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

most of the complexes. The circular depressions have disappeared and the membranes uniformly show a random distribution of membrane-associated particles again (Fig. 3b).

Conventional thin-section electron microscopy of duplicate pellets confirmed the calcium-induced granule aggregation and subsequent disaggregation upon EDTA treatment (Figs. 2a and 3a), confirming earlier results with granules isolated on continuous sucrose gradients (*3*). The membranes generally have a wrinkled appearance, which can be attributed to shrinkage during the complete dehydration in alcohol prior to embedding. In the freeze-fracture preparations, in contrast, the water is only partially replaced by 30 percent glycerol. In most instances the contact area between aggregated granules shows a continuous pentalaminar membrane measuring 130 to 140 Å. Its central dark line is somewhat broader and more electron-opaque than the outer ones (Fig. 2a, inset).

It has been assumed that the particles on freeze-fractured membrane faces represent proteins in the fluid mosaic model of the membrane (*8*). Alterations in *p*H, electrolyte concentration, temperature, and other parameters can induce translational movements of particles, as demonstrated in both plasma and organelle membranes (*9*). Such rearrangements have also been found in systems where membrane fusion takes place; for example, in virus-induced erythrocyte fusion (*10*) and in trichocyst secretion from *Paramecium* (*11*). Calcium in low concentrations induces a congregation of membrane-associated particles in Golgi-derived secretory vesicles isolated from rat liver (*12*).

In a comprehensive study of mucocyst secretion in *Tetrahymena*, Satir *et al.* (*13*) showed that particles of the membrane of the mucocyst tip rearrange to form an annulus that matches a preexisting rosette of particles in the plasma membrane. During the short interval between attachment and discharge, the rosette progressively enlarges.

We interpret the calcium-induced particle-free areas and surrounding collections of membrane-associated particles at sites where chromaffin granules are in contact as the result of similar phenomena and as a first step in the exocytotic process. These features are in accordance with the proposition by Ahkong *et al.* (*14*) that membrane fusion involves a displacement of proteins so that the lipid

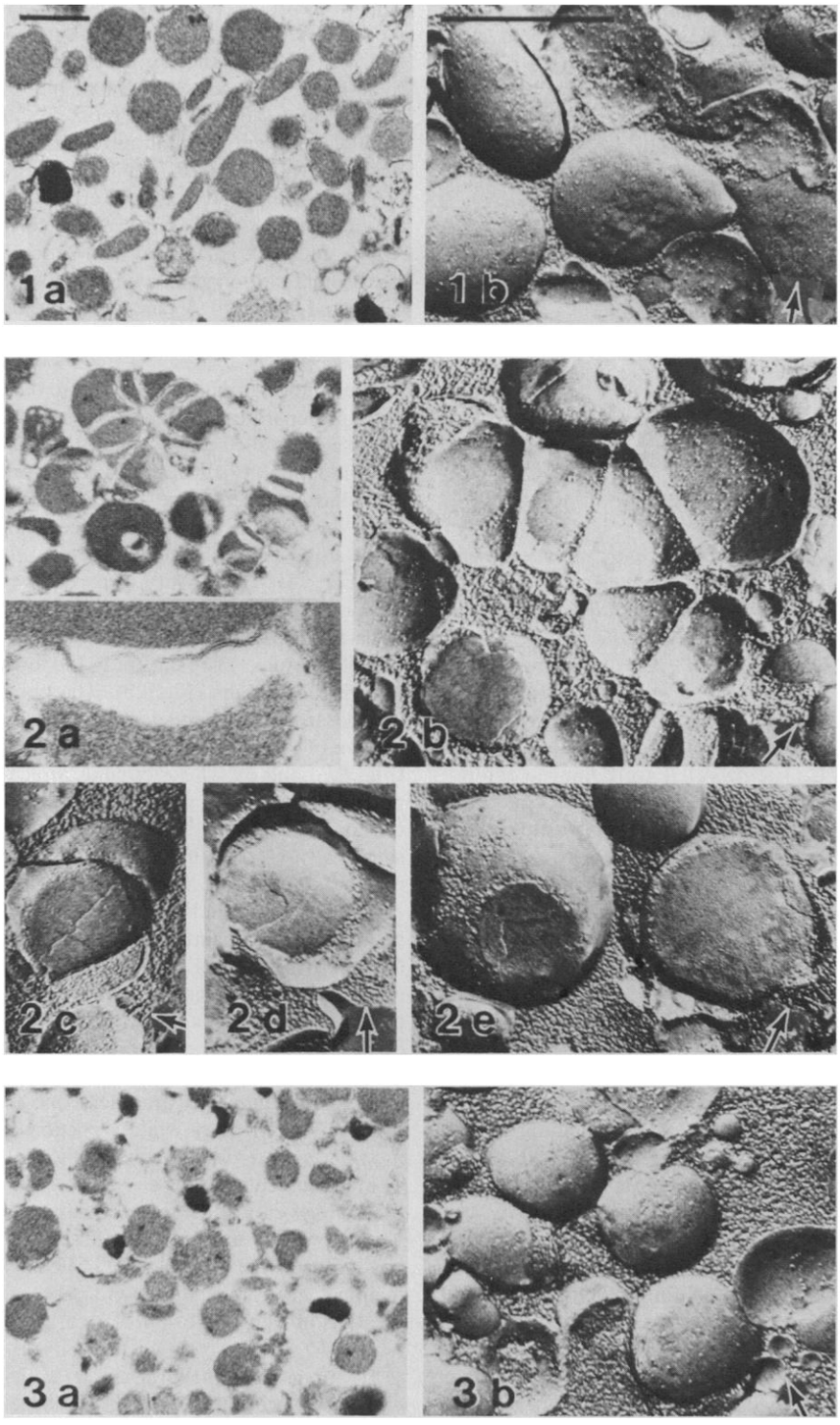

Fig. 1. Normal isolated chromaffin granules from the adrenal medulla are round to oval [(a) ultrathin secretion, × 20,000] with a random distribution of membrane-associated particles on both the PF and EF faces [(b) freeze-fracture preparation, × 50,000]. Scale bars, 0.5 μm. Arrow in (b) indicates direction of platinum shadowing. Fig. 2. Calcium-induced aggregation complexes of granules and displacement of core material are shown in ultrathin section (a, × 20,000). The wrinkled pentalaminar, partially tangentially cut membrane between adjacent granules measures 130 to 140 Å in diameter (inset, × 110,000). Aggregation of granules is also seen in freeze-fracture preparations (b, × 50,000). The particle-free EF and PF faces of large attachment areas are shown in (c), (d), and (e). Note the remaining membrane fragments, indicating tight adhesion. Arrows in (b) to (e) indicate direction of platinum shadowing. Fig. 3. Granules incubated in calcium followed by EDTA are disaggregated and core structure changes are reversed (a, × 20,000), and the membranes show a random distribution of particles again (b, × 50,000). Arrow in (b) indicates direction of platinum shadowing.

bilayers can come into close contact, allowing the subsequent fusion to take place. The calcium ions are effective in establishing a tight contact between adjacent chromaffin granules with fusion of the external membrane leaflets, resulting in a pentalaminar structure. Similar membrane formations have also been observed between adjacent secretory granules of mast cells (*15*) and of pancreatic islet cells (*16*). It appears that additional factors are involved in the completion of the fusion process beyond the stage of reversibility.

In some systems where exocytotic release involves preformed sites on the inner surface of the cell membrane, the frequency of exocytotic figures as seen in freeze-fracture preparations can be increased by various stimuli: in neuromuscular junction, electrical stimulation in the presence of calcium (*17*); in central synapses of lamprey, electrical stimulation and potassium depolarization in the presence of calcium (*18*); in central synapses of rat, lack of anesthesia (*19*); in mossy fiber endings of rabbit hippocampus, epileptic convulsion (*20*); in neurohypophysis, electrical stimulation or exposure to cold (*21*); and in *Paramecium*, ionophores in addition to calcium (*22*).

While all these examples represent intact biological systems, the identification of specific fusion factors will require further biochemically defined in vitro experiments.

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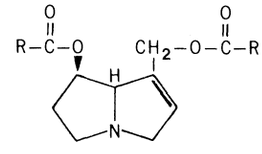
Pyrrolizidine Alkaloids: Their Occurrence in Honey from Tansy Ragwort (*Senecio jacobaea* L.)

Abstract. *The hepatotoxic alkaloids known to occur in tansy ragwort* (Senecio jacobaea *L.) are also present in honey produced from the nectar of this species. These alkaloids, which include senecionine, seneciphylline, jacoline, jaconine, jacobine, and jacozine, are potentially carcinogenic, mutagenic, and teratogenic and may pose health hazards to the human consumer.*

The hepatotoxic pyrrolizidine alkaloids present in local tansy ragwort (*Senecio jacobaea* L.) have been demonstrated conclusively to be present in honey produced from the nectar of this plant. Certain liver ailments and other diseases in humans in developing nations have been attributed to the consumption of foods and herbal medicines prepared from pyrrolizidine alkaloid–containing plants. We report here that human exposure to the pyrrolizidine alkaloids through food products is a very real possibility in the United States.

Tansy ragwort is a weed introduced to maritime regions of both western and eastern North America from Europe (*1*, *2*). The toxicity of *S. jacobaea* is well known and is due to a mixture of pyrrolizidine alkaloids which include senecionine, seneciphylline, jacobine, jaconine, jacoline, and jacozine (*2–5*). All six of these alkaloids are cyclic diesters of the 1,2-dehydropyrrolizidine ring system (**1**). Values for the median lethal dose (LD_{50}) of the alkaloids in tansy ragwort are around 100 mg/kg on the basis of animal experiments (*2*).

The consumption of foods and herbal medicines contaminated with pyrrolizidine alkaloids results in acute veno-occlusive lesions which progress to liver cirrhosis (*5*). The Budd-Chiari syndrome, which is manifested by hepatic vein occlusions in native South African populations is apparently also related to the consumption of bread containing *Senecio* flour (*5*). More important, however, are the animal experiments that have shown that certain pyrrolizidine alkaloids are carcinogenic (*6*), mutagenic (*2*), and teratogenic (*8*).



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Blooming of *S. jacobaea* occurs from the middle of July through September in western Oregon and Washington. During this time there is a general dearth of nectar and pollen in other entomophilus species, and tansy ragwort is actively foraged upon by honey bees (*Apis mellifera* L.).

We attempted to discover whether the endogenous alkaloids in tansy ragwort are shunted through the nectar secretory process and ultimately deposited in the

Table 1. Percentage of tansy ragwort pollen and concentration (expressed as parts per million) of pyrrolizidine alkaloids found in honey samples from the Pacific Northwest.

Honey sample	Geographical source	Average tansy ragwort pollen (%)*	Concentration of pyrrolizidine alkaloids (ppm)†
1	Elma, Washington	2.6 ± 0.7	1.1 and 1.4
2	Beaverton, Oregon	0.8 ± 0.1	0.3 and 0.4
3	Toledo, Oregon	1.9 ± 0.4	1.2 and 2.2
4	Salem, Oregon	0.7 ± 0.4	3.2 and 3.9
Control	Corvallis, Oregon	0.0	0.0

*Average of three replicates. †Uncorrected; two separate determinations.