Biochimica et Biophysica Acta, 643 (1981) 650—658
Elsevier/North-Holland Biomedical Press

BBA Report

BBA 71516

SPECIFICITY OF BINDING OF CHOLESTEROL, STEROID HORMONES AND OTHER COMPOUNDS IN SYNAPTOSOMAL PLASMA MEMBRANES, AND THEIR EFFECT ON OUABAIN-SENSITIVE ATPase *

SPYRIDON G.A. ALIVISATOS [a,**], GEORGE DELICONSTANTINOS [a] and GEORGE P. THEODOSIADIS [b]

[a] *Department of Physiology, University of Athens Medical School, Athens 609*
[b] *Department of Opthalmology, University of Athens Medical School, Athens 607 (Greece)*

(Received December 8th, 1980)

Key words: Cholesterol binding; Glucoside; Aliphatic compound; Steroid hormone; (ATPase)

Summary

The effects of various steroids and some aliphatic compounds on biological membranes (synaptosomal plasma membranes) were studied. It was shown that:

(1) Very dilute aqueous 'solutions' ($1 \cdot 10^{-7}$ M to $1 \cdot 10^{-5}$ M) are satisfactory for a study of such phenomena.

(2) When using amphipathic substances (e.g., octanol glucoside), the effect on membranes will depend on the relative concentrations of the reactants. At large concentrations of the amphipaths they act as detergents, solubilizing the membrane constituents through formation of mixed micelles, whereas at small concentrations such as those mentioned above, they would bind onto the membranes.

(3) The presence of a glycon in glycoside (e.g., cholesterol glucoside, dodecanol glucoside, etc.) is a convenient method of increasing their solubility in water. The sugar moiety does not contribute in their binding at specific sites.

(4) The specific activity of the functional parameter used in this study, i.e., the ouabain-sensitive ATPase, rises after 3 h preincubation with cholesterol or

* Abstracts of this work were presented at the meeting of the Hellenic Biochemical Biophysical Society (Athens, 1980), the 3rd Balkan Biochemical Biophysical Days (Belgrade, 1980) and the 13th FEBS Meeting (Jerusalem, 1980).

** To whom inquiries should be addressed.

Abbreviations: ATPase, the synaptosomal plasma membrane ouabain-sensitive adenosine triphosphatase (EC 3.6.1.3).

testosterone at least 2.5-fold above its original value. At this level, testosterone binds on synaptosomal plasma membranes in approximately 30-times smaller quantities per mg protein as does cholesterol. In the presence of both testosterone and cholesterol the effect of the two compounds is much higher. The glucocorticoid cortisol evokes a milder effect upon the specific activity of ouabain sensitive ATPase.

(5) Estrone and progesterone evoke decrease of the specific activity of the ATPase of synaptosomal plasma membranes from young dog brain (approx. 50%). This effect of the two hormones is thus opposite to that of cholesterol.

(6) At the membrane level, the steroid structure is not absolutely necessary to evoke enzymatic functional changes. Aliphatic compounds like dodecanol or its glucoside imitate the action of cholesterol or testosterone, while octanol or its glucoside simulate the action of progesterone.

(7) Accordingly, cholesterol and testosterone can be mutually excluded from their binding sites at proper relative concentrations, while octanol or its glucoside may exclude progesterone.

We previously observed that binding of cholesterol onto synaptosomal plasma membranes of mouse, rabbit, dog or man, or its binding onto plasma membranes of Purkinje heart cells of young dogs in situ, evokes very significant functional changes of various integral proteins of these membranes, including the ouabain-sensitive ATPase of synaptosomal plasma membranes and the electrophysiological proteins [1]. Changes have also been observed in the function of adenylate cyclase of synaptosomal plasma membranes and of many integral proteins of the outer and inner mitochondrial membranes [2,3]. More recently we studied the kinetics of the binding of cholesterol onto synaptosomal plasma membranes, and concluded that it follows a cooperative process [4]. Our findings, at least with respect to functional changes evoked by binding, have been recently confirmed and extended to include sarcoplasmic membranes of muscle [5].

Cholesterol and octanol were Merck, A.G., products. Dodecanol and steroid hormones (cortisol, oestrone, progesterone and testosterone) were purchased from the Sigma Chemical Co. (St. Louis, MO). Radioactive compounds, i.e., ^{14}C-labeled cholesterol, cortisol, oestrone, progesterone and testosterone, all at specific radioactivities greater than 50 Ci/mol, and D-[U-^{14}C]glucose, specific radioactivity greater than 230 Ci/mol, were all purchased from the Radiochemical Centre, Amersham, U.K. Other chemicals used in this study were of the finest chemical purity commercially available. Purification of cholesterol and its radioactive analogue was as described previously [4]. Most other steroids were tested on 0.25 mm thick precoated TLC plates (Merck), and found satisfactory for our experiments. In cases where many impurities were present, the compounds, isolated on the chromatography plates, were scraped off, eluted and stored at -20°C, as previously [4].

Cholesterol, dodecanol and octanol glucosides were synthesized according to the method of Helferich and Mueller [6]. Dodecanol and octanol glucosides, however, were crystallized according to the method of Baron and Thomson [7]. Crystallized cholesterol-α-D-glucoside (4 mg) was dissolved in 50 ml water

(i.e., $1.45 \cdot 10^{-4}$ M). An aliquot (1.5 ml) was used for the determination of the sugar moiety by the periodate spectrophotometric procedure, as described previously [8,9]. Another 25 ml aliquot was made 2 M with HCl, and refluxed for 2 h. It was then reduced in the flash evaporator to 1 ml, and 0.1 ml was used for cholesterol determination [10]. The ratio of sugar to cholesterol was found to be 1.05 (expected: 1.00). The ^{14}C-labeled analogue, prepared from [4-^{14}C]-cholesterol (250 μCi, using one half of the total prescribed quantity of the steroid) [6], was a product of specific radioactivity of 68 cpm per $1 \cdot 10^{-9}$ mol.

The radioactive sugar-containing analogues were prepared starting with 1 mCi D-[U-^{14}C]glucose. The specific radioactivity of glucoside was 35 cpm per $1 \cdot 10^{-9}$ mol, and that of actanol glucoside was 87 cpm per $1 \cdot 10^{-9}$ mol. Analytical work was along the same lines as with the cholesterol glucoside, with the exception of the dodecanol and octanol moieties, the identification of which was established from the identity of functional results obtained with dodecanol and octanol in synaptosomal plasma membranes.

The cholesterol glucoside was of the *a* configuration, due to a Walden inversion occurring during its synthesis. This, however, did not alter its binding or its functional effects on membranes (see below). Apparently, the configuration at the 3 position of the steroid plays a role at different sites of its action (e.g., at the cell nucleus).

Aqueous 'solutions' of all these compounds were prepared as described previously for cholesterol [2]. Synaptosomal plasma membranes were prepared and stored as described previously [3]. The same methodology [4] was used for the preincubations of the synaptosomal plasma membranes with the respective compounds and for the measurement of their binding.

The ouabain-sensitive ATPase activity of synaptosomal plasma membranes was tested [11], in the majority of the experiments, in aliquots of the final suspension of membranes after ultracentrifugation of the preincubated mixture. Acetylcholinesterase activity of the synaptosomal plasma membranes [12] and lactate dehydrogenase [13] of the soluble fraction (supernatant of 100 000 $\times g$) of the whole brain homogenate were also tested for possible effects of the steroids upon their specific activity.

The large differences of the binding of cholesterol and its glucoside, and the dodecanol glucoside on the one hand and binding of cortisol, octyl glucoside, oestrone, progesterone and testosterone on the other, are shown in Fig. 1. It is noteworthy that in all instances the concentrations of these compounds ($1.375 \cdot 10^{-5}$ M) and the quantities of synaptosomal plasma membranes (0.35 mg/ml) in the preincubation mixtures were the same. For the sake of easy comparison, all results are expressed in terms of a power regression ($y = a \cdot x^b$), which does not necessarily coincide always with the 'best fit' curve. This was done in order to accomodate an instantaneous binding after mixing the reactants. This 'instantaneous' deposition of radioactivity from the labeled compounds onto synaptosomal plasma membranes is different in nature from the subsequent binding. We are led to this conclusion by the absence of functional changes of integral proteins occurring during this minute period of (initial) time. The selected type of presentation, however, allows the reader to form his own opinion with respect to a considerable portion of the tightly 'bound' radioactivity. Another approach would be to subtract the zero-time intercept

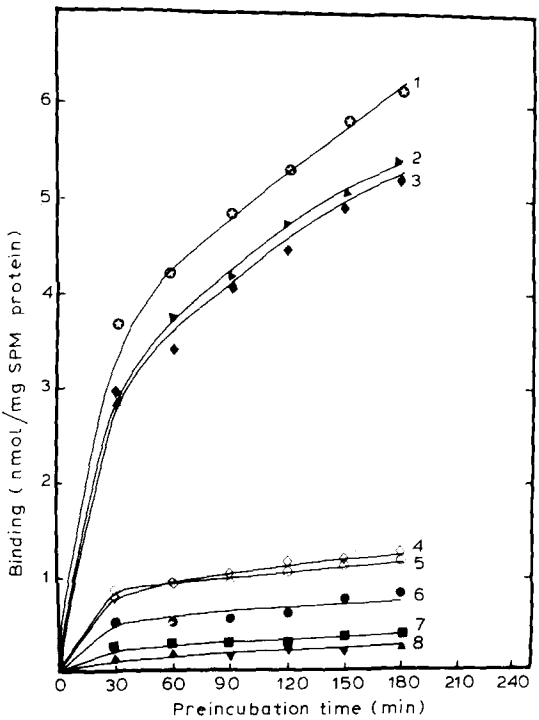

Fig. 1. Binding of various compounds onto synaptosomal plasma membranes (SPM). Aqueous 'solutions' of all labeled compounds were at 13.75 μM. Synaptosomal plasma membranes proteins were at quantities of 0.35 mg/ml of incubation mixture, buffered with 5 mM Tris-HCl/0.15 M NaCl (pH 7.4). Incubations at 37°C; aliquots were withdrawn at times indicated in the abscissa. The indicated values represent averages of numerous determinations. They are expressed in terms of a power regression ($y = ax^b$). Curves: 1, dodecanolglucoside, $r^2 = 0.99$; 2, cholesterolglucoside, $r^2 = 0.97$; 3, cholesterol, $r^2 = 0.98$; 4, cortisol, $r^2 = 0.93$; 5, octylglucoside, $r^2 = 0.92$; 6, progesterone, $r^2 = 0.83$; 7, estrone, $r^2 = 0.99$; 8, testosterone, $r^2 = 0.98$.

of a linear regression, a procedure that agrees perfectly with our experimental data (binding and functional changes). However, all coefficients of determination for power regressions are above 0.92 (Fig. 1). Dodecanol and octanol are not included in this Figure, since these two compounds, as used in our studies, were not labeled. It may be assumed, however, from results of the influence of their binding upon the ouabain-sensitive ATPase activity, that this binding would be the same as that of their respective glucosides. This happens also when either (labeled) cholesterol or its glucoside are used (Fig. 1).

The effects of the binding of these compounds upon the main functional parameter used in this study, i.e., the specific activity of the ATPase, are shown in Fig. 2. It is evident that testosterone, which binds in approximately 30-times smaller quantities as compared to the binding of cholesterol, is the most active of the compounds tested for their activity on ATPase in this study. The response of ATPase in this case is sigmoid (Fig. 2A). This was verified in several experiments and it is probably due to the natural paucity of specific binding sites for this steroid in synaptosomal plasma membranes. Of the other compounds in the same Fig. 2, cholesterol and its glucoside have an action qualita-

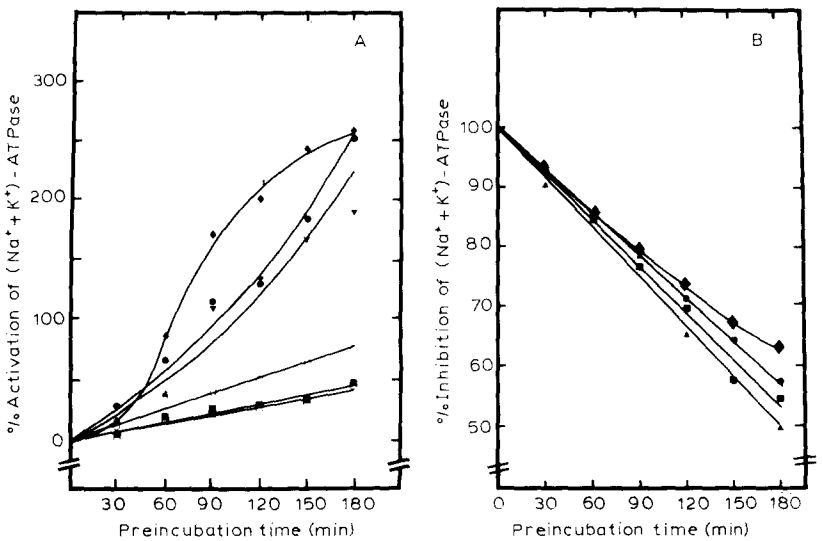

Fig. 2. Changes of the ouabain sensitive ATPase activity evoked by the binding of various compounds on synaptosomal plasma membranes. Conditions as in Fig. 1. In A: ♦———♦, testosterone (sigmoid); ●———●, cholesterol, $y = ae^{bx}$, $r^2 = 0.94$; ▲———▲, cholesterol glucoside, $y = ae^{bx}$, $r^2 = 0.94$; ○———○, cortisol, $y = a + bx$, $r^2 = 0.97$; ■———■, dodecanol glucoside, $y = a + bx$, $r^2 = 0.93$; □———□, dodecanol, $y = a + bx$, $r^2 = 0.95$. In B, ♦———♦, progesterone (1), $y = ae^{bx}$, $r^2 = 0.99$; ●———●, octanol (2), $r^2 = 0.99$; ■———■, octanol glucoside (3), $r^2 = 0.98$; ▲———▲, estrone (4), $r^2 = 0.96$. Curves 2—4 are linear.

tively similar to that of testosterone. The response of ATPase at this binding level is exponential ($y = a \cdot e^{bx}$). The glucocorticoid cortisol, as well as dodecanol and its glucoside, evokes a smaller and approximately linear response on the enzyme activity (Fig. 2A). All these differences in the shape of the curves denote that the changes of ATPase activity are most probably due to (a) the number of specific binding sites and (b) the capability of a given compound to cross the boundary of the membranous structure. For example, cholesterol has many binding sites in synaptosomal plasma membranes, and the initial portion of the curve, which we are now studying, acquires the shape of a power function, which might have been converted to sigmoid if this compound had not started to incorporate into the membranous structure (nonspecific binding). Following this trend of thought, one might expect that at higher concentrations, if feasible, the graph of response to cortisol, dodecanol and its glucoside would also follow a curved path, as above.

The binding of all other compounds, shown in Fig. 2B, evoke decrease of the specific activity of the ATPase. The observed effects of steroids manifest themselves only upon integral proteins (i.e., proteins embedded in the membranous bilayer). Thus, there is no effect of cholesterol upon cholinesterase activity, a peripheral enzyme (Table I). There is also no effect upon the soluble lactic dehydrogenase. The fact that all bona fide integral proteins studied so far in our laboratory are influenced by the steroids could further support the idea that cholinesterase is indeed a peripheral enzyme [14].

Fig. 3A shows that cholesterol and testosterone can mutually exclude each

TABLE I

EFFECT OF CHOLESTEROL ON THE ACETYL CHOLINESTERASE (EC 3.1.1.7) ACTIVITY OF SYNAPTOSOMAL PLASMA MEMBRANES

The values given are the means ± S.D. of five different determinations. Experimental details as in Fig. 1.

	Acetylcholinesterase activity (ΔA at 412 nm/mg synaptosomal plasma membrane protein per min)
Cholesterol-treated membranes	0.1452 ± 0.0068 *
Controls (untreated membranes)	0.1490 ± 0.0085

* Not statistically significant from controls ($P < 0.05$).

other from their binding sites. Apparently, the exclusion limits to even greater extent specific sites available for binding of testosterone and prevents any possible escape of it into the structure of the membrane. Thus, the binding curve acquires its sigmoid character. It should be noted that if the zero-time intercept were subtracted as shown in the figure, the regression in the Hill equation would jump from a determination coefficient, r^2, of 0.93 to one of 0.99. The increase of the specific activity of ATPase by testosterone alone is linear (or

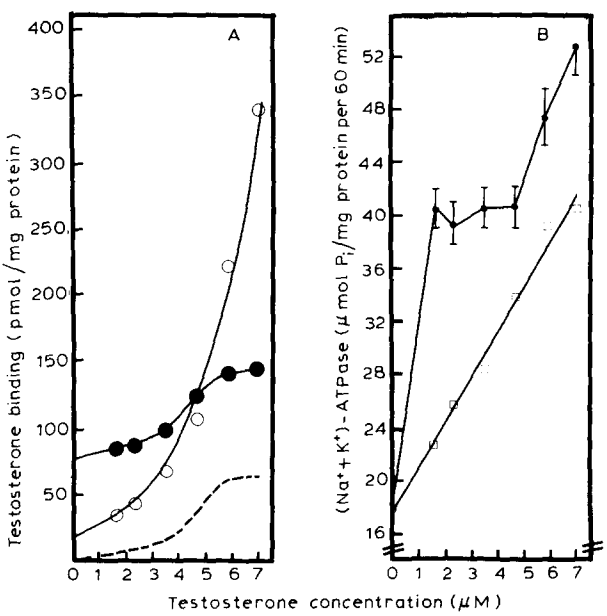

Fig. 3. A. Binding of testosterone alone (○———○) ($y = ae^{bx}$, $r^2 = 0.98$) or in the presence of $7 \cdot 10^{-6}$ M cholesterol (●———●) ($r^2 = 0.93$) onto synaptosomal plasma membranes. Testosterone concentrations as indicated in the absissa; synaptosomal plasma membrane proteins (0.35/ml); incubations were for 180 min; the dotted line ($r^2 = 0.99$) represents a linear translocation of the testosterone-cholesterol mutual exclusion by subtracting the zero time intercept; r^2 of the two lower curves is the linear determination coefficient of the corresponding Hill equation. B; Changes of the ouabain-sensitive ATPase activity evoked by testosterone alone (□———□) ($r^2 = 0.99$) or by testosterone in the presence of $7 \cdot 10^{-6}$ M cholesterol ●———●. The curves in A and B are representatives of five determinations. Bars in B represent the range of deviations from five different determinations.

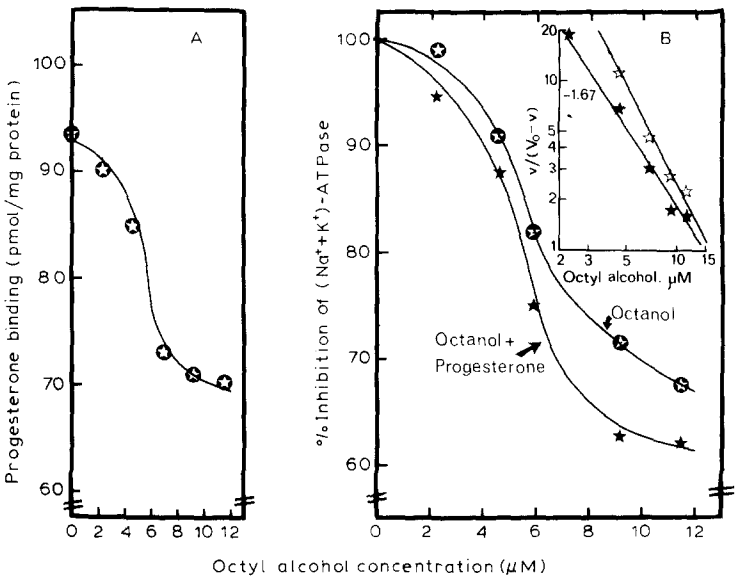

Fig. 4. A. Mutual exclusion of progesterone and octanol; octanol at the concentrations indicated in the absissa; progesterone concentration $0.5 \cdot 10^{-6}$ M; synaptosomal plasma membranes proteins (0.35 mg/ml). For this curve, $r^2 = 0.95$ and slope (n) = 1.63. B. Inhibition of ouabain-sensitive ATPase activity by changing concentrations of octanol alone, or in the presence of $0.5 \cdot 10^{-6}$ M progesterone. The insert shows the Hill plots of the two sigmoid curves. The determination coefficients (r^2) for the two straight lines in the insert are 0.98 and 0.99 respectively; the slopes are −1.67 (★) and −1.98, respectively.

slightly sigmoid, Fig. 3B). Cholesterol, however, if present together with testosterone, changes drastically the shape of the curve representing the rise in specific activity of the enzyme. At higher concentrations of testosterone the specific activity rises to much higher values.

The exclusion of progesterone by octanol is shown in Fig. 4A. In this instance, octanol does not create such a large instantaneous binding as does cholesterol after mixing of the reactants (cholesterol or octanol) with the membranes (see above and Fig. 1). The present protocol (Fig. 4) involves a constant concentration of labeled progesterone ($0.5 \cdot 10^{-6}$ M), with changing concentrations ($(2-12) \cdot 10^{-6}$ M) of octanol. The results, although opposite (i.e., decrease of the ATPase activity), are very similar to those obtained with testosterone. Thus, the curve representing the drop of the specific activity of the ATPase acquires fully its sigmoid character in the presence of octanol. Furthermore, since both octanol and progesterone plus octanol show sigmoid curves, it is easy to determine the Hill coefficient for these allosteric phenomena. The change in the Hill coefficient is somewhat minimal (from −1.67 to −1.99). It thus differs from results of Farias et al. [15] related to the regulation of allosteric membrane-bound enzymes from mammalian and bacterial membranes through changes in their lipid composition. These results indicate that there is very little relation of the bound progesterone or octanol with the changes of the membrane fluidity.

Finally, the lack of effect of cholesterol in attempts to compete with progesterone for its binding sites (mutual exclusion by steroid hindrance) [16] is indi-

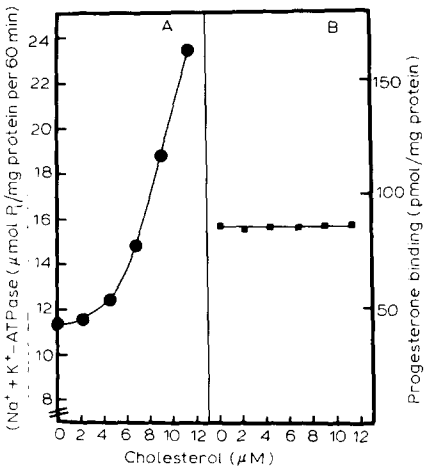

Fig. 5. A. The evoked effect on ouabain-sensitive ATPase activity of cholesterol at concentrations indicated in the absissa in the presence of $0.5 \cdot 10^{-6}$ M progesterone. Notice that the effect of cholesterol prevails. $y = ae^{bx}$, $r^2 = 0.98$. B. Absence of exlusion of progesterone ($0.5 \cdot 10^{-6}$ M) binding onto synaptosomal plasma membranes (0.35 mg protein/ml) by changing concentrations of cholesterol. $\bar{x} = (8.57 \pm 0.03) \cdot 10^{11}$.

cated by the constancy of the radioactivity from labeled progesterone in the presence or absence of cholesterol (Fig. 5). It is evident, however, that when both progesterone and cholesterol are bound in their respective sites, the effect of cholesterol prevails. Apparently, binding sites effecting an increase of the specific activity of ATPase exert stronger influence as compared to those effecting its depression.

Specificity of binding, at the synaptosomal plasma membrane level and with the ouabain sensitive ATPase as the only functional parameter, may be classified into two groups: cholesterol, dodecanol, cortisol and testosterone, which all evoke increase of the specific activity of the ATPase; and octanol, progesterone and estrone, which all evoke decrease of the ATPase activity.

Obviously, no attempt is made here to correlate some of the physiological activities of the hormones [17] with their effects on synaptosomal plasma membranes. It is not attempted, either, to imply that octanol, which evokes a slightly stronger effect upon ATPase as compared to progesterone or estrone, may have hormonal activities. More studies are required, both with integral proteins at the membrane level of target organs and at the level of intact animals (production of oestrus, etc.).

It should also be noted that the authors are fully aware of the fact that steroid hormones are carried in the plasma by specific receptors and that the classical idea of regarding their physiological activity involves the whole apparatus of protein neosynthesis, from the nucleus on [17—19]. We do not attempt to shake these views. Our purposes is to study the general behaviour of membranes, and the system selected, aqueous solutions of the steroid, etc., presents a number of advantages for the simplicity of application of mathematical treatment, etc.

Results reported here confirm our previous observations on the functional

effects of cholesterol upon integral proteins [1—3]. We have already studied the effect of cholesterol, using as a functional parameter the adenylate cyclase, the activity of which is considerably depressed [1,3].

It is worth mentioning that the glycon component of glucosides is necessary only to increase the water solubility of these compounds, as happens in the cardiac glucosides (Ref. 4 and unpublished results). Finally, the seemingly lower capability of octanol glucoside, as compared to cholesterol, to bind into synaptosomal plasma membranes, is rather due to more rapid reversibility of its binding. The more water soluble the compound (like some general anaesthetics), the more reversible its binding would be (unpublished results).

It would be appropriate to close this discussion by mentioning that octanol or dodecanol glucosides have repeatedly been used in biological studies to dissolve membranes [20]. It is of course expected that the effects of a certain amphipath would depend on the relative concentrations of itself and the other reactant (membranes). At large concentrations, octanol glucoside forms micelles. Together, in a mixture with membranes, octanol glucoside may form mixed micelles with membrane constituents, acting as a 'solubilizer'. At relative small concentrations, it preferably binds onto the membrane, producing effects like these reported here.

This work was partially supported by funds from the University of Athens and a grant of the Greek National Research Foundation, in support of Dr. G. Deliconstantinos. One of us (S.G.A.A.) wishes to thank the Academician Professor (of Gynecology) N. Louros for useful discussions during the course of this work.

References

1 Alivisatos, S.G.A., Nikitopoulou, G., Papaphilis, A., Molyvdas, A.P., Tsiboukidou, L., Kamper, E. (1978) Fed. Proc. 37, 516
2 Alivisatos, S.G.A., Papastavrou, C., Drouka-Liapati, E., Molyvdas, A.P. and Nikitopoulou, G. (1977) Biochem. Biophys. Res. Commun. 79, 677—683
3 Papaphilis, A. and Deliconstantinos, G. (1980) Biochem. Pharmacol. 29, 3325—3327
4 Alivisatos, S.G.A., Deliconstantinos, G., Papaphilis, A. and Theodosiadis, G. (1981) Biochim. Biophys. Acta 643, 642—649
5 Madden, I.D., Chapman, D. and Quinn, P.J. (1979) Nature 279, 538—540
6 Helferich, B. and Mueller, W.M. (1973) Chem. Ber. 106, 715—716
7 Baron, C. and Thompson, T.E. (1975) Biochim. Biophys. Acta 382, 276—285
8 Alivisatos, S.G.A., Ungar, F., Lukas, L. and LaMantia, L. (1960) J. Biol. Chem. 235, 1742—1750
9 Alivisatos, S.G.A. and LaMantia, L. (1960) Biochem. Biophys. Res. Commun. 2, 164—168
10 Johnson, S.M. (1979) Anal. Biochem. 95, 344—350
11 Dunham, E.T. and Glynn, I.M. (1961) J. Physiol. 156, 274—293
12 Ellman, G.L., Coourtney, D.K., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—95
13 Bergmeyer, H.U. and Bernt, E. (1974) in Methods in Enzymatic Analysis (Bergmeyer, H.U., ed.), Vol. 2, pp. 574—579, Academic Press, New York
14 Singer, S.M. and Nicholson, G.L. (1972) Science 175, 720—731
15 Farias, R.N. (1980) Adv. Lipid Res. 17, 251—283
16 Changeaux, J.P., Thiery, J., Tung, Y., Kittel, C. (1967) Proc. Natl. Acad. Sci. U.S.A. 57, 335—341
17 Mueller, G.C., Gorski, J. and Aizawa, Y. (1961) Proc. Natl. Acad. Sci. U.S.A. 47, 164—169
18 Mueller, G.C., Herrnanen, A.M. and Jervell, K.J. (1958) Recent Prog. Hormone Res. 14, 95—129
19 Jensen, E.V. (1979) Pharmacol. Rev. 30, 477—491
20 Rosevear, P., VanAken, T., Baxter, J. and Ferguson-Miller, S. (1980) Biochemistry 19, 4108—4115