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Calcium-Binding of Synaptosomes Isolated from Rat Brain Cortex

IV. Effects of Ruthenium Red on the Co-Operative Nature of Calcium-Binding

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Summary. Ruthenium red combines with isolated synaptosomes, resulting in strong inhibition of their Ca^{2+}-binding. In isotonic saline media, however, the dye-induced inhibition of Ca^{2+}-binding is significantly greater than that expected for the amount of bound dye and Hill's exponent of the Ca^{2+}-binding decreases to 1 with an increase in the amount of the dye bound. On the other hand in isotonic mannitol-sucrose solution, inhibition of synaptosomal Ca^{2+}-binding brought about by the dye is proportional to the amount of dye bound. Based on these results, the effects of the dye on the co-operative nature of synaptosomal Ca^{2+}-binding is discussed.

It has been well documented that ruthenium red inhibits the binding or uptake of Ca^{2+} by subcellular organelle such as mitochondria (Moore, 1971; Vasington, Gazzotti, Tiozzo & Carafoli, 1972; Rossi, Vasington & Carafoli, 1973), synaptic plasma membrane (Madeira & Antunes-Madeira, 1973; Kamino, Inouye, Ogawa, Uyesaka & Inouye, 1975*a*) and sarcolemma (Madeira & Antunes-Madeira, 1974). Furthermore, Sottocasa, Sandri, Panfili, de Bernard, Gazzotti, Vasington & Carafoli (1972) and Gomez-Puyou, de Gomez-Puyou, Becker and Lehninger (1972) demonstrated the inhibitory effect of the dye on Ca^{2+}-binding by some Ca^{2+}-binding protein. As reported previously (Kamino, Uyesaka & Inouye, 1974), Ca^{2+}-binding of synaptosomes was of a co-operative nature having Hill's exponent of about 3.4. Thus it is expected that the dye provides a useful tool for elucidating the nature of such a co-operativity. In the present study, we investigated the effect of ruthenium red on the mode of Ca^{2+}-binding with synaptosomes isolated from rat brain cortex.

Materials and Methods

Preparation of Synaptosomes

Synaptosomes were prepared from rat brain cortex as described elsewhere (Kamino, Inouye & Inouye, 1973). To prepare synaptosomal suspensions in isotonic solution of NaCl, KCl or mannitol (0.225 M)-sucrose (0.1 M), the pellets separated from the stock suspension were suspended in an appropriate buffered solution after washing with the suspending medium by resuspension and centrifugation (12,000 × *g* for 20 min). The pH of suspending media was adjusted to 7.3 with 20 mM Tris-HCl buffer.

To estimate the particulate concentration, the content of suspensions was assayed by the method of Lowry, Rosebrough, Farr and Randall (1951) with bovine serum albumin as the standard.

Determination of Ca^{2+}-Binding

As described previously (Kamino *et al.*, 1974) free Ca^{2+} concentration in synaptosomal suspension was determined from the difference in absorbance at 507 and 542 nm, $\Delta A_{507-542}$, of Ca-murexide complex measured with a dual wavelength spectrophotometer (Hitachi Type 356) and the bound Ca^{2+} was estimated from the difference between the concentration of the cation added and remaining free. As shown in Fig. 1, in the difference spectrum of the Ca^{2+}-murexide *vs.* the free murexide in the presence of ruthenium red of 100 μM, the isosbestic point (λ_i) and the wavelength (λ_m) for the minimum ΔA shifted from 507 to 485 nm and from 542 to 532 nm, respectively. But a single linear correlation between $\Delta A_{485-532}$ and Ca^{2+} concentration was maintained in the presence of the dye for a given murexide concentration. Since λ_i and λ_m were dependent on the dye concentration, we determined the pair of λ_i and λ_m in the presence of ruthenium red of given concentration beforehand which satisfied a linear relation between $\Delta A_{\lambda_i-\lambda_m}$ and Ca^{2+} concentration. Applying such a ΔA (for instance $\Delta A_{504-542}$ in the presence of 25 μM ruthenium red), the bound Ca^{2+} in the dye could be determined just as in its absence.

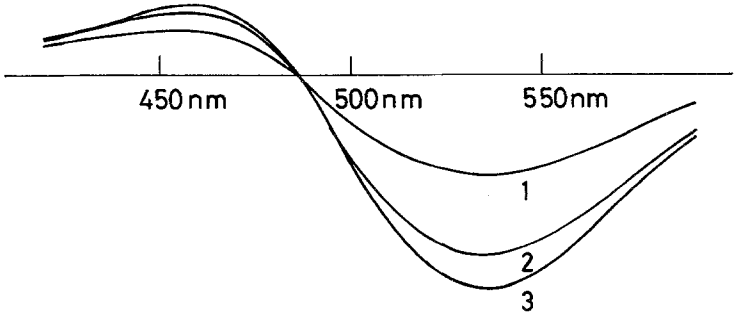

Fig. 1. In the presence of ruthenium red (100 μM), the difference spectrum of the Ca^{2+}-murexide *vs.* the free murexide spectra were obtained by adding 60 μM (spectrum 1), 120 μM (spectrum 2) and 240 μM (spectrum 3) $CaCl_2$ to the measuring cuvette, which contained 100 μM murexide in 170 mM NaCl solution containing 20 mM Tris-Cl buffer (pH 7.3). Intensity of absorbance is an arbitrary unit

Determination of Binding of Ruthenium Red

Binding of ruthenium red was determined by incubating synaptosomes (0.2～0.5 mg protein/ml) with various concentrations of the dye for 10 min at 22°～24 °C. The suspension was spun down at 12,000 × *g* for 30 min, the free dye was determined spectrophotometrically at 536 nm on the supernatants thus separated as made by Moore (1971) and Madeira and Antunes-Madeira (1974). The amount of the dye bound with synaptosomes was calculated from the quantity of the dye added and the concentration remaining in the supernatants. Ruthenium red was purchased from Chroma-Gesellschaft Schmid & Co. Since the absorption spectra of the dye solution was identical with those described by Luft (1971), it was used without further purification.

Estimation of Hill's Exponent and Dye-Induced Inhibition

According to our previous study (Kamino, Uyesaka, Ogawa & Inouye, 1975*b*) binding of Ca^{2+} with synaptosomal Ca-binding sites is expressed as

$$M + n\,Ca^{2+} \rightleftharpoons M\,Ca_n \tag{1}$$

and

$$\log\{\bar{Y}/(1-\bar{Y})\} = n \log y - \log K_c \tag{2}$$

where M denotes a Ca-binding site of synaptosomal membrane, y the free Ca^{2+} concentration, K_c the dissociation constant of M-Ca-complex and $\bar{Y}$ stands for fractional saturation of the Ca-binding sites with Ca^{2+}. With murexide titration, the value of y and that of bound Ca^{2+} at a given y can be evaluated and so the total binding capacity of synaptosomes can be estimated, and $\bar{Y}$ is obtained. As shown below, the binding capacity was markedly depressed in the presence of ruthenium red in the suspending media. Denoting the free dye concentration as x and the binding capacity at x as S_x, the inhibitory effect of the dye was expressed as

$$I(x) = S_o - S_x / S_0 \tag{3}$$

where S_o denotes Ca^{2+}-binding capacity at $x = 0$. It is easily derived from Eqs. (1) and (2), that

$$\frac{y^n}{K_c} = \frac{\bar{Y}}{1 - I - \bar{Y}} = \frac{\bar{Y}'}{1 - \bar{Y}}, \quad \bar{Y}' \equiv \frac{\bar{Y}}{1-I} \tag{1'}$$

and

$$\log\{\bar{Y}'/(1-\bar{Y}')\} = n \log y - \log K_c. \tag{2'}$$

Thus, evaluating the value of n graphically from Hill plots based on Eqs. (2) and (2'), the effect of the dye on the Hill's co-operativity parameter, n, was examined, while the dye-induced inhibition was followed by using Eq. (3).

Denoting fractional saturation of the Ca-binding site with the dye as $\bar{X}_R$, Hill plot for the dye binding is obtained just as Eq. (2)

$$\log\{\bar{X}_R/(1-\bar{X}_R)\} = n \log x_R - \log K_D \tag{4}$$

where K_D stands for the dissociation constant at synaptosome-dye complex and x_R the free concentration of the dye.

Results

Effects of Ruthenium Red on Ca^{2+}-Binding Curves of Isolated Synaptosomes

Low concentration of ruthenium red brings about an inhibition of the synaptosomal Ca^{2+}-binding; the dye inhibits the maximal binding capacity by about 60% when added in a concentration of 20 μM in both NaCl and KCl media. The typical result in NaCl media is shown in Fig. 2. Such results are quite similar to La^{3+}-induced inhibition of synaptosomal Ca^{2+}-binding reported previously (Kamino *et al.*, 1975*b*). The Ca^{2+}-binding curves appear to become less sigmoidal (more hyperbolic) in their general shape as the dye concentration increases. Using Eq. (2) or (2′) presented above, Hill plot at any x can be constructed, from which the Hill's exponent n in the presence of the dye is graphically estimated. As shown in Fig. 3*a* and *b*, the Hill plots thus constructed are almost linear

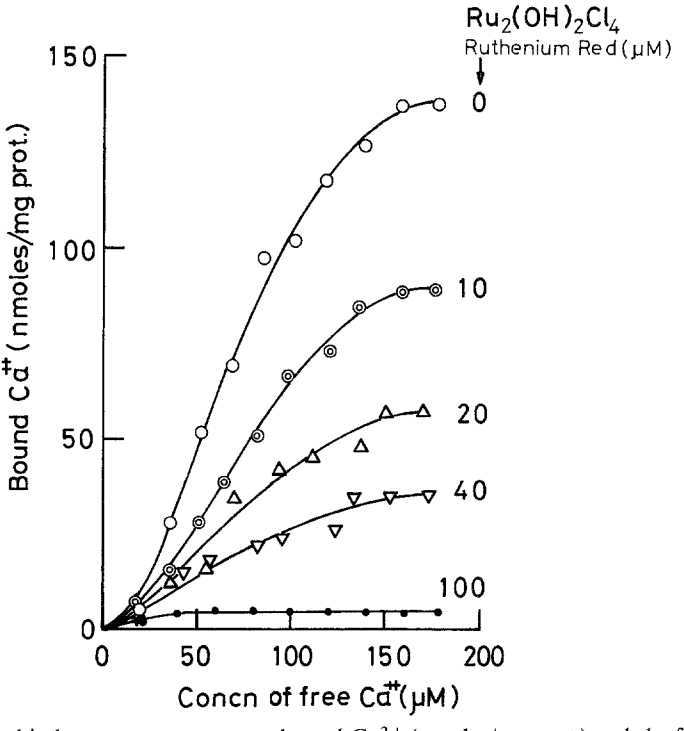

Fig. 2. Relationship between synaptosome-bound Ca^{2+} (nmoles/mg prot.) and the free Ca^{2+} concentration (μM) in suspending media in the presence of ruthenium red. Ruthenium red concentration added in suspension media: 0 μM, ○; 10 μM, ◎; 20 μM, △; 40 μM, ▽; 100 μM, ●. Suspending media: 170 mM NaCl solution containing Tris-Cl buffer (pH 7.3, 20 mM)

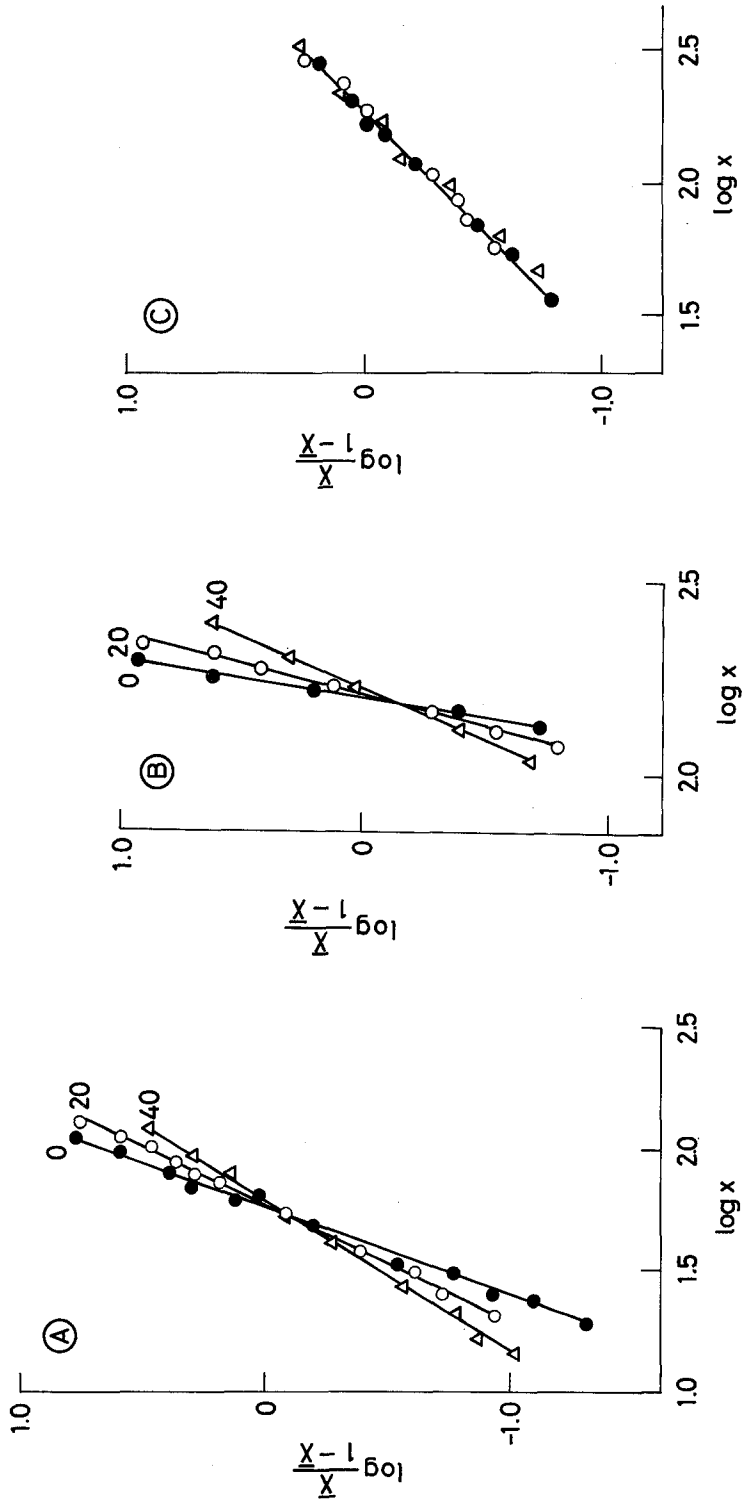

Fig. 3. Hill plots of Ca^{2+}-binding by synaptosomes in the presence of ruthenium red. ●, 0 μM; ○, 20 μM; △, 40 μM of ruthenium red added in suspending media. (*A*) in NaCl media, (*B*) in KCl media, (*C*) in mannitol-sucrose media

and n actually decreases with increasing x. But the value of dissociation constant K_c is hardly affected. In mannitol solution, Ca^{2+} also bound with synaptosomes, and its maximal binding capacity was about 130 nmoles/mg protein. This value was almost the same as that in NaCl or KCl solution. The Ca^{2+}-binding curve in the isotonic mannitol-sucrose was hyperbolic with Hill's exponent $n=1$ in Eq. (2) (Fig. 3*c*), a fact suggesting that the Ca^{2+}-binding of synaptosomes in this medium is not co-operative. The value of K_c in the isotonic mannitol-sucrose is greater than in NaCl media, but less than in KCl. Such a Ca^{2+}-binding was also strongly inhibited by ruthenium red of low concentration, but the Hill plots in the presence of the dye overlap with that in its absence, n remaining unaltered (Fig. 3*c*). Thus, it is concluded that the dye not only causes loss of ability to bind Ca^{2+}, resulting in apparent noncompetitive inhibition of synaptosomal Ca^{2+}-binding at least in the range of Ca^{2+} concentration used, but also exerts some effects on the co-operative nature of the binding sites in saline media.

Binding of Ruthenium Red by Isolated Synaptosomes

Such a powerful inhibitory action of La^{3+}-type (Kamino *et al.*, 1975*b*) induced with ruthenium red in the synaptosomal Ca^{2+}-binding strongly suggests the firm association of the dye with synaptosomes and our data show that this is actually the case.

As shown in Fig. 4*A*, the Scatchard plots of the dye binding in NaCl, KCl and mannitol media practically overlap, with no apparent significant difference between the suspending media as found in its inhibitory effect on the Ca^{2+}-binding. The plot is not linear over the range of concentrations used. The results suggest the presence of different binding sites having different affinities to the dye. But extrapolation to the abscissa of its linear part in the range of low dye concentrations enables estimation of the maximum binding capacity of the highest affinity sites. The value thus obtained is around 130 nmoles/mg protein, a value in fairly good agreement with that of Ca^{2+}-binding (cf. Fig. 2).

Such a finding strongly suggests that the sites with the highest affinity for the dye are the Ca^{2+}-binding sites and their association with the dye is firmer than that with Ca^{2+} ion, resulting in an apparently noncompetitive inhibition of Ca^{2+}-binding. Applying Eq. (4), therefore, a Hill plot was constructed for such a dye binding of high affinity sites. As shown in Fig. 4*B*, it is almost linear, the value of n being around 1, independently of the type

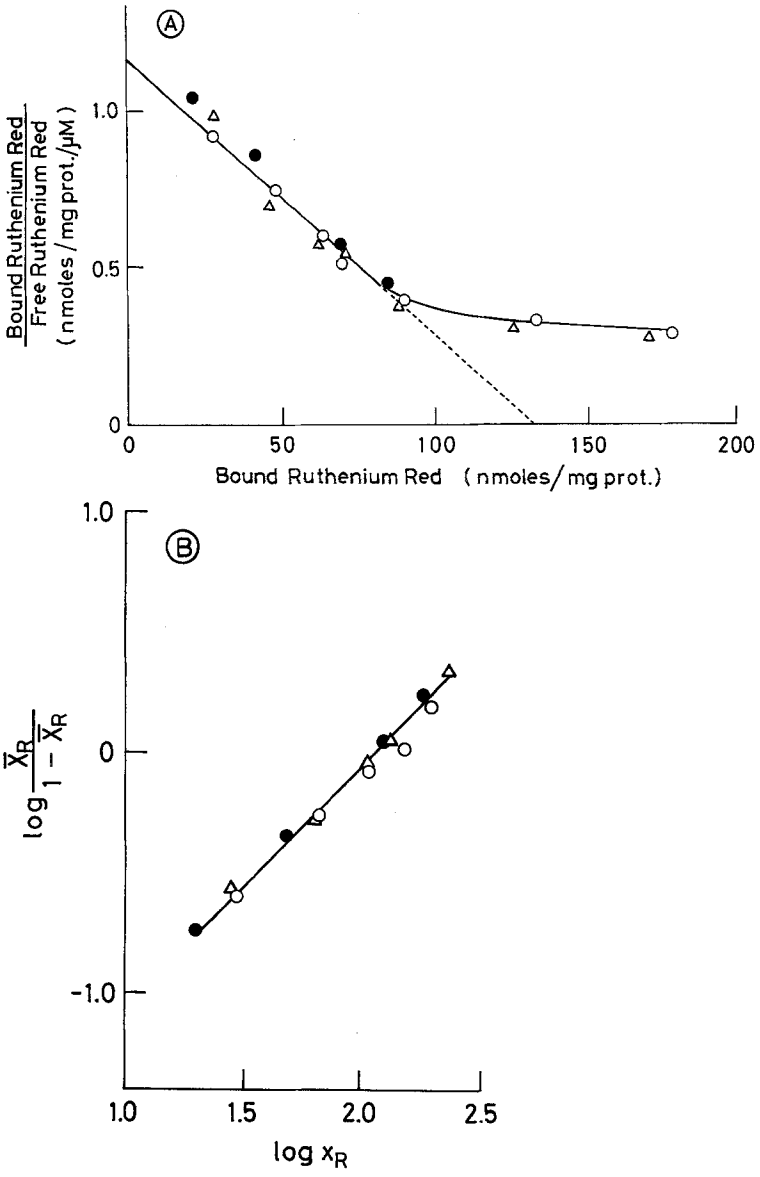

Fig. 4. (*A*) Scatchard plots of ruthenium red binding with synaptosomes in NaCl (○), KCl (△) and mannitol media (●). (*B*) Hill plots of ruthenium red binding by synaptosomes. $\overline{X}_R$ is fractional saturation of bound ruthenium red, x_R is free ruthenium red concentration in μM. Each symbol the same as in *A*

of suspending media, a sharp contrast with the Ca^{2+}-binding which shows the values of n significantly greater than 1 in saline media. If indeed the dye does combine with the synaptosomal Ca^{2+}-binding site, the mechanism of such a binding should be different from that of Ca^{2+}-binding;

rather it appears to be due to adsorption or statistical multiple binding with glycoproteins within the site just as in the case of binding of metal ions with proteins described by Klotz (1946).

With increased concentration of the dye, the greater is the binding with the synaptosomes. Experiments are in progress to acquire data regarding the low affinity sites.

Correlation between the Amount of Ruthenium Red Bound and its Inhibitory Effect on Ca^{2+}-Binding

To obtain further insight to the mechanism with which the dye inhibits Ca^{2+}-binding, we examined correlation of $I(x_R)$, defined by Eq. (3) with fractional saturation, $\bar{X}_R$ in Eq. (4), of the synaptosomes with the dye. The former at a given x_R can be obtained from the results such as presented in Fig. 2, while the latter at any x_R is easily obtained from Fig. 4*B*. The $I-\bar{X}_R$ plots thus constructed are illustrated in Fig. 5.

If the inhibition brought about by the dye results from its occupation of the Ca^{2+}-binding sites by one-to-one kinetics, it is obvious from definitions that

$$I(x_R)=\bar{X}_R. \tag{5}$$

The Figure shows that, in mannitol-sucrose media, such is actually the case. In saline media, however, the plots clearly deviate from Eq. (5);

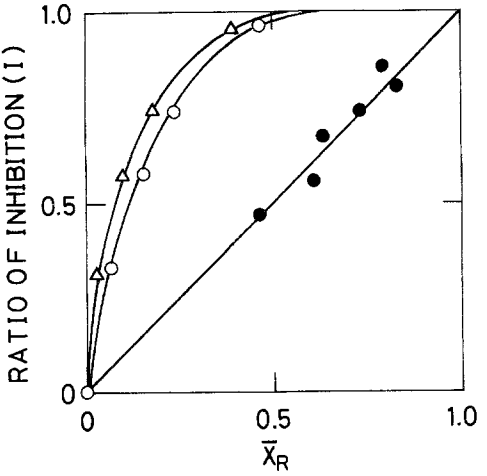

Fig. 5. Relationship between the amount of ruthenium red and its inhibitory effects on Ca^{2+}-binding. I: ratio of inhibition; $\bar{X}_R$ and each symbol the same as in Fig. 4

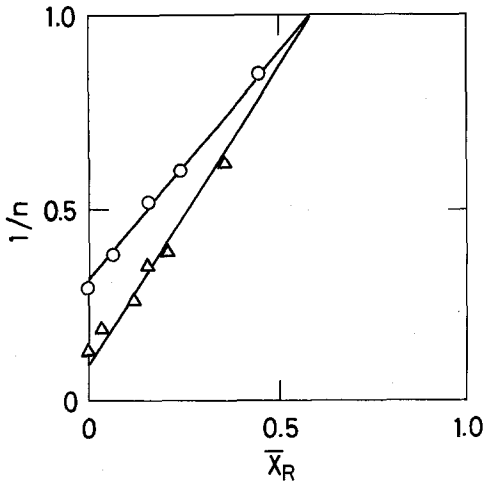

Fig. 6. Relationship between $\bar{X}_R$ and $1/n$ (n: Hill's exponent). Each symbol the same as in Fig. 4

the Ca^{2+}-binding is almost completely inhibited at around 70% saturation of synaptosomes with the dye. Thus, in saline media it is evident that one molecule of the dye bound inhibits binding of more than one molecule of Ca^{2+}. Such a finding suggests the presence of some interaction between the sites which bind the Ca^{2+} and/or the dye. The values of n as high as 3.4 in NaCl and 8 in KCl indicate an interaction between the loci with which Ca^{2+} combines. The above-mentioned finding would be quite plausible, if the dye combines with the same site as Ca^{2+} and the complex thus formed interacts with adjacent sites via a mechanism similar to that concerned with Ca^{2+}-binding.

The effect on the parameter n of the dye is seen in Fig. 6, in which an almost linear relation between $1/n$ and $\bar{X}_R$ is evident. According to Wyman (1968), the apparent free energy of interaction per site, ΔF_I, is proportional to $\left(1-\frac{1}{n}\right)$. Thus, a decrease in ΔF_I of Ca^{2+}-binding sites induced by the dye is proportional to $1/n_x - 1/n_o$, where the subscripts x and o denote the presence of the dye of a concentration x_R and its absence, respectively. Such a decrease may be the result of diminished interaction between the sites and some sites are necessarily affected by binding with the dye. Thus, it seems quite likely that

$$1/n_x - 1/n_o = k\,\bar{X}_R \tag{6}$$

where k is the proportionality constant. It is apparent that Eq. (6) is concordant with the results presented in Fig. 6.

Discussion

Rahamimoff and Alnaes (1973) reported inhibitory action of ruthenium red on neuromuscular transmission, which these investigators explained by formation of the complex firmly bound with Ca-receptors on presynaptic membranes. Using isolated sarcolemma, Madeira and Antunes-Madeira (1974) also observed binding of the dye with membranes. Although detailed analyses concerning nature of the action were not included in these reports, the mode of binding with sarcolemma appears to be, qualitatively at least, similar to that with isolated synaptosomes described herein. Ruthenium red is known to react selectively with acid mucopolysaccharides and related substances in biological tissues (Luft, 1971). Electronmicroscopical studies (Singer, Krishnan & Fyfe, 1972) revealed that the dye could be present on the neuronal membrane inside the myelin sheath. Moreover, the dye was reported to strongly inhibit the binding of Ca^{2+} with glycoprotein isolated from mitochondria (Sottocasa *et al.*, 1972). These reports together with our own data lead to the deduction that ruthenium red combines firmly with Ca^{2+}-binding sites on synaptosomal membranes, in which some protein(s), probably glycoprotein(s), are involved with the combining.

The present study on the inhibitory effect of ruthenium red revealed additional characteristics of Ca^{2+}-binding sites. In nonionic mannitol media, Hill's exponent n is around 1 and the bound dye inhibits binding of Ca^{2+} in the ratio of one to one, while in isotonic saline media n is significantly greater than 1 and one molecule of bound dye inhibits more than one Ca^{2+} to combine with the sites. This finding strongly suggests a conformational effect on the sites of ionic milieus, which results in an interaction between the sites.

The slope of the inhibition curve, $m(=\Delta I/\Delta \bar{X}_R)$, provides an average number of the sites affected by one bound dye at a given $\bar{X}_R$. The values of m computed are plotted against $\bar{X}_R$ in Fig. 7. As expected, m falls down to 0 as $\bar{X}$ increases, while it is $5 \sim 10$ at $\bar{X}_R \to 0$, a value which provides a rough estimate of the size of interacting sites. Hill's exponent of Ca^{2+}-binding reaction, $n(=4 \sim 8)$, is not always equal to, but usually reflects, the size of interacting sites in the positive "homotropic" interaction brought about by Ca^{2+}. Our finding that m and n are nearly in the same magnitude strongly suggests that powerful inhibitory action of ruthenium red on synaptosomal Ca^{2+}-binding is due to negative "heterotropic" interaction between the sites and the number of the Ca^{2+}-binding sites interacting with each other is around $5 \sim 8$.

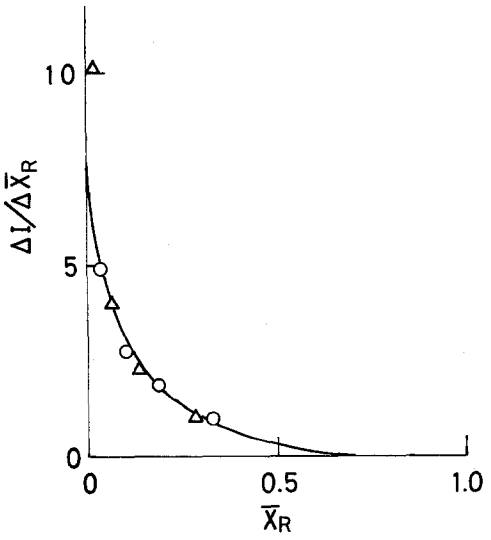

Fig. 7. The plot of $\Delta I/\Delta \bar{X}_R$ as a function of $\bar{X}_R$ constructed from Fig. 5 (*see text*). Each symbol the same as in Fig. 4

The above-stated fact that in isotonic mannitol-sucrose the value of n for Ca^{2+}-binding can be reduced to 1 is strong evidence that all sites are identical. According to Wyman (1968), the free energy of interaction per site at a given $\bar{Y}$, ΔF_I, is therefore proportional to $(1-1/n)$. The rate of decrease in ΔF_I by the dye, $\delta(1/n)/\delta \bar{X}$, would parallel the number of interacting sites affected by the dye, $m\bar{X}_R$, which remains rearly constant independentyl of $\bar{X}_R$ because the m-$\bar{X}_R$ curve is almost hyperbolic (Fig. 7). Thus it is not surprising that $1/n \sim \bar{X}_R$ plot is linear as shown in Fig. 6.

In light of such results, it is very likely that a conformational change in the sites is involved in the Ca^{2+}-binding in saline media. The nonlinear inhibition curve in saline media presented in Fig. 5 can be explained, therefore, by an inhibitory interaction between the sites occupied and unoccupied by the dye.

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