

cis-Unsaturated Fatty Acids Induce the Fusion of Chromaffin Granules Aggregated by Synexin

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ABSTRACT When isolated chromaffin granules were aggregated by synexin (a Ca^{2+} -binding protein present in chromaffin and other secretory tissues) and then exposed to *cis*-unsaturated fatty acids at 37°C , they fused together to form large vesicles. The fusion was monitored by phase and electron microscopy and by turbidity measurements on the granule suspension. Arachidonic acid was the most effective fusogen, whereas *trans*-unsaturated fatty acids, saturated fatty acids, detergents or lysolecithin were inactive. During fusion some of the epinephrine of the granules was released but the soluble core proteins remained trapped in the resulting vesicles. These vesicles swelled to enclose the maximum volume. Although this swelling could be inhibited by increasing the osmotic strength of the medium, it did not appear to depend on the chemiosmotic properties of the granule membranes as it was not influenced by ATP, a proton ionophore, or an anion transport inhibitor.

The regulators of this *in vitro* fusion— Ca^{2+} , synexin, and free, *cis*-unsaturated fatty acids—may be present in the cytoplasm of the chromaffin cell when it is stimulated to release epinephrine and granule proteins by exocytosis. Therefore, this fusion event may be the same that occurs between chromaffin granules undergoing compound exocytosis.

Exocytosis is one of the most frequent events occurring in living cells that requires the fusion of two membranes. It is the basis for the release of many secretory products such as hormones, neurotransmitters, and digestive enzymes, which are stored in membrane-bounded vesicles before release. Historically, a valuable system for the study of this process has been the secretion of catecholamines from the adrenal chromaffin cell. The nature of exocytosis was defined morphologically (13) and biochemically (17) in this system as the discharge of storage vesicle contents but not vesicle membranes. The chromaffin cell was also one of the first secretory systems in which the requirement for Ca^{2+} to activate exocytosis was recognized (15). Recently, a model for the molecular basis of exocytosis has been developed based on data obtained from the chromaffin system (29). This model explains the Ca^{2+} requirement in terms of synexin, a Ca^{2+} -binding protein found in many secretory tissues (7–9, 12, 22). When activated by Ca^{2+} , this protein causes the attachment of isolated secretory vesicles to one another, in a fashion that may be analogous to the interaction between vesicles that occurs during compound exocytosis. The model also defines the breakage of the secretory vesicle membrane and release of its contents during exocytosis as an osmotic

lysis resulting from the ATP-dependent transport of protons and permeant anions into the vesicle interior (5, 10, 24, 31). However, the relationship between those two events has remained poorly defined. In particular, it has not been clear that the regions of contact formed between secretory vesicles by synexin would be the most likely place for the membranes to break, as must occur during compound exocytosis.

This communication describes observations suggesting that, in the presence of small amounts of unesterified, *cis*-unsaturated fatty acids, the regions of contact formed by synexin between chromaffin granules (the secretory vesicles of the adrenal medulla) do indeed break, leading to the fusion¹ of the granules and the formation of larger vesicles retaining some of the granule contents.

¹ In previous communications describing synexin (7, 8) the term "fusion" was used to refer to the formation of close contacts ("pentameric complexes") between chromaffin granule membranes. In the present context it is more consistent with current terminology to reserve the term "fusion" to refer to the breakage of the membrane barriers (i.e., "fission" in Palade's nomenclature [23]) and the formation of the larger vesicles.

MATERIALS AND METHODS

Materials

Chromaffin granules were prepared from bovine adrenal medullary tissue by differential centrifugation in 0.3 M sucrose as previously described (24), or by purification on an isotonic step-gradient of sucrose and Metrizamide (30), collecting the granules at the interface of densities 1.10 and 1.12 g/ml. The behavior of the granules prepared by either method was indistinguishable in the assays described in this report.

Synexin was prepared from bovine liver using essentially the procedures described for the preparation of adrenal medullary synexin: precipitation in 20% ammonium sulfate and gel filtration on Ultragel ACA 34 (LKB Instruments, Inc., Rockville, Md.) (7, 8). However, 2(*N*-morpholino)ethane sulfonic acid (MES)-NaOH, pH 6.0, was substituted for the histidine buffer previously used, and the initial extract was prepared from 250 g of tissue. The synexin obtained from liver has been shown to have the same molecular parameters and interaction with chromaffin granules as had previously been described for adrenal medullary synexin (9). The liver synexin was stored frozen at -20°C at a concentration of $\sim 100\ \mu\text{g}/\text{ml}$ in the gel filtration buffer (0.3 M sucrose, 40 mM MES-NaOH [pH 6.0]).

Fatty acids tested for fusogenic activity were obtained from Sigma Chemical Co., St. Louis, Mo.

Aggregation and Fusion of Chromaffin Granules

Synexin-induced granule aggregation was carried out under the conditions of the assay for synexin activity (7): incubation at 37°C of 1-ml samples containing 10 to 20 μg of synexin, 240 mM sucrose, 30 mM KCl, 32 mM MES-NaOH (pH 6.0), 2.5 mM EGTA, and CaCl_2 of appropriate concentration to give the desired free Ca^{2+} concentration. The granule suspension had an initial absorbance at 540 nm (A_{540}) of 0.3, corresponding to 70–90 $\mu\text{g}/\text{ml}$ of granule protein. After incubation for a minimum of 15 min, the fusogen was introduced as follows: Fusogens were stored as 5 mg/ml solutions in ethanol at -20°C and diluted in sucrose-MES buffer to 100 $\mu\text{g}/\text{ml}$ before each series of experiments; 40–100 μl of the resulting emulsion was added to the granule suspension. Stearic and myristic acids did not form emulsions at 100 $\mu\text{g}/\text{ml}$, so these compounds were introduced in ethanol, taking care that the final ethanol concentration in the granule suspension was $<1\%$.

The turbidity (A_{540}) of granule suspensions was monitored on a Gilford 250 recording spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) equipped with an automatic cuvette positioner, which permitted the intermittent monitoring of four simultaneous reactions.

For phase microscopy, a 40- μl sample of the suspension was examined between a glass slide (Kimble #75023; Kimble Div., Owens-Illinois, Inc., Toledo, Ohio) and a 24 \times 30 mm cover slip (Kimble #75100). The slide and cover slip were scrubbed lightly and rinsed in deionized water before each series of observations in order to remove contaminating fusogens.

For electron microscopy, 5-ml suspensions of granules were fixed by adding 50% glutaraldehyde to a final concentration of 2% and placing the samples on ice. After 15 min the granules were sedimented at 20,000 g for 15 min. The pellets were collected in 1 ml of 2% glutaraldehyde and resedimented by centrifugation in a Beckman Microfuge (Beckman Instruments, Inc., Palo Alto, Calif.). The pellets were postfixed in 1% osmium tetroxide, before dehydration and embedding. Sections were stained with aqueous lead citrate and uranyl acetate and examined by conventional transmission electron microscopy.

Release of Protein and Epinephrine from Fusing Granules

5 min after granule fusion was induced, suspensions were centrifuged at 20,000 g for 15 min at 4°C and the supernatant fractions assayed for protein and epinephrine content. Protein was assayed by the method of Lowry et al. (19) using bovine serum albumin as standard, after precipitation of protein in cold 10% TCA. In addition, some protein determinations were performed using the Bradford method (4). Synexin showed poor color development in the Bradford assay, relative to chromaffin granule proteins, and it was found that when bovine serum albumin was used as a standard for chromaffin granule proteins (26) and bovine gamma globulin or ovalbumin was used as a standard for the protein

content of the synexin preparations (7), results consistent with the Lowry method (with serum albumin standard) were obtained. Epinephrine was assayed by the fluorometric trihydroxyindole method at pH 2 (2).

As an alternative to the centrifugation procedure, granule suspensions were chromatographed on PD-10 columns (Pharmacia Fine Chemicals, Piscataway, N. J.) containing Sephadex G-25, to separate free epinephrine from the granules and membranes.

RESULTS

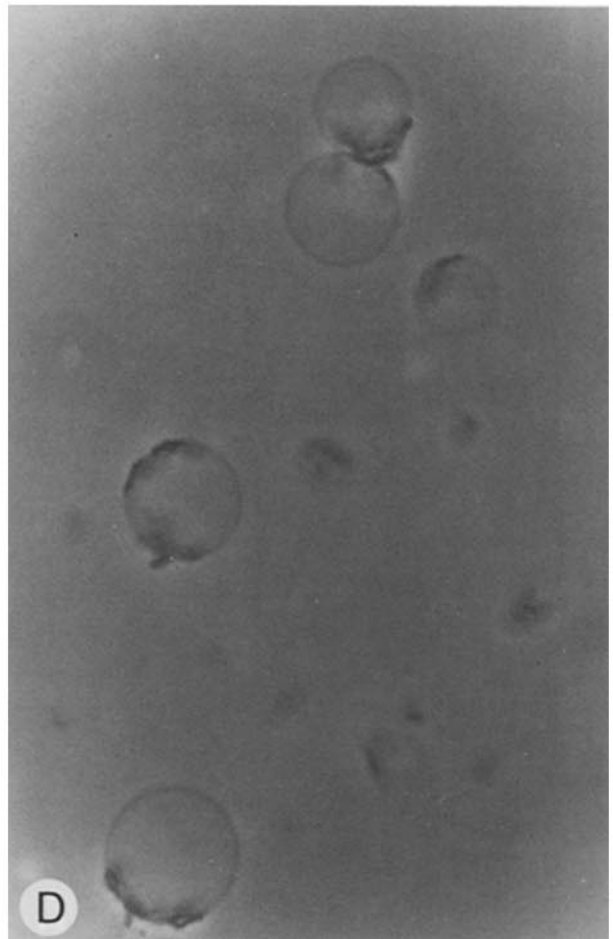
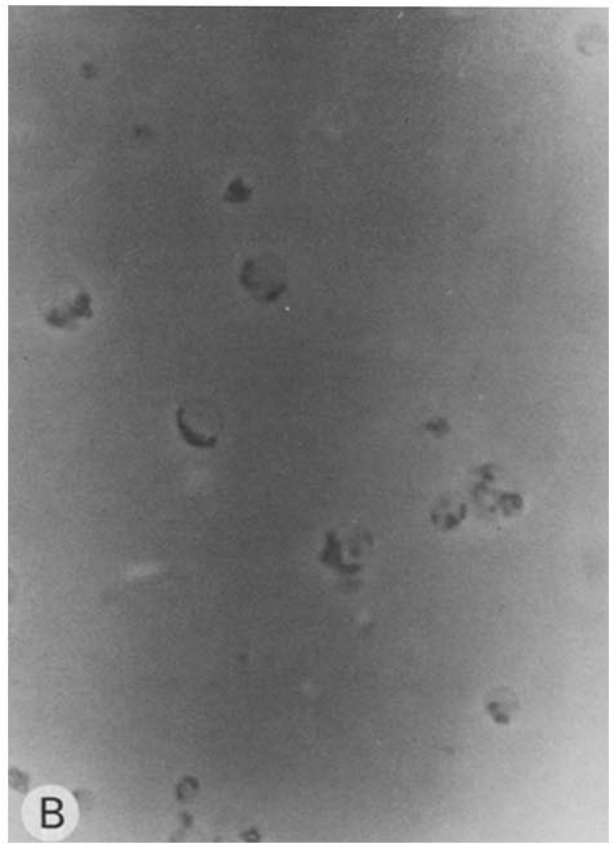
Initial Observation of Chromaffin Granule Fusion

The fusion of chromaffin granules was first observed in the phase microscope during a careful examination of large clumps of granules aggregated by synexin (similar to those in Fig. 1 A). This approach was inspired by an electron micrograph of an isolated chromaffin cell that had been exhaustively stimulated to secrete epinephrine by the secretagogue veratridine (this micrograph is published in reference 28). The cytoplasm of this cell contained vacuoles that appeared to have been formed by the fusion of large numbers of cytoplasmic chromaffin granules in the process of compound exocytosis. Subsequent examination of thick sections of cells similarly treated revealed that these vacuoles were of sufficient size to be readily seen in the light microscope. This observation suggested that, if such an interaction between granules could be induced to occur in a cell-free system, the formation of similar structures might be monitored by phase microscopy. Accordingly, a search for *in vitro* conditions that would induce fusion between large numbers of chromaffin granules was initiated using synexin to bring the granules into close contact.

Particularly large clumps of chromaffin granules could be grown *in vitro* by incubating the granules with high concentrations of synexin (10–20 $\mu\text{g}/\text{ml}$) and saturating concentrations of Ca^{2+} (1 mM) at 37°C for longer than 15 min (Fig. 1 A). During examination in the phase microscope these clumps were seen, over the course of several minutes, to undergo a remarkable transition to form large, spherical vesicles similar to those in Fig. 1 B–D. The course of this event was reminiscent of the appearance of a handful of soap suds fusing to form larger bubbles—characterized particularly by small, sudden, fusion events that caused the clump to jump suddenly one way then another. However, in the case of soap bubbles the enclosed volume does not increase, whereas the volume enclosed in the fusing granules apparently increased as the large vesicles seemed to swell from the clumps. As the phase-lucent vesicles swelled, the attached phase-dense clumps decreased in size. These observations seemed consistent with the interpretation that the clumped granules had fused and that their membranes had been rearranged to form the surfaces of the large vesicles.

However, the fusion phenomenon was not reliably reproducible, occurred to different extents on different portions of the microscope slide, never occurred in a test tube, but only when the clumps were examined in the microscope, and could be completely prevented by washing the microscope slide and cover slip in distilled water or acetone before observation. Apparently, the glass slide or slip was providing an essential factor for fusion. The exact nature of this factor has been difficult to determine because of the proprietary nature of the

FIGURE 1 Visualization of chromaffin granule fusion in the phase microscope. (A) Clumps of granules that have been aggregated by incubation with synexin and Ca^{2+} for ~ 40 min. (B–D) Vesicles of various sizes formed after further incubation of the preparation for ~ 15 min in the presence of 4 $\mu\text{g}/\text{ml}$ arachidonic acid. Graticule marks are 10 μm apart.



processes involved in the manufacture of microscope slides and cover slips. However, this fortuitous observation led to the survey of potentially fusogenic compounds reported below.

Survey of Compounds for Fusogenic Activity

Because the fusion of chromaffin granules that was initially observed apparently resulted from an exogenous factor introduced by the microscope slide or cover slip, a survey was conducted to see if a similar effect could be induced by compounds recognized as having fusogenic potential in cellular systems. A particularly useful guide in selecting compounds was the work of Ahkong et al. on the fusion of erythrocytes (1). The compounds that were tested are listed in Table I. Each compound was analyzed in two tests: the effect of the compound on the turbidity of the granule suspension and the effect on the appearance of granule aggregates in the phase microscope.

Fig. 2 illustrates the application of the turbidity assay to fusion in the presence of arachidonic or oleic acid, which were found to be effective fusogens (see below). The reaction was started by adding the granules to the synexin and Ca^{2+} . The aggregation of granules was permitted to proceed for 15 min and this aggregation was manifested as an increase in turbidity (Fig. 2, traces *a*, *b*, and *c*). When a *cis*-unsaturated fatty acid was added, the turbidity of the suspension immediately fell because of the formation of the transparent vesicles from the dense clumps of granules and, also, probably to a lesser extent, because of some release of material from the granules (this latter effect was also seen in the absence of synexin, when the granules were not aggregated; Fig. 2, trace *d*). The decline in turbidity was largely complete in 5 min, and the extent of the decline appeared to correlate with the extent of formation of large vesicles observable in the microscope (detergentlike molecules, e.g., SDS or lysolecithin, were an exception in that they caused granule lysis and a decline in turbidity but never formed observable vesicles). The decline in turbidity from the aggregated state (in which the turbidity was usually ~160% of the initial turbidity of the unaggregated suspension) to the state 5 min after adding the fusogen is given in Table I as a quantitative characteristic of the effect of the compound on the suspension. Although this parameter was useful for screening compounds for fusogenic activity, it must be emphasized that it is not a direct measure of actual granule fusion because lysis or disaggregation can also contribute to the turbidity decline.

Of the compounds tested, the only effective fusogens found were the naturally occurring, *cis*-unsaturated fatty acids. Arachidonic acid was the most effective, causing microscopically observable fusion at a minimum concentration of 2 $\mu\text{g}/\text{ml}$ (6.6 μM) in the presence of 80 $\mu\text{g}/\text{ml}$ granule protein (50 $\mu\text{g}/\text{ml}$ of granule lipid[16]). Doubling the granule concentration doubled the threshold concentration of arachidonic acid needed for fusion. The series of 18 carbon *cis*-unsaturated fatty acids—linolenic, linoleic, and oleic—had an effectiveness that decreased as their degree of saturation increased. Higher levels of the less effective acids (e.g., 10 $\mu\text{g}/\text{ml}$ of oleic acid) were able to cause the same degree of fusion as lower levels of the most effective (4 $\mu\text{g}/\text{ml}$ of arachidonic acid).

A comparison of the fusogenic activity of several structural and conformational isomers of oleic acid strikingly suggested that a particular stereochemistry was critical to induce fusion. Oleic acid has a double bond between the 9th and 10th carbons in the *cis* configuration. If this bond is in the *trans* configuration (elaidic acid), no fusion occurs. If the *cis* bond is moved closer

TABLE I
Compounds Tested for Fusogenic Activity *

Compound	Con-	Δ Tur-	Vesicle
	cen-	idity‡	
	tra-	in 5	forma-
	tion	min	tion§
	$\mu\text{g}/\text{ml}$	%	
1. Arachidonic acid	1	22	0
[all <i>cis</i> 5,8,11,14 eicosatetraenoic acid (20:4)]	2	31	+
	4	81	++++
2. Linolenic acid	4	49	++
[all <i>cis</i> 9,12,15 octadecatrienoic acid (18:3)]			
3. Linoleic acid	4	47	++
[all <i>cis</i> 9,12 octadecadienoic acid (18:2)]			
4. Oleic acid	4	44	++
[<i>cis</i> 9 octadecenoic acid (18:1)]	10	78	++++
5. Palmitoleic acid	4	41.3	++
[<i>cis</i> 9 hexadecenoic acid (16:1)]			
6. <i>cis</i> -Vaccenic acid	4	34	+
[<i>cis</i> 11 octadecenoic acid (18:1)]			
7. Erucic acid	4	25.5	+
[<i>cis</i> 13 docosenoic acid (22:1)]			
8. Petroselenic Acid	4	0	0
[<i>cis</i> 6 octadecenoic acid (18:1)]	10	4	0
9. Elaidic Acid	4	0	0
[<i>trans</i> 9 octadecenoic acid (18:1)]	10	0	0
10. Methyl arachidonate	4	0	0
11. Methyl oleate	4	0	0
12. Glycerol mono-oleate	4	0	0
13. Lauric acid	4	0	0
14. Myristic acid	5	0	0
15. Palmitic acid	4	0	0
[hexadecanoic acid (16:0)]			
16. Stearic acid	5	0	0
[octadecanoic acid (18:0)]			
17. Stearyl amine	4	0	0
18. Retinol	4	0	0
19. α -Tocopherol	4	0	0
20. Prostaglandin E ₂	4	0	0
21. Lysolecithin	4	0	0
(from egg yolk)	10	51	0
22. SDS	4	12	0
23. Benzalkonium chloride	4	0	0
	8	0	0

* Compounds were added to ~80 $\mu\text{g}/\text{ml}$ of granule protein after aggregation by synexin for 15 min.

‡ Change in turbidity of the granule suspension induced by the compound after 5 min. Similar to the analysis in Fig. 2, the change is given as a percentage of the A_{540} of the suspension before aggregation. The values given were reproducible to within five percentage units for a single preparation of granules or synexin. The relative order of effectiveness for different compounds was the same for all preparations of granules and synexin.

§ Degree of vesicle formation seen in the phase microscope: +++++, extensive fusion, one or more large vesicles have developed from every clump; ++, moderate fusion, most clumps have developed vesicles but these are smaller, leaving a large part of the clump unfused; +, limited fusion, vesicles are difficult to find, occurring in <5% of the clumps; 0, no fusion, not a single vesicle can be seen.

to the head group, to carbons 6 and 7 (petroselenic acid), no fusion occurs. If the *cis* bond is moved away from the head group to the 11, 12 position (vaccenic acid), the extent of fusion is extremely reduced. In addition, if the head groups of arachidonic acid or oleic acid are methylated, the compounds do not cause fusion. The saturated fatty acids tested, C₁₂ through C₁₈ (lauric, myristic, palmitic, stearic), were all completely ineffective. Stearyl amine, α -tocopherol, and *trans*-retinol—three ad-

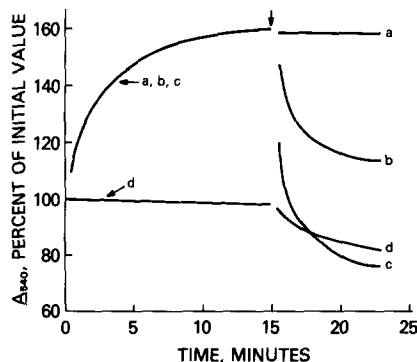


FIGURE 2 Turbidity (A_{540}) of a chromaffin granule suspension undergoing aggregation and fusion. From 0 to 15 min the granule suspension (A_{540} , ~ 0.3 ; $80 \mu\text{g/ml}$ protein) is incubated with (traces a, b, and c) or without (trace d) $15 \mu\text{g/ml}$ synexin. At 15 min $4 \mu\text{g/ml}$ fatty acid is introduced; trace a, elaidic acid (*trans* 18:1); trace b, oleic acid (*cis* 18:1); traces c and d, arachidonic acid (*cis* 20:4).

ditional compounds that Ahkong et al. (1) reported would fuse erythrocytes—were also ineffective. The anionic and cationic detergents, SDS and benzalkonium chloride, were ineffective. Of particular interest was lysolecithin, as it has often been suggested that the detergentlike properties of this molecule might enable it to play a role in natural membrane fusion. However, lysolecithin caused no vesicle formation.

Further Characteristics of the Fusion Reaction

Using arachidonic acid as a standard fusogen, several additional characteristics of the fusion event were examined. The conditions of the standard fusion experiment had been selected to optimize visualization of the formation of large vesicles. However, these extreme conditions were not required for fusion to be detectable in the phase microscope. The concentrations of synexin used in the standard fusion experiments of $10\text{--}20 \mu\text{g/ml}$ were greater than that needed to saturate the turbidity increase used as an assay for synexin activity ($\sim 5 \mu\text{g/ml}$; reference 7). However, these large concentrations did lead to the formation of more extensive granule aggregates and are probably comparable to the *in vivo* concentration of synexin, because the yield of synexin is $10 \mu\text{g/g}$ of tissue (7, 9), and in the chromaffin cells immunofluorescence experiments have demonstrated that the synexin is concentrated in the cytoplasm (9). When the concentration of synexin was reduced in the fusion assay to $2.5 \mu\text{g/ml}$, vesicle formation was still readily apparent.

The level of Ca^{2+} in the standard experiment (1 mM) was sufficient to saturate synexin, which has a K_d for Ca^{2+} of $200 \mu\text{M}$. However, when the entire aggregation and fusion experiment was conducted at $160 \mu\text{M}$ Ca^{2+} , vesicle formation still occurred. When the granules were aggregated in 1 mM Ca^{2+} , and then excess EGTA was added to reduce the free Ca^{2+} concentration to $20 \mu\text{M}$, the turbidity of the suspension dropped 14%, and the size of the granule aggregates was somewhat reduced. The aggregation step was thus only slightly reversible. When arachidonic acid was subsequently added, the remaining, irreversible aggregates fused to form large vesicles.

If arachidonic acid was added at the beginning of the granule aggregation reaction, rather than after aggregation was complete, fusion still occurred. However, the vesicles formed tended to be much smaller, even after prolonged incubation (~ 45 min). Apparently, the growth of larger vesicles by the fusion of smaller, already fused vesicles was a slow process. This may

have been attributable simply to the slower rate of diffusion of vesicles when compared to individual chromaffin granules.

Almost every vesicle seen in the phase microscope, even after extensive fusion and prolonged incubation (up to 2 h), had some phase-dense, clumped material on its border (Fig. 1B–D). Part of this material may have been contaminating mitochondria, which electron microscopic examination (see below) suggested tend to aggregate to, but not fuse with, the chromaffin granules.

The fusion process was temperature dependent. As previously described, the aggregation of chromaffin granules by synexin does not occur at 0°C (7). In addition, however, when the granules were aggregated at 37°C , then placed on ice, the aggregates were stable and subsequent addition of arachidonic acid led to no change in the suspension. When the cooled suspension, containing arachidonic acid, was finally placed in a 37°C spectrophotometer cuvette, fusion immediately occurred.

Raising the pH of the suspension from 6.0 to 7.2 strongly inhibited the rate of fusion. The rate of decline of the turbidity of the suspension, corresponding to fusion, was reduced 10-fold by this pH change. However, the final extent of fusion was the same at the more neutral pH, as was the general appearance of the structures formed.

The fusion event, as monitored by phase microscopy, manifested a specific requirement for synexin. Because of their negative surface charge, chromaffin granules can also be aggregated by strongly basic proteins such as polylysine (this type of aggregation is distinct from synexin-induced aggregation in that it is temperature and Ca^{2+} independent and is inhibited by increasing the ionic strength). When granules were aggregated into large clumps by $4 \mu\text{g/ml}$ of polylysine, subsequent addition of arachidonic acid did not result in the formation of vesicles visible in the phase microscope.

The presence of the nonfusogenic compounds listed in Table I, at the concentrations given, did not prevent the fusion of granules upon the subsequent addition of arachidonic acid. Stearyl amine slightly decreased the rate and extent of fusion induced by arachidonic acid. It is of particular interest that α -tocopherol (vitamin E), which may stabilize membranes (14), and lysolecithin, which may be formed in conjunction with free fatty acids as a result of phospholipase action, did not prevent vesicle formation.

Because the vesicles contained larger volumes than the granule clumps from which they arose, it was of interest to determine whether the chemiosmotic properties that have been described in isolated chromaffin granules (6, 10, 24) could have contributed to this swelling. A possible driving force for the fusion might have been the ATP-dependent pumping of protons and Cl^- ions into the aggregated vesicles, thus increasing their osmotic content and creating a pressure increase that could be relieved by fusion of the granules to form larger, spherical structures. This would have been consistent with the inhibition of fusion seen when the incubation medium was alkalinized. However, the addition of MgATP to the suspension did not enhance vesicle formation or reduce the amount of fusogen required. Although leakage of ATP from the granules might have obviated any requirement for exogenous ATP, the addition of $5 \mu\text{M}$ carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone, a potent proton ionophore, or 1 mM 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid, an inhibitor of anion transport, both of which prevent chemiosmotic events in chromaffin granules (6, 24), had no apparent effect on the

fusion reaction. In contrast, however, when the aggregated chromaffin granules were exposed to a hypertonic shock by adding sucrose to increase the osmolarity of the suspension to 800 mosM, subsequent addition of arachidonic acid did not lead to the formation of the usual large vesicles. Whether membrane fusion, per se, had been prevented was not determined.

It was also of interest to determine whether metabolism of the fatty acids, particularly arachidonic acid, was important for fusion. However, the addition of 100 μ M indomethacin to block any cyclo-oxygenase activity that might have been present did not inhibit the process. In addition, as shown in Table I, prostaglandin E₂, one of the prostaglandins that is formed from arachidonic acid and released from platelets during exocytosis (21), did not induce fusion.

Using arachidonic acid uniformly labeled with ¹⁴C, it was found that, during fusion by 4 μ g/ml of the fatty acid, 93% of the label was apparently bound to the granules, as it could be removed from the medium by centrifugation of the granules. Subsequent resolution of the membrane phospholipids by thin-layer chromatography (3) revealed no incorporation of the fatty acid into phospholipids. Therefore, the effective fusogen appears to have been the unesterified fatty acid incorporated noncovalently into the granule membrane.

Retention of Granule Contents during Fusion

Chemical analyses were performed to determine whether the contents of the chromaffin granules were retained within the new structures formed when fusion occurred. The aggregated and fused granules were sedimented in the centrifuge and the supernatant fractions were assayed for protein and epinephrine content relative to the total epinephrine and soluble protein of undisturbed granules. An example of this type of analysis is presented in Fig. 3. It is necessary to note as controls the amount of release that occurs when the granules are incubated alone, with synexin only, or with arachidonic acid only. 100% of the epinephrine and 80% of the protein in the chromaffin granule are in the core and are potentially soluble. Thus, we expect leakage of protein and epinephrine, as percents of totals, to be in the ratio of 4:5. However, in the presence or absence of arachidonic acid during the 20-min incubation there is a preferential leak of epinephrine. When fusion occurs, there is an additional release of epinephrine above the release occurring in the control samples. In this respect the fusion was apparently leaky. However, in the case of soluble core proteins, the analysis in Fig. 3 suggests that the fusion event may be completely conservative, because no soluble protein escapes beyond control levels.

Because the centrifugation procedure may have been harsh enough to contribute to the leakage of epinephrine, the separation of granules and vesicles from epinephrine was also performed by molecular exclusion chromatography on Sephadex G-25. Using this procedure, however, we also found that fusion was associated with additional epinephrine leakage. Thus, it appeared that the fusion event allowed the selective release of some of the small components of the granule cores, while retaining the larger molecules.

Electron Microscopy of Fused Chromaffin Granules

Chromaffin granules that had been aggregated by synexin for 15 min and then further incubated for 5 min in the presence

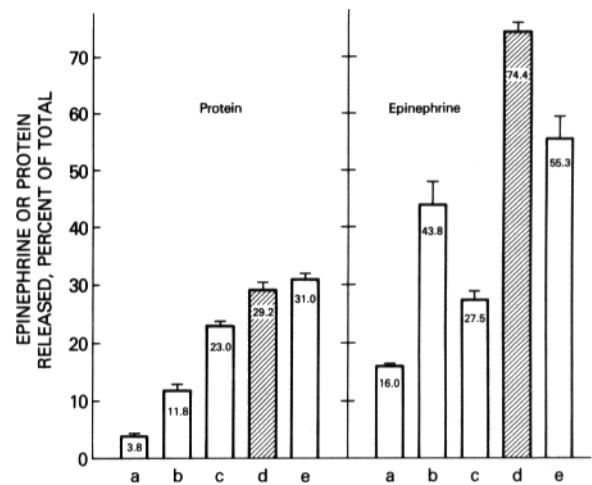


FIGURE 3 Leakage of protein and epinephrine from chromaffin granules undergoing aggregation and fusion. Suspensions containing 80 μ g/ml granule protein were incubated as follows: a, 20 min without synexin or arachidonic acid; b, 20 min without synexin, but with 4 μ g/ml arachidonic acid added at 15 min; c, 20 min with 15 μ g/ml synexin alone; d, 20 min with synexin, with arachidonic acid added at 15 min. Fusion occurs only in case d. Bar e indicates the degree of leakage that should occur if fusion does not induce any leakage above control levels ($e = a + [b - a] + [c - a]$). Leakage of protein in c, d, and e may be overestimated by a maximum of 2% of total because of unbound protein introduced in the synexin preparation (7). The error bars represent the standard deviations of duplicate experiments.

or absence of added arachidonic acid were examined by thin-section electron microscopy. In the absence of arachidonic acid the cores of the aggregated granules remained dense and separated from one another by the closely apposed granule membranes (Fig. 4A). However, in the presence of arachidonic acid most of the granules fused to form larger structures bound by a single membrane and containing flocculent core materials apparently greatly diluted from their original concentration in the dense granules (Figs. 4B and 5). These structures were generally smaller than those illustrated in Fig. 1, because of the shorter incubation time before fusion (15 min vs. 30–45 min). They also appeared “relaxed” or “floppy” instead of spherical, suggesting that they had not yet fully expanded, or that the tensions maintaining the spherical shape may have been relaxed during the fixation and processing of the samples. In micrographs of control samples exposed to arachidonic acid in the absence of synexin, no evidence of granule fusion was seen. Contaminating mitochondria were occasionally seen in the electron micrographs, and they often appeared to have been attached to chromaffin granules by synexin action. However, the mitochondria appeared very resistant to fusion because they usually remained intact, even when seen attached to the membranes of very large vesicles formed by the fusion of chromaffin granules.

When granules were aggregated by polylysine and exposed to arachidonic acid, many empty membrane envelopes were seen (Fig. 6B). These envelopes were often larger than single granule membranes but smaller than the vesicles formed by synexin. This suggested that a limited amount of membrane fusion had occurred but that the resulting structures had broken and released their contents. The inability of granules aggregated by polylysine to form large vesicles without the loss of core materials was consistent with the failure to observe fusion of such aggregates in the phase microscope.

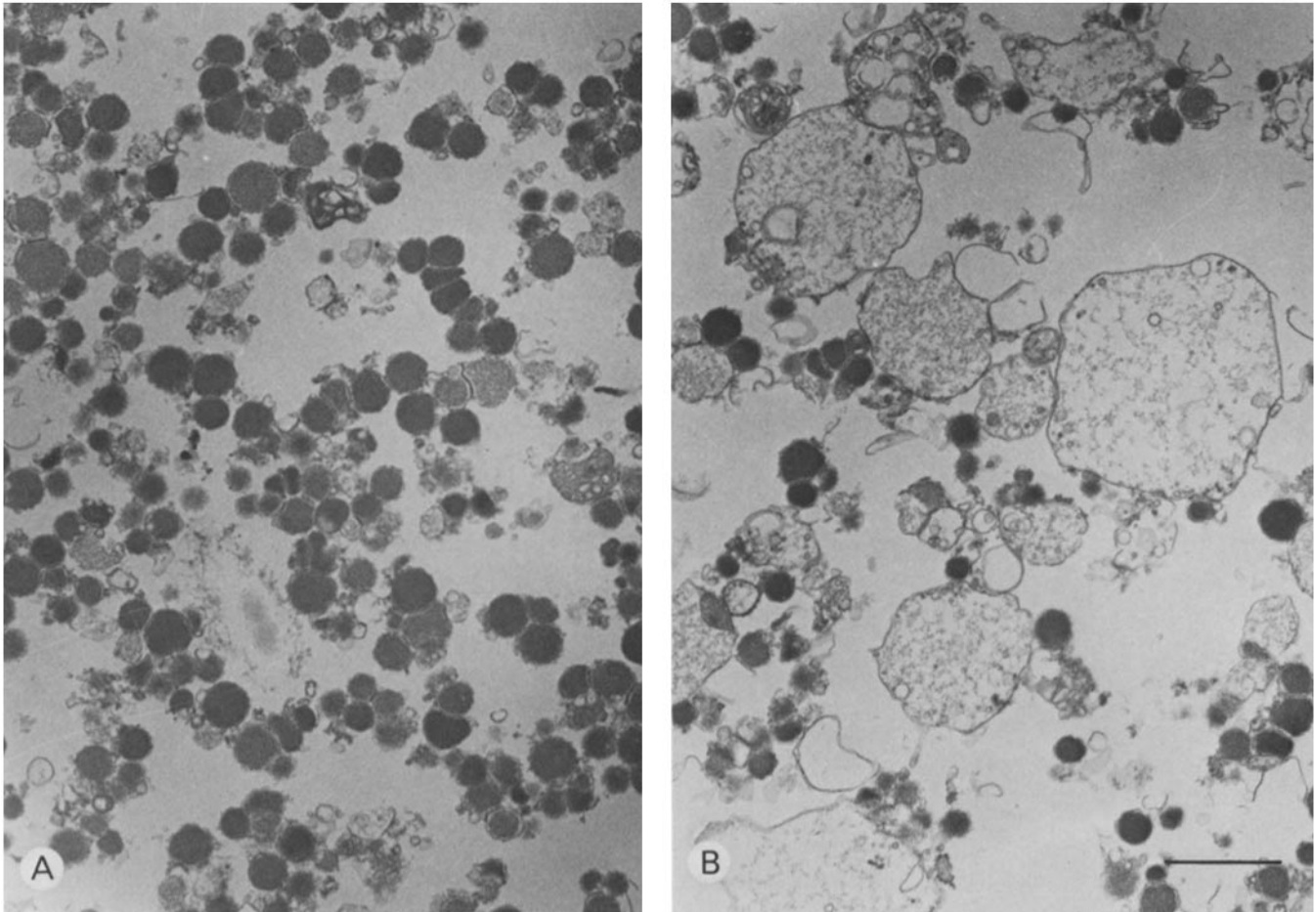


FIGURE 4 Electron micrographs of aggregated (A) and fused (B) chromaffin granules. Suspensions were incubated for 20 min in the presence of synexin, and, in B, arachidonic acid was introduced at 15 min. Bar, 1 μ m.

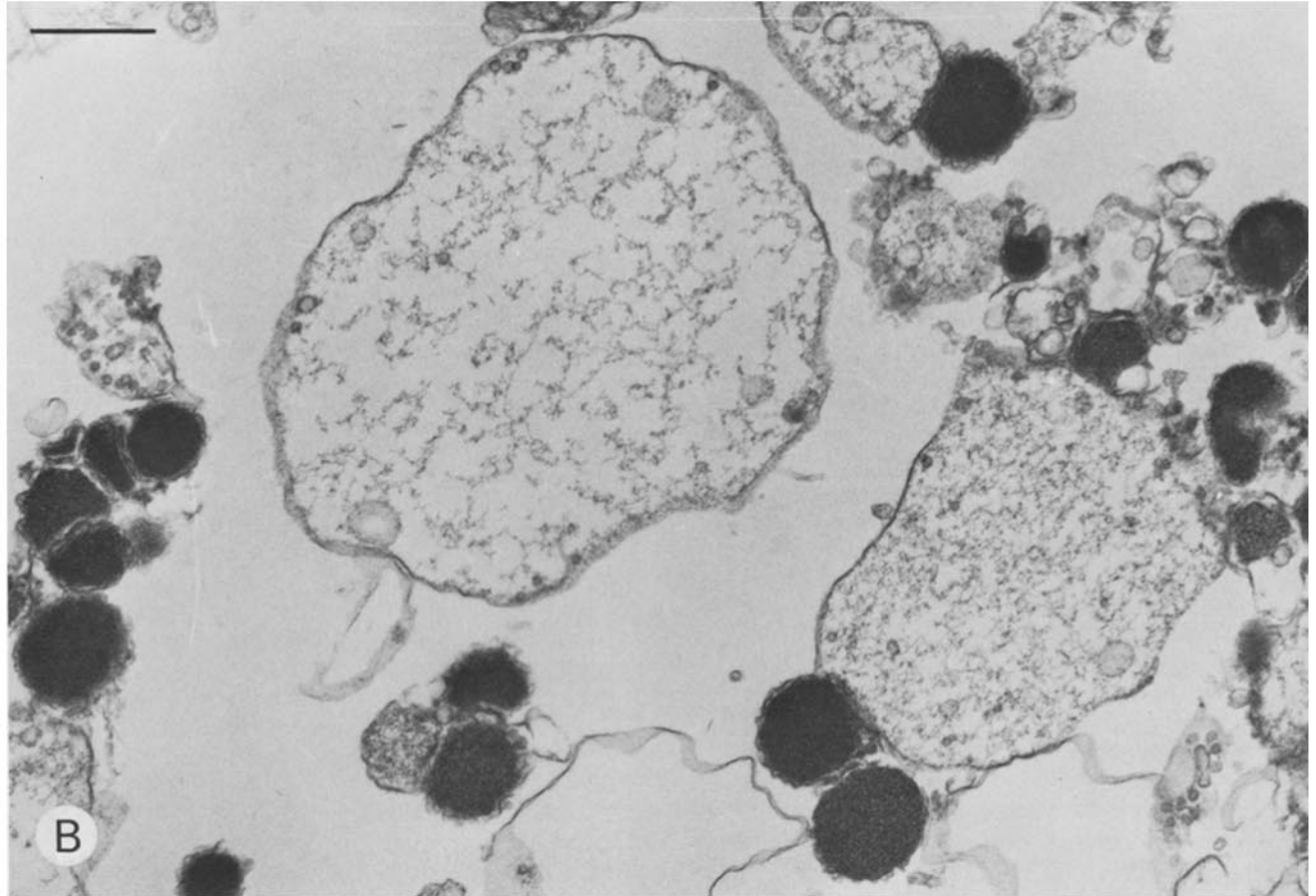
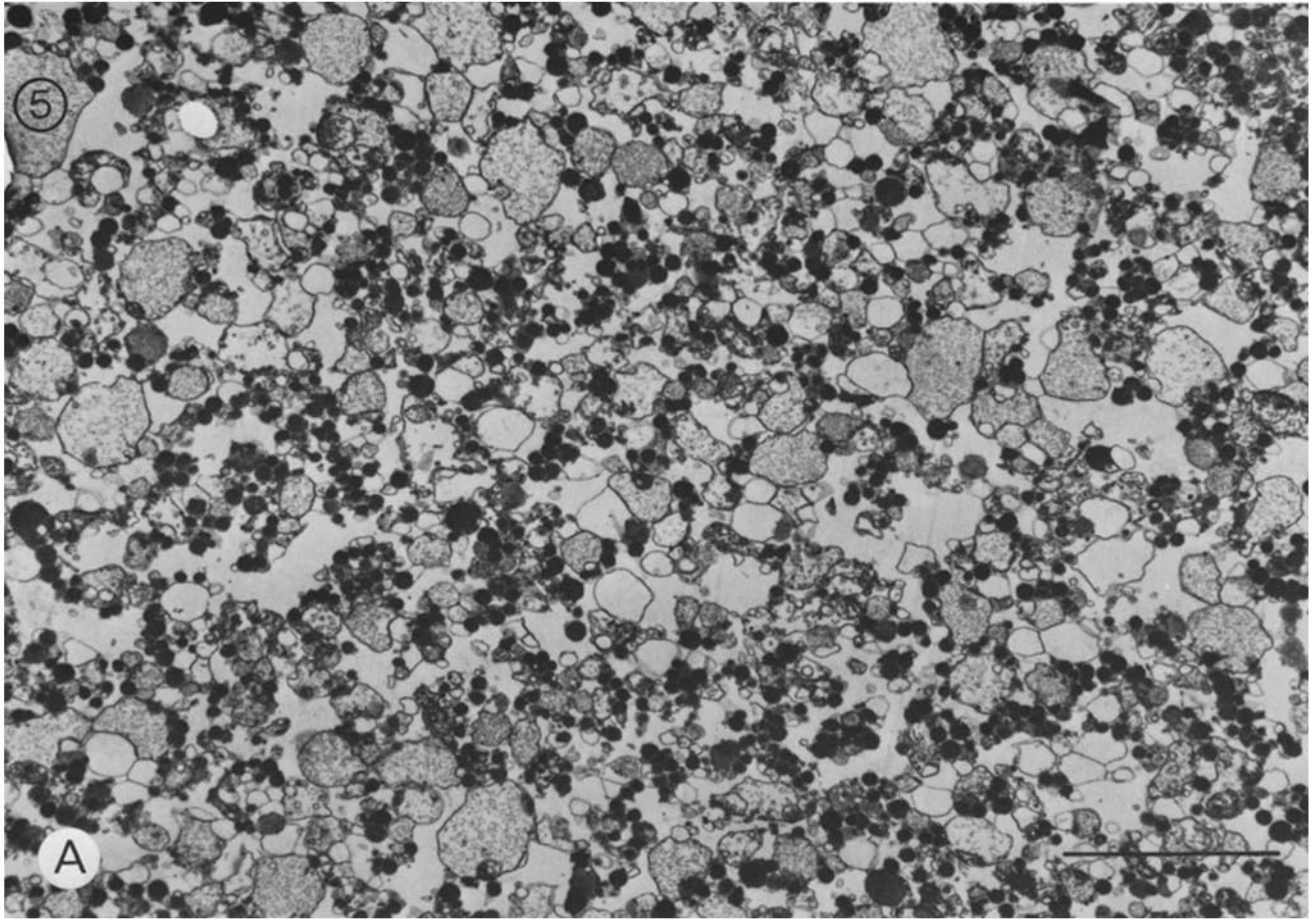
DISCUSSION

This report describes the fusion of chromaffin granules on an extensive scale in a cell-free system, in a manner that may be analogous to the fusion of granules that occurs during compound exocytosis in the adrenal chromaffin cell. The ability to observe this fusion in the phase microscope reduced the need for more sophisticated fusion assays based on chemical mixing of vesicle contents or electron microscopy. However, electron microscopy was essential for confirmation that actual fusion had occurred. The facility of the observation of fusion by light microscopy made it possible to examine in some detail the environmental determinants of the fusion event. The requirements for efficient fusion were found to be those likely to occur in the cytoplasm of the secretory cell when it is stimulated: the presence of synexin, Ca^{2+} , and free (unesterified), unsaturated fatty acids.

The ability of various lipids to act as membrane fusogens has been described previously (see reference 20 for a review). However, the levels of fusogen required in such studies, absolutely or relative to the membrane lipids, are generally one to two orders of magnitude higher than the concentrations of fatty acid used in the present study. Furthermore, the chromaffin granule fusion required only a few minutes, as opposed to up to 1 h for cell fusion. This kinetic difference is probably attributable, at least in part, to the role of synexin in holding the membranes together in close contact. Granule fusion also exhibited a striking specificity, to a degree not seen in previous

fusion studies, for particular *cis*-unsaturated fatty acids. For example, movement or isomerization of the double bond in oleic acid greatly reduced or eliminated its fusogenic potential. The explanation of this specificity is not clear at present. However, it may be relevant that Klausner et al. (18) have suggested that *cis*-unsaturated fatty acids enter different lipid domains in natural and artificial membranes than do *trans*-unsaturated or saturated fatty acids.

The implied requirement for a free, unsaturated fatty acid for membrane fusion to occur in exocytosis might be readily satisfied by the action of a phospholipase A_2 on membrane phospholipids (11, 21). In the case of blood platelets, for example, arachidonic acid is freed from membrane lipids when the platelets are stimulated to secrete serotonin. Some of this arachidonic acid is metabolized to form prostaglandins. The experiments in this paper suggest that some or all, before oxidation, may be involved in membrane fusion. Indeed, the amount of free arachidonic acid formed, relative to the total lipid content of the platelet, may be as high as 8% (21), which is comparable to the amount responsible for the fusion of chromaffin granules in the present study (4–8% relative to total granule lipids). Arachidonic acid is one of the major fatty acids of the chromaffin granule membrane, comprising 19% of the total fatty acids present in phospholipids (oleic acid accounts for 14%, and linoleic for 6% of the fatty acids; reference 33). However, very little is presently known about nonlysosomal phospholipases or about arachidonic acid metabolism in the chromaffin cell.



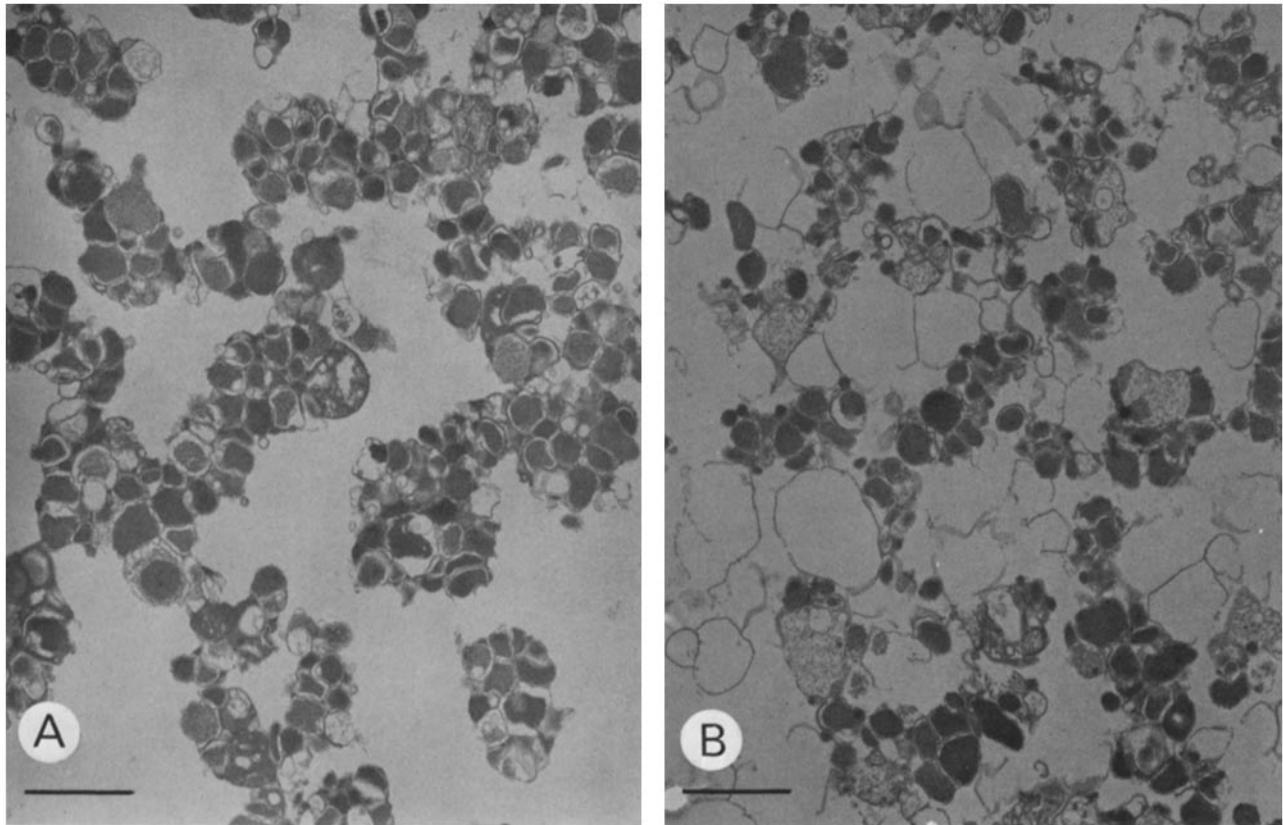


FIGURE 6 Chromaffin granules aggregated by polylysine and exposed to arachidonic acid. Suspensions were incubated for 20 min in the presence of polylysine and, in *B*, arachidonic acid was introduced at 15 min. Limited fusion and extensive membrane breakage is apparent in *B*. Bar, 1 μm .

In addition to specificity for particular fatty acids, the overall aggregation and fusion reaction also demonstrated a specificity for Ca^{2+} , for synexin, and for granule membranes. The Ca^{2+} specificity is accounted for by the specific requirement of synexin for this ion in order to bring the membranes together (7). Aggregation by basic proteins such as polylysine did not lead to the extensive and semiconservative (i.e., not leaky to larger molecules) fusion induced by synexin. Although mitochondria were attached to chromaffin granules by synexin, they very seldom fused with the granules in the presence of arachidonic acid.

It is useful now to review the hypothesis that has been put forward for the roles that synexin and the chemiosmotic properties of chromaffin granules may play in the process of exocytosis (29) and to examine explicitly what modifications to this hypothesis the current observations suggest. When the chromaffin cell is stimulated to secrete epinephrine, the cytoplasmic Ca^{2+} concentration increases. Synexin, which is presumably free in the cytoplasm as a 47,000-dalton monomer at low Ca^{2+} , binds Ca^{2+} and polymerizes to form small 50×150 -Å rods (8). These rods may self-associate, and they may bind to chromaffin granule (7) and plasma membranes (25, 32). By so doing, they bring the membranes into close contact. The low affinity of synexin for Ca^{2+} ($K_d \sim 200 \mu\text{m}$) may conveniently restrict synexin action to the vicinity of the plasma membrane, where Ca^{2+} is at the highest concentration, assuming that it enters from the extracellular medium. At this point in the

process it has been suggested that granules attached to the plasma membrane (or to the membranes of previously ruptured granules) are exposed to the high, extracellular concentrations of Cl^- (27, 29). This anion is then driven into the granule interior as a counter ion to protons being pumped into the granule by the granule membrane MgATPase (10, 24). Consequently, the osmotic strength in the granule increases, and eventually the granule ruptures, releasing its contents. For exocytosis to occur the granule must rupture at the point of contact with the plasma membrane (or with the membrane of a granule that has already released its contents). However, it has been by no means clear that the "pentalamellar structure" formed by the synexin-induced apposition of membranes (7) would be preferentially weak and thus inclined to break.

The implication of the present report is that the point of contact formed between membranes by synexin can indeed become weak and will break if an unesterified, *cis*-unsaturated fatty acid is present. If such an agent is made available by the action of a phospholipase (perhaps Ca^{2+} activated), exocytosis will occur.

It is attractive to visualize that the rupture of granule membranes brought into contact by synexin and destabilized by an unsaturated fatty acid is driven by an increase in osmotic strength within the granule. This could explain the swelling of large vacuolelike vesicles, seen in the present experiments, and the inhibition of the formation of these structures by increased osmotic strength, as well as the osmotic suppression of exocy-

FIGURE 5 Low and high magnification electron micrographs of fused chromaffin granules. Incubation as in Fig. 4 *B*. Bars: *A*, 5 μm ; *B*, 0.3 μm .

tosis, which has been described in several cell types (5, 27, 31). However, in the present experiments, the inability of an uncoupling agent (proton ionophore) or of an inhibitor of anion transport to inhibit the fusion of granules, and the failure of MgATP to accelerate the process raise doubts about the importance of the chemiosmotic swelling of the granules in the fusion event. Nonetheless, it seems clear that some type of swelling (enclosed volume increase) must occur for vesicles to form which are large enough to be seen in the light microscope. Geometric considerations dictate that, when the surfaces of n small spheres are rearranged to form a single large sphere, the volume enclosed in the large sphere is \sqrt{n} times larger than the collective volume of the n small spheres. The largest vesicles formed from fused chromaffin granules were $10\ \mu\text{m}$ in diameter and thus may have been constructed from the membranes of 1,600 granules of $2,500\text{-}\text{\AA}$ diameter, assuming conservation of membrane area. Therefore, the contents of these vesicles were diluted 40-fold. This seems too great a volume increase to be accounted for by the chemiosmotic properties of the membrane within the time-frame of the fusion event or by the osmotic consequence of solubilizing the small molecules trapped in the granule cores. Perhaps the displacement of counterions from negatively charged matrix proteins (chromogranins) has led to their expansion, thus driving the swelling of the vesicles.

In interpreting the present experiments, it should be emphasized that the experimental conditions used were optimized for the visualization of fusion in the phase microscope. Exocytosis *in vivo* may occur as a result of a far more limited degree of fusion. A detailed investigation of granule fusion induced by synexin and *cis*-unsaturated fatty acids under more restricted conditions may now be valuable. Synexin is a widely distributed protein, and *cis*-unsaturated fatty acids are common components of membrane phospholipids. Thus, the detailed study of this event may help to elucidate the mechanism of Ca^{2+} -dependent membrane fusion in other systems as well.

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