Antibody Binding Measurements with Hapten-Selective Membrane Electrodes

Abstract. *Direct antibody-hapten binding measurements were carried out with the use of a hapten-selective membrane electrode. Potentiometric titrations of rabbit antibody to the hapten trimethylphenylammonium ion demonstrate the speed and convenience of the new method for the determination of binding equilibria. Average intrinsic binding constants obtained agree well with those measured by equilibrium dialysis techniques.*

Potentiometric membrane electrodes with selectivities for a wide range of chemical and biological species have been described (*1*). A new possibility of potential importance to immunochemistry involves the use of hapten responsive membrane electrodes for the measurement of antibody-hapten interactions. To demonstrate this concept, we prepared a membrane electrode responsive to the hapten trimethylphenylammonium ion and used it as a direct probe to measure the binding of this hapten to its antibody. Our data show that the electrode method yields average intrinsic binding constants (K^0) in good agreement with values obtained with equilibrium dialysis techniques (*2, 3*). The potentiometric method has, moreover, the advantage of being much more rapid than the dialysis technique and of not requiring the use of radioisotopes.

The trimethylphenylammonium ion electrode is prepared by precipitating the tetraphenylboron salt of the hapten, dissolving this salt in dioctyl phthalate (Eastman Kodak), and polymerizing the liquid material into a polyvinyl chloride membrane at 200°C (*4*). Final membranes (2 mm in diameter and 0.17 to 0.30 mm thick) were attached to an electrode body which was filled with $10^{-2}M$ trimethylphenylammonium chloride solution and fitted with a Ag/AgCl internal reference electrode. All potentiometric measurements were made at 25°C relative to a miniature, saturated calomel reference electrode.

A typical calibration curve of the hapten electrode from 2.5×10^{-5} to $2.5 \times 10^{-7}M$ at *p*H 8.0 after initial conditioning is shown in Fig. 1. Although the calibration becomes nonlinear at hapten concentrations below $10^{-6}M$, useful measurements can still be made at even lower concentrations because of the excellent time stability of the electrode. Selectivity measurements (*5*) yielded a selectivity of hapten to sodium ion of 3.3×10^3 and a negligible response to magnesium ion. As a consequence, magnesium chloride was used to adjust the ionic strength ($0.15M$) of all working solutions. Specially purified rabbit γ-globulin antibody to trimethylphenylammonium ion was provided by the Roswell Park Memorial Institute (Buffalo, New York). The antibody, kept in tris-HCl buffer (*p*H 8), had a total protein concentration of 13.27 mg/ml, as determined by optical measurements at 280 nm. Potentiometric titration of 0.66 mg of antibody (nominal concentration 1.65 mg/ml) with $5 \times 10^{-4}M$ hapten in buffer (*p*H 8) yelds the typical titration curve shown in Fig. 2. Calculation of antibody concentration from titration end points, assuming a molecular weight of 160,000 for rabbit γ-globulin (*6*) and two binding sites per antibody molecule (*7*), results in analytical values within 20 percent of the nominal concentration.

Analysis of the binding equilibria from titration curves is achieved by converting each titration point to free hapten concentration via the calibration curve, computing the hapten bound as the difference between the total and free concentrations and by applying the mass action equation (*7*)

$$\frac{1}{b} = \frac{1}{KA^0}\left[\frac{1}{c}\right] + \frac{1}{A^0} \qquad (1)$$

where b is the concentration of bound hapten, c is the concentration of free hapten, A^0 is the antibody site concentration, and K is the binding constant. In Fig. 3 we show a typical graphical analysis presented as a graph of $1/b$ as a function of $1/c$; from the intercept, the antibody site concentration, A^0, is determined as $1.65 \times 10^{-5}M$ (± 4 percent), which is in good agreement (20 percent) with the total protein content determined spectrophotometrically. In order to determine binding constants, it is desirable to recast the data in the form of a Hill plot (*8*) (Fig. 4). The average intrinsic binding constant K^0, defined as the reciprocal of the free hapten concentration at which one-half of the antibody sites are occupied, is found where $\log r/(n - r)$ equals zero (where r is the number of moles of hapten bound per mole of antibody and n is the number of binding sites per antibody molecule).

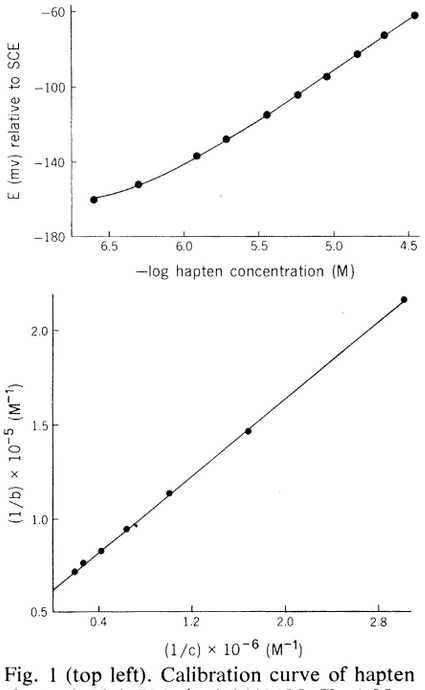

Fig. 1 (top left). Calibration curve of hapten electrode ($0.017M$ tris, $0.044M$ $MgCl_2$, $1.25 \times 10^{-4}M$ EDTA buffer medium adjusted to *p*H 8 with HCl). *E*, the potential, is expressed relative to a saturated calomel electrode (*SCE*).

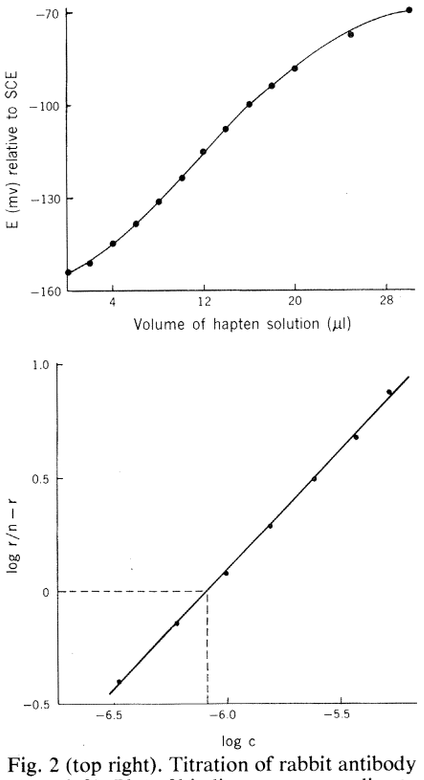

Fig. 2 (top right). Titration of rabbit antibody with hapten ($5 \times 10^{-4}M$) at *p*H 8.0.

Fig. 3 (bottom left). Plot of binding curve according to Eq. 1. Fig. 4 (bottom right). Hill plot of binding data; c is the concentration of free hapten, r is the number of moles of hapten bound per mole of antibody, and n is the number of binding sites per antibody molecule.

Four separate determinations carried out in this manner yielded K^0 values ranging from 1.02×10^6 to 1.78×10^6 with an average value of $(1.38 \pm 0.33) \times 10^6$ liters per mole. These values are in good agreement, despite differences in experimental conditions, with three previous studies, in which equilibrium dialysis techniques were used to determine K^0 values of 8.7×10^5 liters per mole (*2*), 1.1×10^6 liters per mole (*3*), and 2.01×10^5 liters per mole (*2*), respectively.

The response time of the hapten electrode is sufficiently rapid to yield stable potentials in 1 to 3 minutes even at the low hapten concentrations involved. As a result, an entire binding curve can be constructed in less than 1 hour, with the consumption of less than 1 mg of antibody. Moreover, the technique does not require the labeling of haptens with tracer radioisotopes. In view of the considerable saving in cost and effort involved, the membrane electrode method may offer an attractive alternative to traditional methods for the study of hapten-antibody interactions, particularly since new membrane electrodes are now being developed in increasing numbers.

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Calcium-Induced Displacement of Membrane-Associated Particles upon Aggregation of Chromaffin Granules

Abstract. *Isolated chromaffin granules incubated in 10 millimolar calcium chloride aggregated, forming contact sites with a pentalaminar membrane structure. These circular attachment sites were free of membrane-associated particles, which accumulated at the periphery. Incubation in 20 millimolar ethylenediaminetetraacetic acid reversed these changes, which are regarded as initial events in the membrane fusion reaction.*

Secretion from exocrine, endocrine, and nervous tissue involves extrusion of a preformed product from membrane-limited granules or vesicles that fuse with the plasma membrane as well as with each other. The critical role of calcium in this process of exocytosis and in membrane fusion in general has been supported by abundant evidence [for reviews see (*1*)].

The adrenal medulla, with its relatively large chromaffin granules, has proved to be suitable for morphological studies of exocytosis, including the freeze-etching technique (*2*). Isolated chromaffin granules may serve as an experimental model since they have shown reversible aggregation and striking core structure changes in the presence of calcium (*3*). In the work reported here we used this system to examine the distribution of membrane-associated particles during the aggregation induced by calcium ions.

Bovine adrenal glands were cooled on ice and used within 1 hour of slaughter. All biochemical work was done at 0° to 4°C. The medullae were minced and homogenized in 0.3*M* sucrose containing 10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, *p*H 7.0. The granule fraction was obtained by low- and high-speed centrifugation and was further purified by centrifugation through isosmotic sucrose-metrizamide gradients as described in (*4*). Monoamine oxidase activity in these samples was very low, and mitochondrial profiles were rarely seen.

Purified chromaffin granules were incubated in a medium containing 10 m*M* $CaCl_2$, 1 m*M* KCl, 0.26*M* sucrose, and 10 m*M* Hepes buffer at *p*H 7.0. This concentration of calcium was found to be optimal for fusion in a preliminary series. After 30 minutes one half was fixed with Hepes-buffered glutaraldehyde at a final concentration of 1 percent. The other half received 20 m*M* ethylenediaminetetraacetic acid (EDTA) and was further incubated for 15 minutes before fixation. Control granules were fixed after incubation in 1 m*M* KCl only. All batches were divided into one portion for freeze-fracturing and one for thin-section transmission electron microscopy. The centrifuged pellets were slightly less than 1 mm thick and always cut or broken transversely so that the whole depth could be examined. After up to 2 days of fixation, the pellets for freeze-fracture were washed with 6.8 percent sucrose in 0.2*M* cacodylate buffer, *p*H 7.4, for 3 hours, incubated in cacodylate-buffered 30 percent glycerol, and kept at 4°C up to 4 weeks. The length of storage has no effect on the ultrastructure of glutaraldehyde-fixed specimens (*5*). Small blocks were rapidly frozen in Freon 22 at −150°C and kept in liquid nitrogen. The material was fractured in a Balzers BA 360 M vacuum microtome and etched for 10 seconds at −100°C and 5×10^{-7} torr. The surface was shadowed with 22 to 23 Å platinum-carbon at an angle of 45° and replicated with 250 Å carbon. All grids were examined in a Siemens IA electron microscope.

The control granules are round to oval and are separate from each other (Fig. 1a). Replicas of freeze-fractured pellets exhibit a smooth surface with a random distribution of membrane-associated particles on both the PF and EF faces [nomenclature according to Branton *et al.* (*6*)] (Fig. 1b). The density of particles is higher at the PF faces.

Calcium treatment results in aggregation of granules. They cluster in the form of branched chains (Fig. 2b). The granules possess large round depressions suggestive of large areas of mutual contact (Fig. 2, d and e). These circular attachment sites either are planar or bulge into the adjacent granule. The contact area itself is always free of particles on both membrane faces. At its margin the particles aggregate around the circumference, resulting in a rosette-like appearance (Fig. 2, b, d, and e). Elsewhere the intramembranous particles are randomly distributed as in the control. The membranes within the contact area are very tightly apposed. In some freeze-fracture images of aggregated granules, one of them is broken away while a portion of its membrane is left attached (Fig. 2c). The attachment area often shows membrane fragments of the formerly attached vesicle, where the process of fracturing has exposed the outer leaflet. Such remaining membrane fragments are indicative of tight adhesion of two membranes (*7*).

Treatment of the aggregated granules with EDTA results in disaggregation of