MOLECULAR PHARMACOLOGY, 7, 530–537

# The Use of a Cholinergic Fluorescent Probe for the Study of the Receptor Proteolipid

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(Received February 3, 1971)

SUMMARY

A trimethylammonium derivative, 1-dimethylaminonaphthalene-5-sulfonamidoethyltrimethylammonium perchlorate, was synthesized and used as a fluorescent probe for the study of the receptor proteolipid extracted from the electric organ of *Electrophorus electricus*.

A special technique based on the preferred partition coefficient of the proteolipid for organic solvents and of the trimethylammonium derivative for water was developed. Such a method permits the accurate and rapid titration of the proteolipid with the cholinergic fluorescent probe and also the study of the action of other drugs competing for the binding sites.

A cooperative type of interaction between the fluorescent trimethylammonium derivative and the receptor proteolipid was observed, and the competition of acetylcholine, dimethyl-*d*-tubocurarine, and decamethonium for the binding sites of the fluorescent probe was demonstrated.

## INTRODUCTION

Special proteolipids (hydrophobic lipoproteins) have been extracted from different gray structures of the central nervous system and shown to have high affinity for binding drugs active in synaptic transmission, such as dimethyl-*d*-tubocurarine (1), atropine (2), 5-hydroxytryptamine (3), and several adrenergic blocking agents (4, 5). Such hydrophobic proteins were found to be associated mainly with the synaptic structures and particularly with the nerve ending membranes and were designated "receptor proteolipids" (1). More recently a proteolipid was isolated and purified from the electric organ of *Torpedo* and *Electrophorus* and shown to bind $^{14}$C-acetylcholine and other cholinergic drugs *in vitro* (6). A saturation study of this proteolipid with acetylcholine suggested the presence of a single binding site of high affinity for a proteolipid molecule, with an assumed molecular weight of 40,000 daltons, having a dissociation constant of $1 \times 10^{-7}$ M and a group of low-affinity sites with a dissociation constant of $1 \times 10^{-5}$ M (7). In such

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[2] The work carried out at Buenos Aires was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas and the National Institutes of Health (5 R01 NS 06953-05).

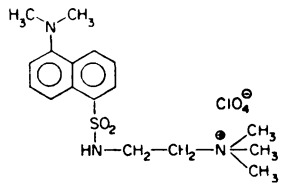
binding studies each titration point required the use of at least two columns of Sephadex LH-20, with and without proteolipid, which made the work rather long and painstaking.

In the present work advantage was taken of the use of a fluorescent probe having a cationic head similar to that of acetylcholine and with a preferred partition coefficient for water. This technique permitted a rapid and reliable study of the fluorophore–proteolipid receptor interaction, and of the competitive effect of other cholinergic drugs on such binding. A general technique for measuring the binding of drugs to hydrophobic biological substances may be based on the principles set forth in this investigation.

## METHODS

*Separation and purification of proteolipids.* Specimens of *Electrophorus electricus* were kept frozen at −30°. The electroplaques were dissected and homogenized in distilled water (10%, w/v) in a Waring Blendor for three 1-min periods at the highest speed. The homogenate was lyophilized and stored under vacuum over a desiccant. One gram of lyophilized electroplaques, equivalent to 12.5 g of fresh tissue, was homogenized in 15 ml of chloroform–methanol (2:1 by volume) for 1 min in an Ultra-Turrax apparatus (Karl Kolb, Frankfurt). After standing at room temperature for 5 min, the extract was filtered through Whatman No. 2 filter paper. The residue was washed with chloroform–methanol (5 ml; 2:1 by volume), the volume of the extract was noted, and a volume of chloroform equal to half that of the extract was added. This was then evaporated under vacuum at room temperature to a final volume of 5 ml. After standing at room temperature for 20 min, the extract was loaded onto a Sephadex LH-20 column (2.1 × 18 cm) that had been equilibrated overnight in chloroform. The standard elution procedure previously reported (8) was modified slightly as follows: chloroform, 80 ml; chloroform–methanol (15:1), 30 ml; and chloroform–methanol (4:1), 70 ml. The eluate was monitored at 278 nm with an LKB Uvicord ultraviolet absorption meter at a flow rate of 0.5 ml/min and collected into 2–5-ml fractions. The chromatographic pattern showed three peaks of proteolipid in the chloroform and two peaks in chloroform–methanol (4:1) [see Fig. 1 of De Robertis *et al.* (7)]. Peak 3 showed the highest affinity for binding of acetylcholine and thus was called the receptor peak. In this peak the lipid phosphorus to protein ratio was about 0.2, and the protein yield was approximately 220 μg.

*Synthesis of fluorescent probe.* A fluorescent trimethylammonium derivative, 1-dimethylaminonaphthalene-5-sulfonamidoethyltrimethylammonium perchlorate, was used as a probe. Its chemical formula is



Mol wt 434.5

For the synthesis of the compound, 1.6 g of 1,5-dimethylaminonaphthylsulfonyl chloride were refluxed with 0.53 g of unsymmetrical dimethylethylenediamine (Aldrich Chemical Company) in 50 ml of acetone for 1 hr. The resulting hydrochloride was neutralized with a solution of 0.6 g of potassium carbonate dissolved in 1 ml of water and refluxed for 30 min. Then 1.5 ml of methyl iodide were added slowly and refluxed for 1 hr. Additional acetone was added, and the solution was poured into 250 ml of 3 M sodium perchlorate. The resultant crystals (2.3 g) were filtered and washed with a saturated $NaHCO_3$ solution. After recrystallyzation from water, the crystals were washed with ether. Melting point range: 182.5–184.5°.

ELEMENTAL ANALYSIS

| | *Calculated* | *Found* |
|---|---|---|
| | % | % |
| C | 46.84 | 46.73 |
| H | 6.01 | 5.91 |
| N | 9.64 | 9.52 |
| S | 7.36 | 7.40 |
| Cl | 8.13 | 8.22 |
| O | 22.02 | 22.22[a] |

[a] By difference.

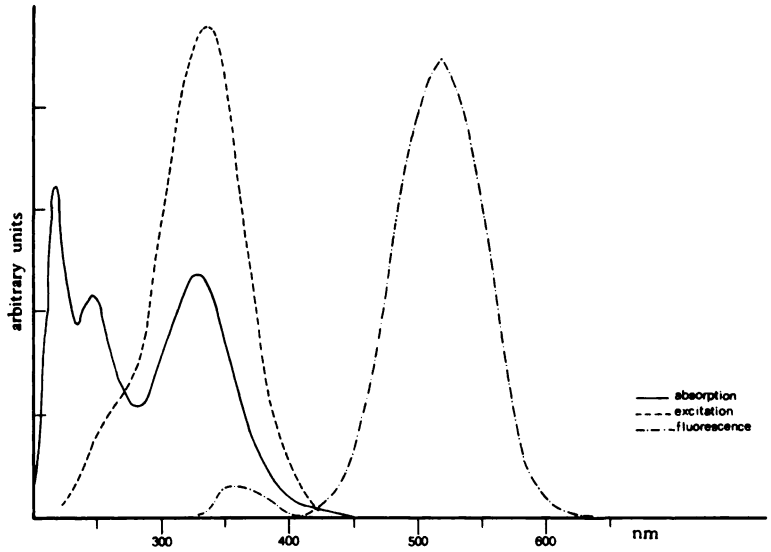
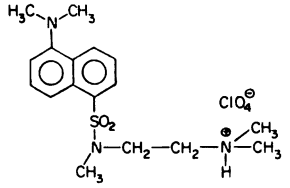



FIG. 1. *Absorption and technical fluorescence spectra of 1-dimethylaminonaphthalene-5-sulfonamidoethyltrimethylammonium perchlorate in water saturated with chloroform*
See the text for further description

N—H stretching vibrations for tertiary amine salts were not observed in the infrared region, eliminating the possibility of the synthesized compound having the structure



*Method for study of binding.* The excitation and fluorescence spectra of the probe are illustrated in Fig. 1. The main excitation region corresponds to the long-wave absorption band of this compound, which has a maximum at 340 nm. In the experiments the wavelength chosen for excitation was 370 nm. The emission band is, as expected, the mirror image of the 340 nm absorption band, having its maximum at 520 nm. The wavelength region of the emission chosen for the measurements of the concentration of the fluorescent probe was 540 nm. The wavelengths of excitation (370 nm) and emission (540 nm) were very well separated, so that corrections for scattered exciting light were minimized. The spectra were not corrected for the variation of the output of the xenon arc with wavelength, for the transmission of the excitation monochromator in the case of the excitation spectrum, or for the transmission of the fluorescence monochromator and photomultiplier response in the case of the emission spectrum. However, such corrections were not relevant in our case, because we simply used the total emission excited at a given wavelength as a

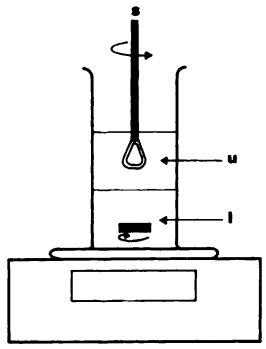



FIG. 2. *Apparatus used for study of fluorescent probe–proteolipid interaction*
A small cylinder was placed on a magnetic stirrer which kept the 2 ml of the lower phase containing the proteolipid sample (*l*) under constant agitation. Another stirring device (*s*), made of Teflon, was set in the 2-ml upper aqueous phase (*u*).

means of determining the concentration of the fluorophore in water. All the measurements were made with an Aminco-Bowman spectrofluorometer and registered on an *xy* Aminco-Bowman recorder. Standard curves for the fluorescent probe in chloroform-saturated water were made in concentrations ranging between $8 \times 10^{-8}$ and $1.8 \times 10^{-5}$ M. The partition coefficient of the compound between water saturated with chloroform and chloroform saturated with water at 18° was 31:1 in favor of the water.

As shown in Fig. 2, the experimental procedure used throughout consisted in placing a sample of 2 ml of proteolipid, containing about 30 μg of protein per milliliter of chloroform, in the bottom of a small cylinder. Such a proteolipid was washed several times with 2 ml of water saturated with chloroform for a period of 30–60 min, until the fluorescence in the upper phase reached a constant value. This value was deducted from all the subsequent measurements. When equilibrium was achieved, the fluorescent compound was added to the upper aqueous phase under constant stirring of both phases for 5 min. The solution of the fluorescent probe was $1.6 \times 10^{-4}$ M, and the additions were made 1–10 μl at a time. After each addition the fluorescence measurement was made in 1 ml of the upper phase, and this phase was returned to the cylinder before the next addition.

Competition experiments were performed using acetylcholine chloride, dimethyl-*d*-tubocurarine hydrochloride, and decamethonium bromide. These three compounds showed no intrinsic fluorescence in the wavelength used. The titration with the fluorescent probe in the presence of the competing drug was carried out as indicated above.

## THEORY OF THE METHOD

Two phases, with volumes $V_u$ and $V_l$, respectively, are equilibrated. The lower phase contains a binding agent (proteolipid) which is insoluble in the upper phase. Let $M$ moles of a ligand $X$ be introduced so that it becomes partitioned between the two phases. The amount of ligand $X$ in the lower phase, $M_l$, is made up of two parts, one free and the other bound to the proteolipid.

$$M_l = ([X_b]_l + [X_f]_l) \cdot V_l \qquad (1)$$

where $[X_b]_l$ is the bound concentration of $X$ in the lower phase, $[X_f]_l$ is the free concentration of $X$ in the lower phase, and $V_l$ is the volume of the lower phase. In the upper phase the amount of ligand is

$$M_u = [X_f]_u \cdot V_u \qquad (2)$$

where $[X_f]_u$ is the free concentration of $X$ in the upper phase and $V_u$ is the volume of the upper phase.

If an equilibrium between the phases has been established,

$$\frac{[X_f]_u}{[X_f]_l} = S \qquad (3)$$

in which $S$ is the partition coefficient.

$$M = M_u + M_l = [X_b]_l \cdot V_l + [X_f]_u \left( V_u + \frac{V_l}{S} \right) \qquad (4)$$

Since $M$, the total amount of ligand added, is known, a simple determination of $[X_f]_u$ is sufficient to determine $[X_b]_l$, when $S$ is known from a previous determination.

The method becomes particularly sensitive if $S$ is a large number, in which case the ligand in the upper phase is virtually the total free ligand and the ligand in the lower phase in virtually the total bound ligand. Moreover, in such cases the smallest concentration of free ligand in equilibrium with bound ligand that can be determined by the method of measurement becomes decreased by the factor $S$, thus extending by this factor the range of dissociation constants that may be estimated.

*Plotting and interpretation of results.* When When $[X_f]_l$ and $[X_b]_l$ are known for a fixed medium and temperature, the equilibrium is completely determined. There are various ways in which the results may be plotted according to the needs of the case (see ref. 9). We favor, for reasons given elsewhere (10, 11), the plot of $-\log [X_f]$ against $[X_b]$. The former quantity is proportional to the chemical potential of the ligand, so that the plot gives directly the concentration bound at each potential and is a direct thermodynamic description of the system. In a

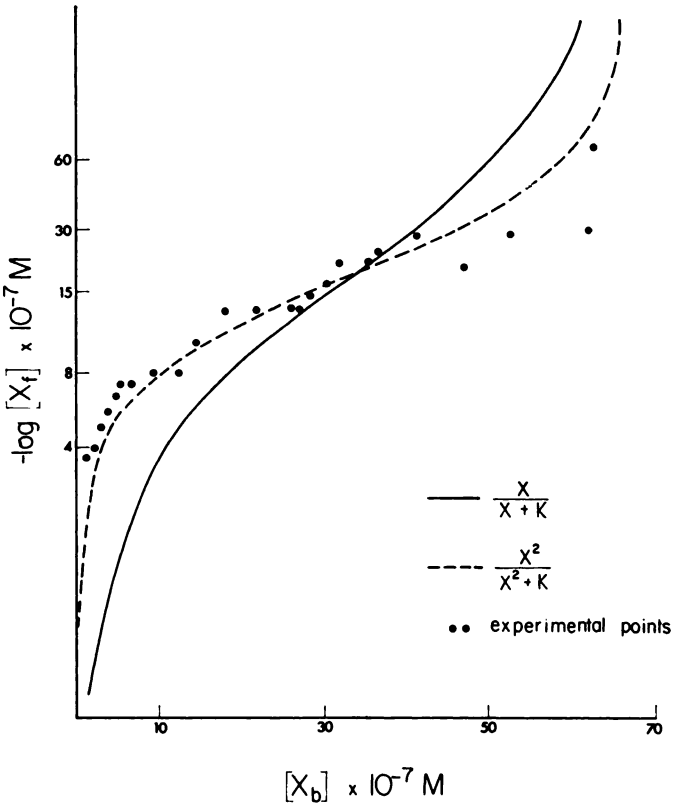



FIG. 3. *Plot of $-\log$ free ligand $[X_f]$ in upper phase against concentration of bound ligand $[X_b]$ in lower phase*

The points represent the experimental values. The continuous curve (——) was computed for a simple type of binding (see Eq. 5), and the discontinuous one (- - -), for cooperative binding of pairs of sites (Eq. 6).

system with two sets of binding sites (multiple binding), if the sites have dissociation constants separated by a large interval (in practice 100:1), two titration regions separated by an intermediate saturation region are observed. If the dissociation constants are close, the resolution into separate regions is no longer possible, but in either case the logarithmic span or interval in -log $[X_f]$ between 10% and 90% total saturation is larger than 1.81 units. The exact value of 1.81 units corresponds to equal, independent sites (simple binding). In cases of cooperative binding, that is, when the addition of ligand facilitates further addition, the logarithmic span is always less than 1.81 units. Such measurements thus give a first indication of the type of binding: simple, multiple, or cooperative.

## RESULTS AND DISCUSSION

Figure 3 shows a plot of the experimental values of -log $[X_f]$ against $[X_b]$. From these and similar titration curves we have attempted to determine (a) the stoichiometry of the reaction of the fluorescent probe with the proteolipid and (b) the type of binding process. In considering the possible sources of error it is necessary to bear in mind that a titration curve like that of Fig. 3 must cover at least one to two orders of magnitude in free ligand concentration. The factor of 20 reached is sufficient to define the curve over a span such that comparison with theoretical curves becomes possible. Over this range of free ligand the bound fluorescent ligand varied over a factor of 60, and the ratio of free to bound ligand never exceeded

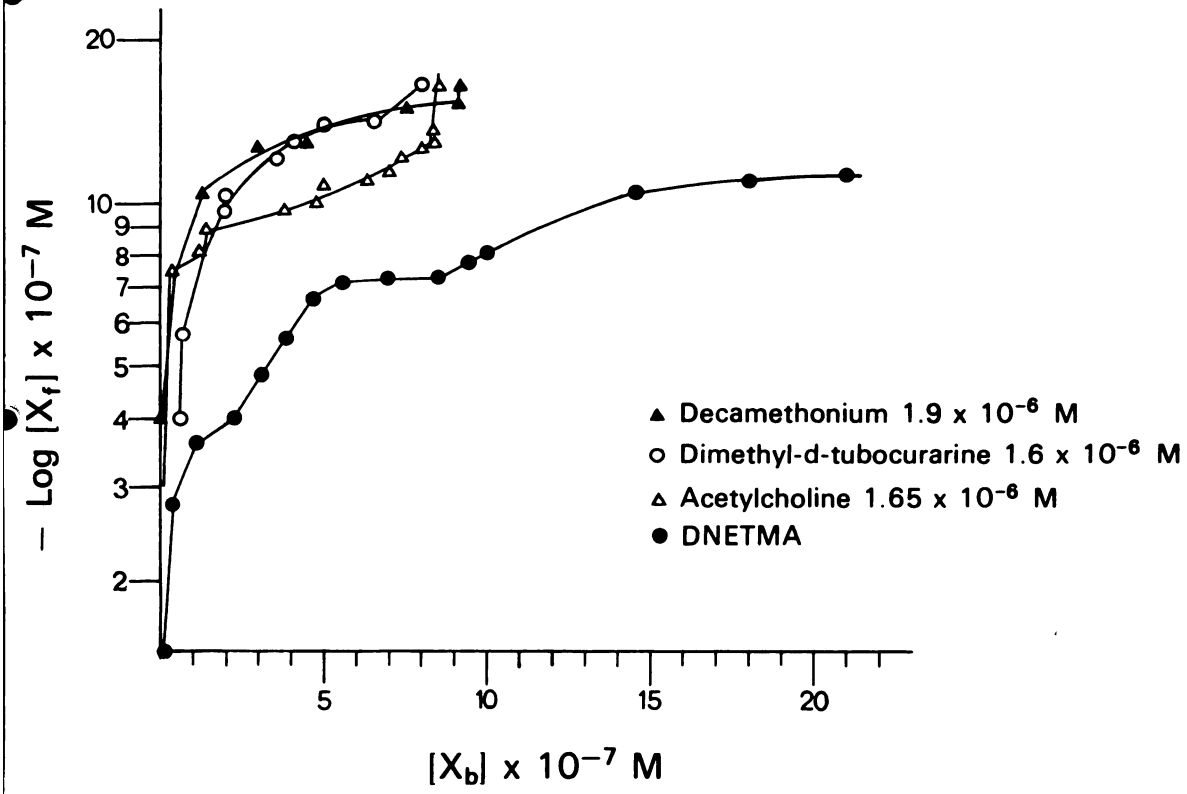



FIG. 4. *Effect of addition of competing drugs on binding of 1-dimethylaminonaphthalene-5-sulfonamidoethyltrimethylammonium perchlorate (DNE TMA) by proteolipid*

Coordinates as in Fig. 3. Notice the smoothed curve fitting the experimental points (●) for the binding of the fluorescent ligands, in comparison with the upward displacement of the curves in the presence of the competing drugs.

4. The largest value of free ligand measured by the fluorescence technique was smaller than $10^{-5}$ M. Therefore optical artifacts due to appreciable reabsorption of fluorescence are negligible.

*Stoichiometry of the reaction.* It is seen in Fig. 3 that an apparent end point of the titration (i.e., saturation) is reached after 1 mole of ligand is bound to approximately 5000 g of proteolipid. This over-all stoichiometry is close to that previously observed for the binding of acetylcholine (7) (1 mole/4000 g of proteolipid).

*Type of binding.* Examination of the experimental values indicates that the central 80% of the titration curve (Fig. 3) occurs over a span considerably shorter than 1.8 log units, so that we must have a case of cooperative binding. To demonstrate the cooperativity we have drawn the theoretical curves corresponding to the equations

$$\frac{[X_b]}{T} = \frac{[X]}{[X] + K} \tag{5}$$

$$\frac{[X_b]}{T} = \frac{[X]^2}{[X]^2 + K} \tag{6}$$

where $T$ is the total concentration of sites.

A value of $K = 2.16 \times 10^{-6}$ M was chosen so that the curves would coincide with the experimental points when half the sites were occupied ($[X_b]/T = \frac{1}{2}$). Equation 5 describes simple binding, while Eq. 6 describes cooperative binding of pairs of sites, so that when one site of the pair is occupied the other is also occupied. It is evident that the simple binding type of curve does not fit at all whereas the cooperative binding of pairs gives a very reasonable fit to the experimental points (Fig. 3). The situation appears

quite different from that obtained in the binding of acetylcholine, in which case De Robertis *et al.* (7) identified one strong binding site per 40,000 g of proteolipid ($K_1$ = $1 \times 10^{-7}$ M) and some 10 weaker sites ($K_2$ = $1 \times 10^{-5}$ M). Attempts to fit the experimental binding curve with a curve corresponding to a strong binding site and a group of weaker but cooperative sites gave a much poorer fit than the one shown in Fig. 3. The difference between the noncooperative binding of acetylcholine and the cooperative binding of the fluorescent ligand is not unexpected in view of the large hydrophobic surface of the fluorophore, which enhances the possibility of stabilizing a nearby site. The accuracy of the measurements is not sufficient to allow a decision as to whether the exponent 2, assumed in Eq. 6, is to be preferred to some higher value. If such were the case, one could conclude that the binding sites do occur in pairs which may bear important structural or functional relations.

*Binding of a competing ligand.* The identity of the proteolipid sites for binding acetylcholine and the fluorescent trimethylammonium derivative may be demonstrated by competition experiments (Fig. 4). When a fixed amount of acetylcholine, dimethyl-*d*-tubocurarine, or decamethonium was introduced into the system, the titration curves of the fluorescent ligand were displaced upward, indicating the molecular displacement of the latter by the competing drug. When the amount added was small, that is, in the region where competition takes place with the strong binding site of acetylcholine, the titration curve was displaced some 0.5 logarithmic unit. In similar regions the titration curve was displaced 0.7 log unit when the competitor was dimethyl-*d*-tubocurarine or decamethonium (Fig. 4). If the partition coefficients of the competitors and of the fluorescent ligand are all strongly in favor of the water phase, the affinity ratios of the ligand and competitors are given to a first approximation by the ratio of ligand concentrations in the water phase that produce the same binding in the absence and presence of the competitor. On this assumption the relative affinities of the fluorescent trimethylammonium derivative, acetylcholine, dimethyl-*d*-tubocurarine, and decamethonium are approximately in the ratio 1:4:5:5, respectively. These crude affinity ratios do not admit a simple interpretation in terms of dissociation constants, not only because of the lack of knowledge of the exact partition coefficients of the competitors but also because of the different characteristics of the binding of the competing ligands: i.e., multiple binding in the case of acetylcholine, cooperative in the case of the fluorescent trimethylammonium derivative.

## CONCLUDING REMARKS

*Differences between fluorophore and acetylcholine.* 1-Dimethylaminonaphthalene-5-sulfonamidoethyltrimethylammonium perchlorate resembles acetylcholine in the ethyltrimethylammonium end, which carries a strong positive charge and which binds anionic sites in the proteolipid. This fluorescent probe lacks the esterophilic region which provides for a secondary mode of interaction for acetylcholine (see ref. 12). The lower affinity of the fluorescent ligand for the proteolipid as compared to that of acetylcholine is thus easily explained. The cooperative binding effect observed for the ligand but absent with acetylcholine is more difficult to explain. It may depend upon the fact that the fluorescent ligand is a bulky hydrophobic molecule that is capable to a much larger extent than acetylcholine of modifying the structure of the proteolipid upon binding.

*Extension of methodology to other studies.* The insolubility of the proteolipid in the water phase and its solubility in organic phases immiscible with water provide an excellent opportunity for carrying out binding studies using partition of the free ligand between the water and the nonpolar phase. The theory of the method developed in the present work can easily be applied to the study of binding of ligands labeled with radioisotopes. Such a method is already being applied in this laboratory to the study of receptor proteolipid–drug interactions.

ACKNOWLEDGMENT

The authors are indebted to Dr. Telma Caputi for her collaboration in the computation of the data.

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