Calcium-Promoted Fusion of Isolated Chromaffin Granules Detected by Resonance Energy Transfer between Labeled Lipids Embedded in the Membrane Bilayer†

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ABSTRACT: Chromaffin granules isolated from bovine adrenal medulla were labeled with small unilamellar vesicles (SUV) made from *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine and/or *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine as donor and acceptor fluorophores. Labeling of granules could be followed by the relief of self-quenching experienced when the labels were incorporated into the granule membrane. The mechanism of incorporation seemed to be fusion of the SUV with the granule membrane although transfer of label could not be ruled out. Granule–granule fusion could be detected either by resonance energy transfer from donor to acceptor fluorophores or by donor quenching, in either of two different experimental designs: by a decrease in donor fluorescence and an increase in acceptor fluorescence when donor-labeled granules fused with acceptor-labeled granules or by an increase in donor fluorescence and a decrease in acceptor fluorescence when granules containing both probes fused with unlabeled granules. Fusion of freshly prepared granules could be initiated by millimolar concentrations of calcium; magnesium was less effective. Mg-ATP had no effect. Fusion was inhibited by potassium glutamate and a variety of organic and inorganic cations and anions, which also inhibited granule–granule aggregation to a lesser extent. The conditions for promotion and inhibition of granule–granule fusion were quite different from those reported by Knight and Baker [Knight, D. E., & Baker, P. F. (1982) *J. Membr. Biol. 68*, 107–140] for exocytosis of granule contents from permeabilized chromaffin cells. We conclude that the membrane fusion seen in this report is activated by a different mechanism.

The chromaffin granules of the bovine adrenal medullary cells release their stores of catecholamines, ATP, and soluble protein into the blood stream by fusion of the storage granule with the cell plasma membrane. This process, termed exocytosis, is one of a growing number of biological processes which involve membrane fusion. It has been suggested that an in vitro model for this process can be found in the calcium-promoted fusion of chromaffin granule membranes with each other (Morris, 1974; Edwards et al., 1974; Creutz, 1981; Morris et al., 1983).

In a series of reports, our laboratory has been exploring the morphology and the kinetics of the processes involved in the aggregation and fusion of the isolated granules. Although we have been able to observe the fusion of the granules by electron microscopy (Morris, 1974; Morris et al., 1983), this methodology is time consuming an labor intensive. Elaboration of the conditions under which the fusion can take place has been slow.

On the other hand, stopped-flow kinetic studies of the granule membranes have shown that aggregation of intact membranes can be followed by changes in the turbidity of the samples (Morris et al., 1979a) and occurs at near diffusion-controlled rates (Morris et al., 1979b; Morris & Hughes, 1979). This is followed by lateral displacement of (some of) the proteins from the contact region (Schober et al., 1977; Haynes et al., 1979; Morris et al., 1982b).

We have sought to develop a spectroscopic method for real-time study of membrane fusion to complement stopped-flow aggregation measurements. Several methods for assessing the fusion of artificial phospholipid bilayer vesicles have been developed based either on the mixing of the core contents (Ingolia & Koshland, 1978; Wilschut & Papahadjopoulos, 1979; Zimmerberg et al., 1980; Lelkes et al., 1980) or on the interaction of probes placed in the membrane (Vanderwerf & Ullman, 1980; Owen, 1980; Cohen et al., 1980; Schenkman et al., 1981; Kendall & MacDonald, 1982). Of these approaches, the latter is best suited to biological vesicles, which are isolated with their storage contents intact and are sensitive to extensive manipulation. Vanderwerf & Ullman (1980), utilizing resonance energy transfer measurements (Förster, 1951; Stryer, 1978), first demonstrated that fluorescent donors and acceptors attached to the head groups of phospholipids could be used to report the fusion of donor- and acceptor-containing populations of small unilamellar vesicles (SUV).[1] Struck et al. (1981) have similar results from using headgroup-labeled NBD-PE and Rho-PE cosonicated into SUV. In their assay, the donor and acceptor fluorophores are placed in the same SUV at a high enough surface density that appreciable donor quenching occurs, transferring energy to the acceptor. Fusion of probe SUV to non-probe-containing SUV results in a dilution of the probes, increasing donor fluorescence and reducing acceptor fluorescence. The fusion of two probe-containing vesicles is silent, since no change in probe surface density occurs. By following the changes in donor quenching, the extent of fusion can be calculated from the equations of Fung & Stryer (1978). Owen (1980), using the eximer fluorescence from pyrenyl-PE, demonstrated that SUV will fuse to cell membranes, while Struck et al. (1981) found

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[1] Abbreviations: SUV, small unilamellar vesicle(s); PS, phosphatidylserine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rho-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; PL, phospholipid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; LDH, lactate dehydrogenase; DBH, dopamine β-hydroxylase; buffered sucrose, 300 mM sucrose–10 mM HEPES, pH 7.3; TLC, thin-layer chromatography.

most of their SUV attached to the cell membrane without fusing.

We have recently reported that both the aggregation and fusion of artificial phospholipid vesicles could be followed by stopped-flow mixing and that the former is the rate-limiting step (Morris et al., 1983b). In this paper, we report the development of a spectroscopic assay for the fusion of chromaffin granules to each other, using resonance energy transfer between appropriately labeled phospholipids embedded into the granule membrane. The progress of aggregation and fusion can be followed simultaneously by using resonance energy transfer to monitor fusion along with observation of the changes in the turbidity of the suspension to read aggregation rates. Preliminary results concerning conditions under which the granules aggregate and fuse are presented.

Experimental Procedures

Materials

NBD-PE, Rho-PE, and *Escherichia coli* PE were purchased from Avanti Polar Lipids, Birmingham, AL, delivered on dry ice, and stored at −20 °C under argon until the vials were opened. The probe lipid–$CHCl_3$ mixture was diluted to 1.0 mg/mL with cold $CHCl_3$, stored at −20 °C, and used within a month.

Methods

Preparation of Chromaffin Granules. Chromaffin granules were isolated by previously described methods from bovine adrenal glands collected from a local slaughterhouse. For the bulk of these studies, the twice-washed crude granule pellet, previously judged to be >95% pure (Cahill & Morris, 1979), was used. Recent experiments have established that these granules have very little mitochondrial ATPase contamination (Weinbach et al., 1983). In a few cases, lysed resealed ghosts formed from both labeled and unlabeled granules were prepared by the procedure of Apps et al. (1980). All procedures and subsequent handling of the granules were carried out at 0–4 °C.

Protein Determinations. For rapid determination of protein concentrations of the freshly prepared granule suspensions, granules were diluted in cold 0.3 M sucrose–10 mM HEPES, pH 7.3 (buffered sucrose), and the optical density at 320 nm (A_{320}) was rapidly measured. The protein concentration of the samples was calculated from the relation $12.58A_{320} = 1.0$ mg of protein/mL for intact chromaffin granules (Südhof & Morris, 1983). Samples were diluted to 1.0 mg/mL on the basis of this measurement and split into 2.0-mL aliquots for labeling. Protein concentrations of the washed, labeled fractions as well as the starting material were determined by the Bradford (1976) Coomassie blue binding assay using bovine serum albumin as a standard. Differences between the two measurements were less than 5%.

Labeling of Isolated Granules with Fluorescent Phospholipids. (*A*) *Preparation of Probe-Containing Small Unilamellar Vesicles* (*Probe SUV*). Small unilamellar bilayer vesicles were formed from NBD-PE and/or Rho-PE by evaporating 200 μg of probe under nitrogen at room temperature in a 20-mL scintillation counting vial and then removing the last traces of organic solvent by 5-min exposure to 30 millitorr. A 2.5-mL aliquot of buffered sucrose was added and the sample sonicated for 5 min under argon at room temperature with a Heat Systems Model WA375 sonicator set at output 2, continuous 80% duty cycle using a flat tip (Morris et al., 1983b). Metal particles were removed by low-speed centrifugation (final [lipid] = 0.080 mg/mL). The probe vesicles were combined with the freshly prepared granules within an hour after sonication.

The fact that the labeled lipids formed SUV upon sonication was ascertained by sonicating the lipids in the presence of [^{3}H]sucrose. Unincorporated label was removed by chromatography on Sephadex G25 at room temperature, and the peak color fraction was rechromatographed. Trapped volumes of 0.17 and 0.010 μL/μmol for NBD and Rho SUV were determined, respectively. These values are about 0.5 and 0.025 of the expected value of 0.39 μL/μmol for a 250-Å SUV. Since both types of SUV lost activity between chromatographic runs, we attribute this to leakage through the membrane.

(*B*) *Granule Labeling Procedures.* After the protein concentration was rapidly determined by absorption measurement, the granule suspensions were diluted to 1.0 mg/mL protein. Suspensions of probe phospholipid vesicles were rapidly added with gentle vortex mixing to 2.0 mL of granule suspension and the mixtures incubated approximately 14 h at 0 °C. The suspensions were centrifuged 10 min at $30000g_{max}$, and the supernatant was decanted and saved. The pellets were washed 3–5 times by resuspension in 2.0 mL of buffered sucrose and centrifugation as described above. Preliminary tests showed that very little unincorporated label was removed after the third wash. Final pellets were resuspended in 2.0 mL of cold buffered sucrose and stored on ice in the dark until used.

Stability of the probes was assayed by thin-layer chromatography on Whatman 4855-621 plates with 65:25:4 $CHCl_3$:CH_3OH:H_2O as the developing solvent. TLC of the probes before and after sonication shows ~99% of the labeled lipid in a single spot. This result does not change upon storage of sonicated lipids at 0 °C for 2 weeks. Probe NBD incorporated into granule membranes and then extracted by 3 volumes of 2:1 $CHCl_3$:CH_3OH is also ~95% in its original form. A series of two to three other products comprise the remainder. It is possible that these products are due to the extraction methodology (room temperature for 1 h) or to turnover of lipid by enzymes (chromaffin granules are known to be contaminated with lysosomal phospholipase A_2). However, it is also possible that it is the result of the labeling procedures. They are unlikely to be the cause of energy transfer seen in the absence of fusion for the following reasons: (1) When double-labeled granules are mixed with unlabeled granule, no reduction in energy transfer is seen (i.e., no collision-dependent exchange occurs through the medium). (2) When Ca^{2+} is added, the change in energy transfer often exceeds 15–20% of the total increase in donor fluorescence (reduction of donor quenching). The degradation products do not exceed 5% of the total fluorescence and could not account for these results. (3) As the granules age, the concentrations of the degradation products increase while the extent of energy transfer decreases. As noted below, only results from freshly labeled granules are included in this paper.

Chromaffin granule fusion assays were performed by diluting 25–100-mL granule stock suspensions (1.0 mg of protein/mL, stored at 0 °C) with 1.0 mL of 300 mM sucrose–10 mM HEPES, pH 7.3 (buffered sucrose), containing 2.5 mM EGTA preequilibrated to the stated temperature. Initial fluorescence or absorbance measurements were made, and then a small volume of concentrated salt solution in buffered sucrose was added rapidly by hand, and time-dependent measurements were recorded. When the readings had stabilized (1–3 min), a small volume of concentrated $CaCl_2$ or $MgCl_2$ was added, and further changes were recorded. Finally, 10–20 μL of 10% (v/v) Triton X-100 or 30% (v/v) Ammonyx LO was added to disrupt the granule membranes, and the final records were

made. Control experiments, as described in the text, were performed under the same conditions.

Addition of detergent to the membranes does not necessarily give essentially infinite dilution of the probes as claimed by Struck et al. (1981) since they may partition into micelles. However, we have tested the ability of two different detergents to produce this effect and find that after appropriate correction for the quenching of the fluorescence by the detergent itself (1) both detergents give essentially the same increase in fluorescence and (2) above a given detergent concentration threshhold this effect is detergent concentration independent. If the probe were being autoquenched by partitioning into micelles, increasing the number of micelles should decrease the probe concentration within them. This seems not to be the case.

Fluorescence Measurements. Uncorrected fluorescence spectra and time-dependent measurements were made in a thermostated Perkin-Elmer Hitachi Model 650-10S fluorometer after first ensuring that no stray light was entering the sample chamber. Measurements were made by using crossed Polaroid polarizers to reduce scattering artifacts (Struck et al., 1981). Preliminary experiments established that the development of resonance energy transfer was severalfold slower than the aggregation induced by calcium (see below). Therefore, hand-mixing experiments were performed as described previously (Morris et al., 1982b).

Turbidity measurements were made essentially as described previously (Morris et al., 1982b; Südhof & Morris, 1983), using a Beckman Model 24 spectrophotometer connected to a chart recorder.

Miscellaneous Calculations. The concentrations of the probe phospholipids were calculated from the molar extinction coefficients of the probe lipids in ethanol [17 950 M^{-1} cm^{-1} for NBD-PE at 475 nm and 81 065 M^{-1} cm^{-1} for Rho-PE at 547 nm in ethanol (Dr. A. Walter, personal communication)] and agreed to within a few percent with the values supplied by the manufacturer. Calculations of the amount of incorporated probe and the surface density of the probe phospholipids were made from the probe concentration, determined by the fluorescence relative to a known concentration of probe SUV in detergent [after appropriate correction for the detergent solubilization (Struck et al., 1981)], and an assumed lipid:protein ratio of 0.48 mmol/mg as reported by Winkler (1976) and Winkler & Westhead (1980).

Results

Uptake of Label by Granules. Incubation of freshly prepared chromaffin granules with freshly prepared sonicated small unilamellar vesicles made from fluorescent lipids (probe SUV) results in the uptake of the label into the granule membrane. We base our assertion of this phenomenon on the following observations: (A) Probe fluorescence increases when unlabeled chromaffin granules are mixed with probe SUV. (B) The spectra of granules treated with dual-labeled SUV show concentration dependent changes in the emission spectra of the probes. (C) After incubation, a large precentage of the total fluorescence is associated with the chromaffin granules which cannot be removed by extensive washing.

(A) Kinetics of Probe Uptake into Chromaffin Granules. The fluorescence of the labeled phospholipid probes is highly self-quenched when the chloroform is evaporated; hydrating and sonicating the dehydrated material do not improve the fluorescence (Nichols et al., 1981; Hoekstra, 1982a; Tanaka & Schroit, 1983). Probe lipids incorporated into the granule membrane from SUV should show a large increase in fluorescence due to dequenching (relief of self-quenching) by

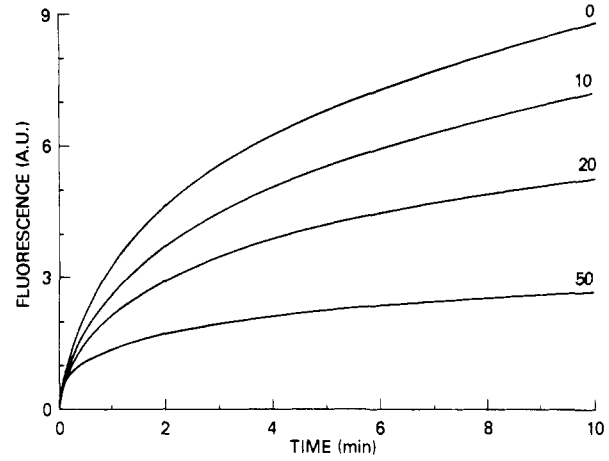

FIGURE 1: Time course of incorporation of probe SUV into chromaffin granule membranes detected by increase in fluorescence. Fresh, unlabeled chromaffin granules (final [protein] = 77 μg/mL) were incubated at 10 °C. At time zero 20 μL of dual-labeled SUV was rapidly added by hand and the rhodamine fluorescence recorded vs. time. Excitation, 570 nm; emission, 590 nm; slits, 6 nm. The scans from top to botton represent SUV (final [lipid] = 1.33 ng/mL) containing 0, 10, 20, and 50% (mol/mol) *E. coli* PE. Since the lipid concentrations were kept constant, the fluorescence intensities have been normalized to the 100% probe PE curve for direct comparison. Similar results (not shown) were generated for incorporation of NBD-PE and Rho-PE SUV.

dilution among the granule lipids. Therefore, the increase in fluorescence of the granule samples after incubation would represent incorporated label. Assuming that granule probe:non-probe PL ratios are so low that self-quenching does not occur, the surface density of the incorporated probe can be calculated (see Methods). Placing probe SUV in ethanol or detergent will disperse the lipids to essentially infinite dilution and relieve the self-quenching (Struck et al., 1981). Thus, detergent extraction can be used to judge the total fluorescence contained in the granule sample, the amount of contamination by unincorporated probe being proportional to the difference between the fluorescence before and after detergent addition.

Figure 1 shows the time-dependent increase in rhodamine fluorescence seen when fresh granules are incubated with dual-labeled probe SUV at 10 °C. Since the total number of fluorophores remains constant during the experiment, we assume that the increase is due to dequenching when probe molecules are added to the chromaffin granule membrane. The increase in fluorescence was shown to be dependent upon the initial concentration of SUV and temperature for both single-labeled and dual-labeled probe SUV (data not shown).

The uptake is dependent upon the lipid composition of the probe SUV. Figure 1 also shows the decrease in the rate and amplitude of incorporation of the initial uptake of label into the granules due to increasing the concentration of phosphatidylethanolamine in the SUV. Other results (not shown) indicate that total uptake was depressed. Also, probe vesicles containing substantial amounts of phosphatidylcholine will not interact with the granule membrane.

(B) Spectral Changes in the Probes after Uptake. Granules incubated overnight at 0 °C with single- or dual-labeled probe SUV and then subjected to three cycles of washing as described under Methods to remove unincorporated label also show large increases in fluorescence, which is further increased when detergent is added. Further washes do not significantly reduce either type of fluorescence associated with the pellet. Such results using dual-labeled probe SUV are shown in Figure 2 along with the changes in the spectra seen upon detergent

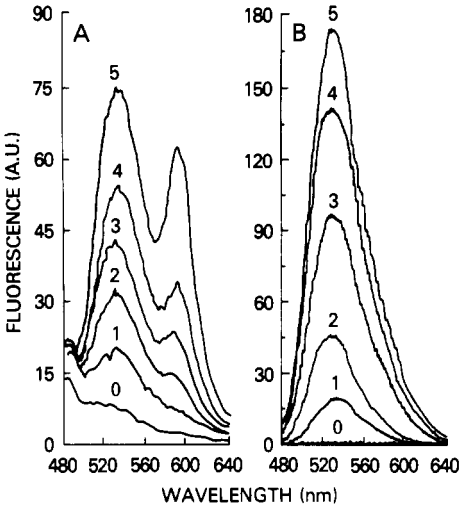

FIGURE 2: Effect of increasing probe SUV concentration on the incorporation of probe PE into chromaffin granule membranes. Chromaffin granules (1.0 mg/mL) in buffered sucrose were incubated with dual-labeled probe SUV for 14 h at 0 °C, washed, and resuspended at 1.0 mg/mL in buffered sucrose. (A) Scans of washed granules (final [protein] = 80 μg/mL) at 25 °C. Excitation, 455 nm; slit widths, 6 nm. Scans from bottom to top represent granules incubated with 0, 1.0, 2.0, 3.0, 4.0, and 5.0 μg/mL probe SUV. (B) The same samples after the addition of 20 μL of a 30% (v/v) solution of the nonionic detergent Ammonyx LO. Note in (A) the relative increase in the peak at 590 nm with increasing probe concentration, reflecting increased resonance energy transfer due to increased surface density of both probes. This effect would be even more pronounced if the background scattering (lowest trace) were subtracted from the other spectra. After detergent addition, the probes are at essentially infinite dilution, no resonance energy transfer takes place, and no change in the shape of the envelope of the spectra is seen.

addition. It should be noted that the incorporated probes show increased donor quenching (as judged by the relative increase of the acceptor peak compared to the donor peak) with increasing amounts of label originally added, showing that both probes are being incorporated into the same granule. Detergent increases the fluorescence of the sample. However, the shape of the spectra of the detergent-treated material is constant; only the absolute amplitudes have increased. This would be the expected result if the donors and acceptors had been confined to relatively close proximity before the detergent treatment had dispersed the probe molecules.

(*C*) *Association of Label with the Granule Fraction after Washing.* The increase in fluorescence seen in Figure 2 when detergent is added is due to dequenching of a previously quenched population of probe molecules. We assume that these are probe SUV which have been carried through the wash procedure. A similar problem was encountered by Struck et al. (1981) when labeling cultured cells and by Tanaka & Schroit (1983) when labeling erythrocytes. As can be seen in Figure 3, this represents 20–50% of the total available fluorescence in the sample. As previously noted, this contaminating material is not easily removed. Only samples with relatively low contamination (20–40%) were used for the fusion experiments described under Demonstration of Granule–Granule Fusion. As we shall see, this contamination is not available for further interactions with the membranes.

Figure 4A shows that the relative incorporation of both probes after overnight incubation at 0 °C is approximately constant, representing 25–40% of the originally added probe. The calculated surface densities are relatively low (less than 1 probe per 100 granule phospholipids), which will produce relatively small changes in fluorescence levels when fusion occurs (Fung & Stryer, 1978). Unfortunately, as can be seen

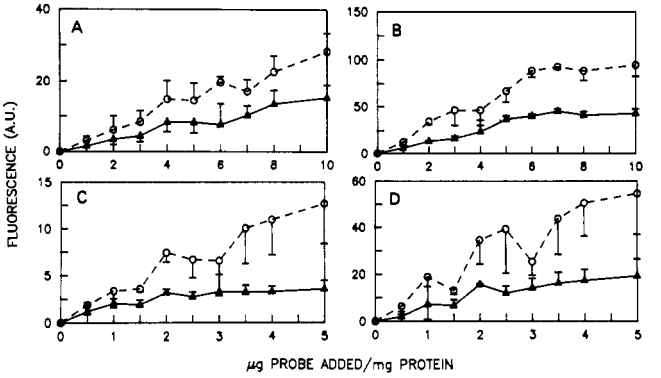

FIGURE 3: Concentration dependence of the increase in incorporation of probe SUV into chromaffin granules and increased contamination of granules by unincorporated probe. Granules were incubated for 14 h at 0 °C, washed, and resuspended at 1.0 mg of protein/mL. Aliquots were resuspended in buffered sucrose at 25 °C, and the fluorescence was recorded. Detergent was added and the increase in fluorescence recorded. (Δ) Before detergent addition; (O) after detergent addition. Results are the means ± SD of three separate experiments. (A) NBD-PE incorporation into chromaffin granules; (B) Rho-PE incorporation; (C) NBD-PE incorporation into dual-labeled SUV; (D) Rho incorporation into dual-labeled SUV.

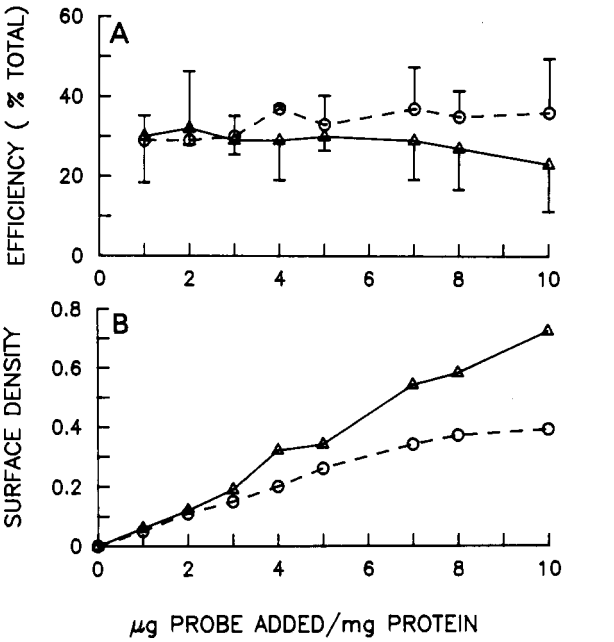

FIGURE 4: (A) Concentration dependence of the efficiency of probe incorporation. Granules (final [protein] = 1.0 mg/mL) were incubated for 14 h at 0 °C with labeled SUV, washed, and resuspended at 1.0 mg of protein/mL. Ordinate: Ratio of probe incorporated into the granule membrane to the total probe added to the incubation. (Δ) NBD-PE; (O) Rho-PE. (B) Surface density of labeled membranes as a function of the original probe SUV concentration. The surface density values, expressed as the number of probe molecules per 100 granule phospholipids, were calculated as described under Methods.

in Figure 3, increasing the initial probe concentration only exacerbates the problem of unincorporated background without improving the probe:PL ratio.

Two sets of controls strongly suggest that the labeling procedure does not harm the storage capacity or the aggregation properties of the granules. We have demonstrated that the fusion of small unilamellar vesicles to each other results in the loss of small molecules sealed within the vesicle cores (Morris et al., 1983b). Catecholamines are stored at 400–600 mM in the chromaffin granule core (Winkler & Westhead, 1980). To demonstrate that probe incorporation does not render the granules leaky to small molecules, advantage was taken of the dequenching of the catecholamine fluorescence

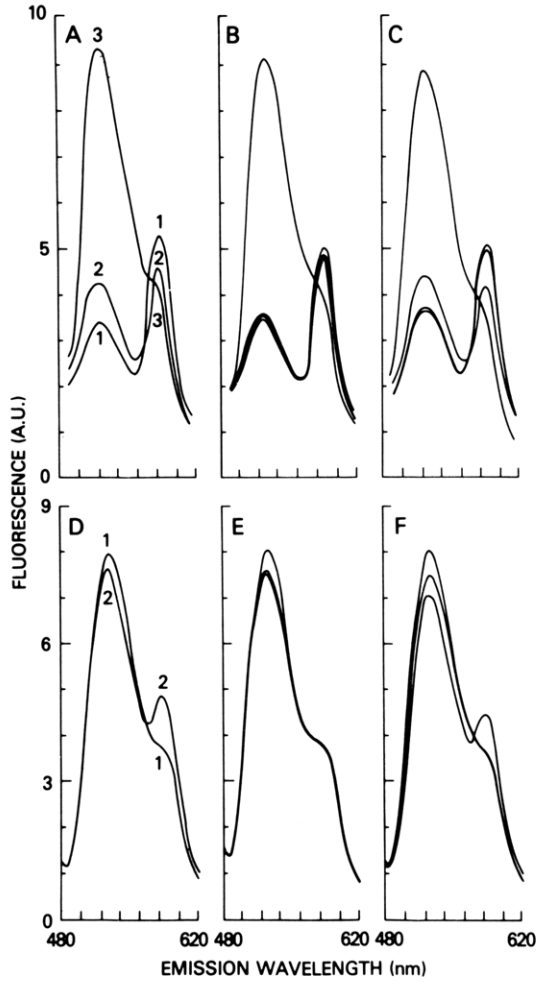

FIGURE 5: Effects of various ions on the fusion of chromaffin granules. Assays were performed as described under Methods. (A–C) Fusion of dual-labeled granules to unlabeled granules: excitation, 460 nm; slits, 6 nm; temperature, 30 °C. (A) Addition of 6.5 mM $CaCl_2$ (trace 2) increases NBD fluorescence (530 nm) and decreases Rho fluorescence (590 nm). Trace 3 shows sample after detergent addition. (B) Addition of 70 mM potassium glutamate does not affect the fluorescence but blocks the calcium-dependent changes. (C) Addition of sucrose (final concentration 415 mM) has no effect. (D–F) Fusion of NBD-labeled to Rho-labeled granules; temperature, 27 °C. (D) Addition of 7.0 mM Ca^{2+} (trace 2) decreases NBD fluorescence and increases rhodamine fluorescence. (E) Addition of 70 mM potassium glutamate has no effect beyond dilution of the sample but blocks the Ca^{2+}-dependent change. Addition of sucrose (F) has no effect. Addition of other inhibitors (Table I) produced results similar to (B) or (E). Addition of Mg-ATP produced results similar to (C) or (F).

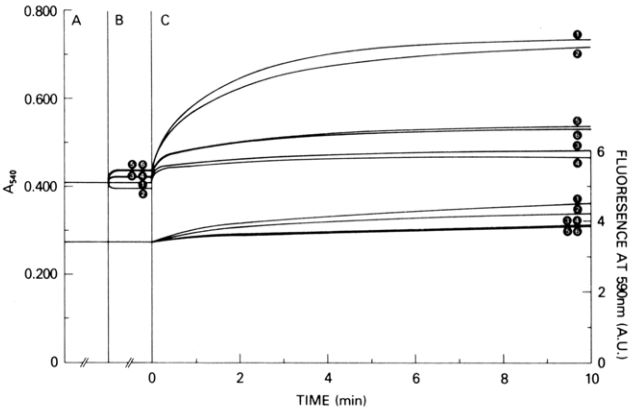

FIGURE 6: Time course of aggregation and fusion of labeled granules. Assays were performed as described under Methods on dual-labeled granules. Final [protein] = 91 μg/mL; temperature = 27 °C. The upper group of traces are the time-dependent changes in absorbance at 540 nm (A_{540}). (A) Starting $A_{540} \simeq 0.410$. (B) Addition of a small volume of perturbing agent: (1) control (no addition; [sucrose] = 300 mM); (2) addition of sucrose (final concentration 420 mM) leads to a concentration-dependent decrease in the signal; (3 and 4) addition of sodium or potassium glutamate produces a small increase in the signal, interpreted as partial aggregation of the granules, similar to KCl-promoted aggregation of granule membranes reported previously (Morris et al., 1979b, 1982b); (5 and 6) addition of KCl or choline chloride produces greater aggregation. (C) Addition of $CaCl_2$ (final concentration 6.5 mM) to the control sample shows the typical large increase in turbidity produced by Ca^{2+} which is not affected by increased osmolarity. Addition of KCl or choline chloride depresses Ca^{2+}-promoted aggregation; addition of potassium or sodium glutamate is highly inhibitory. The lower traces show the effects of ion addition on the development of resonance energy transfer. Excitation, 460 nm; emission, 590 nm; slits, 6 nm. Addition of the perturbants (B) produced only dilution-dependent reduction in the signal. Addition of 6.5 mM Ca^{2+} (C) produced an increase in resonance energy transfer which was depressed slightly by increased [sucrose]. The salts produced a large depression of the development of resonance energy transfer with sodium and potassium glutamate > KCl, choline chloride.

seen when these molecules are released (Johnson & Scarpa, 1976; Morris et al., 1977; Südhof & Morris, 1983). At 10 °C, exchanging the sucrose suspension medium for 0.3 M glycerol, a process which lyses the granules, produced a 6-fold increase in catecholamine fluorescence, while addition of 73 μg/mL probe SUV, which resulted in rapid uptake of the label (cf. Figure 1), showed no detectable increase in catecholamine fluorescence (data not shown).

Calcium will aggregate chromaffin granule membranes at near-diffusion-controlled rates (Morris et al., 1979b). In control experiments, the aggregation rates and amplitudes of hand-mixed calcium-promoted aggregation of untreated granules were essentially identical with those for labeled granules (results identical with Figure 5).

Demonstration of Granule–Granule Fusion. It has been shown that the efficiency of energy transfer in a bilayer membrane will change with changes in surface density of the donors and acceptors (Fung & Stryer, 1978; Struck et al., 1981). Membrane fusion experiments can be performed in either of two ways. The donor and acceptor can be placed into separate populations of granules which upon fusion will result in large reduction in the average distance between donor and acceptor. The donor fluorescence will be quenched and the acceptor enhanced (Vanderwerf & Ullman, 1980). Alternatively, as the surface density of probe molecules is reduced, the average distance between probes will increase, and the efficiency of resonance energy transfer will decrease. If both probes are placed in the same membrane at the beginning of the experiment, the donor will be quenched, and energy will be transferred into the acceptor spectrum. The degree of fusion of labeled vesicles with unlabeled acceptors can be judged by the reduction of resonance energy transfer or the increase of donor fluorescence (Struck et al., 1981). As seen in Figure 6, both of these labeling configurations produced changes in resonance energy transfer consistent with the fusion of granules to each other.

It has been reported that NBD incorporated into phosphatidylserine (PS) vesicles changes its quantum yield in the presence of Ca^{2+}, the changes being interpreted as detecting lateral phase separations (Hoekstra, 1982a,b). In our hands, addition of Ca^{2+} to the single-labeled chromaffin granules produced an increase in the NBD fluorescence and a slight decrease in the Rho fluorescence (Figure 7). These results are opposite in sign to those quoted above for NBD-PE incorporated into PS SUV but are suggestive of interactions of the probes within the plane of a given bilayer, since the rapid kinetics for the Ca^{2+}-induced changes in probe fluorescence in SUV show a concentration-independent rate, which is an

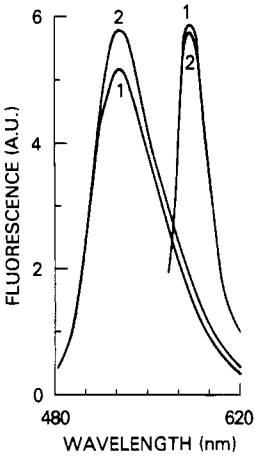

FIGURE 7: Effects of calcium on the single-labeled granules. Addition of 7.0 mM $CaCl_2$ to NBD- or Rho-labeled granules produces a substantial increase in the fluorescence of the former and a slight decrease in that of the latter. [Protein] = 42 mg/mL; temperature = 28 °C; NBD excitation, 460 nm; Rho excitation, 560 nm; slits, 6 nm. (1) Before and (2) after Ca^{2+} addition.

order of magnitude faster than that seen for aggregation. Also, the progress of the reaction does not fit the bimolecular rate reaction model which characterizes SUV–SUV or granule–granule interactions (Lansman & Haynes, 1975; Morris et al., 1979a,b; Haynes et al., 1979a,b). A more extensive analysis of these changes for the labeled granules will be published separately. Since the direct effect of calcium on the incorporated probes could be falsely interpreted as membrane fusion if both probes start out in the same granule, the experiment in which the probes are initially incorporated into separate granules provides the most positive evidence for calcium-dependent fusion but precludes quantitative calculation of the extent of fusion from donor quenching measurements, unless the direct Ca^{2+} effects can be precisely quantitated and subtracted from the total change. Also, any formal analysis of the kinetics of fusion will require controlling these effects. Further tests for the possibilities of artifacts are detailed in the next section.

Tests for Inertness of Unincorporated Label. The presence of significant amounts of contaminating unincorporated probe lipid in the labeled granule preparations could produce changes in the fluorescence signal which may be misinterpreted as fusion of the granules with each other (Figure 8). The worst possibilities would be that the addition of a fusogen such as Ca^{2+} causes the fusion of the unincorporated probe SUV with each other or with the appropriate granule to promote energy transfer in the absence of granule–granule fusion. The former is unlikely since control experiments show no Ca^{2+}-promoted aggregation or fusion of probe SUV. To control for the latter, a series of control experiments were always performed on granules used for fusion experiments:

(1) Granules labeled with either donor or acceptor lipids were incubated at elevated temperatures for the course of time of a normal experiment (10–30 min). Lack of increase in the fluorescence emission with time indicated no further incorporation of SUV into the granule membrane (see Figures 1 and 8C).

(2) To test for the possibility that the previously labeled granules were refractory to further labeling but fresh granules were not, the experiment was repeated in the presence of a 3–5-fold excess of unlabeled granules. Results were always negative for any further addition of contaminating probe SUV to the granule membranes (Figure 8D).

(3) These experiments were repeated with the dual-labeled granules. In this case, addition of labeled SUV to the granule should change not only the quantum yield of the probes but also the energy transfer seen due to the change in probe surface density (cf. Figure 2). Again, all controls were negative.

(4) Experiments 1–3 were repeated with the addition of the fusogen (Ca^{2+}) after the initial readings. As noted in Figure

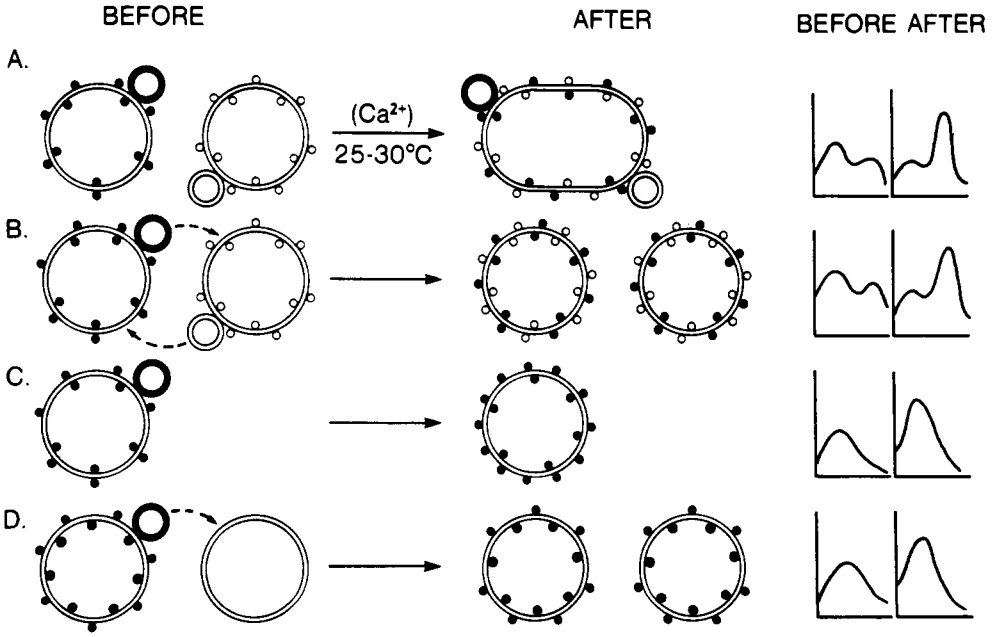

FIGURE 8: Diagrams of possible changes in fluorescence after mixing of labeled granule samples. (A) Chromaffin granules [represented as large hollow spheres, which have either NBD-PE (O) or Rho-PE (●) incorporated into their bilayers] as well as unincorporated, contaminating probe SUV (represented as either light or dark small hollow spheres) are mixed at elevated temperature. The addition of Ca^{2+} fuses the granule membranes, allowing the two probes to greatly decrease their separation distance. The emission spectrum shows quenching of the donor fluorescence and increase of the acceptor fluorescence. (B) the same initial conditions as (A) leads to fusion of the contaminating probe SUV with the granule membranes. Once again, intimate mixing of the two probes leads to donor quenching and resonance energy transfer. (C) Control for fusion of contaminating SUV with the granule membrane. If single-labeled SUV fuse with granules, an increase in fluorescence, due to dequenching of the self-quenched probe molecules, will be seen. (D) Addition of probe to unlabeled granules will also lead to an increase in fluorescence even though the original probe-containing granules are refractory to further interaction with the SUV.

7, NBD fluorescence increases in the presence of Ca^{2+} while Rho fluorescence decreases. The magnitude of these changes was no different if unlabeled granules were present. Thus, we conclude that the results for the single-labeled granules with or without unlabeled granules and for the dual-labeled granules without unlabeled granules were negative for further probe incorporation. Only the dual-labeled granules plus unlabeled granules plus calcium showed energy transfer consistent with fusion of the granule membranes.

Factors Affecting Granule–Granule Fusion. After establishing that any given set of labeled granules was undergoing fusion by the tests outlined above, we tested the effects of various ions on the fusion reaction. As noted in Figures 5 and 6, the presence of 70 mM sodium glutamate, potassium glutamate, sodium chloride, or choline chloride in the buffered sucrose almost completely inhibited the fusion, as did a variety of other salts (data not shown). Organic anions and cations seemed to be more inhibitory than inorganic ions. These ions partially inhibited the aggregation of the granules (Figure 6). The aggregability of granules was not affected by the labeling procedure (data not shown). It is worthwhile noting that the addition of sucrose to increase the osmotic pressure did not in itself inhibit aggregation or fusion. Addition of Mg-ATP did not promote fusion by itself nor did it enhance the Ca^{2+}-promoted fusion.

Discussion

Granule Labeling Procedure. We chose to use the crude granule preparation rather than granules further purified by gradient centrifugation, since our experience has been that granules subjected to dehydration on continuous sucrose gradients or exposed to media such as metrizamide or percoll (Morris & Schovanka, 1977; Schober et al., 1977; Carty et al., 1980) do not fuse as well as those from the untreated preparations. The granules also contain a population of exchangeable, peripheral proteins (Morris et al., 1982b) which may have direct roles in the aggregation–fusion of the granules. These proteins are removed by repeated washes (Morris et al., 1982b) or density gradient purification (Morris et al., 1979b), which suggests that the granules would best be subjected to as little manipulation as possible during the labeling procedure. As noted below, lysed resealed granule ghosts will not fuse. Therefore, we chose to incorporate the probe SUV by direct interaction of the probe vesicles with the granules at 0 °C as the least invasive and traumatic method. The use of derivatizing agents or phospholipid exchange proteins would have involved the addition of foreign material plus the problem of removing it before further experiments could be performed. Also, the exchange proteins may only label the outer leaflet of the bilayer, and the derivatives would almost certainly label the membrane proteins as well as the lipids. While this may be desirable under some circumstances, it was not deemed so for a general method to test for membrane fusion.

It has been suggested that the mechanism for addition of acyl chain labeled probe phospholipids from SUV to cell membranes is via exchange rather than fusion (Pagano et al., 1981a,b; Nichols & Pagano, 1981). However, several observations argue for fusion of the head-group-labeled probe SUV used in this study to the granule membrane. The initial rates of incorporation are very rapid and become progressively slower. Up to 40% of the added probe lipids are eventually incorporated into the granule membrane. Exchange of this magnitude would lead to relief of fluorescence self-quenching in the SUV which was not seen (the supernatants from the wash steps contained essentially no fluorescence until detergent was added). However, it could be that only the contaminating probe SUV exchange lipids; those left in the supernatant have exchanged little to none of their probe.

Co^{2+} ions will quench both probes at low concentrations via collisional quenching. Co^{2+} also gains access to the interior of SUV and will eventually quench all available fluorescence. This is not true for larger vesicles formed by reverse-phase evaporation where Co^{2+} quenches only the fluorescence on the external leaflet of the bilayer unless bilayer integrity is disrupted, e.g., by freezing and thawing (S. J. Morris and R. Blumenthal, unpublished results). Addition of 8–50 μM Co^{2+} quenched 60–70% of the available probe fluorescence in the granules; the remainder was not quenched by 350 μM Co^{2+}. A 250-Å diameter SUV will have 65–70% of its lipid in the outer leaflet of its highly curved bilayer. Fusion of the probe SUV with the granule membrane such that the two external leaflets fuse and the two internal leaflets fuse would leave 65–70% of the probe on the outside of the granule membrane. Exchange of phospholipids between probe SUV and the granule membrane without "flip-flops" would leave 100% of the probe on the external leaflet of the granule membrane. The fluorescence of contaminating probe SUV is already nearly completely quenched and would be unaffected by the added Co^{2+}. Thus, the Co^{2+} quenching result argues in favor of fusion of SUV with the granule membrane as the probe incorporation mechanism.

Conditions for Granule–Granule Fusion. The resonance energy transfer seen when the two populations of granules are mixed and calcium is added is due to fusion rather than exchange of probes incorporated into the granule membrane. We can reach this conclusion because of the following two reasons: (1) the reaction deteriorates with time, and more importantly, (2) the reaction disappears if lysed, resealed ghosts are prepared from the labeled granules. These ghosts will reestablish their pH and potential gradients and transport catecholamines against a concentration gradient (Apps et al., 1980). Since aggregation by calcium is not diminished by this treatment (Morris et al., 1982a), it is hard to imagine a mechanism which would poison passive lipid–lipid exchange processes.

In a recent study, we demonstrated that aggregation was the rate-limiting step for the fusion of PS–PE vesicles (Morris et al., 1983b). Figure 5 demonstrates that hand-mixed fusion reactions seem to follow a slower time course than aggregation of the granules. However, a stopped-flow study comparing the aggregation and fusion rates will be required to define any congruence. We have previously noted in stopped-flow studies of aggregation of lysed resealed granule membranes that KCl and NaCl caused limited aggregation of the membranes but only slightly inhibited calcium promoted aggregation (Morris et al., 1979b). Out present results on intact granules, which show that a varitey of ions reduce both aggregation and fusion, are quantitatively different from the previous study and will require a stopped-flow analysis of the aggregation and fusion kinetics of the intact granules. The results of the inhibition of both aggregation and fusion of intact granules are qualitatively similar, however, and suggest that it is the reduction in the former which underlies the reduction of the latter.

The fusion reported here takes place under substantially the same conditions as those previously observed by electron microscopy, i.e., 1–10 mM Ca^{2+} in a low ionic strength buffer; Mg^{2+} is about 25% as effective as Ca^{2+}, and Mg-ATP has no effect. These conditions are far from the physiological conditions for exocytotic release seen in experiments with intact adrenal medullary chromaffin cells (Amy & Kirshner, 1980; Kilpatrick et al., 1981). Recently, Knight & Baker (1982) have reported that cells permeabilized by repeated subjection

to short square wave pulses at the membrane breakdown potential can be rendered permeable to small (M_r <1000) molecules while retaining larger molecules such as the cytoplasmic marker LDH. These cells respond to increasing [Ca^{2+}] by releasing catecholamines and the intragranular marker DBH with little to no loss of LDH. The half-maximal [Ca^{2+}] for this phenomenon is ~1 μM. The release is supported by glutamate; small inorganic cations like Cl^- are inhibitory. Mg-ATP greatly stimulates the release, Mg^{2+} alone is inhibitory. As noted in Figures 5 and 6 and related text, we found a variety of monovalent organic cations, especially glutamate, inhibited our fusion. We find no requirement for Mg-ATP. Mg^{2+} is about 4-fold less effective than Ca^{2+} in promoting fusion of isolated granules. It is obvious that conditions for the two experiments are quite different, and we are left with the conclusion that they are proceeding by different mechanisms. The requirement for Mg-ATP in granule–cell membrane fusion suggests an enzymatic process which is not necessary in vitro or can be overcome by the presence of larger amounts of calcium. That the fusion is abolished in the lysed, resealed ghosts strongly suggests that some component necessary to fusion is lost or denatured by this process.

The assay for fusion described here should allow for rapid screening of conditions affecting the aggregation and fusion reactions in vitro and testing of agents such as synexin (Creutz et al., 1978, 1979; Morris & Hughes, 1979; Morris et al., 1982c; Hong et al., 1981, 1982; Odenwald & Morris, 1983) or other proteins (Südhof et al., 1982; Lampe & Nelsestuen, 1982; Sundler & Wijklinder, 1983; Blumenthal et al., 1983; Kuhmar et al., 1982) which lower the calcium requirement for membrane vesicle aggregation and fusion. The assay is amenable to stopped-flow rapid mixing, which will allow a formal kinetic analysis of the reactions involved, and should prove to be a valuable tool for exploring a number of membrane fusion systems.

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Registry No. NBD-PE, 91632-07-4; Rho-PE, 78346-67-5; Ca, 7440-70-2; sodium glutamate, 142-47-2; potassium glutamate, 19473-49-5; sodium chloride, 7647-14-5; choline chloride, 67-48-1.

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Identification and Synthesis of a Naturally Occurring Selenonucleoside in Bacterial tRNAs: 5-[(Methylamino)methyl]-2-selenouridine†

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ABSTRACT: *Escherichia coli*, *Clostridium sticklandii*, and *Methanococcus vannielii* synthesize ^{75}Se-labeled amino acid transfer ribonucleic acids ([^{75}Se]tRNAs) when grown with low levels (≈1 μM) of $^{75}SeO_3^{2-}$. When *E. coli* [^{75}Se]tRNA was digested to nucleosides and analyzed by reversed-phase high-performance liquid chromatography, a single selenonucleoside accounted for 70–90% of the ^{75}Se label in the bulk tRNA. This nucleoside was shown to be indistinguishable in a number of its properties from authentic 5-[(methylamino)methyl]-2-selenouridine. Preparation of the authentic selenonucleoside was accomplished and the synthetic compound characterized by its UV and ^{1}H NMR spectral properties. The new selenonucleoside also accounted for 40–60% of the ^{75}Se found in [^{75}Se]tRNA from *C. sticklandii* or *M. vannielii*. Each of these anaerobic bacteria contains one additional selenonucleoside in their tRNA populations distinct from 5-[(methylamino)methyl]-2-selenouridine. Pure seleno-tRNA^{Glu} isolated from *C. sticklandii* contains one 5-[(methylamino)methyl]-2-selenouridine and one 4-thiouridine per tRNA molecule.

It is well established that selenium is an essential component of several bacterial enzymes and of mammalian glutathione peroxidase (Stadtman, 1980). Selenium also has been shown (Chen & Stadtman, 1980) to be a specific constituent of another class of macromolecules, the amino acid transfer ribonucleic acids (tRNAs). A selenium-containing tRNA^{Glu} from *Clostridium sticklandii* has been purified (Ching & Stadtman, 1982), lysine- and glutamate-accepting tRNAs of *Escherichia coli* have been shown to contain selenium (Wittwer, 1983), and recently, we have found extensive incorporation of selenium in several tRNA species from *Methanococcus vannielii* (Ching et al., 1984). Lability of the incorporated selenium in these tRNAs to treatment with CNBr, KBH_4, or iodoacetate and its relative stability at pH 9 (a condition used to deesterify aminoacylated tRNAs) suggested the selenium was present in a modified nucleoside. We now report the isolation of a prominent selenium-containing nucleoside from several bacterial seleno-tRNAs and its identification as 5-[(methylamino)methyl]-2-selenouridine. The synthesis of this new, naturally occurring nucleoside also is described.

Experimental Procedures

Growth of Bacteria and Isolation of ^{75}Se-Labeled tRNA. Wild-type *E. coli* strain WG1 was obtained, grown in minimal glucose/salts medium, and labeled with $^{75}SeO_3^{2-}$ or $^{75}SeO_4^{2-}$, and ^{75}Se-labeled tRNA was isolated as described by Wittwer (1983). Selenium-enriched [^{75}Se]tRNA was prepared from bulk tRNA (isolated from cells incubated with 0.85 μM $^{75}SeO_3^{2-}$ and 8 mM SO_4^{2-}) by successive chromatography on Sepharose 4B (Holmes et al., 1975) and the RPC-5 reversed-phase system of Pearson et al. (1971) at pH 4.5 and then at pH 7.5 using conditions described previously (Wittwer, 1983). *C. sticklandii* was cultured in $^{75}SeO_3^{2-}$-supplemented media, and ^{75}Se-labeled tRNAs were isolated from the radioactive cells (Chen & Stadtman, 1980; Ching & Stadtman, 1982). In some experiments, the *C. sticklandii* tRNA population was labeled with ^{75}Se in cultures prevented from further growth by the addition of chloramphenicol. Pure ^{75}Se-labeled tRNA^{Glu} from *C. sticklandii* was isolated as described by Ching & Stadtman (1982). Preparations of ^{75}Se-labeled *M. vannielii* cells and purification of ^{75}Se-labeled tRNAs from this organism were as described by Ching et al. (1984).

Reversed-Phase High-Performance Liquid Chromatography (HPLC) Analysis of Nucleosides. Up to 3 A_{260} units of tRNA was rapidly hydrolyzed with nuclease P1 and bacterial alkaline phosphatase as described by Gehrke et al. (1982).

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