Stimulated Secretion of Lysosomal Enzymes by Cells in Culture*

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Leonard Warren
From the Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104

F9 mouse teratocarcinoma and PyS-2 cells in culture incubated with monovalent cations in buffered sucrose solution (0.25 M) can secrete as much as 40% of their total lysosomal enzymes into the medium within 30 min. Longer incubation does not lead to further loss of enzyme, suggesting that only a certain fraction of lysosomes is capable of discharge. The simultaneous presence of sucrose and cation, each at the respective optimal concentrations of 0.25 and 0.15 M, is required for lysosomal discharge (i.e., twice isoosmolarity). The cells remain fully viable. Sodium ions are more effective than lithium and potassium ions, whereas amines and divalent cations are less effective. Other sugars including glucose can replace sucrose to varying extents.

Secretion is accompanied by a rapid short-lived rise in the level of cAMP. Forskolin as well as agents that activate G protein such as cholera toxin, AlF₃, and vanadate ions also increase the rate of secretion. Sucrose-Na⁺ stimulation takes place independently of changes in influx or efflux of calcium ions or changes in the levels of extracellular or free intracellular calcium ions. Neomycin, an inhibitor of phospholipase C, has little effect on secretion. Our results suggest that the secretion observed is mediated by a cAMP-dependent mechanism involving G proteins. Calcium ions and phospholipase C appear to play little or no part in the activation process.

Lysozymes, found both intracellularly and extracellularly, are capable of degrading lipids, carbohydrates, proteins, and nucleic acids. These enzymes play an important role in normal metabolic turnover, in the degradation of hormones and other molecules, and in defense against infection.

Secretion is generally quite slow in culture, especially as cells reach confluence, and only a small percentage of the total lysosomal enzyme content is lost from the cell each day (1). The rate of enzyme release from the cell can be increased moderately by a variety of agents, including weak bases such as aliphatic amines and NH₄Cl, the extent of increase depending on the concentration of the agent and on the cell type (1-5). The agents appear to exert their effect after being disrupted in the lysosomes (6) and prelysosomal compartments where they elevate the pH, enhance binding of hydrolyses to receptors, inhibit receptor utilization, and thereby disrupt the intracellular pathway for transport of acid hydrolases. Newly synthesized enzymes may be directly secreted (7). Sucrose stimulates secretion in organ culture of bone and cartilage (8), and sucrose together with NH₄ ions stimulate secretion in human fibroblasts (9). Release of lysosomal enzymes can also be effected by exposure of cells to immune complexes (10-12), zymosan, small fiber asbestos (13), and dextran sulfate (14).

In the present study it is shown that inorganic monovalent cations play an essential role in the secretory process since cells in culture, not specialized to secrete, bathed in 0.25 M sucrose solution and exposed to inorganic cations, such as Na⁺, can secrete more than a quarter of their lysosomal enzymes within 30 min without loss of viability. This rapid rate of secretion has facilitated the study of factors involved in the process and is somewhat analogous to the explosive exocytosis of granules from mast cells (15) and cortical granules from sea urchin eggs (16). The complications of uptake, synthesis, and degradation of lysosomal enzymes in studies lasting hours and days have been eliminated. By using this system, it has been shown that the secretion of lysosomal enzymes is dependent on cAMP and G protein function and is unaffected by shifts in concentration of intracellular calcium ions or on the activity of phospholipase C.

While the underlying stimulatory process of the sucrose-Na⁺ procedure is not fully understood, it provides a convenient method to study a secretory mechanism, applicable to many kinds of normal and pathological cells.

**EXPERIMENTAL PROCEDURES**

*Materials—All chemicals used were of reagent grade. Sodium iodocetate was from Aldrich. The following compounds were obtained from Sigma: para-nitrophenyl derivatives of phosphate, α- and β-D-galactose, α-fucose, α-D-mannose, and β-D-N-acetylglucosamine, sodium orthovanadate, trifluoperazine-2 HCI, promethazine-HCl, chlorpromazine-HCl, Ca⁺⁺ ionophore A23187, N-methylmaleimide, sodium orthovanadate, and all nucleotides including dibutyryl cAMP and β-nicotinamide adenine dinucleotide. Cholera and pertussis toxins were from List Biological Laboratories, Inc. (Campbell, CA); forskolin and 1,9-dideoxyforskolin and ionomycin were from Behring Diagnostics (Fors-2AM was from Molecular Probes, Inc. (Eugene, OR); and Ca⁺⁺Cl₂ was from ICN (Irvine, CA).

*Cell Culture—F9, an established cell line derived from a transplantable mouse teratocarcinoma, and PyS-2, a stable line of nontumorigenic differentiated cells derived from the same tumor (17), CSAT, a mouse hybridoma, BHK21/C13, C13/B4, WI, K16, 2T7D, and KB/B3T3 cells were all grown at 37 °C in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (Hazleton Research Products, Denver, PA), 50 μg/ml penicillin and 100 μg/ml streptomycin. Cultures were routinely checked for mycoplasma by agar plate cultivation and DNA staining every 6 weeks and were discarded after 15-20 doublings.

*Standard Assay—5 x 10⁶ cells in 3 ml of medium were grown in 35-mm plastic dishes (Costar) for 48 h at which time the cell number had increased to 2-3 x 10⁷. All experiments were carried out in duplicate and in some cases in triplicate. Each plate was washed three times with 3 ml of TM Sucrose (0.15 M NaCl, 0.25 M sucrose) and drained, and 0.5 ml of incubation mixture (TM Sucrose or TM Sucrose, 0.15 M NaCl with or without special factors) was added to each plate. Plates were incubated for 30 min at 37 °C on a rocker platform (Belco Glass Co., Vineland, NJ).
moving at 8 cycles/min. The bathing fluids were transferred to 1.5-
lml plastic tubes with pointed bottoms and centrifuged for 3 min in an
Eppendorf centrifuge (13,500 × g), and the supernatants were
removed. In order to determine the total enzyme activity of the
system, cells were scraped off the dishes into 0.5 ml of TM Sucrose.
Cells were transferred to plastic tubes containing pellets from cen-
trifugation of the incubation medium. Plates were then washed
with 0.25 ml of TM Sucrose and the second harvest of cells was transferred
to the first. The cell suspension was homogenized in the plastic tube with
30 vigorous strokes using a pointed Teflon pestle.
In experiments with hybridomas or human granulocytes, cells were suspended in 0.3 ml of TM Sucrose with or without 0.15 M NaCl.
After incubation, tubes were centrifuged for 5 min at 1000 × g, the
supernatants were withdrawn, and 0.3 ml of TM Sucrose was added
to the cell pellet. Cells were subjected to freezing and thawing three
times and sonication for 30 seconds in a Branson Ultrasonic Cleaner.

**Assay for Acid Hydrolases**—The homogenate and supernatant were assayed for N-acetyl-β-D-glucosaminidase and other acid hydrolases using a para-nitrophenyl glycoside substrate. The assay vessel contained 20 mM sodium citrate buffer, pH 4.8, 3 mg of p-nitrophenyl glycoside (or phosphate), and enzyme (100 μl of supernatant or 10 μl of homogenate) in a final volume of 200 μl. After 1.5 h at 37°C, 3 ml of a solution containing 0.3 M NaCl, 0.15 M K2CO3 were added. Tubes were centrifuged at 1000 × g for 5 min, and absorbancies at 400 nm were determined. The percent of the total activity secreted by the cells of each 35-mm dish was calculated from the activities in the supernatants and homogenate. Sodium chloride (0.15 M) and sucrose (0.25 M) added to the assay vessels had no significant effect on enzyme activity (n = 5). Protein was determined by the Lowry method (18).

**Protease** was measured by the extent of solubilization of radioactive material from [14C]hemoglobin. Substrate was labeled by exposure to [14C]formaldehyde followed by reduction with NaBH₄ (19). Lactate dehydrogenase in cell incubation medium and homogenate was measured by the loss of absorbance at 340 nm of NADH upon addition of sodium pyruvate (20).

**Cytosolic Ca2+** was measured in cells grown on coverslips (11 × 22 mm) incubated for 20 min at 37°C in medium containing fura-2AM (5 mM). The coverslip was then washed three times with TM Sucrose and inserted into a holder adapted for a 1-cm cuvette containing stirred TM Sucrose (37°C). The cuvette with the diagonally positioned coverslip was placed in a Spex Fluorolog 1681 0.22m Spectrometry Laboratory Coordinator from the ratios of fluorescence excited by 340 nm to that excited by 380 nm. Calcium ion concentrations were calculated by a DMIB Spectroscopy Laboratory Coordinator from the ratios of fluorescence excited by 340 nm to that excited by 380 nm. Calcium ion concentrations were determined using calibration measurements made in the presence of 2 mM ionomycin and 65 mM EGTA (pH 8.3 (21, 22).

**Efflux Studies**—Cells in each well of a Costar dish (24 wells) were incubated for 15 h in 1.5 ml of calcium-free medium containing 1
mCi of [35S]sodium. Cells were washed twice with 2 ml of TM Sucrose,
and in triplicate wells 1 ml each of TM Sucrose/CaCl₂ (0.15 M), TM Sucrose/NaCl (0.15 M), or TM Sucrose/NaCl (0.15 M), was added. At given times, radioactivity in 0.1-ml aliquots of the medium was determined.

Cyclic AMP was measured in cells growing in 35-mm plastic dishes by radioimmunoassay. Cells were rapidly washed three times with 2 ml of TM Sucrose. One ml of TM Sucrose or TM Sucrose containing NaCl (0.15 M) was added. After incubation, the medium was removed and discarded and 1 ml of either 5% trichloroacetic acid or 0.1 M HCl was added to extract cAMP (23). After 10 min at room temperature, extracts were transferred to a series of tubes. Trichloroacetic acid was removed by three extractions with water-saturated ether (5, 5, and 3 ml), and residual ether was removed by heating at 50°C for 20 min (23). cAMP was assayed with a radioimmunoassay kit purchased from Amersham. Determinations at each time point were carried out in duplicate, and each extract was assayed in duplicate.

**RESULTS**

In the presence of 0.25 M sucrose solution, secretion by F9 cells increased with increasing concentrations of sodium ions, and maximum secretion was observed at approximately 150

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1 The abbreviation used is: EGTA, [ethylenebis(oxyethylenedi-
nitro)nitrilo]tetraacetic acid.
nigrosin was low and about the same as before the cells were washed with TM Sucrose (<3%). The level of protein in the medium remained constant (Figs. 1 and 2), and the cytosolic marker enzyme, lactic acid dehydrogenase which was found in small amounts in the bathing medium, did not increase with time (Fig. 2). The level remained independent of the secretory behavior of the cell (TM Sucrose, 3.7 ± 1.3%; TM Sucrose, 0.15 M NaCl, 3.3 ± 1.6%; n = 18).

Under the phase contrast microscope (× 250), PyS-2 and F9 cells in TM Sucrose or TM Sucrose/NaCl solution had slightly sharper and brighter edges, shrinking slightly and pulling away from each other. They remained attached and presented a cobblestone appearance. Granularity or vacuolization was not evident in cells bathed in either TM Sucrose or TM Sucrose/NaCl. Within 2 h of their return to medium, these cells were indistinguishable from control cells that had remained in culture medium. After 20 h, all cells were heavily confluent. WM-9, a cell which did not secrete, underwent the same series of changes.

To test the viability of cells in the assay, an experiment was carried out under sterile conditions in which vessels were assayed as usual and the number of cells counted. The experimental bathing medium of a second set of dishes was removed, culture medium was added, and the dishes were incubated a further 20 h when the cells were assayed and counted. A third set of plates was not manipulated the first day but was assayed, and the cells were counted on the second day. After four experiments it was found that cells fully retained their ability to divide and to secrete lysosomal enzymes 20 h after the first secretory discharge, indicating that washing and incubating the cells did not result in cell death. However, the level of N-acetyl-β-D-glucosaminidase in cells that had secreted 24 h previously was only half that of controls. Despite this, the cells responded fully to the sucrose-Na⁺ stimulus on the second day, suggesting that they were capable of converting undischarged lysosomes of the first day to a form capable of responding to a secretory stimulus on the second day.

**Other Cations**—Sodium ions were considerably more effective than K⁺ or Li⁺ in stimulating N-acetyl-β-D-glucosaminidase secretion (Fig. 3). Ca²⁺, Mg²⁺, and NH₄⁺ ions, at lower concentrations, resulted in a maximum secretion of 5% of the total enzyme in 30 min. Krebs solution, a physiological salt solution (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 25 mM NaHCO₃), was no more effective in stimulating N-acetyl-β-D-glucosaminidase secretion than Na⁺ alone. Spermine at a concentration of 5 mM was more effective than Ca²⁺, Mg²⁺, or NH₄⁺, but led to only 8% secretion in 30 min. Maximum secretion occurred when the medium was pH 7.0. Secretory activity was reduced by 20% at pH 6.8 and 7.4.

**Requirement for Sucrose or Other Sugars**—The simultaneous presence of sucrose and cation was required for maximum N-acetyl-β-D-glucosaminidase secretion (Fig. 4). In the presence of 0.15 M NaCl, the rate of secretion increased with increasing concentrations of sucrose to a maximum at 0.25 M (isosmolar), and higher concentrations of sucrose (0.4 or 0.5 M) did not increase the rate further (data not shown). The optimal combined concentration of sucrose and NaCl is, therefore, two times isosmolar. Incubation media were tested containing various proportions of sucrose and NaCl whose combined concentration was isosmolar. Secretion took place but was always less than 50% of maximum (data not shown).

Cells (F9 and PyS-2) were washed with solutions of various concentrations of sucrose in the presence of 0.15 M NaCl for the times indicated. Only N-acetyl-β-D-glucosaminidase was measured in the absence of sucrose. Assays for other enzymes—N-acetyl-α-D-glucosaminidase; 0, protein; a, N-acetyl-β-D-glucosaminidase; c, protein; b, lactic acid dehydrogenase; c, N-acetyl-β-D-glucosaminidase, cations absent.

**FIG. 2.** Rate and extent of secretion of lysosomal enzymes. The standard secretion assay described under "Experimental Procedures" was used. F9 cells were incubated in TM Sucrose with and without 0.15 M NaCl for the times indicated. Only N-acetyl-β-D-glucosaminidase was measured in the absence of sucrose. Assays for other enzymes—N-acetyl-α-D-glucosaminidase; 0, protein; a, N-acetyl-β-D-glucosaminidase; c, protein; b, lactic acid dehydrogenase; c, N-acetyl-β-D-glucosaminidase, cations absent.

**FIG. 3.** Ability of various monovalent cations to stimulate N-acetyl-β-D-glucosaminidase (NAGA) secretion by F9 cells. Assay method is described in the text. [Ma NaCl; •, LiCl; C, KCl].

**FIG. 4.** Simultaneous requirement for sodium ions and sucrose. a, cells washed with TM Sucrose; b, cells washed with 10 mM Tris chloride buffer, 0.15 M NaCl, pH 7.1. The standard assay for secretion of N-acetyl-β-D-glucosaminidase (NAGA), described under "Experimental Procedures," was used. S, 0.25 M sucrose; Na, 0.15 M NaCl.
sugars (0.25 M in 10 mM Tris-Cl, pH 7.2, 1 mM MgCl₂) and then incubated for 30 min in the presence or absence of 0.15 M NaCl. The combination of cation and sugars listed in Table I resulted in very active stimulation. Disaccharides such as sucrose, lactose, and cellobiose as well as trisaccharides (melizitose and raffinose) were more effective than glucose. Glycerol, D-xylose, D-mannose, and D-galactose could not substitute for sucrose (data not shown).

**Sensitivity of Enzyme Secretion to Temperature and Metabolic Inhibitors**—The loss of enzymes from F9 cells in the sucrose-Na⁺ system is temperature-dependent as in human fibroblasts growing in culture medium (24). At 22 °C N-acetyl-β-D-glucosaminidase was secreted at approximately half the rate than at 38 °C. Secretion was greatly but not completely inhibited by metabolic inhibitors such as cyanide and azide (5 mM) which inhibited 60%. Sulfhydryl blocking agents such as sodium iodoacetamide (1 mM), sodium iodoacetate (10 mM), and N-ethylmaleimide (5 mM) inhibited approximately 50%. Higher concentrations of inhibitors did not increase inhibition.

**Effect of State of Growth of F9 Cells on Enzyme Secretion**—Among experiments, there was considerable variation from week to week in the maximal percent of total enzyme secreted in 30 min by F9 and PyS-2 cells, although results within each experiment (i.e. within each set of cells) were always consistent. The basis for the variability is unknown. To determine whether the extent of secretion might be influenced by the state of growth of the cell, several dishes of F9 cells were plated, and a set of dishes was assayed in TM Sucrose in the presence or absence of 0.15 M NaCl on each of 4 days. Secretion was highest 48 h after plating, during early log phase. Thereafter, the response to sucrose-Na⁺ declined (later log and plateau phase) (Fig. 1). Based on these results, cells were routinely seeded at 5 × 10⁵ cells/35-mm plastic dish (5.2 × 10⁹ cells/cm²), and the experiment was carried out after 48 h when there were approximately 2–3 × 10⁹ cells per dish. Cells plated at 2 × 10⁶ per dish secreted 25% less enzyme at 48 h after plating than after 24 h.

**Effects of Other Agents on Enzyme Secretion**—Ouabain produced only small variable effects, whereas valinomycin were routinely seeded at 5 x 10⁻⁶ M and vinblastine sulfate for 4 h when there were approximately 1 × 10⁶ cells per dish. Man- nose 6-phosphate and glucose 1- or 6-phosphate (10 mM) had no effect on the secretion of lysosomal enzymes in the standard assay in the presence or absence of 0.15 M NaCl. Secretion

### Table I

**Requirements for sugars in secretion**

| F9 cells | PyS-2 cells |
|----------|-------------|
| -NaCl    | +NaCl       | -NaCl  | +NaCl  |
| None     | 5.1         | 5.9    |        |
| Sucrose  | 2.3         | 5.0    | 35.9   |
| Lactose  | 2.1         | 8.6    | 46.1   |
| Melizitose | 3.3        | 19.3   | 9.7    |
| Raffinose| 2.2         | 27.6   | 7.9    |
| Cellobiose| 2.2        | 27.6   | 4.1    |
| Glucose  | 3.2         | 11.2   | 3.6    |

by F9 cells was not altered when 10% heat-inactivated horse serum (37 °C, 4 h, pH 10) was included in the assay medium. Secretion by human fibroblasts in culture is also unaffected by serum (24).

**Enzyme Secretion as a Function of Cell Type**—Like F9 cells, PyS-2, a mouse parietal yolk sac cell derived by differentiation of a teratocarcinoma cell (17), secreted 15 to 30% of its lysosomal enzyme in the standard assay in the presence or absence of 0.15 M NaCl. Secretion

### Table II

**N-acetyl-β-D-glucosaminidase by various cell types**

| Cell                  | -NaCl | +NaCl |
|-----------------------|-------|-------|
| BHK21/C13             | 2.1   | 11.5  |
| CI3/B4                | 1.6   | 7.5   |
| 3T3D                  | 1.6   | 5.1   |
| KiBalg3T3             | 2.1   | 21.1  |
| Human granulocytes    | 2.4   | 12.7  |
| Human fibroblasts     | 4.2   | 16.2  |
| Bovine aortic endothelium | 5.5  | 11.9  |
| K16 (rat liver)       | 1.9   | 8.5   |
| W8 (rat liver)        | 2.9   | 7.1   |
| Hybridoma (CSAT)      | 1.3   | 8.9   |

of a teratocarcinoma cell (17), secreted 15 to 30% of its lysosomal enzyme in 30 min in a sucrose-Na⁺ system. Table II gives the percentages of total N-acetyl-β-D-glucosaminidase (NAGA) was assayed in duplicate dishes in either 3 ml of TM Sucrose (B) or TM Sucrose, 0.15 M NaCl (C). Total cell protein per dish on each day was also determined (4). In each experiment, the cell number per dish on days 3 and 4 was 6 × 10⁶.

**FIG. 5. Effect of growth state on responsiveness of cells to sucrose-Na⁺ stimulation.** F9 cells were plated in a series of 35-mm dishes (5 × 10⁶ cells, 3 ml of medium) and after 1, 2, 3, and 4 days, the secretion of N-acetyl-β-D-glucosaminidase (NAGA) was assayed in duplicate dishes in either 3 ml of TM Sucrose (B) or TM Sucrose, 0.15 M NaCl (C). Total cell protein per dish of each day was also determined (4). In each experiment, the number of cells per dish on days 3 and 4 was 6 × 10⁶.

**Non-involvement of Calcium Ions in Enzyme Secretion**—An important early step in several exocytotic processes is a rise in intracellular calcium ions (16). To determine whether calcium played a role in our system, we analyzed the rate of enzyme secretion by cells incubated with the calcium iono-
phores ionomycin or A23187 (1-25 μM) in the presence or absence of CaCl₂ (0.2-3.0 mM). In the presence of calcium ions and ionomycin, the rate of secretion by PyS-2 cells was within 5.9% of control rates (n = 3). The corresponding difference in the presence of A23187 was 9.7% (n = 5) for PyS-2 cells and 2.0% for F9 cells (n = 2).

The rates of uptake of ⁴⁶Ca into F9 and PyS-2 cells, bathed in TM Sucrose or TM Sucrose/NaCl (0.15 mM), were found to be similar (<15% difference over 30 min). Sodium ions also had little effect on the rates of efflux of ⁴⁶Ca from these cells.

Free intracellular calcium ion concentrations were measured in F9 and PyS-2 cells growing as monolayers on coverslips, by double-beam spectrophotometry using fura-2AM (21, 22). Levels were measured in TM Sucrose before and after addition of NaCl (0.15 mM) and CaCl₂ (0.5 mM). Upon addition of NaCl, a relatively small short-lived increase in the level of calcium ions occurred, lasting no more than 1 min followed by a decline, sometimes to a level slightly below the original (Fig. 6A). This calcium must have been derived from intracellular stores. Addition of CaCl₂ (0.2-0.5 mM) in the presence or absence of NaCl caused a far greater increase in cytosolic Ca²⁺ levels that persisted for at least 5 min (Fig. 6). Thus, no

![FIG. 6. Effect of extracellular sodium and calcium ions on cytosolic calcium ion levels. Method of determination of cytosolic calcium ion levels is described under "Experimental Procedures." A, PyS-2 cells. Arrow 1 indicates addition of NaCl (0.15 mM); arrow 2 indicates addition of CaCl₂ (0.5 mM). B, F9 cells. Arrow 1 indicates addition of CaCl₂ (0.5 mM); arrow 2 indicates addition of NaCl (0.15 mM).](image)

**TABLE III**

Effect of phenothiazines and TMB-8 on secretion of N-acetyl-β-D-glucosaminidase by F9 cells

The standard assay for secretion of N-acetyl-β-D-glucosaminidase (NAGA) is described under "Experimental Procedures." All vessels contained TM Sucrose without (None) or with the additions indicated, in a final volume of 0.5 ml. db dibutyryl PyS-2 cells were used in experiment A.

| Additions | F9 | PyS-2 |
|-----------|----|--------|
| None      | 3.5 ± 0.7 |   |
| 100 μM db cAMP | 4.0 ± 0.5 |   |
| NaCl 150 μM | 5.1 ± 0.8 |   |
| NaCl 150 μM | 20.9 ± 1.8 |   |
| NaCl 150 μM + 20 μM db cAMP | 26.4 ± 3.9 |   |
| NaCl 150 μM + 100 μM db cAMP | 38.0 ± 4.2 |   |

Mean ± S.E. (n = 5)

**Fig. 7.*** Changes in the levels of cAMP in PyS-² (A) and F9 cells (B) upon addition of 0.15 mM NaCl (Φ). Control cells (C) were incubated in TM Sucrose only. A radioimmunoassay was used to measure cAMP.
correlation appears to exist between secretion and the level of cytosolic calcium ions in the sucrose-Na⁺ system. These results also show that cells in the presence of sucrose, with or without Na⁺, can control the level of cytosolic Ca²⁺. On the other hand, phosphatases such as tritoperoxidase, chlorpromazine, and promethazine caused considerable but not complete inhibition (Table III) at concentrations that did not cause lysis of cells (1 mM tritoperoxidase). Although the phosphatases bound to calmodulin and interfered with several calmodulin-dependent processes (25), including excocytosis (16, 26), they had other effects (27). TMB-8, another calmodulin antagonist (28), did not inhibit secretion at a concentration of 0.01 M but was inhibitory at 0.1 M (Table III).

Involvement of Cyclic AMP—While sodium ions and sucrose alone were sufficient to elicit a secretory response, the process was enhanced by dibutyryl cAMP (Tables IV and V). Addition of Forskolin, a stimulator of adenyl cyclase (29, 30), to F9 and PyS-2 cells almost doubled the rate of secretion, whereas dibutyryl cAMP had little effect (Table IV). Although dibutyryl cAMP was measured in F9 and PyS-2 cells in the presence or absence of sodium ions, sodium ions appeared to cause a brief but definite elevation in the cAMP content of the cells (Fig. 7). Four- to fivefold increases were observed in 1 minute, followed by a fall to control levels within 7 minute. In three experiments, the levels of cAMP in PyS-2 cells, incubated in TM Sucrose or TM Sucrose/NaCl (0.15 M) for 30 minutes, were within 10% of one another.

Role of G Proteins in the Secretion of Lysosomal Enzymes—

Treatment of cells with either cholera or pertussis toxin, which are known to ADP-ribosylate the α-subunit of G proteins (31), enhanced secretion (Table V). Addition of cAMP to cells already stimulated by pertussis toxin did not have a statistically significant effect. Presumably, cholera toxin stimulated Gα whereas pertussis toxin abolished inhibition mediated by G (32, 33). Secretion by glioma cells was stimulated by pertussis toxin, which also enhanced adenyl cyclase activity in these cells (33). When AlCl₃ (30 μM) and NaF (10 mM) were added, forming AlF₄⁻, G protein function was stimulated (34, 35), and in our experiments, secretion was clearly increased (Table VI).

Many activities have been attributed to the vanadate ion (36), including the ability to stimulate G proteins. Adenyl cyclase activity of rat adipose cells was stimulated by vanadate ions (37), and in the present experiment vanadate stimulation secretion whether or not sodium ions and sucrose were present together or singly. In the standard assay, in seven experiments using PyS-2 cells, 1 mM vanadate stimulated secretion by 70% in the presence of NaCl and 75% in the absence of cations. It is unlikely that vanadate acted through its capacity to inhibit phosphatases (36) since NaF (50 mM), which is a phosphatase inhibitor as well as an inhibitor of phosphorylases and kinases (38), caused a 50% reduction of secretion (n = 5).

**Lack of Involvement of Phospholipase C in Enzyme Secretion**—Two lines of experiments suggested that phospholipase C played little or no part in the secretory response in the sucrose-Na⁺ system. Neomycin (0.5–1.0 mM), an inhibitor of phospholipase C (39, 40), did not inhibit secretion (PyS-2 cells, control 31.0 ± 2.3% secretion; with neomycin, 32.5 ± 4.1% secretion; n = 5). Furthermore, cells grown overnight in the presence of the tumor promoter, phorbol myristate acetate (10⁻⁷ M), and assayed in its presence secreted N-acetyl-β-D-glucosaminidase at the same rate and to the same extent as controls. If phospholipase C took part in the process, phorbol ester, an active substitute for diacylglycerol and one of the products of the reaction catalyzed by this enzyme (41), would be expected to exert a noticeable effect.

**DISCUSSION**

In the present study, rapid secretion of lysosomal enzymes by cells in culture was induced by the simultaneous presence of a monovalent cation and a sugar such as sucrose. Na⁺ was the most effective cation. Cells, which usually secrete lysosomal enzymes at a relatively slow rate, could be induced to discharge a third of their total enzyme content in 30 minute. Despite the fact that cells were exposed to unphysiological conditions (2 × isosmolar) for 30 minute, the levels of the cytosolic marker, lactic acid dehydrogenase, and protein in the extracellular medium were unchanged. Cells remained viable and excluded a vital dye. When replaced in growth medium, they divided at an undiminished rate compared with untreated control cultures. After 24 h they were capable of repeating the process. Treated cells retained the ability to control the levels of cytosolic Ca²⁺ and cAMP and to respond in a graded manner to various stimulating and inhibitory substances. The secretory response was highly reproducible and was proportional to the concentration of cation and sucrose until there was no response. Sucrose-Na⁺ so enhanced the usually slow rate of excocytosis of lysosomal enzymes that the mechanisms involved could be conveniently examined in a manner similar to studies on the discharge of granules from mast cells (15) and fertilized eggs (16). Problems of uptake, synthesis, and degradation of enzymes, which might compli-
cate the evaluation of a slow secretory process, were eliminated.

In the present study and using this method, we were able to show that exocytosis of lysosomal enzymes involved G proteins and cAMP and that secretion did not depend on changes in the concentration of cytosolic Ca$^{2+}$ or on the activity of phospholipase C. In this regard, the lysosomal system differs from the exocytotic systems of mast cells (15), fertilized eggs (16), and other secretory systems (42).

This procedure was effective with several types of cells and may be of general applicability. A closer examination of the secretory mechanism of cells from individuals with various forms of lysosomal membrane transport (43, 44) and lysosomal storage disease (45) is possible. Do any of the known genetic defects causing these diseases have significant effects on the secretion of lysosomal contents? For instance, it will be of interest to examine in detail the exocytosis of lysosomal enzymes and free sialic acid from cells derived from individuals with Salla disease in which egress of sialic acid from the lysosome is defective (43).

Only a certain fraction of the total complement of lysosomal enzymes was secreted in 30 min, and no further loss of enzymes from the cells took place with extended incubation. Usually, 20–30% of the total was secreted but was never more than approximately 50%, whatever the stimulus. Up to 60% of the lysosomes, perhaps those fused with secretory vesicles, appeared to be competent to respond. The apparent division of lysosomes into two populations may permit us to define the change(s) in the lysosomal membrane necessary for the lysosomes to move to the surface and fuse with the plasma membrane during the secretory process. Since, cAMP, as we have shown, is an important mediator in lysosomal enzyme secretion, it is possible that a phosphorylation of proteins by activation of protein kinase A is essential. Phosphorylation of membrane proteins during secretion is now being investigated.

Acid phosphatase differed from all the other lysosomal enzymes in that it was not secreted. This observation is consistent with the data of Waheed et al. (46) who showed that the enzyme, transported to the lysosome as a transmembrane protein, was later cleaved to form a soluble enzyme. In PyS-2 and F9 cells cleavage may be delayed or, possibly, the lysosomes responding to the sucrose-Na$^+$ stimulus were recently formed and had phosphatase still attached to the lysosomal membrane.

Although sucrose was used routinely in this study, other sugars appeared to be at least as effective, while still others were inactive. Maximum activity was observed at a concentration of 0.25 M sucrose (isosmolar) in the presence of isosmolar Na$^+$ (0.15 M), i.e., twice overall isosmotic strength. Combined concentrations greater than or less than two times isomolar were not as effective. Although hyperosmolality is probably an important part of the stimulating impulse, the process requires the combined presence of a monovalent cation and a polyhydroxy1 compound such as sucrose. Neither alone, whatever its osmolarity, is effective in inducing secretion.

It is known that sucrose and other sugars with $M > 220$ are taken up by cells (24, 47, 48) and accumulate in the lysosomes. Water drawn into the lysosomes caused swelling and vacuolation of the cell (48, 49). However, we found that cytochalasin B, which inhibited uptake of sucrose into human liver cells in culture (50), had no effect on secretion in our system. It is quite possible that accumulation of sugar and water in the lysosomal vesicle is not associated with secretion. Perhaps, since the incubation period in our assay was relatively brief, secretion may have occurred before significant uptake of sucrose and water took place. It should be noted that d-glucose (M, 180) could substitute for sucrose in our system, despite the fact that this sugar can escape the lysosome and does not lead to vacuolation (6, 41, 51). More long-term effects such as inhibition of endocytosis (52) and an increase in levels of lysosomal enzymes (6, 49, 53), which occurred after several hours of exposure to sucrose, probably did not have a bearing on the sodium-sucrose system described here.

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REFERENCES

1. Jessup, W., Leoni, P., Bodmer, J. L., and Dean, R. T. (1982) Biochem. Pharmacol. 31, 2657–2662
2. Wilcox, P., and Rattray, P. (1979) Biocbrh. Biophys. Acra 586, 442–452
3. Riches, D. W. H., and Stanworth, D. R. (1980) Biochem. J. 188, 933–936
4. Dean, R. T., Jessup, W., and Roberts, C. R. (1984) Biochem. J. 217, 27–40
5. Inmort, M., Zuhladorf, M., Feige, U., Haslik, A., and Von Figura, K. (1985) Biochern. J. 214, 671–678
6. De Duve, C., DeBarsy, T., Poole, B., Trouet, A., Tuikens, P., and Van Hoof, F. (1974) Biochem. Pharmacol. 23, 2495–2531
7. Gonzalez-Noriega, A., Grubb, J. H., Talkad, V., and Syl, W. S. (1980) J. Cell Biol. 85, 839–852
8. Dingle, J. T., Fell, H. B., and Glauser, A. M. (1969) J. Cell Sci. 2, 139–154
9. Kato, T., Okada, S., Oshiehama, T., Inui, K., Yutaka, T., and Tabuchi, H. (1982) J. Biol. Chem. 257, 7814–7819
10. Weissmann, G., Zurier, R. B., Spieler, P. J., and Goldstein, I. M. (1971) J. Exp. Med. 134, 149–165
11. Schorlemmer, H. V., Davies, P., and Allison, A. C. (1976) Nature 261, 48–49
12. Henson, P. M. (1976) in Lysosomes in Biology and Pathology (Dingle, J. T., and Dean, R. T., eds) Vol. 5, pp. 99–126, Elsevier Science Publishing Co., New York
13. Dean, R. T., Hylton, W., and Allison, A. C. (1979) Biochim. Biophys. Acra 584, 57–65
14. Schorlemmer, H. V., Burger, R., Hylton, W., and Allison, A. C. (1977) Clin. Immunol. Immunopathol. 7, 88–96
15. Howell, T. W., Cockcroft, S., and Gomperts, B. D. (1987) J. Cell Biol. 105, 191–197
16. Baker, P. F., and Knight, D. E. (1981) Philos. Trans. R. Soc. Lond. Biol. Sci. 269, 83–103
17. Lehman, J. M., Speers, W. C., Schwartzendruber, D. E., and Pierce, G. B. (1974) J. Cell Physiol. 84, 13–18
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
19. Rice, R. H., and Means, G. E. (1971) J. Biol. Chem. 246, 831–839
20. Bergmeyer, H. U. (1963) in Methods in Enzymatic Analysis, p. 737, Academic Press, New York
21. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
22. Tsien, R. Y., Rink, T. J., and Poenie, M. (1985) Cell Calcium 6, 145–157
23. Brooker, G., Harper, J. P., Terasaki, W. L., and Moylan, R. D. (1979) Adv. Cyclic Nucleotide Res. 10, 1–33
24. von Figura, K. (1978) Exp. Cell Res. 111, 15–21
25. Cooper, D. M. F., Ahijanian, M. K., and Perez-Reyes, E. (1988) J. Cell. Biol. 36, 417–427
26. Levin, R. M., and Weiss, B. (1987) Mol. Pharmacol. 13, 690–697
27. Tilrell, J. G., and Coffee, C. J. (1980) in Cell Modulin-binding Proteins: Structure and Function (Siegel, F. L., Carafoli, E., Krebsinger, R. H., MacLennon, D. H., and Wasserman, R. H., eds) pp. 245–246. Elsevier Science Publishers B.V., Amsterdam
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28. Chiou, C. Y., and Malagodi, M. H. (1975) Pharmacology (Basel) 53, 279–285
29. Pfeuffer, T., and Metzger, H. (1982) FEBS Lett. 146, 369–375
30. Smigel, M. D. (1986) J. Biol. Chem. 261, 1976–1982
31. Johnson, G. L., Kaslow, H. R., and Bourne, H. R. (1978) J. Biol. Chem. 253, 7120–7123
32. Katada, T., Amano, J., and Ui, M. (1982) J. Biol. Chem. 257, 3739–3746
33. Kurose, H., Katada, T., Amano, T., and Ui, M. (1983) J. Biol. Chem. 258, 4870–4875
34. Sternweis, P. C., and Gilman, A. G. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 4889–4891
35. Bigay, J., Deterre, P., Pfister, C., and Chabre, M. (1987) EMBO J. 6, 2907–2913
36. Nechay, B. R., Nanninga, L. B., Nechay, P. S. E., Post, R. L., Grantham, J. J., Macara, I. G., Kubena, L. T., Phillips, T. D., and Nielsen, F. H. (1986) Fed. Proc. 45, 123–132
37. Shwabe, U., Puchstein, C., Hannemann, H., and Sochtig, E. (1979) Nature 277, 143–145
38. Hewitt, E. J., and Nicolas, J. D. J. (1963) in Metabolic Inhibitors (Hochster, R. M., and Quastel, J. H., eds) Vol. 2, pp. 311–321, Academic Press, New York
39. Downes, C. P., and Michell, R. H. (1981) Biochem. J. 198, 133–140
40. Streh, H., Heslop, J. P., Irvine, R. F., Schulz, I., and Berridge, M. J. (1985) J. Biol. Chem. 260, 7309–7315
41. Berridge, M. J. (1984) Biochem. J. 220, 345–360
42. Albert, P. R., and Tashjian, A. H., Jr. (1984) J. Biol. Chem. 259, 15350–15363
43. Renlund, M., Tietze, F., and Gahl, W. A. (1986) Science 232, 769–762
44. Schneeberger, P. J., and Schulman, J. D. (1983) in The Metabolic Basis of Inherited Disease (Stanbury, J. B., Wyngaarden, J. B., Frederickson, D. S., Goldstein, J. L., and Brown, M. S., eds) pp. 1844–1866, McGraw-Hill Inc., New York
45. McKusick, V. A., and Neufeld, E. F. (1983) in The Metabolic Basis of Inherited Disease (Stanbury, J. B., Wyngaarden, J. B., Frederickson, D. S., Goldstein, J. L., and Brown, M. S., eds) pp. 751–777, McGraw-Hill Inc., New York
46. Waheed, A., Gottschalk, S., Hille, A., Kremler, C., Pohlmann, R., Braunke, T., Hausser, H., Geuze, H., and von Figura, K. (1988) EMBO J. 7, 2351–2358
47. Horvath, A. (1973) Biochim. Biophys. Acta 297, 413–422
48. Munro, T. R. (1968) Exp. Cell. Res. 52, 392–400
49. Cohn, Z. A., and Ehrenreich, B. A. (1969) J. Exp. Med. 129, 201–225
50. Wagner, R., Rosenberg, M., and Estensen, R. (1971) J. Cell Biol. 50, 804–817
51. Lloyd, J. B. (1969) Biochem. J. 115, 703–707
52. Warburton, M. J., and Wynn, C. H. (1976) Biochem. Biophys. Res. Commun. 70, 94–99
53. Bernacki, R. J., and Bosmann, H. B. (1971) J. Cell Sci. 8, 399–406