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Subcellular Structures Involved in Internalization and Degradation of Epidermal Growth Factor

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Epidermal Growth Factor (EGF), a small polypeptide which acts as a mitogen for many cell types, has previously been shown to bind to a specific plasma membrane receptor on 3T3 cells. If ^{125}I-EGF is bound to 3T3 cells for one hour at 4°C, it remains predominantly associated with the plasma membrane-containing fractions obtained by subjecting cell supernatants to equilibrium sedimentation on sucrose gradients. When binding is followed by a 10-minute incubation at 37°C, over 50% of the ^{125}I-EGF is associated with two internal membrane-containing peaks having higher densities than the plasma membrane. After one hour at 37°C, over 80% of the ^{125}I-EGF is degraded and removed from the cells.

The most rapidly labeled internal peak corresponds in density to brain-coated vesicles (CVs). Antiserum prepared against coated vesicles from brain precipitates the ^{125}I-EGF in this peak. In addition, CVs containing ^{125}I-EGF can be co-purified from 3T3 cells exposed to ^{125}I-EGF, using brain as a carrier. Several lines of evidence suggest that the other ^{125}I-EGF-labeled intracellular peak is ^{125}I-EGF in lysosomes.

These results provide kinetic and biochemical evidence for a unidirectional pathway for EGF catabolism by 3T3 cells. EGF first binds to the plasma membrane bound receptors, is then moved to the cytoplasm in CVs, and finally appears in lysosomes, where it is degraded and released from the cells. Ten-millimolar NH_4Cl blocks lysosomal hydrolysis of EGF almost completely. Subsequently, EGF internalization is inhibited. This finding suggests that the pathway for EGF internalization and degradation is tightly coupled.

Key words: EGF, coated vesicles, internalization, lysosomes, NH_4Cl, hormone degradation

Abbreviations used: EGF, epidermal growth factor; CV, coated vesicle; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MES, 2-[N-morpholino], ethane sulfonic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ester), N,N'-tetraacetic acid; MES buffer, 0.1 M MES pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$; pNp, p-nitrophenol; Staph A, Staphlococcus aureus; SDS, sodium dodecyl sulfate.

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The receptor-mediated uptake of polypeptide hormones and other physiologically important substances into cells has recently emerged as a membrane transfer process of particular interest. Work by several investigators has implicated coated vesicles in receptor-mediated uptake [1–4]. Coated vesicles, phospholipid-containing vesicles enclosed by an icosahedral protein shell, are thought to be involved in membrane transport in several systems in eukaryotic cells. Pearse has successfully isolated coated vesicles from brain and other tissues [2]. An antibody against coated vesicles has been prepared [5].

Epidermal growth factor (EGF), a 6045-MW polypeptide, is a potent mitogen which, after binding to its receptor, triggers a series of biochemical events culminating in cell division [6–8]. Recently, receptor-mediated uptake of EGF has been studied using electron microscope autoradiography and fluorescent microscopy [9–11]. These studies indicate that EGF binds to specific receptors which appear to be preferentially located in coated regions of the plasma membrane. Recent studies from two laboratories have suggested that a small portion of ^{125}I-EGF forms a covalent bond with the EGF receptor [12, 13]. After binding, the ligand-receptor complexes form clusters which bud off into the cytoplasm as small vesicles. These subsequently fuse with lysosomes where the EGF and its receptor are degraded [6, 7, 14]. Biochemical confirmation of these studies and identification of these morphological compartments have not yet been provided.

In this paper we present biochemical and immunological evidence that coated vesicles are intermediates in EGF internalization. We also confirm biochemically the morphological studies which previously suggested a three-stage pathway for receptor-mediated internalization: Plasma membrane receptors → coated vesicles → lysosomes [7], and present kinetic data for this pathway. Finally, we present evidence which suggests that this pathway is a tightly coupled one.

MATERIALS AND METHODS

Mouse EGF

Mouse EGF was purified from the submaxillary glands of adult male Swiss-Webster mice by the method of Savage and Cohen [15].

Iodination of Mouse EGF

Mouse EGF was labeled with ^{125}I as previously described [6]. The specific activity of the EGF ranged from 24–33 μCi/μg. Labeled EGF was stored at −20°C until used.

Cell Culture

Swiss 3T3 cells, grown on 100-mm tissue culture plates containing 9 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), were subcultured as described previously [6]. The cultures were incubated in a humidified CO_2 incubator and fed every other day until a confluent monolayer of cells was formed. Determination of protein in homogenates of confluent monolayers indicated a variance of less than 10% in total cell protein between plates. 3T3 variant NR-6, which has no functional EGF receptors, was grown as previously described [6].

^{125}I-EGF Binding and Internalization Assay

Confluent monolayers of cells were washed with 3 ml ice-cold Saline A [17] containing 0.1% bovine serum albumin (BSA), prior to the addition of 3 ml ice-cold binding medium. The binding medium consisted of DMEM supplemented with 5% FCS plus 60 ng/ml

labeled EGF. After a one-hour incubation at 4°C, unbound ^{125}I-EGF was removed by washing each dish with six portions (3-ml) of ice-cold Saline A containing 0.1% BSA. The cells were then washed with 3 ml of warm internalization medium (DMEM with 5% FEC) and allowed to incubate in the same for various period of time at 37°C.

After incubation at 37°C in internalization medium, cells were washed with 3 ml ice-cold phosphate buffered saline (PBS) [18]. The cells in each dish were scraped with a rubber policeman into four ml of ice-cold PBS and pelleted by centrifugation at 1,000g for five min in a refrigerated International centrifuge (International Equipment Company, Boston, MA). The pellet was resuspended in 1.0 ml homogenization buffer, consisting of 0.01 M Tris-HCl–1 mM EDTA, pH 7.4. After a five-minute swelling period on ice, the cells were transferred to a tight-fitting Dounce homogenizer (Kontes Co., Vineland, NJ) and homogenized with 10 strokes. The homogenates were adjusted to 0.1 M MES buffer: 0.1 M MES [2-(N-morpholino)-ethane sulfonic acid]; 1 mM ethyleneglycol-bis-(aminoethyl ether)-N,N′-tetraacetate (EGTA); 0.5 mM $MgCl_2$; 0.02% sodium azide, pH 6.5. Each homogenate was then centrifuged at 600g for five minutes at 4°C. Each supernatant was layered on a sucrose gradient containing 2.5 ml each of 20, 30, 40, 50, 55, and 60% sucrose prepared in 0.1 M MES buffer [19]. In some cases, brain-coated vesicles were added as an internal marker prior to centrifugation. The gradients were centrifuged at 50,000g for 16 h in an SW-27 rotor in the Beckman L350 centrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, CA). One-ml fractions were collected manually from each gradient, using an automatic pipettor. The radioactivity in each fraction was measured in a Nuclear Chicago gamma-spectrophotometer (Nuclear Chicago, Des Plaines, IL). Over 95% of the initial radioactivity applied to the gradient is reproducibly recovered after fractionation. The distribution of radioactivity within the gradient was identical in the presence or absence of brain-coated vesicles. For experiments in which NH_4Cl was used, the binding and internalization media, as well as Saline A and PBS, were adjusted to 10 mM NH_4Cl.

Nonspecific binding, as determined by measuring cell-associated radioactivity of 3T3 NR-6 cells (which are missing a functional EGF receptor) after a one-hour incubation with 60 ng/ml ^{125}I-EGF, or of normal 3T3 cells in the presence of 20 μg/ml unlabeled EGF, amounted to less than 2% of the total label bound to 3T3 cells in the absence of unlabeled EGF.

Nature of Cell-Associated EGF

To characterize the nature of the radioactive material in the gradient fractions and internalization medium, samples containing between 5,000 and 10,000 cpm were chromatographed directly on Bio-Gel P10 (200–400 mesh, Bio-Rad, Richmond, CA) as described by Carpenter and Cohen [7]. Duplicate samples were adjusted to 5% trichloroacetic acid and centrifuged at 3,000g for 30 min. The radioactivity associated with the trichloroacetic acid-precipitable and trichloroacetic acid-soluble material was determined after washing the pellet twice with 5% trichloroacetic acid. Since the ratio of radioactivity associated with the high-molecular-weight peak eluting from the column to radioactivity associated with the low-molecular-weight peak was consistently within 5% of the ratio of trichloroacetic acid precipitable to trichloroacetic-acid-soluble radioactivity for duplicate samples, trichloroacetic acid solubility was used as a criterion for EGF degradation in all subsequent experiments.

N-Acetyl-β-glucosaminidase Assay

N-Acetyl-β-D-glucosaminidase [ED 3.2.1.30] was assayed according to the method of Touster et al [20]. Gradient fractions were sonicated on ice with three 10-second bursts using a Sonifier cell disruptor (Heat Systems – Ultrasonics, Inc., Plainview, NY) prior to assay. Two milliters of 0.1 M glycine–0.1 M NaOH were added to stop the reaction after a 30-minute incubation at 37°C. The optical density at 404 nm was measured and compared with standard p-nitrophenol (pNp) in order to determine the amount of substrate hydrolyzed.

Co-Purification of Labeled Coated Vesicles From 3T3 Cells

In order to isolate coated vesicles from 3T3 cells which had been incubated with ^{125}I-EGF or ^{35}S-methionine, a modification of the procedure of Pearse [2] was employed. After labeling, confluent cells were harvested as described above. Cell pellets were frozen, thawed, and resuspended in 10 ml MES buffer. The resuspended cells were combined with 10 gm of calf brain tissue, functioning as carrier, and 30 ml MES buffer and homogenized in a Sorvall Omni-Mixer employing three 10-second bursts at top speed. All operations were performed at 4°C. The supernatant from centrifugation (600g, 5 min) was saved; the pellet was resuspended in MES buffer for homogenization. This homogenate was centrifuged as before and the resulting supernatant was combined with the first. The remaining steps in the co-purification were carried out as previously described [19], except that the third gradient was omitted. Suspensions of coated vesicles were stored in 0.1 M MES buffer for several days at 4°C.

Absorption of Formalized Staphylococcus aureus Cells to 3T3 Cell Supernatants

The 600 g supernatants from five confluent monolayers of 3T3 cells were prepared as described above in a final volume of 1.0 ml MES buffer. A volume of 0.2 ml of 10% (w/v) Staphylococcus aureus (Pansorban, Calbiochem-Behring, La Jolla, CA) was added and the solution was incubated at 4°C for 2 h after vigorous mixing. The cells were collected by centrifugation at 600g for 5 min, washed three times with MES buffer, and resuspended in 0.2 ml MES buffer.

Immune Precipitation of Coated Vesicles From 3T3 Cells

The 600 g supernatants of cells which had been labeled with ^{125}I-EGF were prepared as described above. To 0.5 ml of supernatant was added 20 μl of anti-CV serum, prepared as described previously [5], or pre-immune serum. After a 2-hour incubation at 4°C, we added 0.2 ml of 10% Staphylococcus aureus that had been preabsorbed with 3T3 cell supernatants in order to reduce the nonspecific binding of the labeled 600g supernatants. After a one-hour incubation at 4°C, the solutions were placed on top of 20–60% sucrose gradients and centrifuged to equilibrium.

Determination of Protein Concentration

The method of Lowry et al [21] was used, with samples dissolved in 2% SDS.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [22], using a 7.5% running gel with a 4% stacking gel.

Autoradiography

Slab gels were dried on a Model 224 Gel Slab Drier (Bio-Rad, Richmond, CA) and placed in contact with a piece of Kodak X-Omat film. After a 3-day incubation at room temperature, the film was removed and developed using an X-Omat.

Trypsinization of ^{125}I-EGF-Labeled 3T3 Cells

The medium from cells which had been allowed to internalize ^{125}I-EGF for 10 min was removed and replaced with 3 ml DMEM containing 0.5% trypsin (Sigma Chemical Co., St. Louis, MO). After a 30-minute incubation at 4°C, 3 ml of DMEM supplemented with 5% FCS was added, and the cells were harvested and prepared for centrifugation through sucrose gradients as described above. Control samples were incubated in DMEM without trypsin.

RESULTS

Gradient Analysis of Binding of EGF at 4°C

To characterize the initial site of binding of EGF to 3T3 cells, we added 60 ng/ml ^{125}I-EGF to confluent dishes of 3T3 cells for 60 min at 4°C. These conditions allow saturation binding to the plasma membrane of 3T3 cells, but block internalization as measured by trypsin sensitivity [6]. After removing all unbound ^{125}I-EGF by extensive washing, we harvested the cells at 4°C, prepared a homogenate, and removed nuclei by low-speed centrifugation (600g, 5 min). Between 75–80% of the cell-associated radioactivity is reproducibly found in the post-nuclear supernatant. Over 95% of the ^{125}I-EGF in the post-nuclear supernatant is membrane-associated, as determined by its sedimentation at 100,000g for 10 minutes in a Beckman airfuge.

We then placed the supernatant solution on a 20–60% sucrose step gradient (see Methods), centrifuged it to equilibrium, fractionated the gradient, and measured the radioactivity in each tube. The bulk of the ^{125}I-EGF (84%) associated with the cells at 4°C was present in a fraction at the top of the sucrose gradient (Fig. 1). A small portion of the ^{125}I-EGF [8] was associated with a more dense cellular fraction (fraction 10, Fig. 1). We identify the ^{125}I-EGF in the fraction at the top of the gradient (Peak I) as associated primarily with plasma membrane for the following reasons: a) Binding and fractionation in this experiment have been performed at 4°C, where little or no internalization takes place [6, 7], b) over 90% of 5′-nucleotidase activity sediments in this region of the gradient (data not shown), c) treatment of cells with trypsin for 30 minutes at 4°C removes over 90% of the radioactivity in Peak I (see below), and d) elevation of the temperature to 37°C (which initiates EGF internalization [6, 7]) causes substantial translocation of radioactivity from Peak I to other, trypsin-resistant, subcellular structures (see below). The ^{125}I-EGF in fraction I is only 20–30% sedimentable in the Beckman airfuge after a 16-hour centrifugation of the sucrose gradient, although the original material placed on the gradient

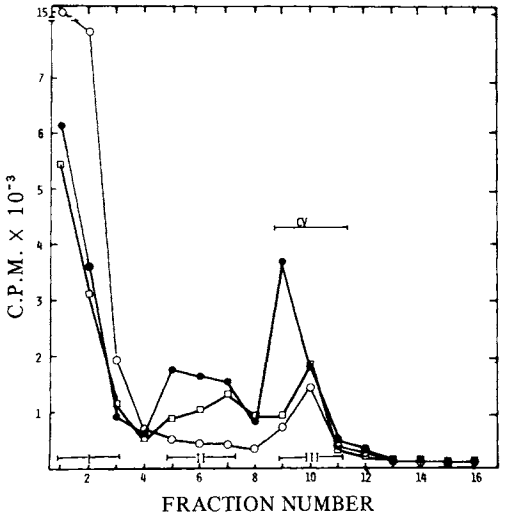

Fig. 1. Time course of ^{125}I-EGF distribution in 20–60% sucrose gradients centrifuged to equilibrium. Six confluent monolayers of 3T3 cells were washed six times and incubated for 60 minutes at 4°C with 3 ml DMEM (5% fetal calf serum) containing 180 ng of ^{125}I-EGF. The plates were then washed six times with ice-cold Saline A containing 0.1% BSA. Cells from two plates were harvested by scraping into 3 ml ice-cold PBS. The plates were rinsed once with 1 ml PBS and the wash combined with the cell suspension. The other four plates were washed once with DMEM at 37°C and incubated for either 10 or 30 minutes in DMEM (5% fetal calf serum). The medium was then removed and the cells were harvested as described above. The rest of the procedure was carried out at 4°C. The two plates for each time point were pooled. The cells from each time point were pelleted by centrifugation at 1,000g for four minutes, resuspended in 1.0 ml of 0.01 M Tris-HCl–1 mM EDTA, pH 7.5, and homogenized with 10 strokes in a tight-fitting Dounce homogenizer. After the homogenates were adjusted to a final concentration of 0.1 M MES by the addition of 0.3 ml of 0.5 M MES, they were centrifuged for 600g for five minutes. The supernatant solutions were collected, and 250 μg of brain-coated vesicles were added. The solutions were placed on top of a 15-ml 20–60% discontinuous sucrose gradient and centrifuged for 16 hours at 50,000g in a Beckman SW-27.1 rotor. The turbid CV-containing band was identified by visual inspection. Sixteen 1-ml fractions were collected from each tube. Radioactivity was measured in a gamma-spectrophotometer. —○—○—, 0 minutes at 37°C; —●—●—, 10 minutes at 37°C; —□—□—, 30 minutes at 37°C.

was over 95% sedimentable by this procedure. This is to be expected for EGF bound to plasma membrane fragments, since some EGF should dissociate during the 16-hour period of centrifugation.

Redistribution of Cell-Bound ^{125}I-EGF Into Intracellular Structures at 37°C

To investigate redistribution of cell-bound ^{125}I-EGF at 37°C, we first incubated cells with saturating levels of radioactively labeled EGF at 4°C, washed the cells in the cold, then shifted to 37°C for 10 and 30 minutes before homogenization and fractionation. After the brief temperature shift, over 95% of the radioactivity in the post-nuclear supernatant was still sedimentable in the airfuge, indicating its association with membrane structures. Results of representative sucrose gradient profiles are shown in Figure 1. Two additional prominent peaks of radioactivity are now present on the gradient (one, Peak III, was in fact observed as a minor peak in the analysis of material bound at 4°C). While a very significant redistribution of radioactivity occurred rapidly after warm-up, over 95% of the

5′-nucleotidase activity remains associated with Peak I, even after 2 hours at 37°C, suggesting that the ^{125}I-EGF in Peaks II and III does not result from plasma membrane contamination.

Trypsinization in the cold of cells incubated at 37°C for 10 minutes prior to homogenization and gradient analysis does not alter the radioactivity associated with Peak II or Peak III. In contrast, ^{125}I-EGF found in Peak I is sensitive to prior trypsin treatment of whole cells (Fig. 2). These data suggest ^{125}I-EGF in Peak II and Peak III is associated with intracellular organelles, while Peak I (trypsin-sensitive) ^{125}I-EGF is associated with plasma membrane.

No appreciable leakage of ^{125}I-EGF or its degradation products occurs from intracellular organelles during the 16-hour centrifugation. If such leakage had occurred, we would have observed appreciable radioactivity in Peak I. However, little radioactivity is observed in Peak I after trypsin treatment of intact cells has removed plasma-membrane-bound ^{125}I-EGF (Fig. 2).

Kinetic Analysis of Redistribution of Cell-Bound ^{125}I-EGF at 37°C

We next monitored changes in the distribution of radioactivity in equilibrium sucrose gradients as a function of time after shifting to 37°C. We looked initially at time points between 0–15 minutes, to examine the time course of the early events involved in translocation of EGF from the plasma membrane to the interior of the cell. At 0 time (ie, without shifting to 37°C), over 80% of the radioactivity is found in Peak I, presumably associated with plasma membrane (Fig. 3). Small but significant amounts of ^{125}I-EGF are pres-

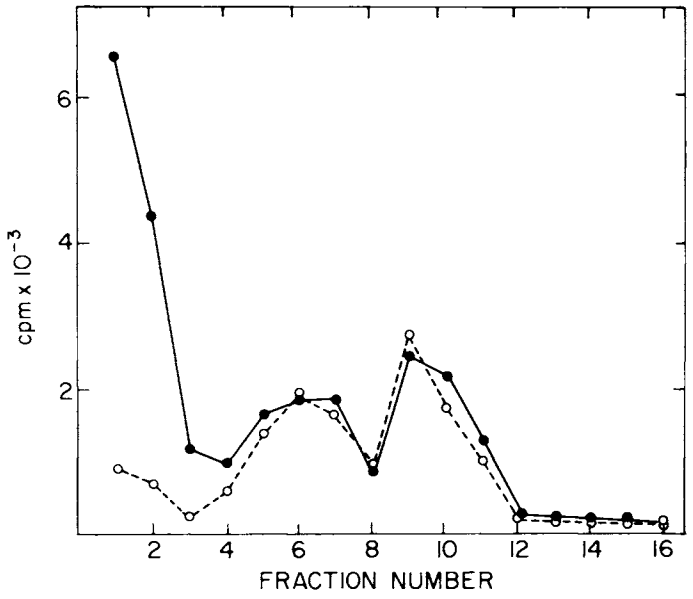

Fig. 2. Effect of trypsin treatment of 3T3 cells on the distribution of cell-associated ^{125}I-EGF in sucrose gradients. Four plates of cells were incubated with ^{125}I-EGF for one hour at 4°C, washed, and incubated at 37°C for 10 minutes. Cells were then placed on ice, washed, and incubated with or without 0.5% trypsin as described in Materials and Methods. The plates were harvested and the cell suspensions analyzed as in Figure 1. –○–○–, plus trypsin; –●–●–, minus trypsin.

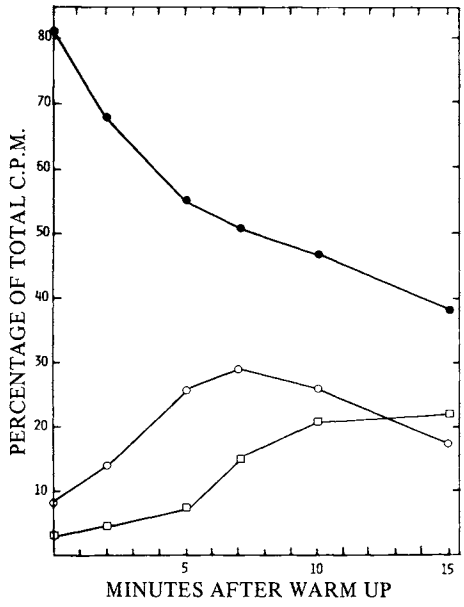

Fig. 3. Distribution of ^{125}I-EGF in sucrose gradients as a function of time after warm-up; 0–15 minutes. Duplicate plates of cells were incubated for 60 minutes at 4°C with 180 ng of ^{125}I-EGF, washed and placed at 37°C for various times. Cells were then washed, harvested, and prepared for analysis. Cell supernatants were placed on sucrose gradients and centrifuged to equilibrium. Fractions were collected and counted as described in Figure 1. The cpm in Peak I (Tubes 1–3), Peak II (Tubes 5–7), and Peak III (Tubes 9–11) were each divided by the total cpm in the gradient to give the percentage of the cpm in each fraction as a function of time. –●–●–, Peak I; –□–□–, Peak II; –○–○–, Peak III.

ent in Peaks II and III. After a short incubation at 37°C, the distribution of radioactivity in the gradient shifts dramatically. The radioactivity associated with Peak III rapidly increases, reaching a maximum between 5–7 minutes, then decreases. The radioactivity in Peak II begins to increase only after 5 minutes, substantially after Peak III begins to accumulate label. Radioactivity in Peak II does not become maximal until 10–15 minutes. By 15 minutes at 37°C, over 60% of the radioactivity initially in Peak I (plasma-membrane-associated radioactivity) has shifted out of this peak.

We next examined redistribution of bound ^{125}I-EGF radioactivity in Peak I, Peak II, Peak III, and in the cell culture medium over a two-hour period, in order to observe the entire process of EGF internalization and degradation. Internalization and degradation of EGF is complete within this time period [6, 7]. In Figure 4, we show the distribution of radioactivity in the three gradient peaks and in the medium as a function of incubation time, out to 120 minutes, at 37°C. Degraded EGF in the medium is measured as trichloroacetic-acid-insoluble radioactivity (see Methods). As in Figure 3, the distribution of ^{125}I-radioactivity changes markedly with time, shifting from Peak I to Peaks II and III, and eventually into the incubation medium as degraded ^{125}I-EGF. Once again (as seen in the early kinetic experiment of Figure 3), radioactive EGF appears to leave Peak I, enter rapidly into Peak III, then leave Peak III after 7–10 minutes and enter Peak II. In this experiment, carried out for a longer time, radioactivity begins to leave Peak II after 15–20 minutes. The amount of degraded ^{125}I-EGF, measured either by trichloroacetic acid precipitation or

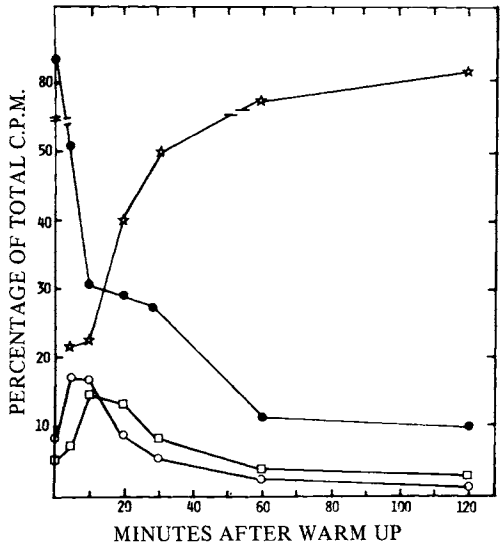

Fig. 4. Distribution of ^{125}I-EGF in sucrose gradients as a function of time after warm-up; 0–120 minutes. Protocols are the same as described in the legend to Figure 3. In addition, the cpm of degraded ^{125}I-EGF from each time point in the 37°C incubation medium was determined by precipitating the protein by the addition of trichloroacetic acid to a final concentration of 10%, removing the precipitate by centrifugation at 3,000g for 30 minutes, and counting the supernatants in a Gamma Counter. The trichloroacetic-acid-soluble cpm were added to the total cpm in each gradient and the percentage of the total cpm in each fraction determined as before. –●–●–, Peak I; –□–□–, Peak II; –○–○–, Peak III; –★–★–, degraded EGF.

gel filtration, does not increase over the first 10 minutes. (At 0 time, approximately 20% of the ^{125}I associated with the cells is not trichloroacetic-acid-precipitable.) By two hours after initiation of 37°C incubation, however, over 80% of the total ^{125}I-EGF has been degraded and is no longer cell-associated.

We have also determined the percentage of trichloroacetic-soluble and -insoluble radioactivity in Peaks II and III after addition of SDS (final concentration of 1%). The trichloroacetic-acid-soluble radioactivity presumably is ^{125}I-EGF, which has been degraded but is still cell-associated. The trichloroacetic-acid-precipitable fraction is intact ^{125}I-EGF. After 37°C incubations of 10 minutes and 20 minutes, 79% and 81% of the ^{125}I-EGF in Peak III is intact. In contrast, 49% of the ^{125}I-EGF in Peak II is trichloroacetic-acid-precipitable at 10 minutes and 20 minutes. These findings suggest, as do the kinetic experiments, that Peak III contains the initial intracellular intermediate in EGF internalization and degradation, while Peak II is a later intermediate.

^{125}I-EGF in Fraction III is Associated With Coated Vesicles

Peak III is the first component to accumulate radioactivity following warming to 37°C, as a consequence of translocation of plasma membrane (Peak I) ^{125}I-EGF. The proposed scheme of EGF transfer of plasma membrane receptors → coated vesicles → lysosomes [7] would thus predict this fraction to be coated vesicles. When we add brain-coated vesicles purified by our previous methods [5, 19] to homogenates of ^{125}I-EGF-labeled 3T3 cells shifted to 37°C, the brain CVs are found to co-sediment with ^{125}I-EGF in Peak III

after equilibrium centrifugation (Fig. 1). These data suggest that the ^{125}I-EGF contained in this peak is associated with an organelle(s) that has the same density as coated vesicles.

Additional evidence that the ^{125}I-EGF in Peak III is associated with coated vesicles was provided by serologic analysis, using our antiserum [5] against CVs. An homogenate, prepared from cells incubated for one hour at 4°C, then warmed to 37°C for 10 minutes, was divided in half. To one half we added anti-CV antiserum; to the other half, pre-immune serum. After a one-hour incubation, we added fixed Staphylococcus aureus cells to each fraction, layered the solutions on sucrose gradients, and centrifuged to equilibrium. The Staphylococcus aureus cells, which act as an immunoabsorbent and bind immunoglobulin and any immunoglobulin-bound antigens, pelleted. The distribution of radioactivity from these gradients is shown in Figure 5. (Radioactivity in the pellet was not measured.) Over 75% of the ^{125}I-EGF radioactivity present in Fraction III was removed by treatment with anti-CV followed by Staphylococcus aureus, compared to the control with pre-immune serum. Moreover, there was no loss of radioactivity from Peak I or Peak II in the anti-CV preparation. These data suggest that 1) the bulk of the ^{125}I-EGF in Peak III is associated with coated vesicles, and 2) coated vesicles containing ^{125}I-EGF are not present in Peaks I or II.

We have also co-purified ^{35}S-methionine-radioactively-labeled 3T3 coated vesicles, using brain as carrier, to demonstrate that CVs from 3T3 cells exhibit similar purification profiles, sedimentation characteristics, and polypeptides as those found for conventional

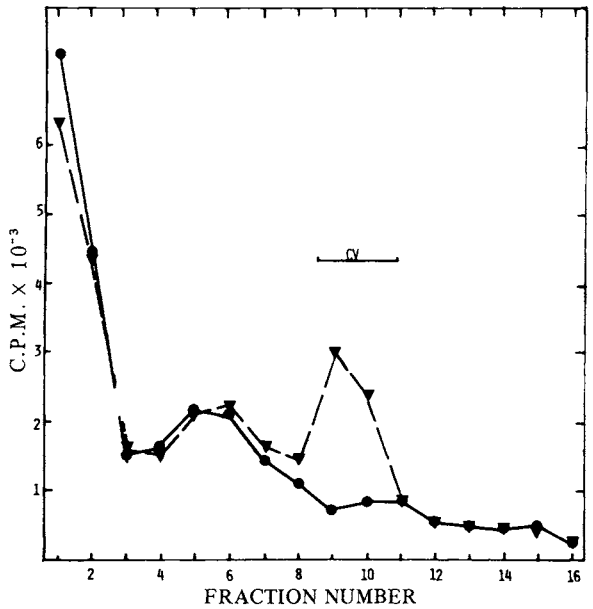

Fig. 5. Distribution of ^{125}I-EGF in sucrose gradients after treatment of cell supernatants with either pre-immune or anti-CV antiserum. A cell supernatant solution was prepared from four plates of 3T3 cells which had been incubated at 4°C with ^{125}I-EGF for one hour followed by incubation at 37°C for 10 minutes. The supernatant was divided in two parts; one half was incubated for two hours at 4°C with 20 μl of pre-immune serum, the other half received anti-CV serum. To each solution was added 20 mg of formalinized Staphylococcus aureus preabsorbed with unlabeled 3T3 cell supernatants as described in Materials and Methods. After an additional one-hour incubation at 4°C, the solutions were placed on two 20–60% sucrose gradients and centrifuged. Gradients were fractionated and analyzed as described in the legend to Figure 1. –▲–▲–, pre-immune serum; –●–●–, anti-CV serum.

brain CVs. Because of limitations in starting material with cultured cells, it was necessary to perform this comparison with carrier brain tissue and biosynthetically radiolabeled 3T3 CVs.

We uniformly labeled 3T3 cells with ^{35}S-methionine, combined the cells with 10 gm of calf brain, and purified the coated vesicles. The procedure we used, modified from our conventional method [19], produces a better yield. The brain-coated vesicles we obtained by this modification appear to be quite pure morphologically (data not shown), and contain the same number and relative amounts of polypeptides, when examined by SDS polyacrylamide gel electrophoresis (Fig. 6a), as do conventionally purified CVs (Fig. 6c). Autoradiography of the gel indicated that clathrin was the major polypeptide co-purified from 3T3 cells using this procedure. Polypeptides of 3T3 coated vesicles corresponding to several other bands in the brain-coated vesicles were also present in the ^{35}[S] autoradiogram (Fig. 6b). This result indicates that the purification method we employ co-purifies coated vesicles from 3T3 cells to a significant extent.

When CVs were isolated (by co-purification with brain CVs) from 3T3 cells labeled at 4° with ^{125}I-EGF and shifted for 0, 10, and 60 minutes to 37°C, the specific activity of the 10-minute CV preparation (406 cpm/mg of recovered CV protein) was fourfold greater than the 0 time (110 cpm/mg) or 60-minute time (94 cpm/mg) CV preparations, again supporting a transient flow of ^{125}I-EGF through this fraction. To demonstrate that the ^{125}I-EGF is not associated with a contaminant in the enriched CV fraction, we incubated the ^{125}I-EGF-associated coated vesicles (co-purified with brain CVs) with either pre-immune or anti-CV serum. Almost all (93%) of the ^{125}I-EGF was precipitated by the immune serum; in contrast, only 20% of the ^{125}I-EGF was precipitated by the pre-immune serum. These data, like those in Figure 5 using homogenates rather than CV-enriched fractions, again suggest the ^{125}I-EGF present in the purified CV fraction is, indeed, in coated vesicles.

To demonstrate that the ^{125}I-EGF in the co-purified CV fraction is *within* a membrane compartment, coated vesicles were treated with trypsin in the presence and absence of SDS. Enzymatic degradation of ^{125}I-EGF was then measured by determining the increase in TCA-soluble radioactivity after trypsin treatment. Only 6% degradation of ^{125}I-EGF in this CV-enriched fraction occurred after treatment with 0.25% trypsin. However, after exposure to SDS, 95% of the ^{125}I-EGF was trypsin-sensitive. The ^{125}I-EGF in this preparation is thus contained in an enclosed membrane compartment. Together with the antibody precipitation studies, these experiments provide evidence that the ^{125}I-EGF from 3T3 cells which co-purified with the brain-coated vesicles is contained inside the coated-vesicle membrane.

Peak II is Associated With Lysosomes

The radioactive EGF which translocates into Peak II is not released when whole cells are exposed to trypsin, suggesting it is also in an intracellular location. Peak II is labeled after Peak III (Figs. 3, 4), suggesting transfer from CVs to Peak II. We have assayed our gradient fractions containing internalized ^{125}I-EGF for N-acetyl-β-D-glucosaminidase (EC 3.2.1.30], a lysosomal marker. Peak II-associated ^{125}I-EGF is coincident with the highest specific activity for this lysosomal marker enzyme (Fig. 7). Also as mentioned previously, over half the ^{125}I radioactivity present in Peak II is soluble in trichloroacetic acid, suggesting a significant portion of the EGF in this intracellular particulate fraction is in a degraded form. Taken together, these findings strongly suggest that the ^{125}I-EGF in Peak II is associated with lysosomes.

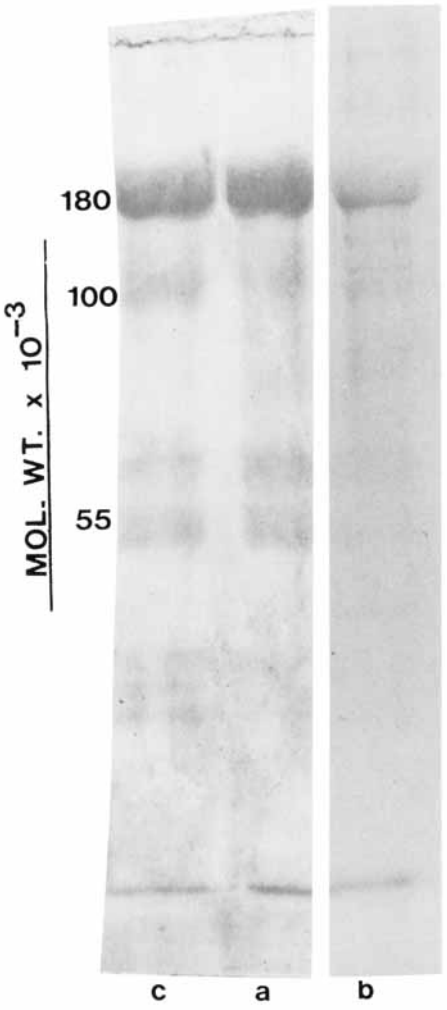

Fig 6 SDS-Polyacrylamide gel electrophoresis of co-purified ^{35}S-methionine-labeled 3T3 and brain-coated vesicles Samples containing 50 μg of protein in 50 μl of sample buffer were prepared as described in Materials and Methods and subjected to electrophoresis on a slab gel for four hours at 35 milliamps. The gel was stained with 0 25% Coomassie brilliant blue, dried under vacuum, and exposed to x-ray film for three days The film was developed in an X-Omat c) Brain CVs purified according to the method of Blitz et al [19] a) Co-purified ^{35}S-methionine-labeled 3T3 and brain CVs (containing 20,000 cpm of ^{35}S-methionine) purified as described in Materials and Methods b) Autoradiograph of (a)

Effects of NH_4Cl on Internalization and Degradation of EGF

NH_4Cl has been demonstrated to be among the most potent inhibitors of cellular EGF degradation, presumably by blocking lysosomal enzyme activity [7]. We have investigated the accumulation of ^{125}I-EGF in the various fractions of our gradients, in the presence of 10 mM NH_4Cl. Figure 8 shows the distribution of ^{125}I-EGF after binding at 4°C and incubation at 37°C for 1 hour, either in the presence or absence of NH_4Cl. It is apparent that there is a great deal more cell-associated radioactivity after 1 hour at 37°C in the presence of 10 mM NH_4Cl than in the absence of this agent.

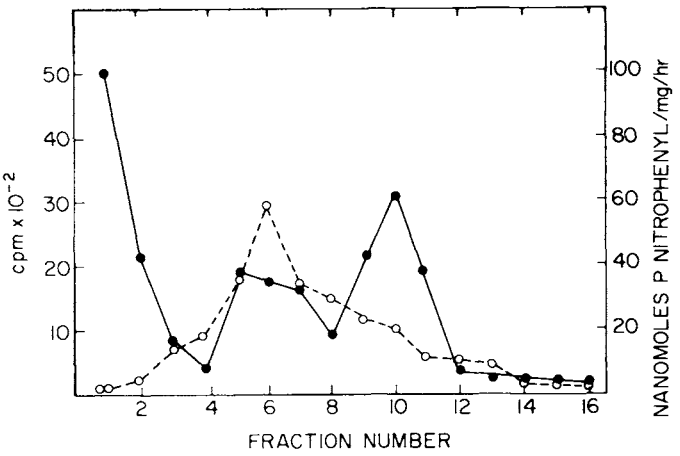

Fig. 7. Distribution of N-acetyl-β-D-glucosaminidase activity and ^{125}I-EGF in 20–60% sucrose gradients. Two plates of cells were incubated with ^{125}I-EGF for 10 minutes at 37°C after a one-hour binding period of 4°C. The cells were harvested and supernatants were prepared and centrifuged through sucrose gradients as described in Figure 1. The amount of radioactivity and enzyme activity were assayed in each fraction. —●—●—, cpm ^{125}I-EGF; —○—○—, p-nitrophenyl/mg protein/hr.

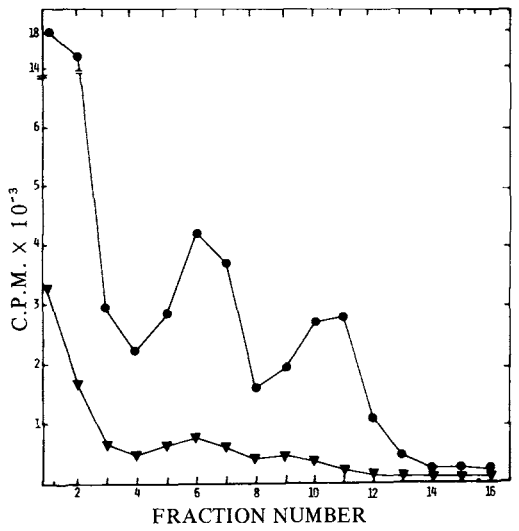

Fig. 8. Distribution of ^{125}I-EGF in sucrose gradients after one-hour binding and one-hour incubation at 37°C in the presence or absence of 10 mM NH_4Cl. Cell supernatants were prepared from two plates of confluent 3T3 cells incubated with ^{125}I-EGF for one hour at 4°C followed by one hour at 37°C with 10 mM NH_4Cl in all solutions. Cell supernatants were also prepared from two plates treated identically, except that no NH_4Cl was present. Gradients were prepared, fractionated, and analyzed as described in Figure 1. —●—●—, plus NH_4Cl; —▲—▲—, minus NH_4Cl.

We have also examined the translocation of ^{125}I-EGF among the gradient fractions and in the medium as a function of time at 37°C in the presence of NH_4Cl (Fig. 9). As can be seen from a comparison of Figures 4 and 9, the distribution of radioactivity in the presence and absence of NH_4Cl is nearly identical after incubation at 37°C for periods up to 10

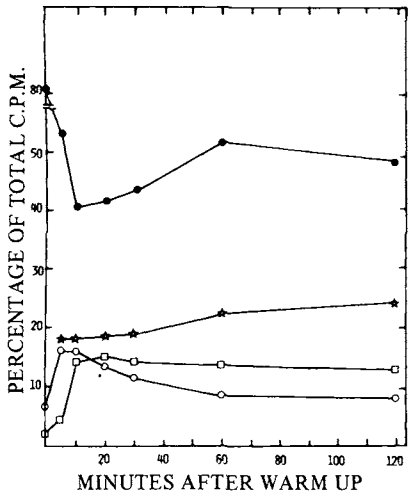

Fig. 9. Distribution of ^{125}I-EGF in sucrose gradients as a function of time after incubation in the presence of NH_4Cl. 3T3 Cells were incubated at 4°C for one hour, washed and placed at 37°C for various times, with the inclusion of 10 mM NH_4Cl in all solutions. For each time point, two plates of cells were harvested, homogenized, and analyzed by equilibrium sucrose gradient centrifugation as described in the legend to Figures 3 and 4. The percentage of cpm in Peaks I–III and as degraded EGF were determined. –●–●–, Peak I; –□–□–, Peak II; –○–○–, Peak III; –★–★–, degraded EGF.

minutes. By this time, there has been a large shift of radioactivity from Peak I to Peaks II and III in both cases. Beyond this time, extensive differences in the distribution of ^{125}I-EGF are observed. Instead of a further decline in the percentage of radioactivity in Peaks I–III, which we see in the absence of NH_4Cl (Fig. 4), we now find that 1) the percentage of radioactivity in Peak III drops slowly, 2) radioactivity in Peak II remains constant, 3) the percentage of radioactivity in Peak I rises, and 4) the percentage of degraded ^{125}I-EGF in the medium increases only slightly with time.

DISCUSSION

Kinetic Analysis of EGF Internalization and Degradation

We conclude, in view of the evidence presented here, that the ^{125}I-EGF in Fractions I, III, and II is associated with plasma membrane, coated vesicles, and lysosomes, respectively; that there exists a strong biochemical argument for a specific, directional intracellular pathway for internalized EGF. Subsequent to binding at 4°C for 1 hour and adjusting the temperature to 37°C, plasma membrane receptor-bound EGF is taken up first into coated vesicles and then into lysosomes. It is then degraded prior to exit from the cell.

Since at most, 15% of the cell-associated ^{125}I-EGF is found in either Peak II or Peak III at any time point, we cannot completely rule out alternate pathways for EGF internalization and degradation. However, in view of the necessarily transitory nature of the association of EGF with either intermediate (coated vesicle or lysosome), this relatively high percentage suggests strongly that the plasma membrane → coated vesicle → lysosome pathway is a major route for EGF metabolism.

The kinetics of EGF internalization reveal several things about the nature of the process. First, approximately 10–15% of the total ^{125}I-EGF appears "internalized" at 4°C with most of the radioactivity associated with Peak III. Coated pits may thus be able to bud off to form coated vesicles at 4°C in this system, albeit at a significantly reduced rate compared with physiological temperatures. Alternatively, this apparent internalization may arise artifactually, as a consequence of homogenization. Coated pits may be broken off, vesiculate, and sediment in the more dense fraction. The work of Gorden et al [9] indicates that approximately one-third of the specifically bound EGF is initially associated with coated pits, which comprise about 2% of the area of the plasma membrane. This result was obtained when binding was carried out for 2 minutes at 37°C or for 2 hours at 4°C. It is likely that the EGF initially bound in the coated pits, as opposed to other regions of the plasma membrane, is the material which is internalized at 4°C (Fig. 1).

The rate of internalization during the first 10 minutes of incubation at 37°C is very rapid, then declines significantly during the next 20 minutes. One reasonable explanation for this finding is that during the one-hour incubation at 4°C, there is an accumulation of coated pits containing ligand-bound receptors on the cell surface. Immediately after warming to 37°C, there is a rapid internalization of essentially all the coated pits as coated vesicles. After 10 minutes at 37°C, very few coated pits may remain at the cell surface; and the rate of internalization decreases, possibly until enough coated pits are reformed to begin internalization again. An alternative explanation for the decreased rate of internalization after 10 minutes may be the necessity of the receptor-ligand complexes to form aggregates, or patches, which then associate with, or serve as nucleation sites, for coated pits. Patching of fluorescent EGF following specific binding has been demonstrated by Maxfield et al [11]. Finally, dissociation of EGF from plasma membrane receptors reduces the opportunity for internalization.

Degradation of EGF, as detected by the appearance of trichloroacetic-acid-soluble radioactivity in the internalization medium, appears to increase in an almost linear fashion from 5–30 minutes after shifting to 37°C. This finding indicates that degradation occurs shortly after the polypeptide reaches the lysosome (Fig. 5). The most dramatic effect of NH_4Cl treatment is on EGF degradation. In the absence of NH_4Cl, there is a very rapid increase in EGF degradation beginning 5–10 minutes subsequent to shifting to 37°C. This increased degradation is not observed in the presence of NH_4Cl, in agreement with the observations of Carpenter and Cohen [7].

There appears to be a constant amount of trichloroacetic-acid-soluble radioactivity present in the medium after warming to 37°C in NH_4Cl-containing medium, constituting about 18% of the total radioactivity in the system. This may be due to the absorption of trichloroacetic-acid-soluble ^{125}I-EGF fragments (which appear to constitute approximately 20% of the radioactivity in the initial iodinated EGF preparation) and its subsequent dissociation in the absence of ^{125}I-EGF during incubation at 37°C. In fact, a considerable amount of trichloroacetic-acid-insoluble radioactivity is also found in the medium, apparently as a result of dissociation of bound ^{125}I-EGF from the plasma membrane, an observation also made by Haigler et al [10]. The dissociated trichloroacetic-acid-insoluble radioactivity also remains relatively constant with time after warming to 37°C, suggesting that the fraction of dissociated whole or degraded EGF remains constant at 37°C within the period of our measurements.

Our results also indicate that NH_4Cl has a delayed effect on EGF internalization. Over half of the counts bound to the plasma membrane are internalized within 10 minutes after shifting to 37°C. Although there appears to be little further internalization, there is a

20% increase in the amount of radioactivity bound to the plasma membrane at later times. This binding may result from the addition of new receptor sites to the plasma membrane, which rebind a portion of the EGF which had previously dissociated from the cell surface. An alternative explanation is that some EGF receptors are actually recycled to the plasma membrane due to the blockage of lysosomal hydrolysis. Finally, some labeled EGF could escape from inactivated lysosomes, find its way to the exterior of the cell, and rebind to unoccupied membrane receptors.

Coupling of Internalization to Degradation

If each step in the internalization of EGF were completely independent, one would predict that NH_4Cl treatment, which blocks lysosomal protein hydrolysis, would lead to an accumulation of essentially all ^{125}I-EGF in the lysosomes after a period of time at 37°C, with a disappearance of plasma membrane binding and coated vesicle containing radioactivity. Alternatively, one might expect to see lysosome and coated vesicle-bound ^{125}I-EGF accumulate in the presence of NH_4Cl, if lysosomal sites are saturable, with a concomitant depletion of plasma membrane binding. In fact, the distribution of radioactivity among the three peaks remains fairly constant between 10 minutes and two hours in the presence of NH_4Cl. If, however, the steps in the internalization process were coupled by a feedback mechanism, disruption of the process at the level of the lysosome would prevent further internalization. This is exactly what we see: cessation of EGF lysosomal degradation and ejection is accompanied by slowed flow through coated vesicles and slowed loss from the plasma membrane.

Several additional findings are consistent with the notion of a coupled pathway of internalization from plasma membrane to lysosome. Anderson et al [24] have recently demonstrated the existence of linear tracks of coated vesicles in several cell types which appear to extend from the periphery of the cell to the perinuclear area. We have recently shown that as many as one-third of the coated vesicles isolated from brain are attached to 100-Å filaments [25]. Coated vesicles from other organs also appear to be complexed with these filaments [D. Sack, R. Fine, unpublished result]. This presumptive cytoarchitecture might serve as a basis for regulation of the plasma membrane → coated vesicle → lysosome pathway. The recent report [26] that treatment of myoblasts with chloroquine, another inhibitor of lysosomal function, causes a sixfold increase in the number of coated vesicles in the cytoplasm as well as a significant increase in the amount of acetyl-choline receptors is also consistent with a coupled pathway.

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REFERENCES

1. Lagunoff D, Curran DE: Exp Cell Res 75:337, 1972.
2. Pearse BMF: Proc Natl Acad Sci USA 73:1255, 1975.
3. Roth TP, Porter KR: J Cell Biol 17:208, 1964.

4. Anderson R, Brown M, Goldstein J: Cell 10:351, 1977.
5. Fine RE, Blitz AL, Sack DH: FEBS Lett 94:59, 1978.
6. Aharonov A, Pruss RM, Herschman HR: J Biol Chem 253:3970, 1978.
7. Carpenter G, Cohen S: J Cell Biol 71:159, 1976.
8. Carpenter G, Lembach KJ, Morrison MM, Cohen S: J Biol Chem 250:4297, 1975.
9. Gorden P, Carpentier J, Cohen S, Orci L: Proc Natl Acad Sci USA 75:5025, 1978.
10. Haigler H, Ash JF, Singer SJ, Cohen S: Proc Natl Acad Sci USA 75:3317, 1978.
11. Maxfield FR, Schlessinger J, Schecter Y: Cell 14:805, 1978.
12. Baker JB, Simmer RL, Glenn KC, Cunningham DD: Nature 278:743, 1979.
13. Linsley PS, Blifeld C, Wrann M, Fox CF: Nature 278:745, 1979.
14. Das M, Fox CF: Proc Natl Acad Sci USA 75:2644, 1978.
15. Savage CR Jr, Cohen S: J Biol Chem 247:7609, 1972.
16. Pruss RM, Herschman HR: Proc Natl Acad Sci USA 74:3918, 1977.
17. Puck TT, Cicciura SJ, Fisher HW: J Exp Med 106:145, 1957.
18. Dulbecco R, Vogt M: J Exp Med 99:167, 1954.
19. Blitz AL, Fine RE, Toselli PA: J Cell Biol 75:135, 1977.
20. Touster O, Aronson NN Jr, Dulaney JT, Hendrickson H: J Cell Biol 47:604, 1970.
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
22. Laemmli UK: Nature 227:680, 1970.
23. Tanabe T, Pricer WE Jr, Ashwell G: J Biol Chem 254:1038, 1979.
24. Anderson R, Vasile E, Mello R, Brown M, Goldstein J: Cell 15:919, 1978.
25. Sack DH, Fine RE, Blitz AL: Fed Proc 37:278A, 1978.
26. Libby P, Bursztajn S, Goldberg AL: Cell 19:481, 1980.