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β-Adrenergic receptor regulation of *N*-linked protein glycosylation in rat parotid acinar cells

(glycoprotein/secretion/neurotransmitter control)

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ABSTRACT **We have investigated the relationship between β-adrenergic receptor stimulation and protein glycosylation and secretion in rat parotid gland cells *in vitro*. The potent β-adrenergic agonist (−)-isoproterenol increases [^{3}H]mannose incorporation into newly synthesized glycoproteins. This effect is enhanced if cells are first preincubated with dolichyl phosphate and is not observed after muscarinic-cholinergic or α-adrenergic stimulation of cells. The increase in [^{3}H]mannose incorporation is abolished by incubation of cells with tunicamycin, suggesting that the glycosylation events being studied involved asparagine-linked oligosaccharides. The extent of increase in glycosylation is dependent on the concentration of (−)-isoproterenol to which cells are exposed. (±)-Propanolol totally abolishes the (−)-isoproterenol-induced increase in [^{3}H]mannose incorporation, in a manner similar to its effects on exocrine secretion. Our findings suggest that β-adrenergic receptor activation has a profound influence on *N*-linked protein glycosylation in rat parotid cells in addition to eliciting exocrine protein release.**

The mechanistic steps required for *N*-linked secretory protein glycoslyation have received considerable attention in the last few years (1). It has been established that oligosaccharide addition, in the form of a polymannosylchitobiosyl unit, occurs via a dolichyl phosphate intermediate on the internal face of the rough endoplasmic reticulum (2–4). Nascent glycoproteins are then segregated in membranous vesicles and transported through the Golgi apparatus en route to their final extracellular destination (5–8). During this period of intracellular translocation prior to secretion, the polymannose oligosaccharide moieties are modified (processed) by the action of several glycosidases and glycosyltransferases, finally yielding "complex" oligosaccharide structures (9, 10). In addition to these mechanistic studies, many investigators have also examined the requirement for complete oligosaccharide addition and processing for proper glycoprotein secretion (11, 12). However, there has been very little study of events that may regulate the extent of *N*-linked protein glycosylation.

Accordingly, we have chosen to investigate possible mechanisms controlling *N*-linked protein glycosylation of secretory proteins. For this we have utilized dispersed rat parotid gland acinar cells *in vitro*. These cells provide a well-studied and convenient model for evaluating exocrine secretion (13). The secretion of saliva is regulated by several neurotransmitters. α-Adrenergic, muscarinic-cholinergic, as well as peptidergic receptors primarily mediate water and electrolyte movement, whereas the β-adrenergic receptor is responsible for most exocrine protein release (14, 15). Many of the proteins secreted from the serous parotid gland are glycoproteins and are thought to have important protective functions within the oral cavity (16).

Several events associated with β-adrenergic agonist induced protein exocytosis have been intensively studied with rat parotid cells *in vitro* (13, 15). Immediately after agonist stimulation of the β-adrenergic receptor, adenylate cyclase activity is increased, marked elevations of cyclic AMP are rapidly detected, cyclic AMP-dependent protein kinase is activated, and receptor-associated phosphorylation of specific parotid proteins occurs. A dramatic increase of protein secretion follows (17, 18).

Two important salivary gland cellular activities, related to protein exocytosis, are protein synthesis and protein processing. Surprisingly little is known about the influence of agents that regulate protein secretion on protein synthesis and protein processing. It was the specific purpose of this study to investigate the relationship between β-adrenergic receptor stimulation and protein glycosylation and secretion in rat parotid gland cells *in vitro*. Our results suggest that β-adrenergic receptor activation has a profound influence on *N*-linked protein glycosylation in these cells in addition to eliciting exocrine protein or glycoprotein release.

EXPERIMENTAL PROCEDURES

Materials. Male Wistar rats, 3 months of age, were purchased from Harlan Sprague–Dawley. All animals were allowed water and NIH-Purina laboratory chow ad lib. Collagenase (CLSPA lot no. S2C481) was purchased from Worthington. Bovine testicular hyalurondiase (type I-S), bovine serum albumin (fraction V), (−)-isoproterenol, (±)-propranolol HCl, (−)-epinephrine bitartrate, carbamoylcholine chloride, and dolichyl phosphate were purchased from Sigma. D-[2-^{3}H]Mannose (24.3 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) and L-[^{14}C]leucine (350 mCi/mmol) were obtained from Amersham. Ham's F12 medium was purchased from the National Institutes of Health media unit. Tunicamycin (lot no NCI-2) was a gift of the Natural Products Branch, National Cancer Institute.

Tissue Preparation. Rats were sacrificed between 9:30 and 10:00 a.m. Parotid glands were removed quickly, trimmed of connective tissue and fat, and minced finely. Enzymatically dispersed parotid cell aggregates were obtained in a fashion similar to that described (18, 19). Briefly, tissue from two rats was suspended in 10 ml of enzyme solution: collagenase (96 units/ml) and hyaluronidase (0.2 mg/ml) in Ham's F12 medium containing 0.56 mM glucose (low-glucose medium). Tissue was incubated in a metabolic shaker at 37°C for 60 min. Resulting cell aggregates were dispersed gently by pipetting and were gassed with 95% O_2/5% CO_2 at 20-min intervals during the incubation. The physiological and pharmacological responses of cell aggregates prepared in this manner have been well characterized (18–20). After enzyme digestion, cell aggregates were

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Table 1. Effect of neurotransmitters on rat parotid acinar cell protein production and glycosylation

Treatment	[^{14}C]Leucine incorporation		[^{3}H]Mannose incorporation		
	cpm/μg of DNA	% of control	cpm/μg of DNA	% of control	^{3}H/^{14}C
Control	38,142 ± 8,471	100	2,442 ± 512	100	0.064
Isoproterenol	50,367 ± 3,199	132	7,634 ± 3,311	337	0.152
Epinephrine	48,691 ± 8,232	128	5,960 ± 2,785	253	0.122
Carbamoylcholine	28,153 ± 6,725	74	2,674 ± 524	105	0.095

Data are the average ± SEM of three separate experiments (two rats per experiment).

washed three times with 5 ml of Ham's F12 low-glucose medium containing 0.022% bovine serum albumin and resuspended in fresh Ham's F12 medium, low-glucose or leucine-free, when [^{3}H]mannose or [^{14}C]leucine, respectively, was used.

Incubation Procedures. Cell aggregates were preincubated for 1 hr at 37°C in the presence or absence of dolichyl phosphate. Dolichyl phosphate supplementation was accomplished by dissolving the lipid in dimethyl sulfoxide prior to the incubation. The final concentration of dimethyl sulfoxide in all incubations was 0.01% (vol/vol). After the preincubation period the neurotransmitter (−)-isoproterenol, (−)-epinephrine bitartrate, or carbamoylcholine, in a final concentration of 10^{-5} M or varying concentrations for dose–response studies, was added simultaneously with either [^{3}H]mannose (25 μCi/ml) or [^{14}C]-leucine (2 μCi/ml). When the effect of tunicamycin on *N*-linked glycosylation was studied, the cell aggregates were preincubated in the presence of tunicamycin (1 μg/ml) for 15 min prior to adding neurotransmitter and [^{3}H]mannose. For β-adrenergic blocking experiments, cells were preincubated with 5×10^{-5} M (±)-propranolol prior to adding agonist and either [^{3}H]mannose or [^{14}C]leucine as above. In all experiments incubation was then continued for an additional 2 hr. At the end of each incubation, cells were separated from the medium by centrifugation (15 sec at 40 × *g*), medium was saved on ice, and the cells were washed three times with phosphate-buffered saline. Cells were homogenized with a Brinkman Polytron (setting 5,10 sec), and the incorporation of radiolabeled leucine or mannose into protein was determined as 10% CCl_3COOH-insoluble material in medium and cell homogenates separately. CCl_3COOH-insoluble material was collected on Millipore filters (HA, pore size = 0.45 μm), washed three times with 10% CCl_3COOH, and dissolved in 10 ml of Filtron X (National Diagnostics), and radioactivity was determined by liquid scintillation spectrometry.

Other Analytical Procedures. The DNA content of cellular homogenates was determined by the diphenylamine method as described by Richards (21).

RESULTS

Incubation of rat parotid cells with [^{3}H]mannose resulted in low levels of incorporation of the carbohydrate precursor into newly synthesized glycoprotein. However, if cells were exposed simultaneously to (−)-isoproterenol, a potent β-adrenergic agonist, marked enhancement of [^{3}H]mannose incorporation was observed (Table 1). When (−)-epinephrine, a mixed α- and β-adrenergic agonist, was present in the incubation medium, protein glycosylation was also elevated but not to the extent of that seen with (−)-isoproterenol. In contrast, cells exposed to carbamoylcholine, a muscarinic-cholinergic agonist, showed no such increase in [^{3}H]mannose incorporation.

In another experiment the affect of α-adrenergic receptor stimulation [10^{-5} M (−)-epinephrine and 5×10^{-5} M (±)-propranolol] on protein glycosylation was evaluated. No difference in the ratio of [^{3}H]mannose to [^{14}C]leucine incorporation in stimulated cells (0.083) compared to control cells (propranolol alone, 0.086) was observed. Stimulation at the β-adrenergic receptor also resulted in increased protein synthesis (as judged by [^{14}C]leucine incorporation into CCl_3COOH-insoluble material), but the effect was not as pronounced as that seen with mannose (Table 1, [^{3}H]mannose/[^{14}C]leucine ratios). When cells were supplemented with dolichyl phosphate, which previously has been shown to elevate basal levels of *N*-linked protein glycosylation without affecting glycoprotein secretion (22), a similar response pattern by parotid cells, to neurotransmitter stimulation, was observed (Fig. 1). However, the increase in [^{3}H]-mannose incorporation after (−)-isoproterenol stimulation was

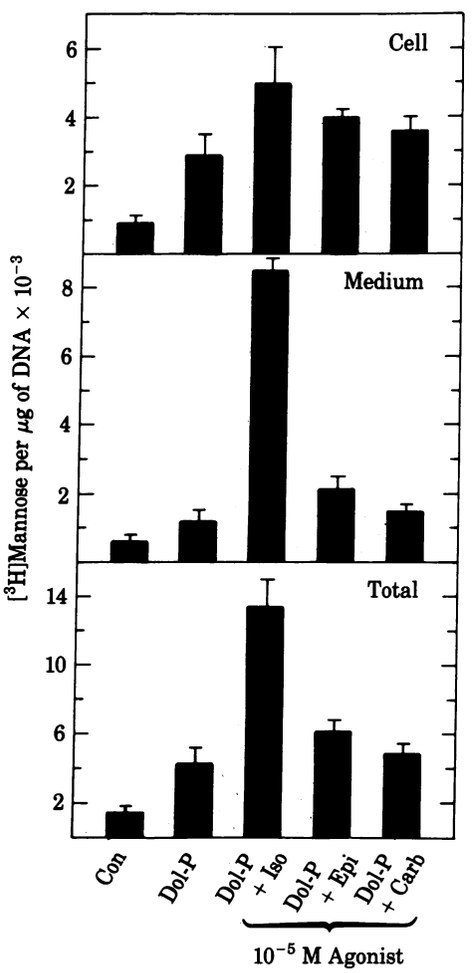

FIG. 1. Effect of neurotransmitter agonists on the incorporation of [^{3}H]mannose into newly synthesized glycoproteins of rat parotid acinar cells. Data are the average ± SEM of four experiments and represent CCl_3COOH-insoluble radioactivity in the incubation medium (secreted proteins) and cell homogenates. Con (control); Dol-P (dolichyl phosphate, 100 μg/ml); Iso (10^{-5} M isoproterenol); Epi (10^{-5} M epinephrine); Carb (10^{-5} M carbamoylcholine).

Table 2. Effect of isoproterenol on the extent of glycosylation of secreted and cellular proteins

Treatment	[^{3}H]Mannose/[^{14}C]leucine		
	Medium	Cell	Total
Dolichyl phosphate (100 μg/ml)	1.56 ± 0.46	0.085 ± 0.01	0.128 ± 0.02
+ (−)-Isoproterenol (10^{-5} M)	0.25 ± 0.03	0.304 ± 0.04	0.254 ± 0.02

Data are the average ± SEM of nine separate experiments, in which the % secretion of [^{14}C]leucine-labeled protein was 4.1 ± 0.7% for controls and 50.8 ± 6.5% for (−)-isoproterenol.

significantly greater than that seen in cells incubated without the polyprenyl phosphate. Therefore, for all subsequent studies parotid cells were preincubated with dolichyl phosphate.

An alternative explanation to the conclusion that β-adrenergic stimulation increases parotid protein glycosylation would be that activation of the β-adrenoreceptor stimulates glycoprotein release, which is accompanied by increased synthesis of secretory protein. If the secreted protein were more heavily glycosylated, the [^{3}H]mannose/[^{14}C]leucine ratio could increase indirectly. As is shown in Table 2, this is not the case. Under control conditions, secreted proteins are more heavily glycosylated than cellular proteins. However, with cells exposed to (−)-isoproterenol, secreted and cellular proteins show comparable levels of glycosylation *and*, importantly, the overall level of protein glycosylation (total: medium and cellular) is twice that of controls.

That the [^{3}H]mannose incorporation stimulated by (−)-isoproterenol was indeed reflective of elevated *N*-linked glycosylation was tested by incubating cells in the presence or absence of tunicamycin. As is shown in Fig. 2, tunicamycin at 1 μg/ml substantially blocks [^{3}H]mannose incorporation into parotid proteins. This concentration of tunicamycin inhibits *N*-linked protein glycosylation, [^{3}H]mannose incorporation, by 90% without any alteration in protein synthesis; [^{14}C]leucine incorporation is 100% of control levels (22).

The increase in [^{3}H]mannose incorporation due to (−)-isoproterenol was totally dependent on the concentration of β-adrenergic agonist present in the incubation medium (Fig. 3). [^{3}H]Mannose incorporation increased in a dose-dependent fashion, reaching maximal levels of about 3-fold basal values at 10^{-5} M agonist, whereas half-maximal incorporation occurred at about 10^{-7} M agonist. A further test that the (−)-isoproterenol effect was indeed mediated through the parotid β-adrenergic receptors was to evaluate the ability of a β-adrenergic antagonist to inhibit the (−)-isoproterenol-induced response. As shown in Table 3, (±)-propranolol completely inhibited the effects of β-adrenergic agonist stimulation on [^{3}H]mannose and [^{14}C]leucine incorporation. The incorporation of both isotopic precursors into newly synthesized protein was reduced to basal levels as was the [^{3}H]mannose/[^{14}C]leucine ratio. Also the effect of (±)-propranolol on protein glycosylation was similar to the effect of the β-adrenergic antagonist on protein secretion (Fig. 4), decreasing (−)-isoproterenol induced exocytosis by about 80%.

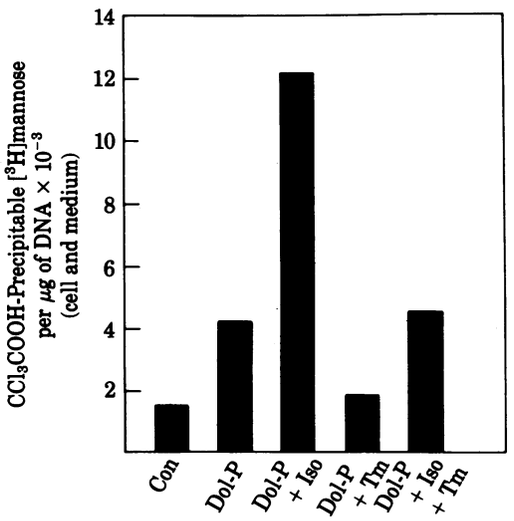

FIG. 2. Effect of tunicamycin (Tm, 1 μg/ml) on dolichyl phosphate (Dol-P, 100 μg/ml)- and dolichyl phosphate (100 μg/ml) with (−)-isoproterenol (Iso, 10^{-5} M)-stimulated elevation of [^{3}H]mannose incorporation into newly synthesized rat parotid glycoproteins. Data are the average of two experiments and represent total (cell and medium) CCl_3COOH-insoluble radioactivity.

DISCUSSION

The data presented in this communication strongly support the hypothesis that β-adrenergic stimulation of rat parotid gland protein secretion also has profound, direct effects on *N*-linked protein glycosylation in these cells. (−)-Isoproterenol substantially increased [^{3}H]mannose incorporation into newly synthesized glycoprotein. The effect appeared to be specific to the pure β-adrenergic agonist because studies on modulation of [^{3}H]mannose and [^{14}C]leucine incorporation into parotid proteins by an α-adrenergic stimulus (epinephrine with propranolol) and a muscarinic-cholinergic agonist (carbamoylcholine) show a different pattern of response from that of (−)-isoproterenol. Indeed this is not surprising because distinct signal transduction mechanisms would be involved. In the parotid gland, stimulation of α_1-adrenergic and muscarinic-cholinergic receptors would elicit responses mediated by changes in Ca^{2+} mobilization (13, 23, 24), whereas binding to α_2-receptors (which are about equivalent in number to the α_1-receptor in this tissue;

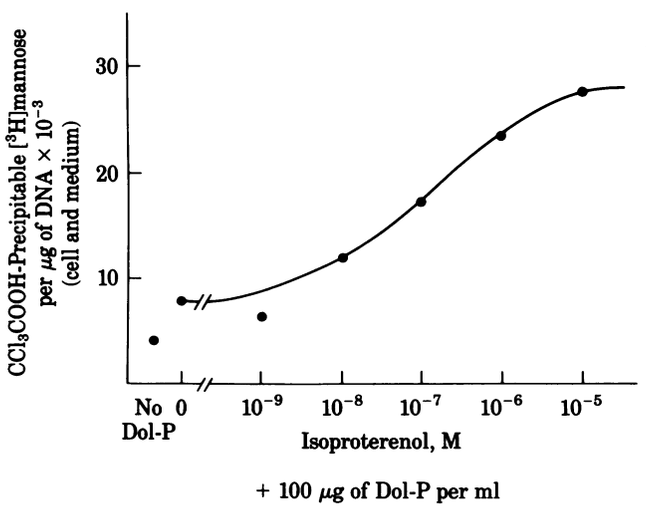

FIG. 3. Effect of various concentrations of (−)-isoproterenol on [^{3}H]mannose incorporation, in the presence of 100 μg of dolichyl phosphate (Dol-P) per ml, into newly synthesized rat parotid glycoproteins. Data are the average of two experiments and represent total (cell and medium) CCl_3COOH-insoluble radioactivity.

Table 3. Effect of (±)-propranolol on isoproterenol-induced alterations in rat parotid cell protein production and glycosylation

Treatment	[^{14}C]Leucine incorporation, cpm/μg of DNA	[^{3}H]Mannose incorporation, cpm/μg of DNA	^{3}H/^{14}C
Control	34,380 ± 2,058	2,084 ± 79	0.061
Dolichyl phosphate (100 μg/ml)	32,890 ± 1,856	5,608 ± 628	0.171
+ (−)-Isoproterenol (10^{-5} M)	48,862 ± 5,605	13,314 ± 1,449	0.273
+ (±)-Propranolol (5×10^{-5} M)	33,540 ± 3,958	4,659 ± 2,336	0.139

Data are the average ± SEM of three separate experiments (two rats per experiment).

refs. 19 and 20) could inhibit β-adrenergic increases in adenylate cyclase (25). (−)-Epinephrine, a mixed α- and β-adrenergic agonist, also increased protein glycosylation but to a lesser extent than (−)-isoproterenol. This order of potency is comparable to the ability of these agonists to elicit cyclic AMP production and amylase release in these cells (17).

This line of reasoning would suggest that the effects on protein glycosylation seen in this study are likely mediated by a cyclic AMP-related mechanism. Consistent with this suggestion is our observation that the enhanced glycosylation is dependent on the concentration of (−)-isoproterenol to which the cells were exposed and that half-maximal responses occurred at an agonist concentration (10^{-7} M) in reasonable agreement with the activation of parotid cyclic AMP-dependent protein kinase, (−)-isoproterenol-induced protein phosphorylation, and exocrine protein secretion (18). Furthermore, the effect is totally abolished by the β-adrenergic antagonist (±)-propranolol in a fashion consistent with its inhibition of cyclic AMP-related events in these cells (17, 18, 26).

Although β-adrenergic-induced enhancement of *N*-linked protein glycosylation was observed in cells preincubated both with or without dolichyl phosphate, the total amount of [^{3}H]-mannose incorporated was doubled when polyprenyl phosphate supplementation was performed. Such an observation supports the possibility that dolichyl phosphate levels may be an important limiting factor in *N*-linked protein glycosylation. This notion has previously been suggested by Carson *et al.* (27).

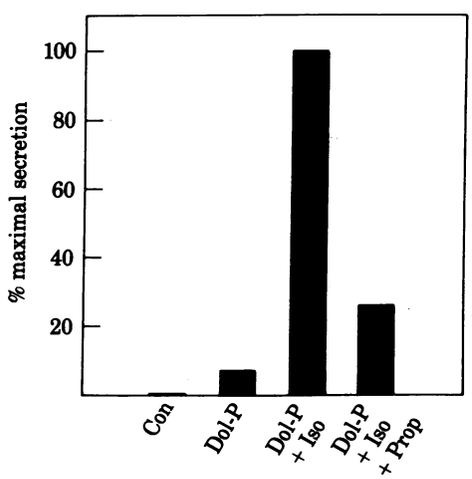

FIG. 4. Effect of (±)-propranolol (Prop, 5×10^{-5} M) on (−)-isoproterenol (Iso, 10^{-5} M)-induced secretion of [^{3}H]mannose-labeled glycoproteins from rat parotid acinar cells. Data are the average of three experiments. Secretion was calculated as 100× (CCl_3COOH-insoluble radioactivity in medium/CCl_3COOH-insoluble radioactivity in cell and medium). Control (Con) secretion for the 2-hr incubation period (37%) was subtracted from data of experimental conditions. Maximal secretion (100%) represents net secretion from (−)-isoproterenol stimulation (74%) minus control levels. Dol-P (100 μg of dolichyl phosphate per ml).

It is interesting that although dolichyl phosphate supplementation alone elevates [^{3}H]mannose incorporation in parotid cells, it does not appear to increase protein secretion (Figs. 1 and 4 and ref. 22), whereas, with (−)-isoproterenol, substantial effects are observed in both responses. This suggests that β-adrenergic stimulation may act separately from, or in addition to, polyprenyl phosphate availability.

Indeed the mechanism(s) by which rat parotid cells have increased *N*-linked glycosylation in response to β-adrenergic stimulation remains obscure. Studies of both [^{3}H]mannose and [^{14}C]leucine incorporation suggest that (−)-isoproterenol increased protein synthesis but elevated glycosylation substantially beyond that predicted based on merely increased protein levels—i.e., the [^{3}H]mannose/[^{14}C]leucine ratio is increased greatly over controls (Tables 1–3). Thus, although some increase in [^{3}H]mannose incorporation may reflect production of new glycoproteins, much of the increase appears to represent glycosylation of new asparagine sites or increased sugar addition to oligosaccharides at the same asparagine sites. An additional explanation of our data would be that β-adrenergic stimulation altered precursor pool sizes or turnover resulting in increased [^{3}H]mannose specific activity. Although we have no data at present to directly address this possibility, we have demonstrated that (−)-isoproterenol has no effect on sugar transport in these cells (unpublished observations).

Although there has been considerable advancement over the last several years in understanding the mechanism of *N*-linked protein glycosylation (1), as noted earlier, little progress has been made in understanding the regulatory aspects of these processes. Our data clearly demonstrate that activation of the β-adrenergic receptor can greatly influence *N*-linked protein glycosylation. Because in the parotid gland this receptor is linked to the adenylate cyclase system, it is tempting to suggest the possibility of a generalized cyclic AMP-dependent regulatory site in *N*-linked carbohydrate processing, which could be mediated through a variety of receptor types (e.g., parathyroid hormone, prostaglandin E, dopamine), all coupled to adenylate cyclase.

Some of the ideas for these studies were generated during a leave spent by one of us (B.J.B.) in the laboratory of Dr. W. J. Lennarz at The Johns Hopkins University School of Medicine. We wish to thank Dr. Lennarz for his encouragement in this work and many helpful comments during this study and on this manuscript.

1. Lennarz, W. J. (1980) *The Biochemistry of Glycoproteins and Proteoglycans* (Plenum, New York).
2. Hanover, J. A. & Lennarz, W. J. (1980) *J. Biol. Chem.* **255**, 3600–3604.
3. Hanover, J. A. & Lennarz, W. J. (1982) *J. Biol. Chem.* **257**, 2787–2794.
4. Das, R. C. & Heath, E. C. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3811–3815.
5. Palade, G. E. (1976) *Science* **189**, 347–358.
6. Bielinska, M. & Boime, I. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 1208–1212.

7. Strous, G. J. A. M. & Lodish, H. F. (1980) *Cell* **22**, 709–717.
8. Sabatini, D. D., Kriebich, G., Morimoto, T. & Adesnik, M. (1982) *J. Cell Biol.* **92**, 1–22.
9. Tabas, I. & Kornfeld, S. (1979) *J. Biol. Chem.* **254**, 11655–11663.
10. Tulsiani, D. R. P., Hubbard, S. C., Robbins, P. W. & Touster, O. (1982) *J. Biol. Chem.* **257**, 3660–3668.
11. Hickman, S. & Kornfeld, S. (1978) *J. Immunol.* **121**, 990–996.
12. Struck, D. K., Siuta, P. B., Lane, M. D. & Lennarz, W. J. (1978) *J. Biol. Chem.* **253**, 5332–5337.
13. Butcher, F. R. & Putney, J. W., Jr. (1980) *Adv. Cyclic Nucleotide Res.* **13**, 215–249.
14. Young, J. A. & van Lennep, E. W. (1979) in *Membrane Transport in Biology*, eds. Giebish, G., Tosteson, D. C. & Ussing, H. H. (Springer, Berlin), Vol. 4, pp. 563–674.
15. Baum, B. J., Ito, H. & Roth, G. S. (1983) in *Adrenoreceptors and Catecholamine Action—Part B*, ed. Kunos, G. (Wiley, New York), pp. 265–294.
16. Mandel, I. D. (1980) *CRC Crit. Rev. Clin. Lab. Sci.* **12**, 321–366.
17. Butcher, F. R., Goldman, J. A. & Nemerovski, M. (1975) *Biochim. Biophys. Acta* **392**, 82–91.
18. Baum, B. J., Freiberg, J. M., Ito, H., Roth, G. S. & Filburn, C. R. (1981) *J. Biol. Chem.* **256**, 9731–9736.
19. Ito, H., Baum, B. J., Uchida, T., Hoopes, M. T., Bodner, L. & Roth, G. S. (1982) *J. Biol. Chem.* **257**, 9532–9538.
20. Ito, H., Hoopes, M., Baum, B. J. & Roth, G. S. (1982) *Biochem. Pharmacol.* **31**, 567–573.
21. Richards, G. M. (1974) *Anal. Biochem.* **57**, 369–376.
22. Kousvelari, E. E., Grant, S. R. & Baum, B. J. *Exp. Cell Res.*, in press.
23. Putney, J. W., Jr. (1976) *J. Pharmacol. Exp. Ther.* **198**, 375–384.
24. Putney, J. W., Jr. (1977) *J. Physiol. (London)* **268**, 139–149.
25. Oron, Y., Kellogg, J. & Larner, J. (1978) *FEBS Lett.* **94**, 331–334.
26. Ito, H., Baum, B. J. & Roth, G. S. (1981) *Mech. Ageing Dev.* **15**, 177–188.
27. Carson, D. D., Earles, B. J. & Lennarz, W. J. (1981) *J. Biol. Chem.* **256**, 11552–11557.