Table I. Values for the Conditional Binding Constants for Immobilized and Dissolved Calcein (Expressed as $\log_{10} K$)

	immobilized calcein		dissolved calcein	
metal	pH 5.15	pH 6.95	pH 5.15	pH 6.95
Cu(II)	9.4	12.4	9.2	12.3
Co(II)	5.9	8.0	5.5[a]	8.2[a]
Ni(II)	6.6	11.1	6.2	9.5

[a] pH 5.05 and 7.05, respectively.

depending on the pH of the measurement. The values are larger than the binding contants for Chelex-100 (*14*) where the binding group is an immobilized iminodiacetate group. The reason calcein binds more strongly than Chelex-100 is that the hydroxy group ortho to the iminodiacetic acid is also involved in complexation. Calcein is a tetradentate ligand while iminodiacetate is only a tridentate ligand. For comparison, Table I includes the values of binding constants for dissolved calcein determined in the same way. They are similar to those for immobilized calcein indicating that immobilization does not interfere with complexation. Although one of the hydroxy groups ortho to the iminodiacetate group must have reacted with cyanuric chloride in the immobilization step, the other is still available.

Because the binding constants are so large, immobilized calcein would be an effective reagent for preconcentrating metal ions for subsequent analysis. It has the additional convenient property that one can tell if it is saturated with transition metal ions by observing whether or not it fluoresces. For use as a preconcentrating reagent, it would be desirable to increase the amount of immobilized calcein per gram of substrate.

Use of Immobilized Calcein as a Sensor. We originally immobilized calcein for use as the reagent in an optical sensor that would respond to transition-metal ions. When immobilized calcein is placed on the end of a bifurcated fiber optic and placed in solution, fluorescence decreases as a function of added metal ion (Figure 3). However, because the binding is so strong, the response is not reversible. The reagent is completely extracting metal ion from solution at these pHs.

To use immobilized calcein in a reversible sensor, it will be necessary to work at lower pHs where the conditional formation constant is smaller or to use calcein in a complexing medium where it will tend to extract a smaller percentage of metal ion.

Another approach to using immobilized calcein as a sensor is to form a nonfluorescent complex and then to add a nonquenching metal ion that displaces the metal in from the nonfluorescent complex. For example, when Zn(II) is added to a sensor in which immobilized calcein has been reacted to form the Co(II) complex, an increase in fluorescence is observed as Zn(II) displaces Co(II).

Immobilized Calcein as a Reusable End Point Indicator. The sensor based on calcein can be used to determine end points of complexometric titrations. For example, Cu^{2+} was titrated with EDTA at pH 7.0. At the equivalence point in this titration a large increase in calcein fluorescence is observed, since a very slight excess of EDTA is sufficient to pull the Cu^{2+} away from the immobilized indicator.

Registry No. Calcein, 1461-15-0; cyanuric chloride, 108-77-0; cellulose, 9004-34-6; nickel, 7440-02-0; copper, 7440-50-8; cobalt, 7440-48-4.

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Reaction of Gibbs Reagent with Para-Substituted Phenols

Sir: Gibbs reagent (2,6-dichloro-*p*-benzoquinone 4-chloroimine) is used as a reagent for the detection of phenol derivatives (*1*). The reagent adds to the para position of the phenol ring to give an indophenol. Indophenols are brightly colored and undergo a dramatic color change with pH due to the ionization of the phenolic proton. Remarkably, many phenol derivatives bearing substituents at the para position react readily with Gibbs reagent. Dacre has summarized the literature on this "nonspecific" Gibbs reaction (*2*). A variety of phenol derivatives bearing alkoxy, aldehyde, halogen, or other groups in the para position were found to give colored products with Gibbs reagent. For each product, λ_{max} was listed and ϵ_{max} was calculated, based on the assumption of quantitative conversion of the phenol derivative to an indophenol. The structures of the resulting products were not assigned by Dacre (*2*). Here, we show that the reaction of para-substituted phenols with Gibbs reagent occurs via loss of the para substituent. The resulting colored product is thus identical with that formed by phenol itself. The wide variations in ϵ_{max} noted by Dacre (*2*) represent varying yields of product rather than differences in molar absorptivity.

EXPERIMENTAL SECTION

Gibbs reagent and 2,6-dichloroindophenol were obtained from Fisher Scientific and phenol derivatives from Aldrich Chemical Co. or other commercial sources and were used without further purification. Reagents were prepared as stock solutions (5 mM) in ethanol. Aliquots (0.25 mL) of Gibbs reagent and phenol derivative were mixed in borate buffer, pH 9.2, 24.5 mL, and the optical spectrum of the blue indophenolate anion was recorded after 2 h. An authentic sample of 2,6-dichloroindophenol was

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Table I. Yield of Dichloroindophenol from the Reaction of Gibbs Reagent with Various Para-Substituted Phenols

compound	para substituent	yield, %
phenol	H	60
p-fluorophenol	F	78
p-chlorophenol	Cl	55
p-bromophenol	Br	52
p-iodophenol	I	45
p-cresol	CH_3	18
p-methoxyphenol	OCH_3	64
p-ethoxyphenol	OCH_2CH_3	63
p-propoxyphenol	$O(CH_2)_2CH_3$	65
p-butoxyphenol	$O(CH_2)_3CH_3$	62
p-phenoxyphenol	OC_6H_5	63
p-cyanophenol	CN	0
4-hydroxybiphenyl	C_6H_5	0
p-ethylphenol	C_2H_5	0
p-propylphenol	C_3H_7	0
p-hydroxybenzoic acid	COOH	0
p-nitrophenol	NO_2	0

prepared in borate buffer containing 2% ethanol; the optical spectrum was recorded and ϵ_{max} was calculated (λ_{max} = 602 nm, $\epsilon_{max} = 2.02 \times 10^4$). The yields of 2,6-dichloroindophenol obtained by the reactions between Gibbs reagent and various para-substituted phenols were calculated from measurements of absorbance at 602 nm (λ_{max}).

Similar samples of these reaction mixtures were treated with a few drops of concentrated HCl to convert the anion to the pink neutral indophenol. The indophenols were extracted with ethyl acetate, concentrated by evaporation, and studied by thin-layer chromatography (TLC) using silica gel plates (Analtech).

RESULTS AND DISCUSSION

We studied a series of para-substituted phenols. The intensity of color formed by reaction with Gibbs reagent varied greatly; results are given in Table I. For each case in which a product could be isolated, the product was identical with the dichloroindophenol formed by the reaction of Gibbs reagent with unsubstituted phenol. This was verified by identity of λ_{max} of the indophenolate anions (602 nm) and identical mass spectra. The products cochromatographed with authentic 2,6-dichloroindophenol on TLC plates in two different solvent systems: $CHCl_3$, R_F 0.18; ether/*n*-hexane (50/50), R_F 0.44. Thus, the observed variations in absorbance of the reaction mixtures must be due to variations in yield of the single product, rather than to variations in molar absorptivity of a series of different products (as suggested earlier (2)). The most easily displaced substituents were halogeno, alkoxy, and phenoxy groups. Methyl substitution (*p*-cresol) gave a low yield of 2,6-dichloroindophenol, and longer alkyl groups gave no detectable colored product.

In the case of phenols bearing both an alkoxy group in the para position and an alkyl substituent at another position, the product with Gibbs reagent will probably be an alkyl-substituted derivative of 2,6-dichloroindophenol. Thus, the products obtained with 2-*tert*-butyl-4-methoxyphenol, 2-*tert*-butyl-4-ethoxyphenol, and 2-*tert*-butyl-4-propoxyphenol all have the same λ_{max} (2); presumably, all three phenols would yield 2′-*tert*-butyl-2,6-dichloroindophenol.

The present investigation was prompted by a study of the reaction of para-substituted phenols with benzidine in a peroxidase/H_2O_2 system (3). This enzymatic system oxidizes benzidine to benzidine diimine, which reacts with phenol to give products analogous to indoaniline (4). The similarity between benzidine diimine and dichlorobenzoquinone monoimine (the likely reactive form of Gibbs reagent (5, 6)) has been noted previously (7). We observed that substituents giving rise to good anionic leaving groups were readily displaced from the phenol ring during the reaction with benzidine diimine, giving a product identical with that formed with phenol itself. Undoubtedly, a similar mechanism occurs in the reactions of Gibbs reagent (see Figure 1). Analysts using Gibbs reagent for the detection of phenolic compounds should be aware of the possibility of displacement of substituents (particularly alkoxy or halogen) from the para position. Identification of the resulting indophenol will not be sufficient to determine the structure of the phenol derivative, since the group in the para position has been lost.

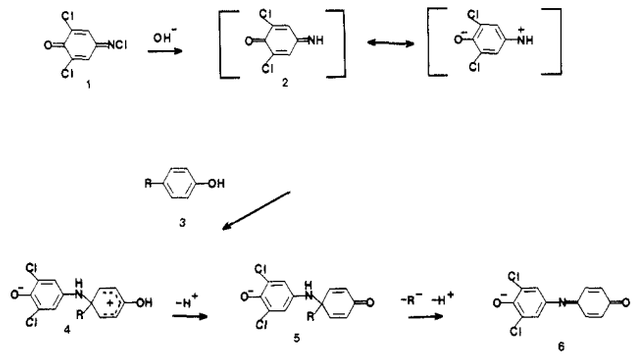

Figure 1. Mechanism of the reaction of Gibbs reagent with para-substituted phenols. Solvolysis of Gibbs reagent (1) yields dichlorobenzoquinone monoimine (2), which attacks the para position of the phenol (3). The adduct (4) deprotonates and the resulting intermediate (5) loses H^+ and R^- to form 2,6-dichloroindophenol (6). (In the case of R = H, intermediate (5) is oxidized to (6) by reaction with a second molecule of (2).)

Registry No. Phenol, 108-95-2; *p*-fluorophenol, 371-41-5; *p*-chlorophenol, 106-48-9; *p*-bromophenol, 106-41-2; *p*-cresol, 106-44-5; *p*-methoxyphenol, 150-76-5; *p*-ethoxyphenol, 622-62-8; *p*-propoxyphenol, 18979-50-5; *p*-butoxyphenol, 122-94-1; *p*-phenoxyphenol, 831-82-3; Gibbs reagent, 101-38-2; dichloroindophenol, 956-48-9.

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