Journal of Chromatography, 248 (1982) 461–468
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,126

Note

Separation of *cis*- and *trans*-3,3'-bis(trimethylammoniomethyl)azobenzene salts (Bis Q salts) by reversed-phase high-performance liquid chromatography

ANNE DELCOUR and DEREK J. CASH*
Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY (U.S.A.)
BERNARD F. ERLANGER
Department of Microbiology, Columbia University, New York, NY (U.S.A.)
and
GEORGE P. HESS
Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY (U.S.A.)
(Received June 18th, 1982)

The high specificity of biological processes allows reactions to be initiated by rapid, light-induced changes in molecular shape of the ligand. This promises to be a useful technique for studying fast biological reactions. For example, the light-induced *cis–trans* isomerization in azobenzene derivatives[1–3] can be used for the *in situ* generation of ligands causing the chelation of metal ions[4], or the inhibition of acetylcholinesterase[5,6], the inhibition of acetylcholine receptor[7] and the activation of acetylcholine receptor[8–15]. The *cis* isomer of 3,3'-bis(trimethylammoniomethyl)azobenzene (Bis Q)[8] (Fig. 1) is an inactive precursor of *trans*-Bis Q which induces acetylcholine

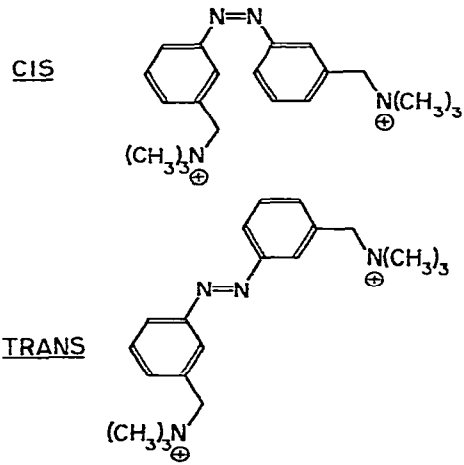

Fig. 1. The *cis* and *trans* configurational isomers of 3,3'-bis(trimethylammoniomethyl)azobenzene cations (Bis Q). *Cis* is converted to *trans* by irradiation at 420 nm and *trans* to *cis* by irradiation at 320 nm. *Cis* slowly reverts to *trans* thermally. The observation of one chromatographic elution peak each for *cis* and *trans* indicates that the rotational isomers, with respect to the C–N bonds, are relatively rapidly equilibrated. One rotamer of each configurational isomer is shown, the *syn–anti* with respect to the azo bond. The other rotamers are the *syn–syn* and the *anti–anti*.

0021-9673/82/0000–0000/$02.75

receptor-mediated transmembrane ion flux and desensitization of the receptor in membrane preparations from the electric eel, *Electrophorus electricus*[22–26]. The use of the rapid *in situ* photolytic formation of *trans*-Bis Q for the study of acetylcholine receptor in rigorous experiments has been until now limited by the availability of pure *cis*-Bis Q.

Acetylcholine itself has been separated by high-performance liquid chromatographic (HPLC) methods[16–18]. Here we report that *cis*- and *trans*-Bis Q can be separated by a simple procedure of reversed-phase HPLC[19] using sodium chloride in the eluent which gives directly a solution suitable for use with biological membranes. The separation can also be achieved with hydrophobic pairing ions. In addition the dependence of the separation on salt concentration and sample concentration was investigated.

EXPERIMENTAL

An octadecylsilane bonded silica column (μBondapak C_{18}, Waters Assoc.) (30 cm × 7.8 mm I.D.) was used with a Waters 6000 A solvent delivery system, U6K loop injector and 440 ultraviolet absorbance detector at 254 nm. The molar extinction coefficients are $\varepsilon_{254} = 6300$ (*cis*) and 2600 $l \cdot mol^{-1} \cdot cm^{-1}$ (*trans*). The mixture of *cis*- and *trans*-Bis Q was prepared by irradiation of *trans*-Bis Q (bromide or iodide in aqueous solution) with a xenon arc lamp through a band filter, $\lambda_{max} = 300$ nm, or with a nitrogen laser, $\lambda = 337$ nm. The manipulations were performed using a darkroom safelight (Sylvania red striplight, F4OR Lifeline used as a roomlight or Kodak No. I filter).

RESULTS AND DISCUSSION

Examples of the separation of *cis*- and *trans*-Bis Q with chloride (Fig. 2) and pentanesulfonate (Fig. 3) are shown in the chromatograms. Sodium chloride and potassium chloride give similar results. These separations can be performed easily with basic equipment without requiring the use of a solvent gradient. The *cis* isomer elutes first in all cases. Steric hindrance between the phenyl groups in this configuration causes the molecule to be skewed about the azo bond through a large angle whereas the *trans* isomer is approximately planar. The observed single peaks for *cis*- and *trans*-Bis Q indicate that the rotational isomers with respect to the C–N bonds are relatively rapidly equilibrated in these conditions. These are the three different rotamers, *syn-syn*, *syn-anti* and *anti-anti* with respect to the azo bond, for each configuration. Whether the contribution of these isomers is kinetically or thermodynamically controlled is pertinant to their use as specific ligands in experiments with, for example, acetylcholine receptor.

The elution volume decreases with increasing salt ($M^+ Cl^-$) concentration to a limiting value (Fig. 4), and there is a corresponding improvement in the separation of the isomers. The dependence of the chromatographic capacity factor[19] and the separation factor on the salt concentration are given in Table I. It is apparent that the partition of the *cis* isomer onto the column is less favorable than that of *trans*, and relatively less susceptible to decrease due to increasing salt concentration. The elution volume decreases with increasing quantity of Bis Q injected, to an extent which

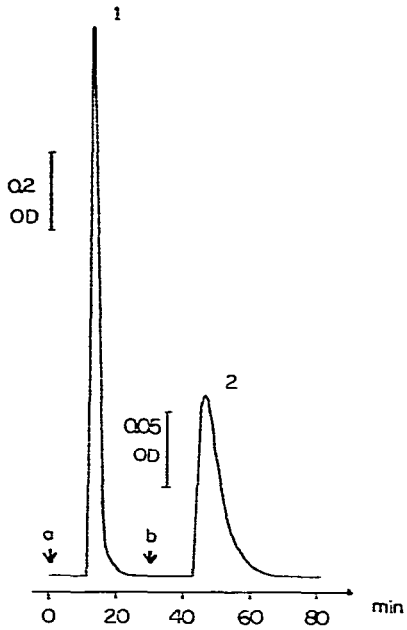

Fig. 2. Elution of *cis*- (1) and *trans*- (2) Bis Q with 500 m*M* sodium chloride in 10% aqueous ethanol, pH 6.5. Total Bis Q (0.69 mg Bis Q chloride containing 60% *cis* and 40% *trans*) was injected at a with 2 absorbance units giving full scale deflection. At b the sensitivity was changed to 0.5 a.u.f.s. Flow-rate was 2 ml/min and the void volume was 10 ml.

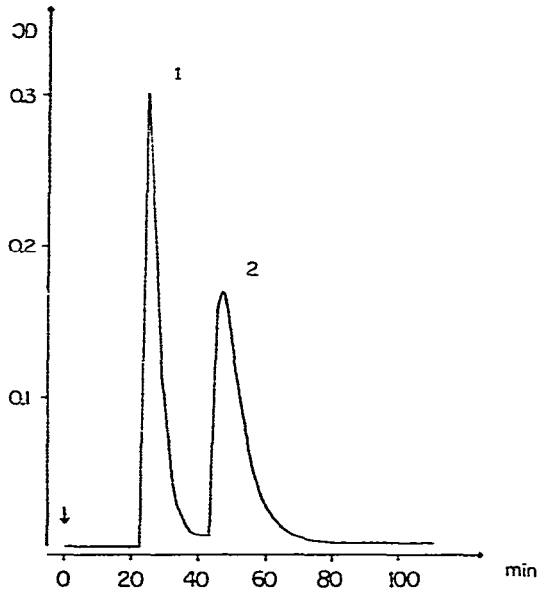

Fig. 3. Elution of *cis*- (1) and *trans*- (2) Bis Q with 50 m*M* sodium pentanesulfonate in 25% aqueous ethanol, pH 6.5. Total Bis Q (0.69 mg chloride salt containing 27% *cis* and 73% *trans*) was injected. Flow-rate was 2 ml/min and the void volume was 8 ml.

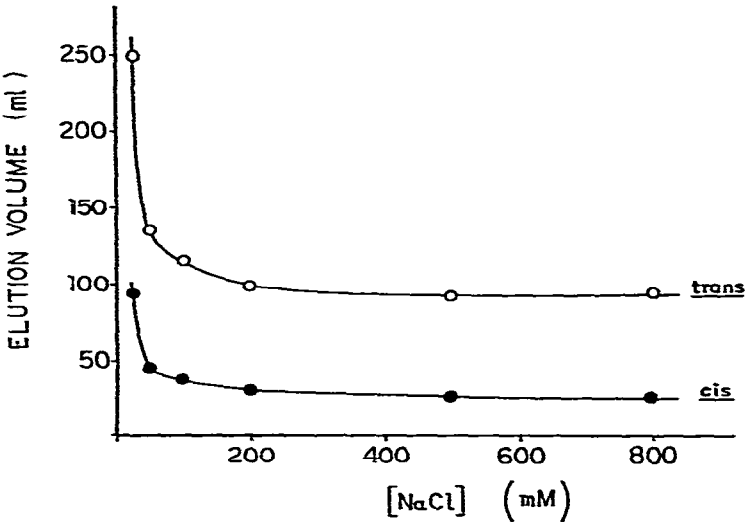

Fig. 4. Dependence of elution volume of *cis*- and *trans*-Bis Q on the concentration of sodium chloride in 10% aqueous ethanol, pH 6.5. The curves correspond to the injection of 0.6 mg *cis*-Bis Q and 0.4 mg *trans*-Bis Q together at each salt concentration. The void volume is 10 ml.

TABLE I

THE CAPACITY RATIOS FOR *cis*- AND *trans*-Bis Q AND THE SEPARATION FACTOR AS A FUNCTION OF SALT CONCENTRATION IN 10% AQUEOUS ETHANOL pH 6.5

Capacity ratio, $k' = (V_t - V_0)/V_0$; separation factor, $\alpha = (V_t - V_0)/(V_c - V_0) = k'_t/k'_c = D_t/D_c$. The void volume, the elution volume for *cis*-Bis Q and the elution volume for *trans*-Bis Q are represented by V_0, V_c and V_t respectively. The separation factor gives the ratio of the capacity ratios, k'_c and k'_t, and the distribution coefficients onto the column, D_c and D_t, for the two isomers[19].

NaCl (mM)	*Capacity ratio*		*Separation factor, α*
	k'_c	k'_t	
25	8.3	24	2.9
50	3.7	12.6	3.6
100	2.7	10.7	3.9
200	2.0	9	4.5
500	1.5	8.3	5.5
800	1.4	8.4	6.0

decreases with increasing salt concentration, as shown in Fig. 5. In addition to these dependencies on salt concentration and sample quantity, the elution volume decreases with increasing ethanol concentration. Although the elution volume of the peak is significantly retarded, with for example decreasing sample quantity, the trailing edge is not retarded, and the peaks lie underneath those with greater sample quantity. This is probably due to the ion pairing equilibria involved[19]. However these results do not rule out aggregation of the dye, which can alter its chromatographic behavior[20].

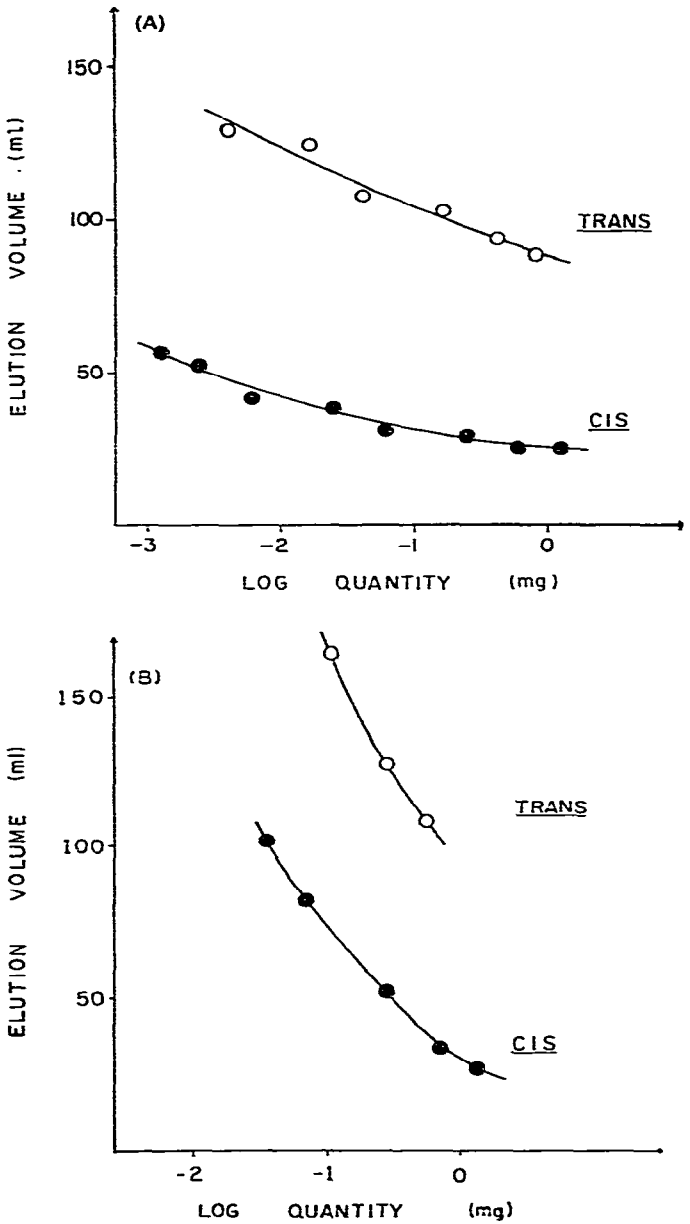

Fig. 5. Dependence of elution volume on quantity of sample injected in 500 m*M* sodium chloride (A) and 50 m*M* sodium chloride (B), in 10% aqueous ethanol, pH 6.5. The void volume is 10 ml.

It is known that for a stable chromatographic system with high sample loading, a high concentration of pairing ion is necessary and that elution peaks may be improved by the addition of further reagents[19]. For our preparative purposes we wished to avoid the addition of a second hydrophobic cation, particularly a quaternary ammonium derivative, or other cosolvents. We required a simple procedure with the

minimum post chromatographic manipulation in mild conditions, giving a solution which does not significantly affect the properties of the biological membrane or acetylcholine receptor, apart from the specific effect of Bis Q. The salt concentration could be reduced to 50 m*M* NaCl to facilitate the preparation of the solution required after chromatography. Up to 20 mg Bis Q could be separated with this size (7.8 mm) column with this solvent. The alcohol could be removed by evaporation at room temperature. To remove all the salt, the effluent was lyophilised or evaporated completely and the Bis Q extracted from the mixed salts with ethanol. For analytical purposes the use of a high salt concentration (*e.g.*, 500 m*M* NaCl) in the chromatography solvent is suitable.

The absorption spectra of pure *cis*- and *trans*-Bis Q are shown in Fig. 6. Besides their rapid photolytic conversion, these isomers are slowly interconverted thermally

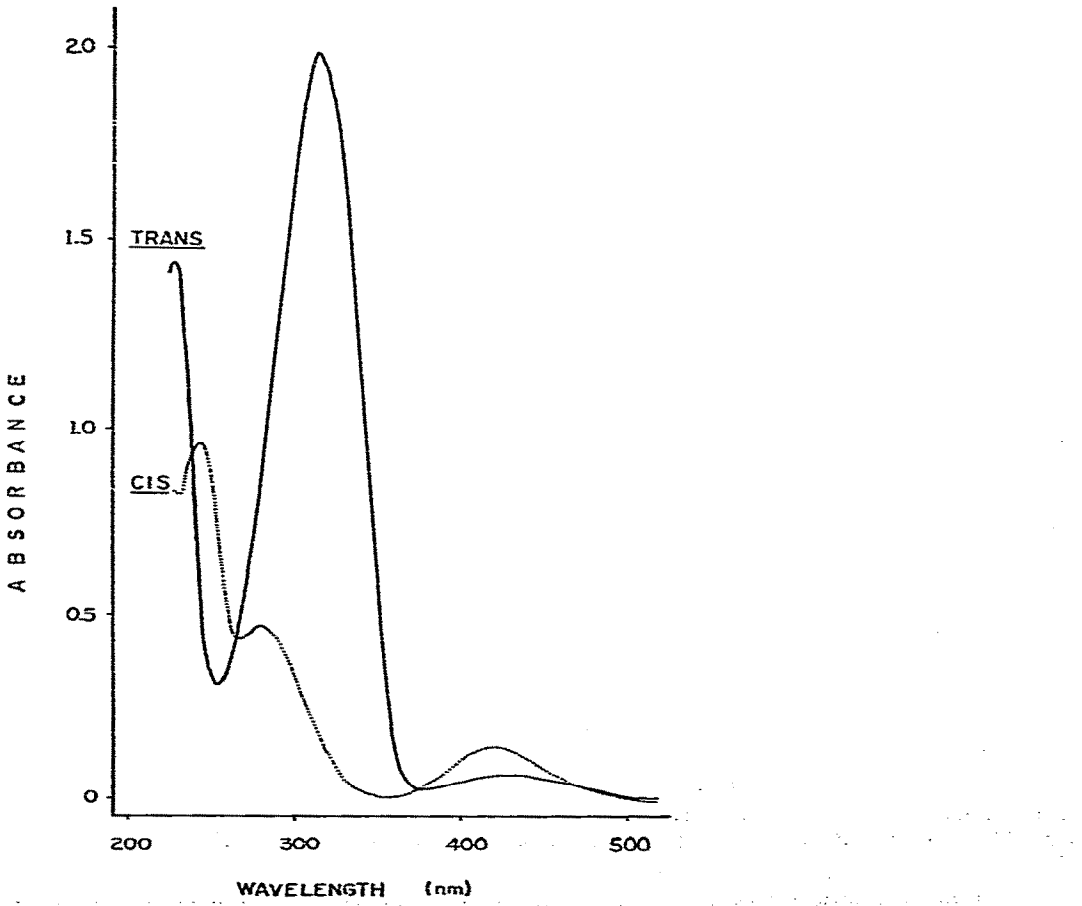

Fig. 6. Absorption spectra of *cis*- and of *trans*-3,3′-bis(trimethylammoniomethyl)azobenzene cations (Bis Q). Bis Q is 1.15×10^{-4} *M* in eel Ringer's solution (168 m*M* NaCl, 5 m*M* KCl, 3 m*M* $CaCl_2$, 1.5 m*M* $MgCl_2$, 1.5 m*M* sodium phosphate buffer, pH 7.0). A useful isobestic point is at $\lambda = 266$ nm, $\varepsilon = 3600$ M^{-1} cm^{-1}. In a mixture of *cis* and *trans* isomers, [Bis Q_{total}] = $A_{\lambda=266}/3600$ *M* and [*trans*-Bis Q] = $(A_{\lambda=318} - 1150\,[\text{Bis } Q_{total}])/15820$ *M*.

at room temperature[21], to an equilibrium mixture containing *ca.* 80% *trans*-Bis Q, with a relaxation constant, $(k_c + k_t) = 0.01\ day^{-1}$ in eel Ringer's solution (Fig. 7). Thus *cis*-Bis Q turns to *trans* at a rate of 0.8% per day and *trans* turns to *cis* at a rate of 0.2% per day. The samples should be freshly prepared with the mildest post chromatographic treatment and when necessary stored deep frozen.

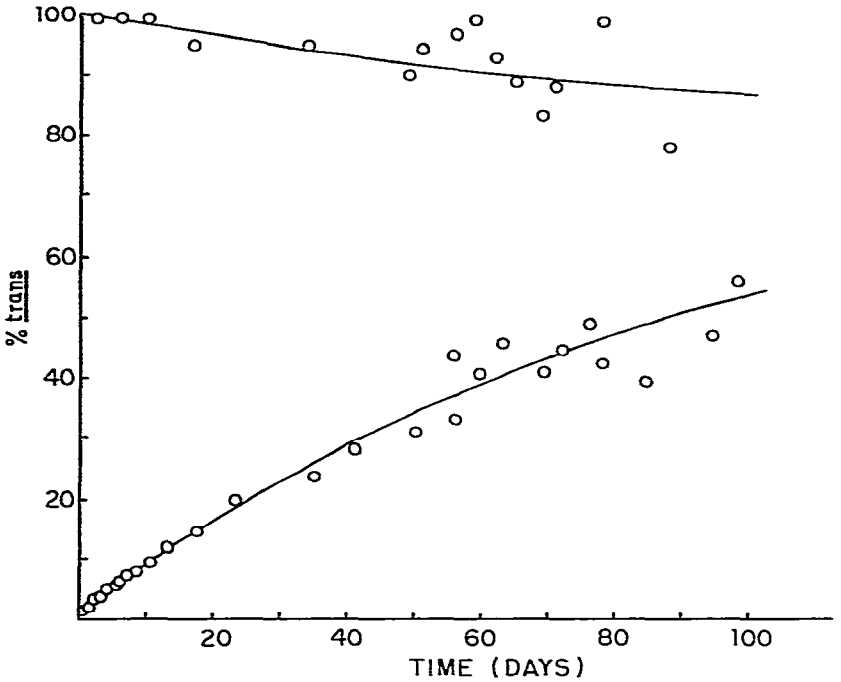

Fig. 7. Thermal interconversion of *cis*- and *trans*-Bis Q at 23°C in eel Ringer's solution (168 m*M* NaCl, 5 m*M* KCl, 3 m*M* $CaCl_2$, 1.5 m*M* $MgCl_2$, 1.5 m*M* sodium phosphate buffer, pH 7.0). The points are experimental. The lines are first order relaxation to an equilibrium solution containing 80% *trans*-Bis Q with a relaxation constant, $(k_c + k_t) = 0.011\ day^{-1}$. $k_{cis \to trans} = 0.008\ day^{-1}$; $k_{trans \to cis} = 0.002\ day^{-1}$. The data are from analysis of the absorption spectra.

ACKNOWLEDGEMENTS

We thank Dr. N. H. Wassermann for providing us with samples of Bis Q, Professor A. Lewis for the use of his chromatography equipment and darkroom and Professor A. C. Albrecht for the use of his nitrogen laser. This work was supported by NIH grants NS 15581 (B.F.E.) and DA 0.2445 (G.P.H.).

REFERENCES

1 P. P. Birnbaum and D. W. G. Style, *Trans. Faraday Soc.*, 50 (1954) 1192.
2 G. Zimmerman, L.-Y. Chow and U.-J. Paik, *J. Amer. Chem. Soc.*, 80 (1958) 3528.
3 R. L. Ross and J. Blanc, in G. H. Brown (Editor), *Techniques of Chemistry, Vol. III, Photochromism*, Wiley-Interscience, New York, 1971, p. 471.
4 M. Blank, L. M. Soo, N. H. Wassermann and B. F. Erlanger, *Science*, 214 (1981) 70.

5 J. Bieth, S. M. Vratsanos, N. H. Wassermann and B. F. Erlanger, *Proc. Nat. Acad. Sci. U.S.*, 64 (1969) 1103.
6 J. Bieth, N. H. Wassermann, S. M. Vratsanos and B. F. Erlanger, *Proc. Nat. Acad. Sci. U.S.*, 66 (1970) 850.
7 W. J. Deal, B. F. Erlanger and D. Nachmansohn, *Proc. Nat. Acad. Sci. U.S.*, 64 (1969) 1230.
8 E. Bartels, N. H. Wassermann and B. F. Erlanger, *Proc. Nat. Acad. Sci. U.S.*, 68 (1971) 1820.
9 N. H. Wassermann, E. Bartels and B. F. Erlanger, *Proc. Nat. Acad. Sci. U.S.*, 76 (1979) 256.
10 H. H. Lester and H. W. Chang, *Nature (London)*, 266 (1977) 373.
11 M. M. Nass, H. A. Lester and M. E. Krouse, *Biophys. J.*, 24 (1978) 135.
12 H. A. Lester, M. E. Krouse, M. M. Nass, N. H. Wassermann and B. F. Erlanger, *J. Gen. Physiol.*, 75 (1980) 207.
13 M. E. Krouse, M. M. Nass, J. M. Nerbonne, H. A. Lester, N. H. Wassermann and B. F. Erlanger, in D. B. Satelle, L. M. Hall and J. G. Hildebrand (Editors), *Neurotransmitter and Hormone Receptors in Insects*, Elsevier/North-Holland, Amsterdam, 1980, p. 17.
14 H. A. Lester, M. M. Nass, M. E. Krouse, J. Nerbonne, N. H. Wassermann and B. F. Erlanger, *Ann. N.Y. Acad. Sci.*, 346 (1980) 475.
15 H. A. Lester and J. M. Nerbonne, *Annu. Rev. Bophys. Bioeng.*, 11 (1982) 151.
16 S. Eksborg and B.-A. Person, *Acta Pharm. Suecica*, 8 (1971) 205.
17 S. Eksborg and B.-A. Person, in I. Hanin (Editor), *Choline and Acetylcholine: Handbook of Chemical Assay Methods*, Raven Press, New York, 1974, p. 181.
18 S. Eksborg and G. Schill, *Anal. Chem.*, 45 (1973) 2092.
19 E. Tomlinson, T. M. Jefferies and C. M. Riley, *J. Chromatogr.*, 159 (1978) 315.
20 D. J. Cash, *J. Chromatogr.*, 209 (1981) 405.
21 J. S. Huebner and W. E. Varnadore, *Photochem. Photobiol.*, 35 (1982) 141.
22 M. Kasai and J.-P. Changeux, *J. Membr. Biol.*, 6 (1971) 1, 34, 58.
23 G. P. Hess, D. J. Cash and H. Aoshima, *Nature (London)*, 282 (1979) 329.
24 D. J. Cash, H. Aoshima and G. P. Hess, *Proc. Nat. Acad. Sci. U.S.*, 78 (1981) 3318.
25 H. Aoshima, D. J. Cash and G. P. Hess, *Biochemistry*, 20 (1981) 3467.
26 D. J. Cash and G. P. Hess, *Anal. Biochem.*, 112 (1981) 39.