |

[a] n.a. = not applicable due to poor convergence.

In the case of PHY, the highest constant is also obtained with the $\beta$-CD, followed by $\gamma$- and α-CD. PHY differs from 3-AP in the presence of the protruding amino group, what in principle would permit a deeper penetration in the cavity and hence an improved stability. However, the association constant between 3-AP and $\beta$-CD is somewhat higher than that obtained with the same host and PHY as guest, so there must be additional factors involved.

***3.3.3. $^1$H NMR and 2D ROESY Measurements.*** NMR experiments are compulsory in order to gain insight on the topology of the complexes. In this case, the results are limited by the low binding constants. Thus, the 1D-NMR spectra of 3-AP with the three natural CDs (molar ratio 1:1, 5 mM) show tiny changes in the chemical shifts of the CDs. In the case of the PHY, ROESY spectra were measured with the three natural CDs (molar ratio 2:1 for $\beta$-CD and 4:1 for α- and $\gamma$-CDs, 3 mM in PHY) but only the experiment carried out with $\beta$-CD showed intermolecular NOEs (Figure 6). These NOEs correspond to the scalar interaction between the aromatic protons of PHY with the inner H3 and H5 of the CD. According to the relative intensity of the signals, the protons of the PHY which are closer to the heterocyclic ring (Ha and Ha′, Figure 6) interact with H5 at the primary rim of the CD, whereas Hb and Hb′ are closer to the H3 of the secondary rim. With such poor NOEs, it has not been possible to extract reliable interatomic distances

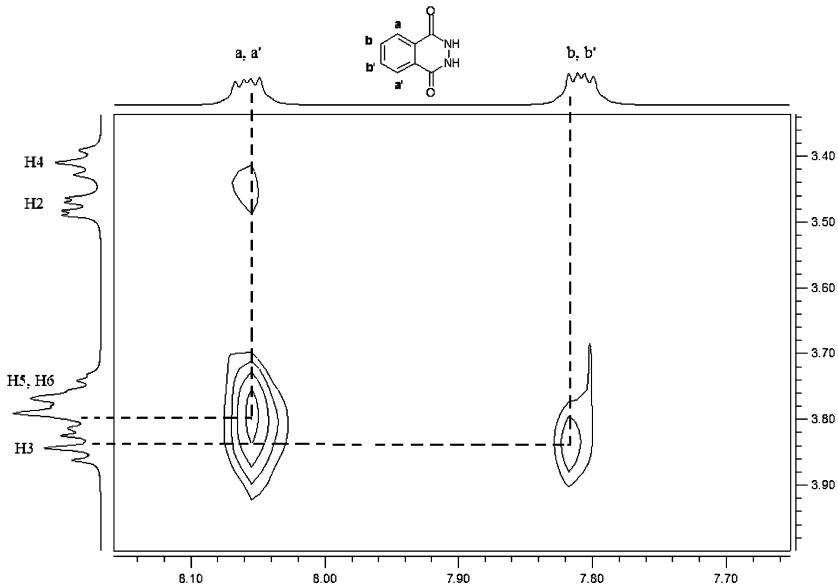



**Figure 6.** Partial view of the 2D ROESY spectrum for the PHY: $\beta$-CD system ($1 \times 10^{-2}$ M PHY, $5 \times 10^{-3}$ M $\beta$-CD).

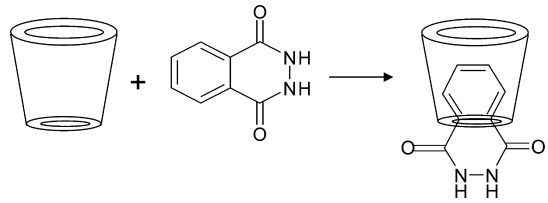


**Figure 7.** Scheme of the inclusion complex between PHY and $\beta$-CD.

via molecular modeling procedures.[24] Figure 7 illustrates qualitatively the most likely tridimensional structure of the complex between PHY and $\beta$-CD according to these data. The inclusion complex established between PHY and $\beta$-CD takes place by the primary ring of the CD. The p$K_a$ of natural CDs are 12.33, 12.20, and 12.08, at 25 °C, for α-, $\beta$-, and $\gamma$-CD, respectively.[53,54] At the alkaline working conditions, the primary OHs are not ionized but those of the secondary rim are (H2 and H3), which have a stronger acidic character. Thus, the widest rim of CDs will be negatively charged in comparison to the neutral primary side. In this case, the entrance in the cavity is less likely to occur by the charged secondary rim. Thus, in spite of the smallest width of this rim, the entrance of the PHY in the CD is energetically favored by this part of the macrocycle. The fact that 3-AP does not show any important intermolecular NOEs even having an association constant with $\beta$-CD comparable to that of PHY must be explained for the presence of the amino substituent. This group involves a certain steric hindrance for the inclusion of 3-AP by the narrowest ring of the CD, but also the establishment of hydrogen bonding with the nonionized hydroxyls, turning out the inclusion of this molecule shallow. Bearing in mind that the intensity of a NOE peak depends on the intermolecular distance, $r$, by $r^{-6}$, it is clear that a shallower inclusion of 3-AP with $\beta$-CD must reduce dramatically the intensity of the signals. Thus, the tiny values of the NOEs must not be related to a lack of interactions between both molecules, although it makes certainly difficult the explanation about what parts of the host and guest interact. The balance of forces involved (hydrogen bonding and van der Waals) seems to be more favorable for the $\beta$-CD, which has the most appropriate dimensions, than for the other CDs.

## 4. Conclusions

The presence of natural CDs in solutions of LUM or ISOL increases notably the CL emission upon oxidation, the highest changes occurring with Co(II) as the catalyst. The incorporation of CDs to the chemiluminescent reaction raises the sensibility of LUM to detect Co(II) in a 1000-fold with $\gamma$-CD, although it is $\beta$-CD the cyclodextrin that produces the highest relative changes in the luminescence of both LUM and ISOL. The other catalysts studied, Fe(III), hemoglobin, and blood, require larger amounts of oligosaccharide to enhance the emitted intensity. Most common derivatives of $\beta$-CD (methylated and hydroxypropylated $\beta$-CD) as well as a soluble copolymer of $\beta$-CD−epichlorhydrin are not so efficient as the natural CDs, the HP-$\beta$-CD being the comparatively most efficient derivative. In the case of blood, the addition of CDs extends the duration of the LUM emission, of interest in forensic applications of the luminol test for revealing blood stains. No intensity enhancement has been detected, on the contrary, when using a commercial kit, although the CDs help to stabilize the mixing solution, diminishing the self-oxidation of the reactant. The process by which the enhancement of luminescence takes place must be ascribed mainly to the complex formed between the intermediate of the reaction, 3-AP (in the case of luminol) and the cyclodextrin, rather than to the luminescent reactant itself. The enhancement in CL when using glucose is much lower than with CDs, which implies that the cyclic structure of these oligosaccharides plays a key factor in the booster effect of these oligosaccharides in the CL reactions. The low binding constants measured by steady-state fluorescence when considering a 1:1 stoichiometry in the same conditions of the reaction indicate that the interactions are generally weak, especially with the reactants. 3-AP provides measurable binding constants, $\beta$-CD being the most stable association and following the same trend as the CL, which suggests that it is the stabilization of the intermediate that is responsible of the emission enhancement. In the case of PHY, the association constants obtained for the three natural CDs reveal also that the most stable inclusion occurs with $\beta$-CD, followed by $\gamma$- and α-CD. In spite of the low binding constants, ROESY experiments point to a shallow inclusion of the charged intermediate inside the cavity that place by the primary rim of the CD, in accordance with the ionization of the secondary ring of the CDs due to the high alkalinity of

the media. The topology of the complex between PHY and $\beta$-CD demonstrates that the presence of the amino group in LUM, ISOL, and 3-AP involves a steric hindrance for the entrance of these molecules by the primary ring.

Future investigations are in course aimed at the study of luminescent species that may form complexes with higher affinities for the CDs, on the basis that superior yields of CL can be obtained.

**Acknowledgment.** The authors acknowledge financial support from Projects CTQ-2006-14933/BQU, UCM-BSCHGR58/08/921628, and MAT2007-65752 and from the Gobierno de Navarra for the doctoral grant for R.M.. They also acknowledge the collaboration of Dr. M. D. Molero and Dr. E. Saez-Barajas of the CAI de RMN (UCM) with the NMR measurements.

## References and Notes

(1) Oldham, P. B.; McCarroll, M. E.; McGown, L. B.; Warner, I. M. *Anal. Chem.* **2000**, *72*, 197R–209R.
(2) Khramov, A. N.; Collinson, M. M. *Anal. Chem.* **2000**, *72*, 2943–2948.
(3) Olesen, C. E. M.; Martin, C. S.; Mosier, J.; Liu, B.; Voyta, J. C.; Bronstein, I. *Methods Enzymol.* **2000**, *305*, 428–450.
(4) García-Campaña, A. M.; Baeyens, W. R. G. *Chemiluminescence in analytical chemistry*; Marcel Dekker Inc.: New York, 2001.
(5) Xu, H.; Duan, C. F.; Zhang, Z. F.; Chen, J. Y.; Lai, C. Z.; Lian, M.; Liu, L. J.; Cui, H. *Water Res.* **2005**, *39* (2−3), 396–402.
(6) Albrecht, H. O. *Z. Phys. Chem.* **1928**, *136*, 321–330.
(7) White, E. H.; Kagi, H. H.; Hill, J. H. M.; Zafiriou, O. *J. Am. Chem. Soc.* **1964**, *86*, 940–941.
(8) White, E. H.; Bursey, M. M. *J. Am. Chem. Soc.* **1964**, *86*, 941–942.
(9) Merényi, G.; Lind, J. S. *J. Am. Chem. Soc.* **1980**, *102*, 5830–5835.
(10) Lind, J.; Merényi, G.; Eriksen, T. E. *J. Am. Chem. Soc.* **1983**, *105*, 7655–7661.
(11) Merényi, G.; Lind, J.; Eriksen, T. E. *J. Am. Chem. Soc.* **1986**, *108*, 7716–7726.
(12) Merényi, G.; Lind, J.; Eriksen, T. E. *J. Biol. Chem.* **1990**, *5* (1), 53–56.
(13) White, E. H.; Bursey, M. M. *J. Org. Chem.* **1966**, *31*, 1912–1917.
(14) Ishida, J.; Takada, M.; Yakabe, T.; Yamaguchi, M. *Dyes Pigm.* **1995**, *27* (1), 1–7.
(15) Lee, J.; Seliger, H. H. *Photochem. Photobiol.* **1972**, *15* (2), 227–237.
(16) Klopf, L. L.; Nieman, T. A. *Anal. Chem.* **1983**, *55*, 1080–1083.
(17) Uzu, T.; Sasaki, S. *Org. Lett.* **2007**, *9*, 4383–4386.
(18) Barni, F.; Lewis, S. W.; Berti, A.; Miskelly, G. M.; Lago, G. *Talanta* **2007**, *72* (3), 896–913.
(19) Qian, K. J.; Zhang, L.; Yang, M. L.; He, P. G.; Fang, Y. Z. *Chin. J. Chem.* **2004**, *22* (7), 702–707.
(20) Thorpe, G. H. G.; Kricka, L. J. *Methods Enzymol.* **1986**, *133*, 331–353.
(21) White, E. H.; Roswell, D. F. *Acc. Chem. Res.* **1970**, *3*, 54–62.
(22) Brundrett, R. B.; White, E. H. *J. Am. Chem. Soc.* **1974**, *96*, 7497–7502.
(23) Yoshida, H.; Ureshino, K.; Ishida, J.; Nohta, H.; Yamaguchi, M. *Dyes Pigm.* **1999**, *41* (3), 177–182.
(24) Sainz-Rozas, P. R.; Isasi, J. R.; González-Gaitano, G. *J. Photochem. Photobiol. A* **2005**, *173* (3), 319–327.
(25) Szejtli, J. *Pure Appl. Chem.* **2004**, *76* (10), 1825–1845.
(26) Khan, A. R.; Forgo, P.; Stine, K. J.; D'Souza, V. T. *Chem. Rev.* **1998**, *98*, 1977–1996.
(27) Woolf, E. J.; Grayeski, M. L. *J. Lumin.* **1987**, *39* (1), 19–27.
(28) Kojo, S.; Tokumaru, S.; Kishida, E.; Tsukamoto, I. *Clin. Chem.* **1992**, *38* (5), 788.
(29) Teranishi, K.; Komoda, A.; Hisamatsu, M.; Yamada, T. *Carbohydr. Res.* **1998**, *306* (1−2), 177–187.
(30) Teranishi, K.; Nishiguchi, T.; Ueda, H. *Carbohydr. Res.* **2003**, *338* (9), 987–993.
(31) Grayeski, M. L.; Woolf, E. J. *J. Lumin.* **1985**, *33* (2), 115–121.
(32) Mitani, M.; Sakaki, S.; Koinuma, Y.; Toya, Y.; Kosugi, M. *Anal. Sci.* **1995**, *11*, 1013–1015.
(33) Yuan, D. Q.; Lu, J.; Atsumi, M.; Yan, J. M.; Kai, M.; Fujita, K. *Org. Biomol. Chem.* **2007**, *5* (18), 2932–2939.
(34) Karatani, H. *Chem. Lett.* **1986**, *3*, 377–380.
(35) Cao, Z. J.; Lau, C. W.; Lu, J. Z. *Analyst* **2004**, *129* (12), 1262–1266.
(36) Voicescu, M.; Ionita, G.; Vasilescu, M.; Meghea, A. *J. Inclusion Phenom. Macrocyclic Chem.* **2006**, *54* (3−4), 217–219.
(37) Bluestar Forensic Home Page; http://www.bluestar-forensic.com (accessed September 28, 2009).
(38) Hwang, T. L.; Shaka, A. J. *J. Magn. Reson. Ser. A* **1995**, *112* (2), 275–279.
(39) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63* (1), 207–213.
(40) Marquette, C. A.; Blum, L. J. *Anal. Bioanal. Chem.* **2006**, *385* (3), 546–554.
(41) Rose, A. L.; Waite, T. D. *Anal. Chem.* **2001**, *73*, 5909–5920.
(42) Burdo, T. G.; Seltz, W. R. *Anal. Chem.* **1975**, *47*, 1639–1643.
(43) Atwood, J. L.; Davies, J. E. D.; Macnico, D. D.; Vögtle, F. *Comprehensive Supramolecular Chemistry*; Pergamon: Tarrytown, NY, 1996.
(44) Norkus, E. *J. Inclusion Phenom. Macrocyclic Chem.* **2009**, *65*, 237−248.
(45) Geibelmann, A.; Klüfers, P.; Kropfgans, C.; Mayer, P.; Piotrowski, H. *Angew. Chem., Int. Ed.* **2005**, *44* (6), 924–927.
(46) Russell, N. R.; McNamara, M. *J. Inclusion Phenom. Mol. Recogn. Chem.* **1989**, *7* (4), 455–460.
(47) Zhang, S.; Ju, H. *Anal. Chim. Acta* **2003**, *475* (1−2), 163–170.
(48) Macarak, E. J.; Kumor, K.; Weisz, P. B. *Biochem. Pharmacol.* **1991**, *42* (7), 1502–1503.
(49) Szejtli, J. *Carbohydr. Polym.* **1986**, *6*, 35–49.
(50) Proescher, F.; Moody, A. M. *J. Lab. Clin. Med.* **1939**, *24*, 1183–1189.
(51) Sainz-Rozas, P. R.; Sánchez, M.; Isasi, J. R.; González-Gaitano, G. *Appl. Spectrosc.* **2002**, *56* (11), 1490–1497.
(52) Sainz-Rozas, P. R.; Isasi, J. R.; Sanchez, M.; Tardajos, G.; González-Gaitano, G. *J. Phys. Chem. A* **2004**, *108*, 392–402.
(53) Gelb, R. I.; Schwartz, L. M.; Bradshaw, J. J.; Laufer, D. A. *Bioorg. Chem.* **1980**, *9* (3), 299–304.
(54) Gelb, R. I.; Schwartz, L. M.; Laufer, D. A. *Bioorg. Chem.* **1982**, *11* (3), 274–280.

JP909707X