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The quinoid structure is the molecular requirement for recognition of phthaleins by the organic anion carrier at the sinusoidal plasma membrane level in the liver

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Sulfobromophthalein electrogenic uptake into rat liver plasma membrane vesicles was shown to admit only the quinoid, trivalent anion. The minimum requirement for this electrogenic process has been investigated in rat liver plasma membrane vesicles by using Thymol blue, a pH-indicator phthalein occurring either as a neutral, phenolic molecule or as a quinoid, monovalent anion. It has been found that Thymol blue is taken up electrogenically, in accordance with Michaelis-Menten kinetics. Parallel inhibition experiments have shown that both sulfobromophthalein and Thymol blue electrogenic uptakes are performed by the same carrier. It is, therefore, concluded that the phthalein structure recognized for transport is the quinoid molecule, with the dissociated acidic function on the benzene ring. Moreover, inhibitions by rifamycin-SV and bilirubin suggest that there exists a common uptake system for bilirubin, phthaleins and other anions. Taurocholate, on the contrary, does not appear to be involved in the same process.

Introduction

The liver performs the removal of a variety of organic anions from plasma and provides for their disposal into the bile. Some of these are products of metabolism, e.g., bilirubin or bile acids. Others may be drugs or xenobiotic dyes employed in diagnostics for the assessment of liver function, notably sulfobromophthalein [1]. We have been interested in understanding the molecular events of plasma membrane bilirubin translocation from sinusoidal blood into the liver. The low water-solubility of bilirubin is such that it restricts its application for in vitro studies. The water-soluble phthalein dye, sulfobromophthalein, shares part of its hepatic metabolism with bilirubin, has visible spectral and pH-indicator properties, making it a valuable tool for both in vivo and in vitro studies. Dissociation of the phenolic proton shifts the phthalein from the colorless phenolic divalent anion to a purple quinoid trivalent one.

Sulfobromophthalein transport in the liver is performed by bilitranslocase, an integral plasma membrane protein localized at the sinusoidal pole of hepatocytes [2–4]. A spectrophotometric technique based on the pH-indicator properties of sulfobromophthalein was applied to the study of sulfobromophthalein transport in liver plasma membrane vesicles. It was concluded that the process is electrogenic and involves sulfobromophthalein as a quinoid trivalent anion [5].

The aim of this study was to determine the minimum structural requirement for such an electrogenic translocation.

To this purpose, thymol blue has been used. This dye is a pH-indicator phthalein, which occurs either as a neutral, phenolic compound or as a

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mono-anionic, quinoid one. We have found that Thymol blue is transported electrogenically into vesicles. In addition, Thymol blue has been shown to share the same carrier as sulfobromophthalein for intravesicular transport. It is, therefore, concluded that the minimum structural requirement for phthalein uptake includes a quinoid form with either a free carboxylate or sulfate.

Materials and Methods

The following reagents have been used: Thymol blue (Riedel-DeHaen, Seelze-Hannover, F.R.G.); sulfobromophthalein, Na salt (Serva, Heidelberg, F.R.G.); valinomycin (Boehringer, Mannheim, F.R.G.); Rifamycin-SV (5,6,9,17,19,21-Hexahydroxy-23-methoxy-2,4,12,16,18,20,22-heptamethyl-2,7-(epoxypentadeca[1,11,13]trienimino)naphtho-[2,1-b]furan-1,11(2*H*)dione 21-acetate) (Dow Lepetit, Milan, Italy); bilirubin (Sigma, St. Louis, USA); taurocholate, Na salt (Sigma, St. Louis, USA); Bio-Rad Protein Assay (Bio-Rad, Munich, F.R.G.); all other reagents are commercially available analytical-grade chemicals. Valinomycin was dissolved in methanol (0.5 mg/ml); Rifamycin-SV was dissolved in 0.1 M potassium phosphate buffer (pH 7.4)/methanol (2 : 1); 0.125–2 mM bilirubin was dissolved in dimethylsulfoxide just before use.

Plasma membrane vesicles were prepared from Wistar albino female rat liver, according to Van Amelsvoort et al. [6]. The final pellet was suspended in 10 mM Hepes (pH 7.4)/0.25 M sucrose, and stored in liquid nitrogen. Vesicles, thawed at 37 °C and diluted 1 : 1 with 0.15 M NaCl, were kept in ice.

Phthaleins movements were followed by a Sigma ZWS II dual-wavelength recording spectrophotometer at room temperature. In view of the fact that the differential absorption coefficient for the dye can be largely influenced by small variations of pH, temperature and ionic strength, accurate measurements can be performed only if this parameter is determined for each set of samples.

The tests were started by the addition of 50 µl vesicles (0.5–1.8 mg protein) to 1.95 ml medium, containing 0.1 M potassium phosphate buffer (pH 8.1), 0.05 M KCl, the dye, solvents and inhibitors as indicated in the legends to the figures. 12 s thereafter, 5 µl valinomycin were added to the cuvette, by threading the needle of a calibrated glass syringe through a hole in the spectrophotometer's cell cover. To ensure reproducibility of the mixing time in the cuvette, a magnetic stirrer was set at the bottom of the cell and addition of valinomycin was performed with the help of a home-made device whereby the piston of the syringe is driven by a spring.

The actual free concentration of phthalein after vesicles addition to the test mixture can be calculated by subtracting the differential absorption change in the absence from that in the presence of the dye. This was necessary in order to take into account the possible absorption and initial entry of the dye into the vesicles.

Thymol blue spectra have been recorded by a Spectracomb 601 spectrophotometer (Carlo Erba, Milano).

Protein determination was performed by the Bio-Rad protein assay, taking bovine γ-globulin (standard I) as the standard.

Results

Fig. 1 shows Thymol blue and sulfobromophthalein structures. The two dyes are analogous in that both undergo pH-dependent opening of the sultonic (for Thymol blue) and lactonic (for sulfobromophthalein) ring, and subsequent shift

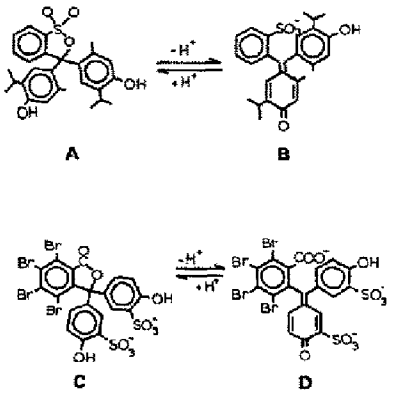

Fig. 1. Structural formulas of protonated (A) and deprotonated (B) Thymol blue and protonated (C) and deprotonated (D) sulfobromophthalein, respectively.

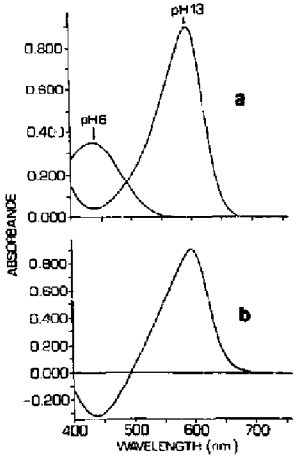

Fig. 2. (a) Spectra of Thymol blue at pH 6.0 and 13.0. (b) Thymol blue difference spectrum (pH 13–pH 6). Experimental conditions: 31.5 μM Thymol blue in either 0.1 M potassium phosphate buffer (pH 6.0) or 0.1 M NaOH; sample volume: 3 ml; room temperature.

from the phenolic structure to the quinoid one. Sulfobromophthalein, however, is charged either as a phenol and as a quinoid, whereas Thymol blue is charged only when occurring as a quinoid molecule.

Panel a in Fig. 2 shows the absorption spectra of Thymol blue at pH 6 and 13, respectively. In view of the pH-indicator properties of Thymol blue, it is expected that its movement from an alkaline compartment into a neutral one can be recorded photometrically, as already shown for sulfobromophthalein [5]. Wavelength pairs are chosen on the basis of the difference spectrum shown in Fig. 2b. The first choice would be 595–650 nm, to restrict the measure to the peak in alkali. Higher sensitivity of the technique could be expected, however, employing $\lambda_{max}-\lambda_{min}$, 595–440 nm.

We have investigated the effect of valinomycin addition to vesicles suspended in a Thymol blue solution (Fig. 3). The experimental conditions chosen are no K^+ and pH 7.4 inside vesicles, 150 mM K^+ and pH 8.1 outside. Absorption changes are recorded at both wavelength pairs mentioned above. The positive-inside membrane potential evoked by valinomycin addition is followed by decrease in absorption at both wavelength pairs used (traces a and c). As predicted, the decrease is larger at 595–440 nm, than at 595–650 nm. A drop of the signal occurs, however, only in the presence of Thymol blue (traces b and d), and not in its absence (traces b and d). To check whether valinomycin could affect absorption by itself, valinomycin has been added to the Thymol blue solution before vesicles. Trace e shows that this is not the case and that absorption decreases only as a consequence of vesicle addition. The ionophore affects the light-scattering properties of the suspension only to a negligible extent (traces b and

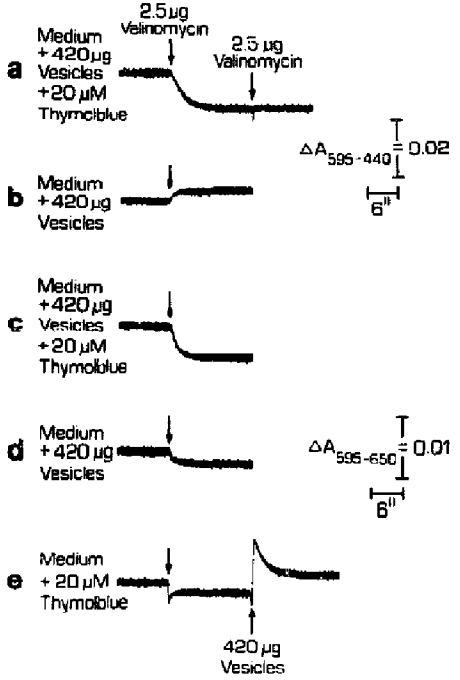

Fig. 3. Effect of valinomycin addition to vesicles suspended in a buffered Thymol blue solution. Experimental conditions: medium: 0.1 M potassium-phosphate buffer (pH 8.1) and 0.05 M KCl; final volume: 2 ml; $t = 20°C$. Traces a and b were recorded at the wavelength pair 595–440 nm; the others at 595–650 nm.

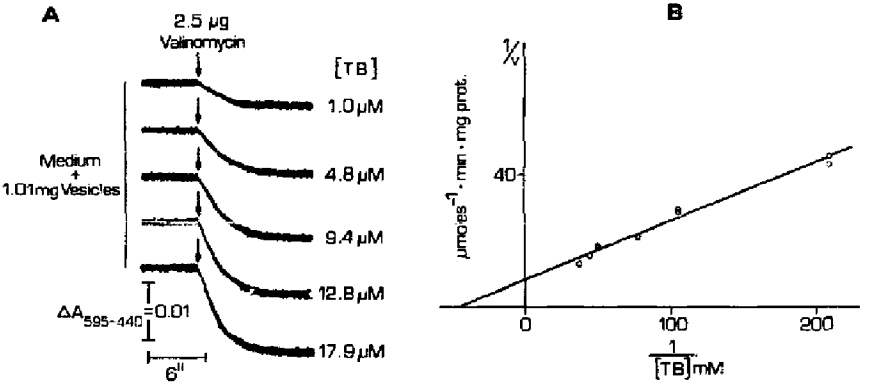

Fig. 4. (A) Concentration dependence of valinomycin-induced Thymol blue uptake by vesicles. (B) Double-reciprocal plot of valinomycin-induced Thymol blue uptake rate. Experimental conditions as in Fig. 3. Wavelength pair 595–440 nm. TB = Thymol blue.

d). On the basis of both this and previous experimentation [5], it can be concluded that decreased absorption is to be taken as the result of Thymol blue entry into the neutral, intravesicular compartment.

Were this a carrier mediated process, it should exhibit saturation kinetics. Fig. 4A shows the variation of both the rate and the extent of valinomycin-induced absorption decrease as a function of Thymol blue concentration. From the Lineweaver-Burk plot (Fig. 4B), an apparent K_m of 21.4 µM and a V_{max} value of 124.5 nmol/min per mg protein have been derived.

The data shown in Figs. 3 and 4 indicate that Thymol blue uptake is electrogenic. The unavoidable conclusion is, therefore, that only the quinoid anion is eligible for transport. Such a restriction was also found for the electrogenic uptake of sulfobromophthalein [5]. If the same transport system is responsible for the electrogenic uptake

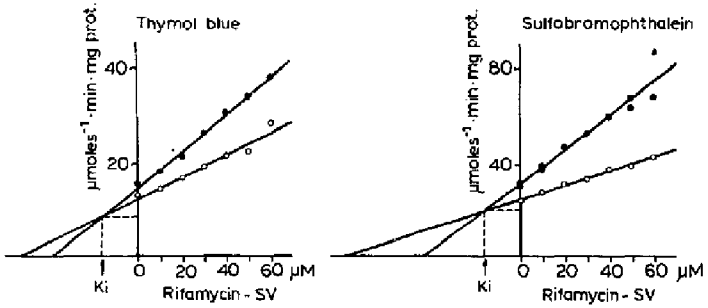

Fig. 5. Dixon plot of inhibition by rifamycin-SV of valinomycin-induced phthalein uptake. Experimental conditions: medium composition as in Fig. 3; 1.77 mg protein; 4 µg valinomycin; dye concentrations were for Thymol blue 31.0 µM (closed symbols) and 39.9 µM (open symbols); for sulfobromophthalein 9.9 µM (closed symbols) and 16.5 µM (open symbols) final volume: 2 ml; $t = 25°C$. The linear correlation coefficients, r, computed were 0.998 (upper curve) and 0.978 (lower curve) for Thymol blue transport and 0.996 (upper curve) and 0.995 (lower curve) in the case of sulfobromophthalein. Wavelength pair: 595–440 nm (Thymol blue) and 580–514 nm (sulfobromophthalein).

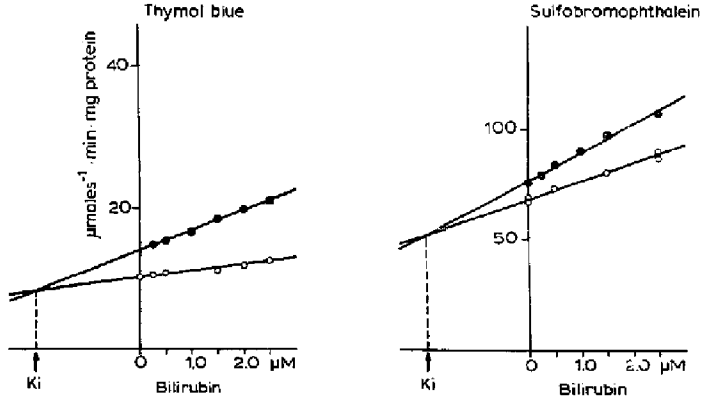

Fig. 6. Dixon plot of inhibition by unconjugated bilirubin of valinomycin-induced phthalein uptake. Experimental conditions: medium composition: as in Fig. 3; 1.01 mg protein; 2.5 μg valinomycin; dimethylsulfoxide 0.25% (v/v); dye concentrations were for Thymol blue 32.6 μM (closed symbols) and 40.5 μM (open symbols); for sulfobrophthalein 10.1 μM (closed symbols) and 15.5 μM (open symbols) final volume: 2 ml; $t = 24°$ C. The linear correlation coefficients r computed were 0.999 (upper curve) and 0.968 (lower curve) for Thymol blue transport and 0.994 (upper curve) and 0.990 (lower curve) in the case of sulfobromophthalein. Wavelength pair: 595–440 nm (Thymol blue) and 580–514 nm (sulfobromophthalein).

of both dyes, it may be inferred that the minimal requirement for the recognition of both phthaleins is the negative charge resulting from the pH-dependent opening of the lactonic or sultonic ring (Fig. 1). To test the hypothesis that the same carrier is involved in both processes, parallel inhibition experiments have been performed. We have selected three potential inhibitors, such as rifamycin-SV bilirubin and taurocholate.

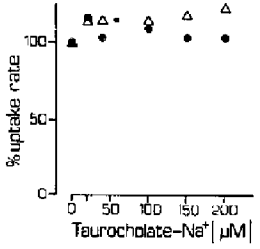

Fig. 7. Effect of taurocholate on valinomycin-induced uptake rate of either sulfobromophthalein or Thymol blue. Experimental conditions: medium composition: as in Fig. 3; 10.1 μM sulfobromophthalein (●); 33.1 μM Thymol blue (△); 0.507 mg protein vesicles; 2.5 μg valinomycin; final volume 2 ml; $t =$ 25°C. Wavelength pair: 595–440 nm (△) and 580–514 nm (●).

Fig. 5 shows the competitive inhibition on the electrogenic transport of both phthaleins exerted by rifamycin-SV. Interestingly, the K_i values are identical, being 16.36 μM for Thymol blue and 16.40 μM for sulfobromophthalein.

Data obtained from inhibition experiments with bilirubin are shown in Fig. 6. Also bilirubin displays competitive inhibition of the electrogenic transport of both Thymol blue and sulfobromophthalein, with K_i values of 1.99 μM and 1.94 μM for the two dyes.

Unlike rifamycin-SV and bilirubin, taurocholate has failed to inhibit either Thymol blue or sulfobromophthalein electrogenic transport (Fig. 7), under the prevailing experimental conditions. It is worth mentioning that the presence of Na^+ in the medium does not affect the result (not shown).

Discussion

The technique used in this and previous studies [4,5,13] contributes to the characterization of organic anions uptake in the liver. Minor adjustments of it allow a number of phthaleins to be used as probes in the study of the process. When sulfobromophthalein is taken up electrogenically,

it is not clear whether the phenolic, divalent anion or the quinoid, trivalent one is the transported species. Both species are charged, hence suitable for an electrogenic process. However, analysis of steady-state data obtained from reported experiments [5] led to the conclusion that only the quinoid form of sulfobromophthalein is recognized for electrogenic plasma membrane translocation. Further definition of the molecular structure undergoing translocation may be achieved by employing analogues such as Thymol blue. This phthalein suits this purpose, since at the extravesicular pH, Thymol blue occurs as a mixture of both neutral (phenolic) and sulfonic (quinoid) molecules.

The direct recording of Thymol blue electrogenic uptake provides kinetic measurements, indicating that only the charged form, i.e., the quinoid, sulfonic anion, is involved in this process.

Inhibition experiments have shown that the electrogenic transport of both Thymol blue and sulfobromophthalein occurs most likely via the same carrier. This conclusion is based on the fact that the K_i values found in the case of both bilirubin and rifamycin-SV are the same for the two transported dyes, as expected for a common carrier.

These data, together with those previously obtained with sulfobromophthalein [5], suggest that the two factors critical for transport are both the quinoid structure and the charge born by the acidic function undergoing intramolecular esterification upon acidification. This charge is not neutralized during transport, a conclusion arrived at on different grounds [7], but is, rather, a precise requirement thereof. It is tempting to conclude, in addition, that the two sulfonic groups on the phenolic rings of sulfobromophthalein are neither a necessary nor a sufficient condition for the electrogenic transport.

An interesting finding is that both bilirubin and rifamycin-SV act as competitive inhibitors for the same translocator. An obvious corollary would be that bilirubin and rifamycin-SV may compete for the same site. This conclusion would agree with earlier results [8,9], which also showed that unconjugated hyperbilirubinemia brought about in vivo by rifamycin-SV may be ascribed to competition for a plasma membrane carrier [9]. The hypothesis of a common carrier for the transported species had already been discussed by Laperche et al. in 1979 [10].

Bilirubin competitive inhibition of both Thymol blue and sulfobromophthalein transport suggests that phthalein translocation occurs at the bilirubin uptake site. This result is in agreement with previous experimental data supporting the existence of a common uptake route for sulfobromophthalein and bilirubin [11]. In particular, they add to the conclusion that in vivo bilirubin uptake is performed by bilitranslocase [12] and contribute to ascribe to this carrier the electrogenic uptake of phthaleins in vesicles.

The K_i values found for bilirubin deserve some comments, however. In an aqueous system, at the pH used in our experiments, unconjugated bilirubin concentration cannot be expected to exceed 70–80 nM [13]. However, the presence of membranes and trace amounts of dimethylsulfoxide allows bilirubin to occur dispersed at nominal concentrations much higher than those simply in the buffer used. A word of caution should, therefore, be added against attributing absolute meaning to the values found. The latter, however, remain a clear indication that the system for the translocation of the two phthaleins is the same and is inhibited by bilirubin.

The failure of taurocholate to inhibit the electrogenic uptake of phthaleins in vesicles, shown in Fig. 7, goes along with a similar result (unpublished data), obtained in isolated hepatocytes, by using the experimental technique previously described [14]. The failure of taurocholate to inhibit sulfobromophthalein uptake by isolated hepatocytes has also been reported [7]. All this supports the view that bilirubin, sulfobromophthalein and a family of other anions are taken up by the liver through a system different from that of the bile salts [11,15–20]. While sulfobromophthalein and bilirubin have been shown to interfere with bile acid uptake [21–25] and binding to membrane proteins [26], previous [7] and present data suggest that the contrary does not occur. In contrast, inhibition of sulfobromophthalein uptake by taurocholate has been reported by Laperche et al., at very low dye concentrations [27]. On the other hand, when long-lasting accumulation, rather than initial rate of uptake, of dibromosulfophthalein is

measured in isolated hepatocytes, taurocholate displayed an inhibitory action [25]. Storing may, however, be totally unrelated to the initial uptake process.

From these data we conclude that Thymol blue is transported electrogenically across plasma membrane via a carrier common to sulfobromophthalein. This process is inhibited competitively by both rifamycin-SV and bilirubin.

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