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# Heterogeneities in Acetylcholine Receptor from *Torpedo* Species

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Micro-heterogeneity of acetycholine receptor is described: preparations differ in their proportions of low- and high-affinity acetylcholine binding sites, with $K_d$ for acetylcholine of 11 and 562 nM respectively. The low-affinity is absent from fresh membranes or unpurified Triton X-100 exstracts, but is the major component of pure or oxidized preparations, and is therefore considered an artifact. Positive cooperativity in all high-affinity preparations is described, and used to explain how a $K_d$ of 11 nM is compatible with a physiological half-response at 1 μM. Oligomeric variations in *Torpedo* species are described. *T. californica* has two major oligomers, H and L, and two minor ones, HH and LL. The principal form is considered to be H; its molecular weight was estimated by $D_2O$ experiments as 535 000. It is a dimer of L, and a hexamer of LL. It is is proposed that HH is a nonspecific aggregate, formed especially at low detergent concentrations.

Research with acetylcholine receptor has had more than its share of disagreements between different laboratories. This paper presents evidence that at least some of these problems may be related to heterogeneities in *Torpedo* receptor preparations. I shall first discuss what might be called micro-heterogeneity.

For several years, our laboratory has stressed the fact that the Scatchard plot of binding of acetylcholine to receptor preparations is frequently curvilinear. Figure 1 shows an example, involving a Lubrol-solubilized preparation

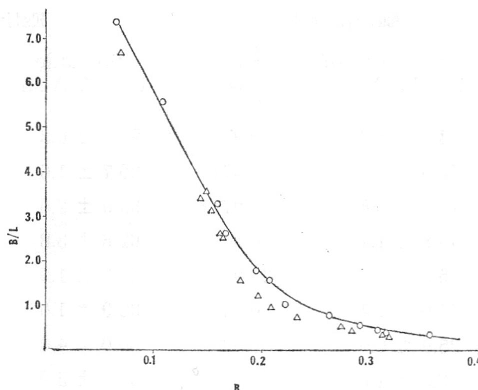



Fig. 1. Binding (B, in nmol g-1) of acetylcholine at various concentrations (L) to a Lubrol WX-extract of long-stored lyophilized *T. marmorata* electroplax membranes.

of stored *Torpedo marmorata* electroplax membrane. Such a plot might arise from negative cooperativity, or it might represent the sum of the activities of two quite separate sites. We have previously provided evidence that the sites are separate, based upon studies of acetylcholine binding at high and low acetylcholine concentrations which indicate two different behaviours under these two different conditions. Perhaps the most persuasive was the observation (Fig. 2) that the time of heat denaturation was quite different for these two

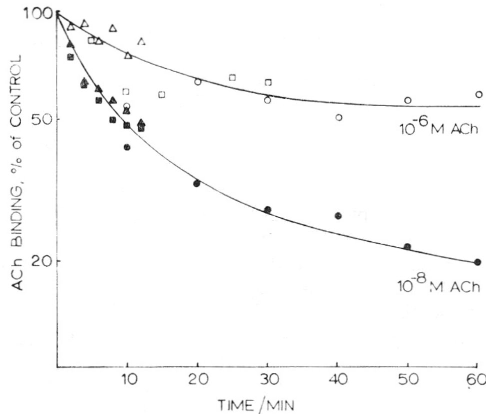



Fig. 2. Loss of acetylcholine-binding activity at 40 °C to a preparation as in Figure 1.

conditions. Other differences were observed with respect to sensitivity to pH and various drugs. Making the assumption that the curved Scatchard plot did indeed represent two non-interacting sites, we were able to calculate (Table I) the relative potency of various drugs against these two sites. The calculations are based upon the assumption of simple competitive inhibition, which is

TABLE I.

*Percent blockade of acetylcholine binding to Lubrol preparations by 100-fold excess of blocking drugs*

| Drug | Estimated | | Estimated | | |
|---|---|---|---|---|---|
| | % Blockade of $10^{-8}$ M Ach | $D_H$ / μM | % Blockade of $10^{-6}$ M ACh | $D_L$ / μM | $D_L$ / $D_H$ |
| Butyryl choline | 88.4 ± 1.0 | 0.022 | 99.7 ± 0.3 | 0.36 | 16 |
| Carbamyl choline | 76.6 ± 0.7 | 0.071 | 86.7 ± 1.6 | 3.56 | 50 |
| Succinyl choline | 76.3 ± 0.6 | 0.077 | 89.9 ± 2.0 | 1.6 | 21 |
| Decamethonium | 49.8 ± 1.4 | 0.34 | 62.6 ± 3.8 | 10.1 | 30 |
| Hexamethonium | 5.5 ± 3.3 | 38.0 | 22.5 ± 1.6 | 45.0 | 1.2 |
| Curare | 54.0 ± 1.4 | 0.14 | 83.9 ± 1.0 | 0.22 | 1.6 |
| Nicotine | 35.9 ± 0.9 | 0.62 | 50.0 ± 4.0 | 14.8 | 24 |
| Hemicholinium | 8.5 ± 1.0 | 3.5 | 52.8 ± 5.9 | 4.5 | 1.3 |
| Atropine | 4.2 ± 0.9 | 512.3 | 16.7 ± 2.9 | 66.8 | 0.13 |

probably an oversimplification, but the trends are in the correct direction. We have used the term high-affinity and low-affinity acetylcholine binding sites to describe these two postulated sites. Table I shows that agonists such as carbamylcholine and nicotine are discriminatory, with affinity 20 to 50 times greater for the highthan the low-affinity site. For acetylcholine itself the ratio is 78. But antagonists such as curare do not discriminate. It is important to note that α-bungarotoxin, like other antagonists, discriminates very poorly between the sites, having a somewhat better affinity for the low-affinity site.

At this point we began to work with fresh *Torpedo californica* electroplax, and found a completely different picture. Whether one works with fresh Triton extracts, or with fresh membrane preparations, one sees only a single kind of acetylcholine binding, and it is of the high-affinity form (Fig. 3). If we now turn to the literature (Table II) we see widely differing reports of

TABLE II.

*Dissociation constants/nM for ACh*

| | High affinity | Low affinity |
|---|---|---|
| (*T. marm.*)[1], crude, 1% Lubrol | 1.4 | 220 |
| (*T. marm.*)[2], crude, 1% Triton | 11 | 562 |
| (*T. calif.*)[3], crude, 1% Triton | 17 | absent |
| Fresh membranes, no detergent | 14 | absent |
| 5-Month-stored, no detergent | 2 | 200 |
| Purified, no added detergent | 17 | 480 |
| (*T. marm.*)[4], fresh membranes, no detergent | 8 | absent |
| (*T. calif.*)[5], purified, no added detergent | absent | 5000 |
| (*T. calif.*)[6], purified, no added detergent | absent | 2300 |
| (*T. marm.*)[7], crude, 1% cholate | 55 | 800 |

dissociation constants for acetylcholine. For stored *Torpedo marmorata,* our high-affinity constants are in the order of 10 nM, and our low-affinity in the order of 1 μM. We now find that fresh material has no low-affinity component, but if it is stored or purified, it develops very substantial amounts of low--affinity material. This made us suspect that the low-affinity material was an artifact of some kind, quite possibly involving oxidation of SH groups. This possibility could explain the striking differences which other workers have found; for instance Weber and Changeux found only high-affinity form in fresh *Torpedo marmorata* membranes, whereas Raftery's group seemed to find only low-affinity forms in purified receptor from *T. californica*. But a few months ago, Changeux's laboratory found that they too were able to detect two kinds of affinity, which fit reasonably well into the high-affinity and low-affinity numbers which we have been reporting.

Confirmatory evidence that the low-affinity form is in fact an artifact is that if one treats fresh Triton extracts with PCMB or by heating at 40 °C

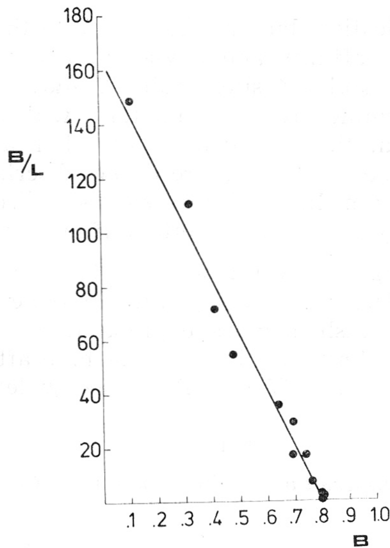



**Fig. 3. Acetylcholine binding to unpurified Triton X-100 extracts of fresh *T. californica* electroplax membranes.**

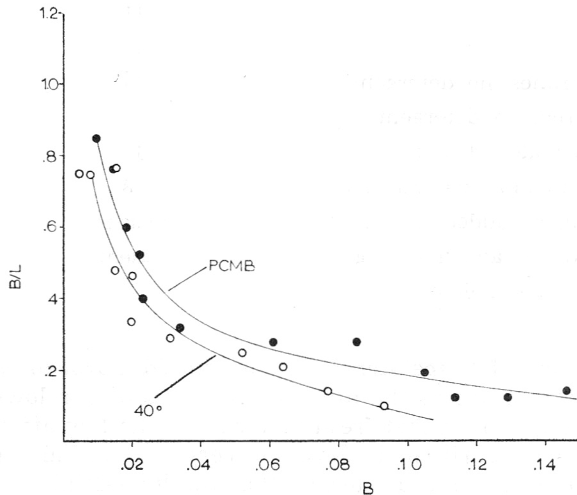



**Fig. 4. Effect of 1 mM PCMB or heat (40 °C, 40 min) upon acetylcholine binding to a preparation as in Figure 3.**

for 40 minutes, one converts it from all-high to mostly-low (Fig. 4). In these figures the high-affinity comprises only 5% of the total binding. Treatment with $10^{-4}$ M DTNB has a similar effect, converting the material to one with only 8% high-affinity.

In three quite different purified preparations from *T. californica*, kindly provided by the Drs. Eldefrawi, high-affinity material was always the lesser part, averaging 32% of the material. Interestingly enough, the purified receptor without added detergent was very insensitive to heat, but if 1% Triton X-100

was added to the pure receptor, which provides very substantial loss of binding activity, then the detergent-treated pure receptor shows extreme heat sensitivity.

We conclude that, with the exception of fresh membrane preparations and fresh detergent extracts, most receptor preparations are heterogeneous. There is a quick measure of the heterogeneity of a preparation, which we call the 6/8 ratio. This is simply the ratio of the amount of acetylcholine bound at $10^{-6}$ M compared with $10^{-8}$ M. In a system which is all high-affinity, with a $K_d$ of 11.3 nM, the 6/8 ratio is small, *i. e.* 2.1; the reason is that the binding is half-saturated at $10^{-8}$ M and almost fully saturated at $10^{-6}$ M, providing a ratio of 2. But if the material is all low-affinity, then the 6/8 ratio (assuming a $K_d$ of 562 nM) is 37, because the preparation is far from saturated at $10^{-8}$ M and mostly saturated at $10^{-6}$ M. Mixed preparations will have 6/8 ratios which are intermediate. Table III shows what an important effect this micro-hetero-

TABLE III.

| | | Preparation A: all high-affinity | Preparation B: all low-affinity |
|---|---|---|---|
| 6/8 Ratio | | 2.1 | 36.6 |
| | $K_i$ low / $K_i$ high | % Inhibition[a] | % Inhibition[a] |
| $10^{-6}$ M carbamylcholine | 50 | 14 | 9 |
| $10^{-6}$ M curare | 1.6 | 7 | 62 |
| $10^{-3}$ M atropine | 0.13 | 2 | 84 |

[a] Calculated for $10^{-6}$ M ACh from: % inhibition $= \dfrac{100\,[I]}{[I] + K_i\,[1 + 10^{-6}/K_d]}$

geneity can have. Using calculated numbers derived from Table I, we see that the sensitivity of an all-high or an all-low preparation to discriminating agents such as carbamylcholine is not much different. The reason is that the inhibition is computed from a formula which involves multiplying the dissociation constant for the drug by the dissociation constant for acetylcholine, so that if both drug and acetylcholine discriminate sharply between the high- and low-affinity sites, the effects balance out. But if we use a non-discriminating drug, or (even more extreme) a counter-discriminatory drug, such as atropine, you see that very different values of inhibition are reported, and different investigators may disagree profoundly about drug effects.

The inhibition picture is confused by the fact that some agents act in unexpected ways. We observed (Table IV) that several polyvalent cations are potent inhibitors of acetylcholine binding, in this case of a Triton-soluble extract of *T. californica*. But an additional effect is upon the 6/8 ratio, which for the control in this study was 1.4, and was increased to 6 by $Fe^{++}$ or $Zn^{++}$. Thus their action takes the form of converting high-affinity to low-affinity material, just as described above for PCMB or DTNB. Perhaps the polyvalent cations should be regarded as denaturants rather than as true inhibitors.

TABLE IV.

*Effect of* 1 mM *Cations on* ACh *Binding to Crude T. Californica in* 1% *Detergent*

| | | % of control | |
|---|---|---|---|
| | | $10^{-8}$ M ACh | $10^{-6}$ M ACh |
| Very Effective | $Cr^{3+}$ | 0.1 | 1 |
| | $Cu^{2+}$ | 0.05 | 2 |
| | $Fe^{3+}$ | 0 | 8 |
| Medium | $Zn^{2+}$ | 9 | 54 |
| | $Fe^{2+}$ | 4 | 24 |
| Poor Effectiveness | $Co^{2+}$ | 96 | 95 |
| | $Mn^{2+}$ | 100 | 96 |
| | $Ni^{2+}$ | 95 | 97 |

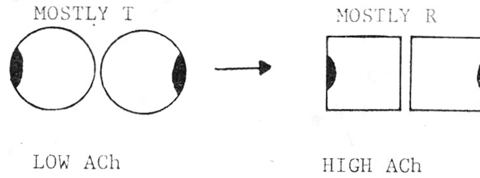
Buffer: Tris with 1% Triton X-100

We now turn to a source of variation which has given us a great deal of trouble, because preparations differ profoundly. Working with fresh Triton extracts, so that low-affinity material is absent, then if one works at sufficiently low acetylcholine concentrations (down to 5 nM) one almost invariably sees positive cooperativity.

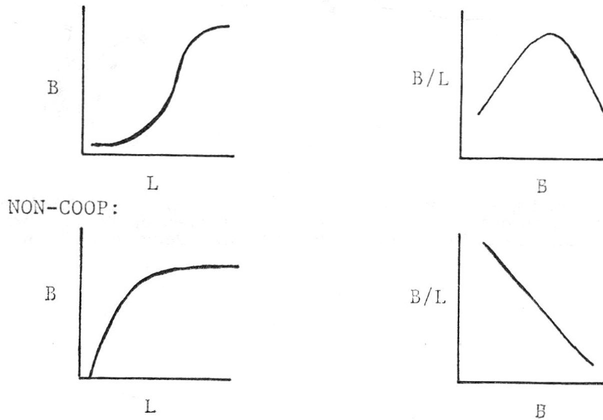
We utilized the Monod—Wyman—Changeux model, which proposes pre-existent R and T forms. Table V is a reminder of some of the definitions involved. The affinity to the R form is higher than that to the T form by a factor $c$. The two forms are in equilibrium, governed by a constant $L$. If one expresses the binding function in such a system by a Scatchard plot, one sees a maximum. I should stress that the dissociation constants for the R and T states are definitely not related to the high- and low-affinity forms that we have just been discussing. Experimentally we observe curves with clear maxima in the Scatchard plot, but the shapes of the curves vary a good deal. Figure 5 shows three preparations selected from a very large number. The points are experimental ones, and the curves are theoretical ones for a tetrameric system, with the values of $c$ and $K_R$ (the dissociation constant for the R form) arbitrarily fixed as indicated, and the $L$ values being differently assigned as shown. The data are thus compatible with the view that the variations in the different preparations involve variations in the value of $L$. It is impossible to escape the conclusion that positive cooperativity is clearly seen. Such a hypothesis also makes sense of an otherwise paradoxical situation; why is it that high-affinity acetylcholine binding, which we believe to be the characteristic form of the native receptor, has a dissociation constant of about 20 nM, when the concentrations of acetylcholine and related ligands to provide half the maximal response in physiological preparations is in the order of 1 μM? If we assume that half of the physiological response occurs when half of the receptors are in the R state, then calculations will show that this condition can occur at concentrations of acetylcholine much higher than the microscopic

TABLE V.

POSITIVE COOPERATIVITY




$$L = \frac{T}{R} \qquad C = \frac{K_R}{K_T}$$




binding constant for the R state. Figure 6 shows one particular theoretical curve, indicating that for a dimer, when $K_R$ is 10 nM the condition for $R$ (the fraction in the R state) being halfmaximal is that the acetylcholine concentration is above $10^{-6}$ M.

One piece of additional evidence supports the view of positive cooperativity acting in accordance with the Monod—Wyman—Changeux model; that is that the inhibition of acetylcholine binding by nicotine follows precisely that predicted for a simple competitive inhibitor, in accordance with the model. But unfortunately curare does not bind in accordance with these simple kinetics, but exhibits half-of-the-site inhibition, just as shown recently for curare inhibition of decamethonium binding[8]. The implications are that the Monod—Wyman—Changeux model is only partially descriptive.

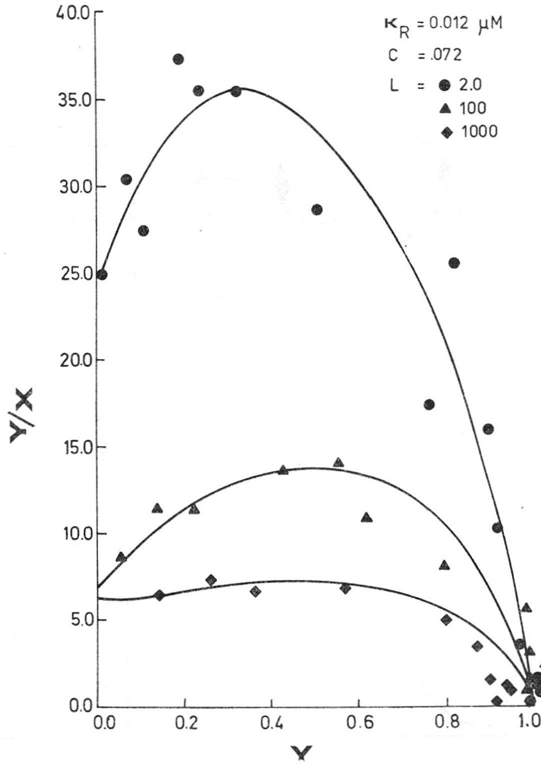



Fig. 5. Binding (X in nmol $g^{-1}$) of low concentrations (Y in μM) of acetylcholine to Triton X-100 extracts of three preparations of fresh *T. californica* electroplax membranes. Points are experimental, lines calculated from Monod—Wyman—Changeux model for three L values.

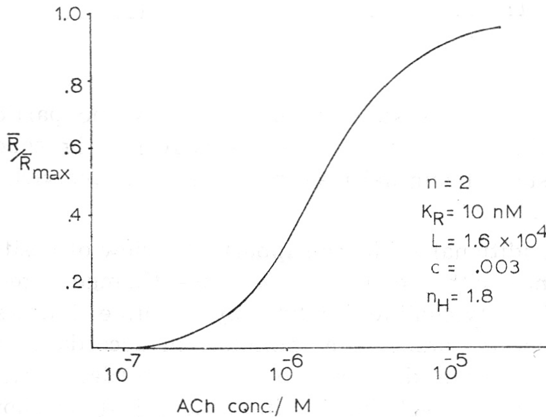



Fig. 6. Calculation from Monod—Wyman—Changeux model of fraction in the R state at various acetylcholine concentrations.

We shall now turn to a different but related problem, which is the oligomeric constitution of *Torpedo* receptors. The following work was done with sucrose density gradient centrifugation at 4 °C, using Triton-extracted electroplax of various *Torpedo* species, and labelling with either iodinated or pyridoxylated α-bungarotoxin. Figure 7 shows our early findings with our stored

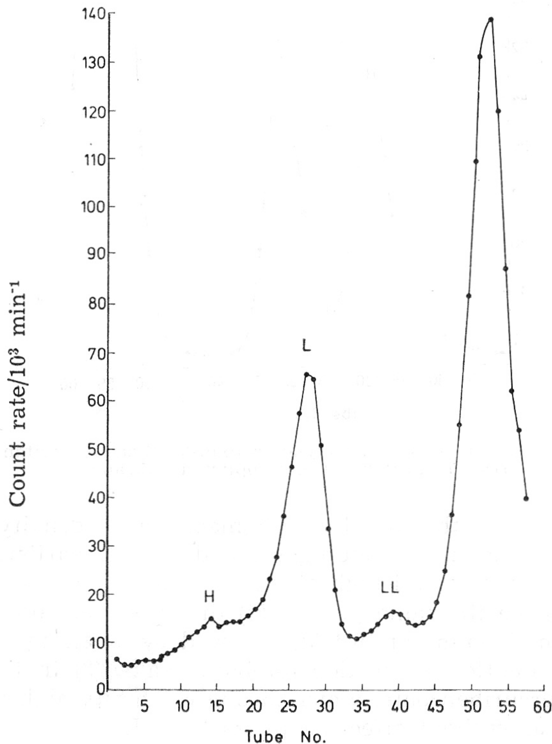



Fig. 7. Sedimentation (pH = 7) of $^{125}$I-toxin-labelled 1% Triton extracts of long-stored lyophilized *T. marmorate* electroplax membranes.

*Torpedo marmorata* material. It is clear that there is one major oligomer only. We also observed a low molecular weight species which we call LL. The large very light peak which you will frequently see on the right-hand side of the elution profile is free toxin, sometimes due to application of toxin in excess, and sometimes due to working with a preparation containing polypyridoxylated toxin, which only bound reversibly to the receptor. Recent work with fresh *T. marmorata* and also *T. ocellata* kindly provided by the Drs. Eldefrawi, showed essentially the same picture. But when we worked with unpurified *T. californica* material, a very different picture emerged (Fig. 8) showing two major oligomers of computed $S_{20,w}$ values of 11.6 and 18.5. Precisely the same situation was seen with highly purified *T. californica* receptor. The proportions of L and H were not affected by varying the relative amounts of toxin and plax over a range of 100-fold. In pure *T. californica* we saw small amounts of another oligomer, a very heavy one which we call HH.

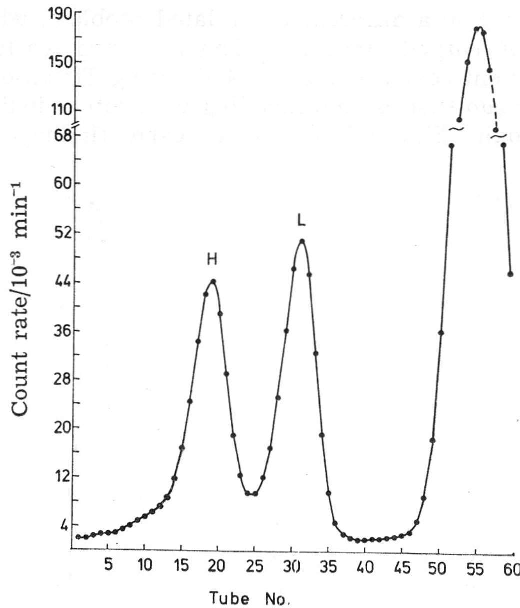



Fig. 8. Sedimentation (pH = 7) of $^{3}$H-pyridoxylated-toxin-labelled 1% Triton X-100 extracts of fresh *T. californica* electroplax membranes.

First let me point out that this L and H macro-heterogeneity has absolutely no connection with the micro-heterogeneity discussed earlier. The evidence was (1) that preparations of all-high-affinity receptor and also of mostly-low--affinity receptor gave the same profile, (2) that pre-incubation with nicotine or curare or carbamylcholine at $10^{-4}$ M, followed by labelling with α-bungarotoxin gave rise to identical protection for both peaks, (3) that treatment with 0.1 mM *p*-chloromercuribenzoate, which strongly inhibits high-affinity binding of acetylcholine, was without effect on peaks H or L.

We now consider the interconvertibility of the oligomers. Heating the preparation for 40 minutes at 40 °C, a procedure which virtually eliminates high-affinity binding of acetylcholine, had virtually no effect on the L peak, but typically halved the H peak and led to a considerable increase in the area of the HH peak (Fig. 9). It would appear that the H peak is subject to aggregation, and is more sensitive to heat than the L peak. When the material was run in gradients at pH = 10 (Fig. 10) there was again a reduction in the H peak without any effect upon the L peak, but in this case the loss from the H peak without any effect upon the L peak, but in this case the loss from the H peak was precisely counterbalanced by a new LL peak, and the s values of the H and LL peaks approached each other, both observations suggesting that the H peak was dissociating to LL units.

By contrast with the effect induced by heat or high pH, both the H and L peaks showed remarkable stability at neutral pH. Figure 11 shows that when the L peak was isolated from a gradient and re-run on the gradient fresh, none of it redistributed to form any other oligomers, nor was there much

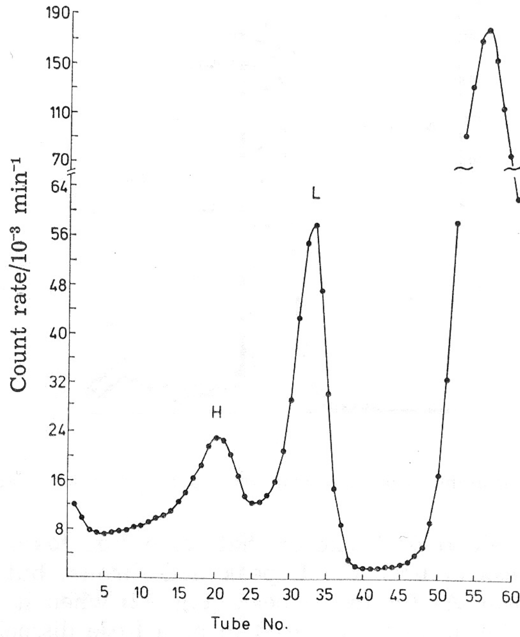



Fig. 9. Effect of heat (40 °C, 40 min) on preparation as in Figure 8.

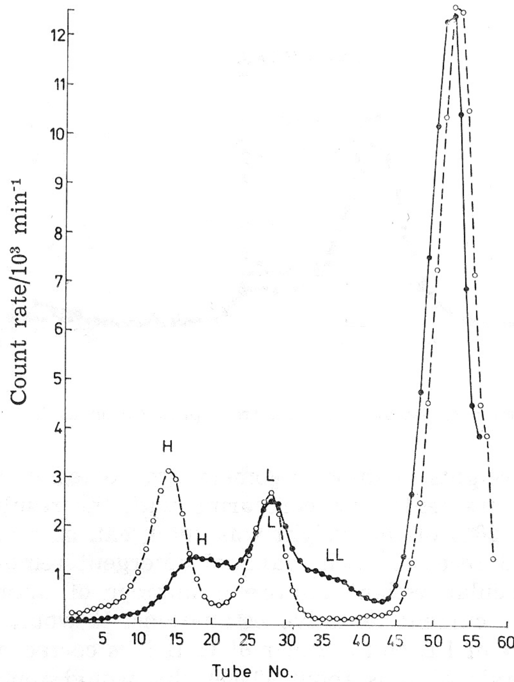



Fig. 10. Effect of sedimenting a preparation as in Figure 8 at pH=10 (●—●—●) or pH=7 ○—○—○).

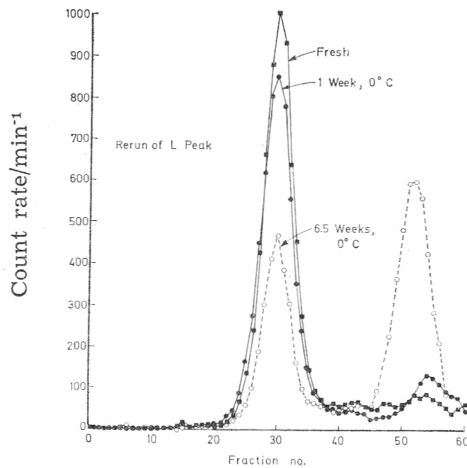



Fig. 11. Re-running isolated L peak from preparation as in Figure 8.

change after one week at 0 °C, except that some free toxin appeared at the right. After 6.5 weeks at 0 °C, the L peak had shrunk, but only by loss of toxin to the free form. As for the H peak (Fig. 12) when it was isolated and re-run in the fresh form, there appeared to be a little dissociation into the L area. In 6.5 weeks, there was also appearance of some free toxin.

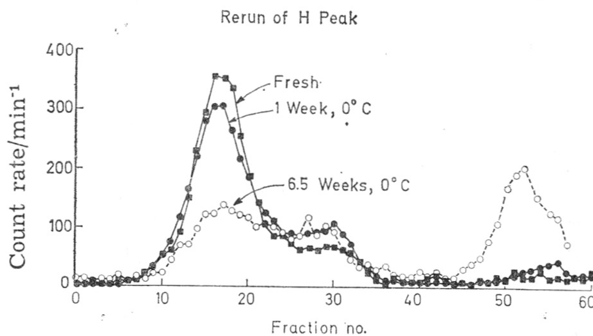



Fig. 12. Re-running isolated H peak from preparation as in Figure 8.

The molecular weights of these oligomers were determined by performing experiments in $D_2O$ gradients, and comparing with the results in $H_2O$. These experiments showed 26% of the weight was detergent, as compared with 10% reported for purified receptor with maximal detergent removal[9]. The precise calculations of molecular weight require a number of approximations, and Table VI. shows our current tentative estimations. It would appear that the H peak is a hexamer of LL and a dimer of L. If it is correct that the minimal binding unit for acetylcholine is about 90 000, this would appear to correspond with LL, and implies that the H peak is a hexamer of LL, and thus quite

TABLE VI.

*Provisional molecular weights as of March 1975*

| | | | |
|---|---|---|---|
| LL = | 89 000 | H = | 535 000 |
| L = | 253 000 | HH = | 1 000 000 |

LL = minimal binding unit
H = a dimer of L, a hexamer of LL
HH = detergent-free artifact

likely to be the active form of the receptor, since hexameric configurations have been reported from several laboratories. We rather suspect that HH and comparable molecular weight material is an artifact of detergent removal. If one runs purified receptor in progressively lower amounts of detergent, not only do the H and L peaks increase their migration, but a good deal of activity shows up in the HH region and at the bottom of the tube, as shown in Figure 13

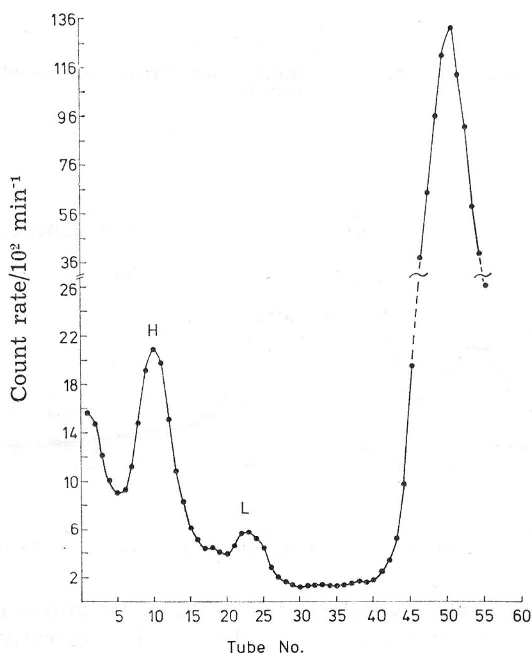



**Fig. 13. Sedimentation of purified receptor as Figure 8, (but highly purified form) and with Triton X-100 at 0.01%.**

for 0.01% Triton. It is a very plausible expectation (Fig. 14) that 1% Triton is able to replace the lipoidal binding which the receptor normally enjoys in the membrane, but that progressive removal of detergent leads to aggregation of receptor molecules by interaction of their hydrophobic areas.

We have recently been successful separating the oligomers by zonal ultracentrifugation (Fig. 15) and are now in a position to study the binding properties of the principal oligomers. If L is a trimer, it is possible that it may show differences in cooperativity in binding, but this remains to be seen.

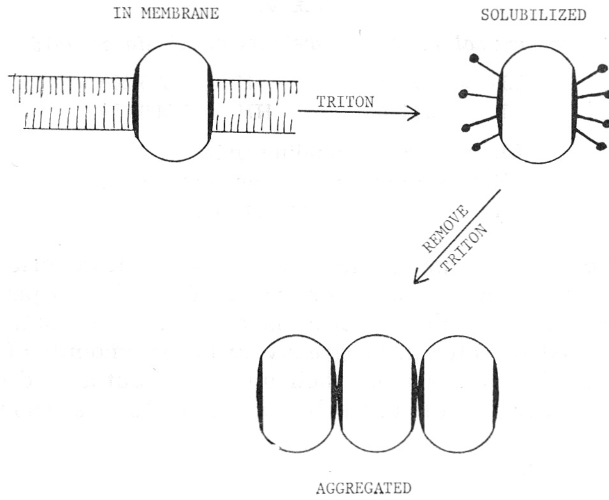



Fig. 14. Diagram of relation between membrane-bound Triton-solubilized and detergent-free receptor.

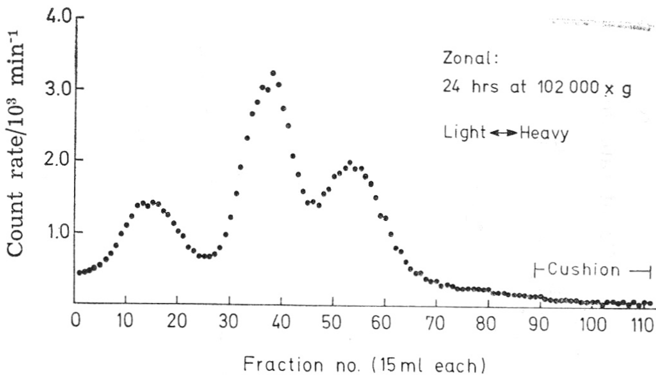



Fig. 15. Zonal ultracentrifugation of preparation as in Figure 8.

In conclusion, possible heterogeneities include variations in the amount of high- and low-affinity, variations in the degree of cooperativity even in preparations lacking low-affinity binding, and variations in the oligomeric constitution of the preparation. We shall need to sort out which of these differences are functional and which are artifactual before we can reconstruct the molecular basis of the transduction process.

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REFERENCES

1. M. E. Eldefrawi, A. T. Eldefrawi, S. Seifert, and R. D. O'Brien, *Arch. Biochem. Biophys.* **150** (1972) 210.
2. R. D. O'Brien and R. E. Gibson, *Arch. Biochem. Biophys.* **165** (1974) 681.
3. R. D. O'Brien and R. E. Gibson, *Arch. Biochem. Biophys.* in press.

4. M. Weber and J.-P. Changeux, *Mol. Pharmacol.* **10** (1974) 15.
5. M. Martinez-Carrion and M. A. Raftery, *Biochem. Biophys. Res. Commun.* **55** (1973) 1156.
6. T. Moody, J. Schmidt, and M. A. Raftery, *Biochem. Biophys. Res. Commun.* **53** (1973) 761.
7. J. B. Cohen, M. Weber, and J.-P. Changeux, *Mol. Pharmacol.* **10** (1974) 904.
8. J.-J. Fu, D. B. Donner, and G. P. Hess, *Biochem. Biophys. Res Commun.* **60** (1974) 1072.
9. S. J. Edelstein, W. B. Beyer, A. T. Eldefrawi, and M. E. Eldefrawi, *J. Biol. Chem.*, in press.

## DISCUSSION

**P. J. Jewess:**

(a) With reference to your table of the inhibition of receptor proteins by multivalent ligands: in view of the possibility of high-low affinity transitions being caused by oxidation, is there any correlation between the redox potential of the cations and the degree of inhibition? (b) Is the same oligomer pattern observed with the purified receptor as well as the α-bungarotoxin-receptor complex?

**R. D. O'Brien:**

(a) I think not. $Zn^{++}$ and $Fe^{++}$, for instance, have similar properties, but quite different redox potentials. (b) We have done a related experiment, in which the unpurified receptor, without toxin, was run on a sucrose gradient, and the fractions assayed for toxin-binding activity by the Franklin-Potter method. The pattern observed was just as for the toxin-labeled receptor.

**I. Silman:**

Could you protect the high affinity form with reducing agents, or could you reverse the effects of DTNB and PCMB with thiol reagents?

**R. D. O'Brien:**

No. We did quite extensive studies attempting to block the effects of heating (40 °C, 40 min), with numerous reducing agents, at various concentrations including very high ones up to $10^{-2}$ M, but no protection was ever observed.

## SAŽETAK

### Heterogenost acetilkolinskog receptora iz vrste *Torpedo*

*R. D. O'Brien i R. E. Gibson*

Opisana je mikroheterogenost acetilkolinskog receptora. Preparacije se razlikuju po odnosu mjesta s niskim i visokim afinitetom za vezivanje acetilkolina; vrijednosti $K_d$ za acetilkolin iznose 11 odnosno 562 nM. Mjesta s niskim afinitetom vezivanja odsutna su u svježim membranama i nečišćenim ekstraktima dobivenim s Tritonom X-100, ali predstavljaju glavni sastojak čistih ili oksidiranih preparata pa se stoga smatraju artefaktom. U svim preparacijama visokog afiniteta utvrđena je pozitivna kooperativnost i njome je razjašnjeno slaganje vrijednosti $K_d$ od 11 nM s fiziološkom reakcijom pri 1 μM. Opisane su oligomerne varijacije u vrsti *Torpedo*. *T. californica* ima dva glavna oligomera, H i L i dva sporedna, HH i LL. Glavnim oblikom smatra se oligomer H; njegova molekularna težina, određena u eksperimentima s $D_2O$, iznosi 535 000. Oligomer H je dimer oligomera L, a heksamer oligomera LL. Postulirano je da oligomer HH predstavlja nespecifični agregat koji se oblikuje poglavito u slabom detergentu.

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