MOLECULAR PHARMACOLOGY, 6, 122–127

The Interaction between Atropine Sulfate and a Proteolipid from Cerebral Cortex Studied by Polarization of Fluorescence

JOSÉ GONZÁLEZ RODRÍGUEZ,[1] JOSÉ L. LA TORRE, AND EDUARDO DE ROBERTIS

Instituto de Anatomía General y Embriología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

(Received September 29, 1969)

SUMMARY

Ultraviolet absorption and fluorescence spectra were recorded from a special proteolipid isolated from the cerebral cortex. This proteolipid has a high binding affinity for various drugs and, when dissolved in chloroform-methanol (4:1 by volume), has a sharp absorption band at 240 mμ and a weaker one at 278–280 mμ. Upon activation at 330 mμ the fluorescence emission is maximal at 380 mμ. The probable reasons for these spectroscopic properties are discussed.

Under the action of increasing concentrations of atropine sulfate there is an increase in polarization of proteolipid protein fluorescence, which follows a sigmoid curve with a Hill number of 3.9. This effect of atropine sulfate can be blocked by acetylcholine and homatropine bromide. These results are discussed in relation to previously reported changes in light scattering. These observations suggest that, under the influence of certain drugs, this receptor proteolipid may undergo molecular aggregation with a high degree of cooperativity.

INTRODUCTION

Previous studies led to the isolation from brain of a special proteolipid (i.e., a lipoprotein soluble in organic solvents) with a high binding affinity for dimethyl-^{14}C-*d*-tubocurarine (1), ^{14}C-serotonin (2), and adrenergic blocking agents (3). This proteolipid is found in the particulate fraction of gray matter and is concentrated in nerve ending membranes (1, 4), which carry the subsynaptic membranes attached (see ref. 5). In the presence of small concentrations of atropine sulfate this proteolipid showed an intense increase in light scattering (6), and the dose-response curves were sigmoid, characteristic of a cooperative interaction (7). Furthermore, the light scattering diminished in amplitude in the presence of acetylcholine, with only a small decrease in cooperativity, while with dimethyl-*d*-tubocurarine, hexamethonium, and succinylcholine there was a considerable increase in cooperativity as measured by the Hill number (n_H) (6).

In the present study the interaction of atropine sulfate with the proteolipid was studied by measuring changes in polarization of protein fluorescence, another method which can reveal conformational changes in protein molecules (8–10). A parallelism between the light scattering response and the changes in polarization of fluorescence was observed in the course of the titration of the proteolipid with atropine sulfate. The

These investigations were supported by grants from the Argentine National Research Council and the National Institutes of Health (NB 06953-04).

[1] Fellow of the Juan March Foundation, Spain.

fluorescence changes could also be partially blocked by different amines.

METHODS

Preparation of proteolipid. Gray matter from bovine cerebral cortex was dissected, lyophilized, extracted with chloroform-methanol (2:1 by volume), and subjected to partition against water (11). The proteolipid was precipitated at 0° with ether, a procedure that eliminates cholesterol, 80% of phospholipids, and about 50% of cerebrosides. It was further purified on a column of Sephadex LH-20 by elution with organic solvents of increasing polarity (12). In general, four peaks of protein were obtained, the last two of which had (a) a low content of lipid phosphorus, (b) a high binding affinity for radioactive drugs (4), and (c) an increase in light scattering with atropine sulfate (6).

Ultraviolet and fluorescence spectra. Ultraviolet spectra of the proteolipid and of tyrosine-tryptophan mixtures were recorded using a Beckman DK-2 double beam spectrophotometer or a manually driven Zeiss PMQ II spectrophotometer. All optical measurements were made using chloroform-methanol (4:1 by volume) as solvent.

Fluorescence was studied with an Aminco-Bowman spectrophotofluorometer provided with a thermostated cell compartment and Glan-Thomson prisms. Fluorescence spectra were registered with an x-y Aminco-Bowman recorder, and the titrations were monitored with a Beckman DB recorder. In order to avoid the influence of Rayleigh scattering during fluorescence measurements, the solutions were activated at 300 mμ instead of 330 mμ (the maximal activation wavelength of the proteolipid) and the emitted light was measured at 380 mμ. The exciting beam was vertically polarized, and the analyzer was alternately set with its electrical vector parallel and perpendicular to that of the plane of polarization of the excitation beam. The apparent polarization of the proteolipid fluorescence, P, was calculated as follows:

$$P = F_{\parallel} - F_{\perp}/F_{\parallel} + F_{\perp}$$

where $F_{\parallel}$ and $F_{\perp}$ are, respectively, the intensities of fluorescence with a parallel and a perpendicular orientation of the analyzer-polarizing prisms. P_0 expresses the polarization of the proteolipid alone, and P the polarization at each point during the titration with atropine sulfate. The increase in polarization of fluorescence is expressed as $P - P_0$. $P_{max} - P_0$ indicates the maximum change in polarization of fluorescence at the end of the titration. Standard and Hill plots (13) of the titration were made, and the Hill numbers (n_H) were determined.

$$n_H = \log[(P - P_0)/(P_{max} - P)]/\log[\text{atropine sulfate}]$$

Light scattering. Light scattering titrations were done with the same instrument as described previously (6). The increase in light scattering was expressed as $T - T_0$, in which T_0 represents the intensity of the scattered light of the proteolipid solution, and T the scattered light intensity at each point in the titration. In control experiments with solvent alone, the addition of atropine sulfate and other drugs, within the concentration range used, did not change the light scattering or the fluorescence, nor did these drugs quench proteolipid fluorescence. Both fluorescence and light scattering intensities were expressed in arbitrary units, and the fluorescence spectra were not corrected.

RESULTS

Ultraviolet spectra. Figure 1 shows an ultraviolet absorption spectrum of the proteolipid from peak III of cerebral cortex (see Fig. 1 of ref. 6). This material shows a strong increase in light scattering with atropine sulfate. The protein, dissolved in chloroform-methanol (4:1), has two main absorption bands. The one at 240 mμ is sharp and of high intensity, while the other, at 278–280 mμ, is wider and weaker. The $E^{1\%}_{1\,cm}$ at 280 mμ varied from one preparation to another, with a mean value of 20. The absorption spectra of tyrosine, tryptophan, and mixtures of these two amino acids were determined with the same solvent. The results indicate that this proteolipid contains both these aromatic residues.

Fluorescence spectra. The excitation spectrum of the same proteolipid measured with the emission wavelength set at 380 mμ shows a shoulder of low intensity at 280 mμ and a maximum at 330 mμ (Fig. 2A). With an

emission wavelength of 430 mμ there is a shoulder at 280 mμ, a shoulder or maximum at 330 mμ, and a maximum at 430 mμ. When activated at 330 mμ, a maximum is observed in the emission spectrum at 380 mμ (Fig. 2B), while activation at 370 mμ results in a maximum at 430 mμ. In Fig. 2 the upper traces correspond to the fluorescence spectra with both prisms in parallel, and the lower ones with prisms perpendicular. The apparent polarization of fluorescence at the 380 mμ emission maximum ranges between 0.01 and 0.1 from one preparation to another.

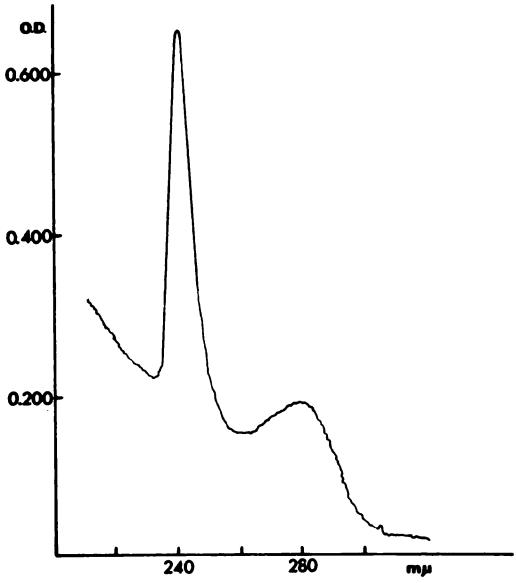

FIG. 1. *Ultraviolet absorption spectrum of a solution in chloroform-methanol (4:1 by volume) of proteolipid from bovine cerebral cortex (peak III of a Sephadex LH-20 column) containing 120 μg of protein per milliliter*

See description in the text.

Reference spectra were recorded with 15.3 μg of tryptophan and 27.2 μg of tyrosine per milliliter, concentrations that are similar to those found in the proteolipid (see below). The reference fluorescence quantum efficiency (F_q) of this solution in water was 226% transmission per microgram of tryptophan, with an optical density of 1.2 when measured against water. When dissolved in chloroform-methanol, the amino acid mixture had an F_q less than 1% transmission per microgram of tryptophan.

Changes in polarization of fluorescence with atropine sulfate. To study the effect of atropine sulfate, 1- or 2-μl aliquots of 10^{-3} M atropine sulfate in chloroform-methanol (4:1) were added to the cuvette, and the value of P was recorded at each point after attaining a maximal response. As shown in Fig. 3, both light scattering and polarization of fluorescence were determined in the same titration. Upon addition of atropine sulfate, there is a progressive increase in $P - P_0$, which rises, following a sigmoid curve, and reaches a saturation level at about 10^{-5} M

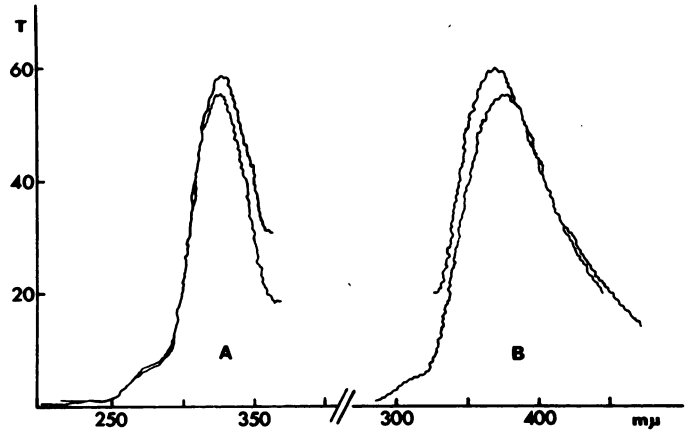

FIG. 2. *Excitation (A) and emission (B) fluorescence spectra of the same proteolipid used in Fig. 1*

The upper traces correspond to the spectra with both prisms in parallel, and the lower ones with prisms in perpendicular alignment.

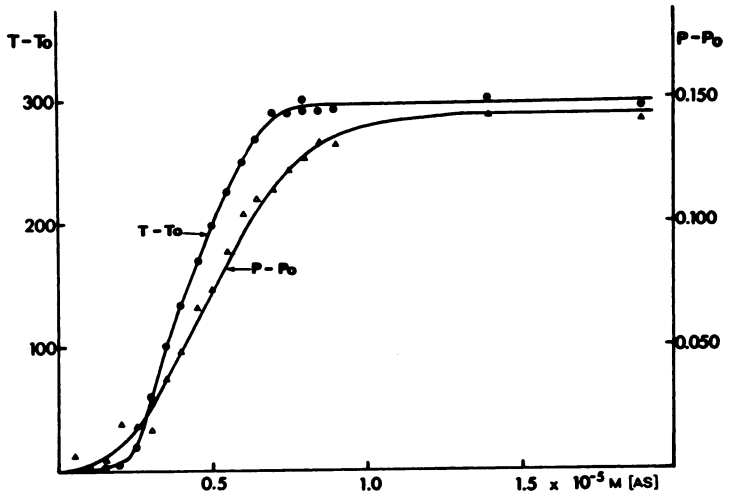

FIG. 3. *Changes in light scattering ($T - T_0$) and polarization of fluorescence ($P - P_0$) of the proteolipid with increasing concentrations of atropine sulfate (AS)*

In each determination, 2 ml of chloroform-methanol (4:1 by volume) containing 40 μg of proteolipid protein were used. Excitation was at 315 mμ, and measurements of light scattering and of polarization of fluorescence were made at 315 mμ and 380 mμ, respectively.

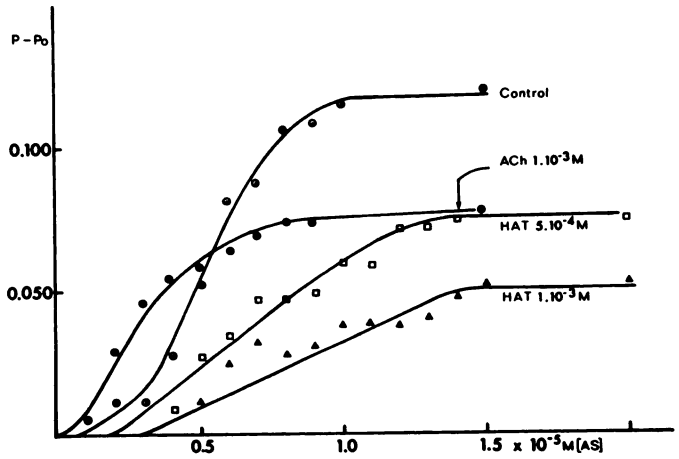

FIG. 4. *Effect of acetylcholine chloride (ACh) and homatropine bromide (HAT) on the changes in polarization of fluorescence of the proteolipid induced by atropine sulfate (AS)*

The concentration of proteolipid protein was 40 μg/2 ml. Excitation was at 300 mμ, and emission at 380 mμ.

drug. In general this curve follows the one obtained with light scattering ($T - T_0$). However, in the latter case, saturation is reached somewhat earlier, at 0.7×10^{-5} M atropine sulfate.

In Fig. 4 it may be observed that acetylcholine (10^{-3} M), added before the beginning of the titration, flattens the sigmoid shape and the saturation level. Acetylcholine decreases the dose of atropine sulfate required to produce half the maximal response. It also reduces the n_H, which indicates a decrease in the degree of cooperativity of the proteolipid–atropine sulfate interaction (Table 1). The effect of atropine sulfate on the polarization of fluorescence can also be

TABLE 1

Changes in Hill number (n_H) corresponding to the experiments described in Fig. 4

Drug	Concentration	n_H
	M	
Control		3.90
Acetylcholine	1×10^{-3}	2.43
Homatropine	5×10^{-4}	4.06
Homatropine	1×10^{-3}	2.88

blocked by homatropine bromide, which at 5×10^{-4} M and 10^{-3} M considerably reduces the amplitude of the atropine sulfate response although it has less effect on the n_H than acetylcholine.

The changes in polarization of fluorescence of the proteolipid could be reproduced with other bivalent amines, such as amphetamine sulfate. We have already reported that the sulfate salts of different amines give a light scattering change similar to that of atropine sulfate (6).

DISCUSSION

As in most proteins (14), the ultraviolet spectrum of this special proteolipid, which has receptor properties, is determined by the content of aromatic amino acids, i.e., phenylalanine, tyrosine, and tryptophan. Unfortunately, to date we lack definite information regarding the exact content of these amino acids in the proteolipid. Preliminary determinations have given the following figures: tryptophan, 10–20 μg; tyrosine, 20–30 μg; and phenylalanine, 140–160 μg/mg of proteolipid protein.

The 330 mμ and 370 mμ bands observed in the excitation and emission spectra of the proteolipid are probably due to the tryptophan residues. At variance with findings on the proteolipid, the control experiments using two of these aromatic amino acids in chloroform-methanol produced very low fluorescence efficiency. Before a more definite interpretation is attempted, however, the fluorescence spectra should be corrected and better knowledge of the amino acid content and the nature of possible prosthetic groups should be obtained. We have observed that the lipid phosphorus, which can be detected in the last peaks of proteolipid of the Sephadex LH-20 column, corresponds mainly to phosphoinositides and phosphatidylserine.

Atropine sulfate showed no fluorescence at the concentrations used. However, when acting upon the proteolipid, it induces a sigmoidal increase in polarization of proteolipid fluorescence. This may be explained by a change in size and shape, leading to an increase in the rotational relaxation time of the proteolipid (8–10). These findings are in general agreement with those previously obtained with atropine sulfate and light scattering (6), which were explained by an increase in particle size. Such a change may be the result of an association of molecular or micellar units into larger assemblies. We suggested (6) that, because of the low polarity of the medium, the atropine sulfate could be little ionized and able to bind simultaneously 2 units of the proteolipid. Preliminary observations from conductometric titrations of proteolipid with atropine sulfate in chloroform-methanol (4:1) indicate an increasing resistivity. Atropine sulfate alone also increases the resistivity of the solvent. These findings suggest that the reaction mechanism is not ionic and may be electrostatic. Preliminary electron microscopic observations also favor the interpretation that the proteolipid–atropine sulfate forms a micellar and aggregating complex.

Another point of considerable interest is the sigmoid shape of the dose-response curve, with an n_H of 3.9, which may express the degree of cooperativity (7) in the atropine sulfate–proteolipid interaction. Changeux and Podleski (15) have found an n_H of 2 for the changes in membrane potential of the electroplax treated with carbamylcholine, and have suggested an analogy between excitable membranes and regulatory enzymes. Our experiments show that the degree of cooperativity can be changed by interaction with other drugs. For example, with acetylcholine there was a tendency to a more hyperbolic type of curve with a reduction in the Hill number (Table 1).

Although both the light scattering and polarization of fluorescence changes are not stereospecific, they show a certain group specificity for various ligands (6). For example, in Fig. 4 it may be observed that there is a striking difference between the effects of acetylcholine and homatropine bromide at the same concentration. That these physicochemical changes are significant

is suggested by our previous studies on the binding of labeled dimethyl-*d*-tubocurarine and other drugs to the proteolipid (1–4). Furthermore, preliminary experiments with equilibrium dialysis using ^{3}H-atropine have shown that at low concentrations, which induce the early changes in polarization of fluorescence and light scattering, there is binding of ^{3}H-atropine to the proteolipid. The binding curves obtained with ^{3}H-atropine also have a sigmoid shape similar to those obtained with light scattering and polarization of fluorescence using atropine sulfate.

These findings with polarization of fluorescence give further support to our previous postulate (1) that in central synaptic membranes there is a special proteolipid having receptor properties for transmitters and other drugs which may be active in synaptic transmission. Upon exposure to drugs, the isolated receptor proteolipid may undergo conformational changes having a high degree of cooperativity.

ACKNOWLEDGMENT

The authors are indebted to Dr. David N. Teller for critically reading the revised manuscript.

REFERENCES

1. E. De Robertis, S. Fiszer and E. F. Soto, *Science* **158**, 928 (1967).
2. S. Fiszer and E. De Robertis, *J. Neurochem.* **16**, 1201 (1969).
3. S. Fiszer and E. De Robertis, *Life Sci.* **7**, 1093 (1968).
4. E. De Robertis, S. Fiszer, J. W. Pasquini and E. F. Soto, *J. Neurobiol.*, **1**, 41 (1969).
5. E. De Robertis, *Science* **156**, 907 (1967).
6. E. De Robertis, J. González Rodríguez and D. N. Teller, *Fed. Eur. Biochem. Soc. Lett.* **4**, 4 (1969).
7. J. Monod, J. Wyman and J. P. Changeux, *J. Mol. Biol.* **12**, 88 (1965).
8. G. Weber, *Advan. Protein Chem.* **3**, 416 (1953).
9. R. F. Chen, *in* "Fluorescence" (G. G. Guibault, ed.), p. 443. Marcel Dekker, New York, 1967.
10. L. Brand and B. Withold, *Methods Enzymol.* **11**, 776 (1967).
11. J. Folch and M. Lees, *J. Biol. Chem.* **191**, 807 (1951).
12. E. F. Soto, J. M. Pasquini, R. Plácido and J. L. La Torre, *J. Chromatogr.* **41**, 400 (1969).
13. T. L. Hill, *J. Chem. Phys.* **20**, 1029 (1952).
14. D. B. Wetlaufer, *Advan. Protein Chem.* **17**, 303 (1962).
15. J. P. Changeux and T. R Podleski, *Proc. Nat. Acad. Sci. U. S. A.* **59**, 944 (1968).