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Selective Protein Transport: Identity of the Solubilized Phosvitin Receptor From Chicken Oocytes

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By two independent methods, the solubilized receptor for phosvitin (PV) has a subunit MW of 116K. Affinity chromatography, showed that only 2 of the more than 25 proteins present in the total detergent solubilized oocyte membrane extract were retained on a PV–agarose column. These proteins of MW of 116K and 100K could be eluted from PV–agarose with free PV. By gel exclusion chromatography, the receptor-^{125}I-PV complexes elute in the void volume of a Biogel A-1.5 column. When these void fractions were assayed by SDS-PAGE only a single protein of MW of 116K was observed in addition to ^{125}I-PV.

Key words: protein transport, phosvitin, receptor, coated vesicles

Selective protein transport, mediated by specific receptors in association with coated pits and coated vesicles is a fundamental cellular process. The vital importance of this transport process is particularly manifest during reproduction. The selective transport of maternal immunoglobulins into the offspring provides the newborn with a passive maternally derived immunity until it becomes immunocompetent. Well studied examples include the illial cells of the rat that sequester maternal IgG from the mother's milk and release it into the neonatal circulation [1, 2]. A different tissue mediates a similar function in the rabbit. In the rabbit, IgG is transported into the developing fetus via the cells of the yolk sac splanchnopleure [3, 4]. In the chicken, IgG crosses into the oocyte via a receptor-mediated mechanism [5, 6].

In addition to IgG, the specific transport of other maternal proteins is also essential for successful reproduction. A particularly well studied example is the selective uptake of vitellogenin via coated vesicles into the developing oocytes of all oviparous animals. In oviparous animals vitellogenin is stored in the oocyte until it is degraded during embryogenesis to provide nutrients for the developing embryo. Well documented studies on the uptake of vitellogenin have been carried out in the mosquito [5, 7], saturnid moths [8, 9], the amphibian Xenopus laevis [10, 11], and the domestic chicken [12, 13].

Receptor-mediated, protein transport has also been well documented in systems unrelated to species reproduction. Low density lipoprotein, a serum cholesterol carrier, appears

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to enter fibroblasts exclusively via a receptor-coated vesicle associated system [14, 15]. Epidermal growth factor [16] and α_2-macroglobulin [17] also appear to be sequestered by a similar mechanism.

The developing chicken oocyte provides an excellent model system in which to study specific protein transport. During the final stages of maturation up to 1 gm of protein per day is transported into the developing oocyte. Kinetic studies in our laboratory have demonstrated the existence of specific receptors for vitellogenin [13], PV [18, 19], IgG [6], LDL and VLDL [20], in association with the oocyte membrane. Morphological studies indicate that virtually the entire oocyte surface is coated, thus implicating coated pits and coated vesicles in the transport process [5, 21, 22].

We have previously characterized the kinetic binding parameters of PV, a 30K dalton subunit of vitellogenin, to isolated oocyte membranes [18]. These studies indicated that PV bound to a specific receptor with a K_D of 3.3×10^{-6} M. We also described experiments that suggested that the membrane-associated receptor could be obtained in a soluble form by extracting the membranes with Triton X-100. In the present report we present evidence tentatively identifying the solubilized receptor as a single polypeptide of MW 116K.

MATERIALS

Biogel A-1.5m, Affi-gel 10, acrylamide, bisacylamide, and molecular weight standards for SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) were obtained from BIO-RAD Laboratories. Carrier-free $Na^{125}I$ was obtained from the Amersham Radiochemical Centre. Phosvitin and Triton X-100 were from Sigma Chemical Co. All other chemicals were of reagent grade and were obtained from commercial sources. Developing chicken oocytes were obtained from a local slaughter house.

All experimental procedures were carried out in an incubation buffer (IB) containing 0.01 M 2-N-morpholinoethane sulfonic acid (pH 6.0), 0.14 M NaCl, 5 mM KCl, 0.83 mM $MgSO_4$, 0.13 mM $CaCl_2$ plus 0.02% sodium azide. Solubilization and chromatography experiments were carried out in IB containing 0.1% (w/v) Triton X-100 (IB-TX).

METHODS

Preparation of Solubilized PV Receptors

Developing oocytes from freshly killed white leghorn laying hens were placed in IB at 0°C. Oocytes approximately 1.5–2 cm in diameter, which are rapidly sequestering vitellogenin, were slit, drained of yolk and returned to ice cold IB. Adherent yolk was removed by gentle shaking in the IB solution. The membrane complex consisting of the oocyte plasma membrane, a fibrous perivitelline layer, a monolayer of follicular epithelial cells, and an acellular basement lamella was dissected free of the overlaying connective tissue and placed in fresh IB. Dissected membranes from 10 oocytes were homogenized in 5 ml of IB containing 1% (w/v) Triton X-100 in a Teflon glass homogenizer. The homogenate was then centrifuged for 60 min at 100,000g, after which the supernatant fraction (soluble extract) was used immediately. All isolation and solubilization procedures were carried out at 4°C.

Affinity Chromatography Procedure

PV–agarose was prepared using Affi-gel 10, a commercially prepared N-hydroxysuccinimide ester of a succinylated aminoalkyl derivative of agarose available from BIO-RAD Laboratories. Affi-gel 10 forms covalent crosslinks to proteins via their free amino groups

with the concomitant release of N-hydroxysuccinimide. PV–agarose used in our experiments was prepared by incubating 200 mg of PV in 10 ml of 0.1 M phosphate buffer (pH 8.0) with 10 ml of Affi-gel 10 resin for 16 h at 4°C. One ml of 1.0 M ethanolamine (pH 8.0) was added to quench any remaining reactive groups for a further 2 h at 4°C. The final product, which contained 9.5 mg of PV bound per ml of packed gel, was placed in a small 15 mm diameter column and washed extensively with IB-TX.

For affinity chromatography experiments, 3 ml of the Triton soluble extract was applied to the column at 4°C. The resin was then washed with 70 ml of IB-TX, and 2 ml eluate fractions were collected. The volume of the washing solution was empirically determined by assaying the protein composition of the eluate fractions by SDS-PAGE. If any proteins were detected by SDS-PAGE in the last fraction of the wash, the wash volume was increased in subsequent experiments. In the experiments described herein no proteins were detectable in the final wash fraction by SDS-PAGE. In our initial experiments we routinely assayed the protein concentration of the eluate fractions by both their optical density at 280 nm and the BIORAD Protein Assay. However, we observed that the protein concentration of the eluate fractions determined by either of these methods would appear to be zero when proteins could be detected by SDS-PAGE. Thus, it was apparent that neither of the assay methods was as sensitive as SDS-PAGE for detecting small quantities of protein. We therefore routinely used SDS-PAGE to assay for the presence of protein components in eluate fractions. Following the 70 ml wash with IB-TX, 40 mg of PV, chicken IgG or chicken serum albumin in 3 ml of IB-TX was applied to the column followed by a further 70 ml of IB-TX. The composition of the eluate fractions was then assayed for protein components by SDS-PAGE. Except for the application of 40 mg of free PV and extensive washing with IB-TX, our PV–agarose columns were not regenerated by the application of low pH or high salt solutions between experiments. Our columns yielded similar results in at least 5 separate experiments using the same PV–agarose.

Gel Exclusion Chromatography Procedures

Glass columns (0.4 X 53 cm) packed with Biogel A-1.5 m and equilibrated with IB-TX at 4°C were used for all gel-exclusion chromatography experiments. One ml samples were applied to the columns and eluted with IB-TX. One ml fractions were collected at a flow rate of 4 ml/h. Vitamin B-12 and blue dextran were added to the samples as internal markers. The protein composition of the eluate fractions was assayed by SDS-PAGE. In order to demonstrate soluble PV binding activity, one ml aliquots of soluble extract were incubated with 10^{-6} M ^{125}I-PV plus or minus a 100-fold molar excess of unlabeled PV for 60 min at 4°C prior to chromatography. ^{125}I-PV was prepared as described previously [18]. ^{125}I activity in the eluate fractions was determined in a well-type gamma counter.

SDS-PAGE Procedure

SDS-PAGE was carried out using the discontinuous buffer system of Laemmli [23]. Fifty μl samples were reduced by incubation with 10% β-mercaptoethanol and then applied to slab gels (0.1 X 16 X 17 cm) and electrophoresed for 16 h at 60 V. Ovalbumin, MW 43K; bovine serum albumin, MW 68K; phosphorylase B, MW 94K; β-galactosidase, MW 116K; and myosin, MW 200K were used as MW standards. Gels were fixed and stained in 500 ml of 0.5% Coomassie blue R in 30% isopropanol–20% acetic acid for 4 h and then destained in 10% isopropanol–10% acetic acid until no further background color could be removed. After destaining, gels were dried and photographed using a yellow filter.

RESULTS

Affinity Purification

We wished to determine if phosvitin (PV) covalently coupled to agarose beads could be used to affinity purify PV receptors from detergent solubilized oocyte membranes. In these experiments, a Triton X-100 extract was applied to a PV–agarose column as described in Methods. After an initial wash with IB-TX, excess free PV was applied to the column in order to competitively displace any PV binding proteins, including the solubilized PV receptor, bound to the PV–agarose. Using SDS-PAGE, the protein composition of the soluble extract, the material that initially washed through the column, and the material that eluted after the addition of free PV were analyzed. The gels (Fig. 1) demonstrate that at least 25 different proteins can be resolved in the soluble extract. Comparison of the material that eluted from the column in the initial wash and the soluble extract indicated that two proteins of MW 116K and 100K are retained on the column. Significantly, these same two proteins elute from the column after the addition of excess free PV. We obtained similar results in five separate experiments. Other proteins were observed to elute after the addition of free PV. However, these appeared to be very minor components and did not appear to be depleted from the material that initially washed through the column. It should be noted that PV is not observed in the gels because it binds Coomassie blue poorly.

Additional experiments were carried out to demonstrate that the elution of the 116K and 100K proteins by PV was the result of a specific interaction between PV and these proteins. When chicken IgG or chicken serum albumin were applied to the PV–agarose column after the application of soluble extract, neither chicken IgG or chicken serum albumin resulted in the elution of the 116K and 100K proteins from the column. Furthermore, the subsequent application of PV to the column resulted in the elution of both the 116K and 100K proteins (data not shown). For these elution experiments, IgG, albumin, and PV were used at the same mg/ml concentrations. In order to verify that the elution of the 116K and 100K proteins from the PV–agarose column was not a simple ionic effect, we attempted to elute these proteins with high concentrations of salt. Washing the PV–agarose column with 1.0 M NaCl did not result in the elution of any detectable protein from the column (data not shown).

In a separate experiment, 40 mg of PV were applied to a PV–agarose column that had not been exposed to the oocyte detergent extract. When the eluent fractions were analyzed by SDS-PAGE, no protein bands were detected by Coomassie blue staining (data not shown).

Gel Exclusion Chromatography

We have previously used gel exclusion chromatography to demonstrate the existence of a soluble PV binding component in Triton X-100 extracts of oocyte membranes [18]. The rationale behind the use of gel exclusion chromatography for these experiments is that soluble receptor–PV complexes must necessarily be larger than free PV itself. The results of a typical gel exclusion chromatography experiment (Fig. 2) show that when detergent extracts, preincubated with ^{125}I-PV, are applied to a Biogel A-1.5m column, three peaks of ^{125}I activity are obtained. The first peak, which eluted in the void volume of this column, was tentatively identified as containing soluble receptor-^{125}I-PV complexes. This peak contained 12% of the eluted ^{125}I activity. The second peak contained 69% of the eluted activity and appears to be free PV because it elutes in the same position and has the same distinctive profile of free ^{125}I-PV. The third peak, which contained 19% of the eluted ^{125}I activity,

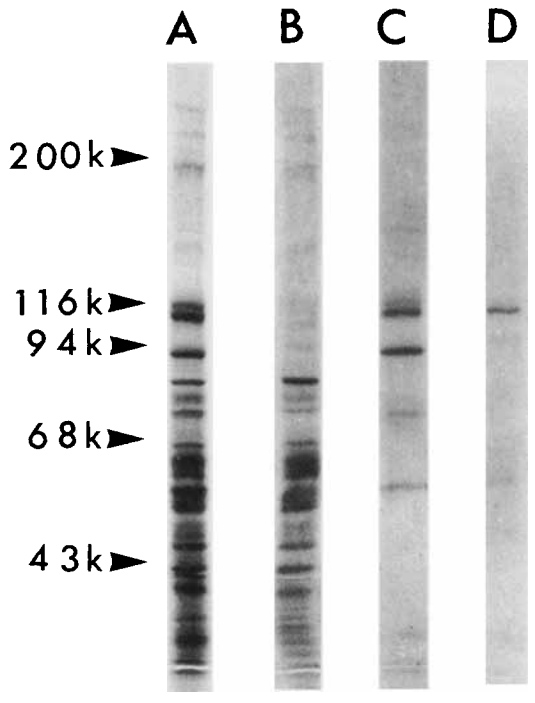

Fig. 1. SDS-PAGE analysis of Triton solubilized extract (A). Solubilized extract that did not adhere to a PV–agarose affinity column (B). Material that was eluted from PV–agarose by the addition of 40 mg of free PV (C). Solubilized extract that eluted in the void volume of a Biogel A-1.5m column (D). SDS-PAGE was carried out on 8–12% gradient slab gels using the discontinuous buffer system of Laemmli [23]. MW standards indicated by arrows are myosin, 200K; β-galactosidase, 116K; phosphorylase B, 94K; bovine serum albumin, 68K; and ovalbumin, 43K.

appears to be a small proteolytic breakdown product of ^{125}I-PV. This material is not observed when ^{125}I-PV is chromatographed separately and does not contain any detectable protein components when assayed by SDS-PAGE. Interestingly, the formation of this peak is not inhibited by the addition of both aprotinin at 100 units/ml and PMSF at 10 μg/ml. In order to demonstrate that the formation of ^{125}I-PV-receptor complexes was specific, a parallel experiment was carried out. In this experiment an aliquot of soluble extract was incubated with 10^{-6} M ^{125}I-PV plus a 100-fold excess unlabeled PV. When this sample was chromatographed over Biogel A-1.5m, only two peaks of ^{125}I activity were observed. Under these conditions no activity was observed to elute in the void volume of the column. The two peaks that were observed in the presence of unlabeled PV corresponded to the free PV peak and the proteolytic fragment peak obtained in the previous experiment. This supports our tentative identification of the void material in the preceding experiment as containing the receptor-^{125}I-PV complexes.

After solubilized extract was incubated with PV and chromatographed over Biogel A-1.5m, the protein composition of the eluate fractions was determined by SDS-PAGE (Fig. 3). The material eluting in the void volume contained one major protein of MW 116K as well as trace amounts of several other proteins. This result, in conjunction with the pre-

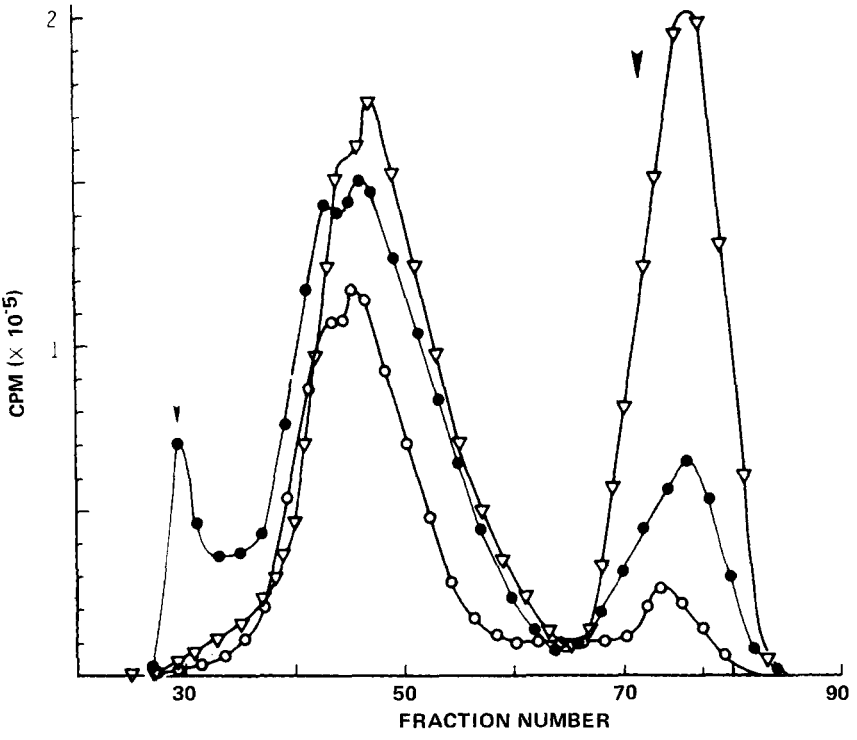

Fig. 2. Assay of ^{125}I-PV binding to soluble extract. One ml aliquots of soluble extract were incubated with 10^{-6} M ^{125}I-PV (2.5×10^6 cpm) in the presence (▽) or absence (●) of 10^{-4} M unlabeled PV for 60 min at 4°C. Following incubation the samples were immediately applied to two identical Biogel A-1.5m columns and eluted with IB-TX as described in Methods. Elution profile of free ^{125}I-PV (1.2×10^6 cpm) (○). The small arrow indicates the elution position of blue dextran, the large arrow indicates the elution position of vitamin B-12. Both blue dextran and vitamin B-12 were routinely included in our samples as internal standards.

vious experiment (Fig. 2) that demonstrated that receptor-PV complexes elute in the void volume, suggests that this 116K protein is in fact the receptor. In addition, this 116K protein corresponds to one of the proteins that was retained by the PV–agarose column and released by the addition of free PV. When the void volume fraction (Fraction 30, Fig. 3) was electrophoresed on the same slab as the affinity purified material, the 116K components obtained by either of these methods appeared to comigrate (Fig. 1).

In 4 separate experiments, we compared the protein composition of the eluate fractions obtained from two identical Biogel A-1.5m columns. One column was used to chromatograph a soluble extract preincubated with PV whereas the other was used to chromatograph a soluble extract preincubated in the absence of PV. In four separate experiments the 116K protein was always observed to elute in the void volume regardless of the presence or absence of PV in the preincubation. In addition, the protein composition of the fractions eluting after the void were found to be the same in both columns, regardless of the presence or absence of PV in the preincubation. Because the exclusion limit of Biogel A-1.5m is approximately 1.5 million daltons for globular proteins we can assume that the 116K protein

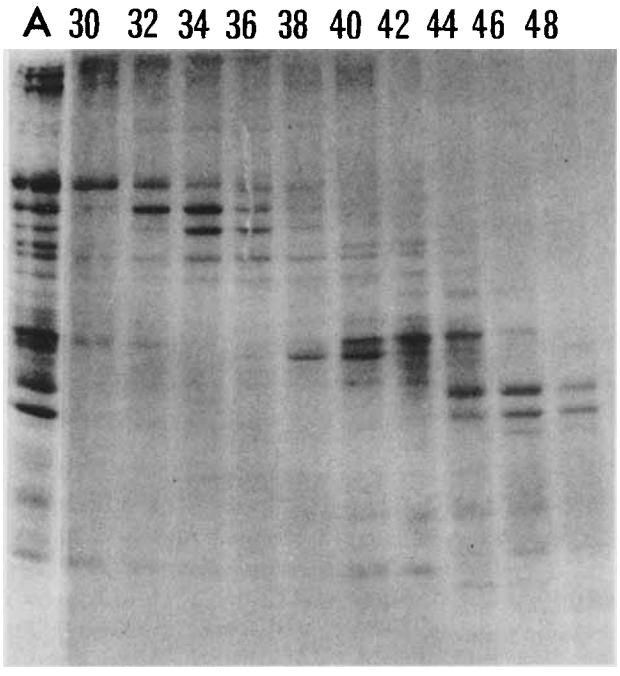

Fig. 3. SDS-PAGE analysis of Biogel A-1.5m eluate fractions. One ml of soluble extract was incubated with 10^{-5} M PV for 60 min at 4°C. Following incubation the sample was immediately applied to a Biogel A-1.5m column and eluted with IB-TX as described in Methods. The eluate fractions were then analyzed by SDS-PAGE. Fraction numbers indicated over appropriate lanes correspond to fraction numbers from related chromatography experiments shown in Figure 2 (void volume is Fraction 30). Lane A contains unchromatographed soluble extract. SDS-PAGE was carried out on a 10% slab gel using the discontinuous buffer system of Laemmli [23].

that elutes in the void volume must be a subunit of a larger complex. Because PV was not required in order for the 116K protein to elute in the void, and no other proteins were present, it may well be that the 116K protein self-associates to form a multimeric receptor complex.

DISCUSSION

Previous reports from our laboratory have shown that both vitellogenin and PV, a proteolytic fragment of vitellogenin, bind to specific receptors associated with isolated oocyte membranes [19]. In addition, we have evidence that both vitellogenin and PV bind to the same receptor [19]. These results lead us to suggest that phosvitin is the component of vitellogenin recognized by the membrane-associated receptor and that this binding is the initial event in the subsequent transport of vitellogenin into the developing oocyte. We have therefore utilized PV as a probe in our continuing studies on the mechanism of vitellogenin transport into developing chicken oocytes. In addition, it should be noted that PV is more readily obtained than vitellogenin and is stable in solution thus making it the ligand of choice.

Recently, we have shown that the nonionic detergent, Triton X-100, can be used to extract the receptor in an active form from isolated oocyte membranes [18]. The soluble form of the receptor was assayed by gel-exclusion chromatography. ^{125}I-PV, preincubated in the presence of Triton X-100 extracts of oocyte membrane, eluted as a higher MW species than free ^{125}I-PV. This effect was not inhibited by the presence of high concentrations of chicken IgG or chicken serum albumin, but was completely inhibited by the presence of high concentrations of unlabeled PV.

In the present report, we have used two independent methods to identify the protein component of the solubilized receptor. One method, affinity chromatography, showed that two proteins with MW of 116K and 100K were capable of binding to PV–agarose. Furthermore, both of these proteins could be competitively displaced from the PV–agarose by the addition of free PV, but not by the addition of IgG or albumin. The second method, gel-exclusion chromatography, took advantage of the observation that ^{125}I-PV-receptor complexes eluted in the void volume of a Biogel A-1.5m column. When the protein composition of the material eluting in the void was assayed by SDS-PAGE, a protein of MW 116K was observed. We believe these results suggest that the 116K protein represents the solubilized form of the membrane-associated PV receptor. Although unlikely, it is also possible that the 116K protein is not the receptor, but rather the receptor may be one of the minor components observed on our gels. For example, if the receptor is a glycoprotein it may stain poorly with Coomassie blue and therefore appear to be a minor component on our gels. Such a possibility will be investigated. Direct evidence supporting our proposition will await the completion of experiments designed to determine whether or not the purified 116K protein will specifically bind PV.

The coelution of the 116K and 100K proteins from the PV–agarose column by PV suggests that both proteins may bind PV or may both be part of a multisubunit complex. However, the results of our gel-exclusion chromatography experiments indicate that only the 116K protein actually binds PV. This suggests that the 116K and 100K proteins may associate with one another, but that the long time and infinite partitioning afforded by gel-exclusion chromatography allowed these two proteins to be separated from one another. Experiments to test this possibility are currently in progress.

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REFERENCES

1. Rodewald R: J Cell Biol 58:189, 1973.
2. Rodewald R: J Cell Biol 71:666, 1976.
3. Sonoda S, Schlamowitz M: J Immunol 108:1345, 1972.
4. Tsay DD, Schlamowitz M: J Immunol 115:939, 1975.
5. Roth TF, Cutting JA, Atlas SD: J Supramol Struct 4:527, 1976.
6. Jackson J, Roth TF: (in preparation).
7. Roth TF, Porter KR: J Cell Biol 20:313, 1964.
8. Telfer WH: J Biophys Biochem Cyto 9:747, 1961.
9. Melius ME, Telfer WH: J Morph 129:1, 1969.
10. Wallace RA, Dumont JN: J Cell Physiol 27(suppl):73, 1968.
11. Wallace RA, Jarad DW: J Cell Biol 69:345, 1976.

12. Schjeide OA, Wilkens M, McCandless RG, Munn R, Peterson M, Carlsen E: Am Zool 3:167–184, 1963.
13. Yusko SC, Roth TF: J Supramol Struct 4:89, 1976.
14. Anderson RGW, Goldstein JL, Brown MS: Proc Natl Acad Sci USA 73:2434, 1976.
15. Anderson RGW, Brown MS, Goldstein JL: Cell 10:351, 1977.
16. Gordon P, Carpenter J, Cohen S, Orci L: Proc Natl Acad Sci USA 75:5025, 1978.
17. Willingham MC, Maxfield FR, Pastan IH: J Cell Biol 82:614, 1979.
18. Woods JW, Roth TF: J Supramol Struct 12:491, 1979.
19. Yusko SC, Roth TF, Smith T: (submitted).
20. Krummins S, Roth TF: J Cell Sci (in press).
21. Perry MM, Gilbert AB, Evans AJ: J Anat 125:481, 1978.
22. Heuser J, Roth TF: (unpublished).
23. Laemmli UK: Nature 227:680, 1970.