# PITFALLS IN THE USE OF LEAD NITRATE FOR THE HISTOCHEMICAL DEMONSTRATION OF ADENYLATE CYCLASE ACTIVITY

ANDRE LEMAY and LEONARD JARETT

From the Divisions of Laboratory Medicine and Metabolism, Departments of Pathology and Medicine, Washington University School of Medicine and Barnes Hospital, St. Louis, Missouri 63110. Dr. Lemay's present address is Department of Pathology, Yale University, New Haven, Connecticut 06510.

ABSTRACT

The biochemistry of the lead histochemical technique for demonstrating adenylate cyclase was studied. The enzyme activity of fat cell plasma membranes, using 5'-adenylyl-imidodiphosphate (AMP-PNP) as substrate, was completely inhibited at $1 \times 10^{-4}$ M $Pb(NO_3)_2$ and yet at $4 \times 10^{-3}$ M $Pb(NO_3)_2$ precipitate could be demonstrated by electron microscopy on both sides of plasma membrane vesicles. No lead-diphosphoimide or lead-phosphate precipitate could be visualized by electron microscopy when the lead was reduced to a level ($2 \times 10^{-5}$ M) which caused only 50% inhibition of the enzyme. A solubility product coefficient of $1 \times 10^{-10}$ M was found necessary to allow precipitation of lead-phosphate complex in the adenylate cyclase medium. Varying the ratio of substrate or dextran relative to the lead failed to protect the inhibition of the enzyme. Increasing concentrations of $\beta$-mercaptoethanol restored the basal and stimulated activity of adenylate cyclase but also prevented the precipitation reaction. Lead at $2 \times 10^{-3}$ M caused the nonenzymatic hydrolysis of AMP-PNP, resulting in the production of small but significant quantities of cyclic AMP and substantial amounts of AMP. This hydrolysis was inhibited by alloxan but unaffected by dextran or NaF. The adenylate cyclase activity of pancreatic islet homogenates and of fat pad capillaries was completely inhibited by lead concentrations equal to or less than those used in histochemical studies (Howell, S. L., and M. Whitfield. 1972. *J. Histochem. Cytochem.* **20**:873–879. and Wagner, R. C., P. Kreiner, R. J. Barrnett, and M. W. Bitensky. 1972. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3175–3179.). The present study shows that the lead histochemical method cannot be used for localization of adenylate cyclase because of the inhibition of the enzyme and artifacts produced by high lead concentrations and the inability to produce a visible precipitate at low lead concentrations which only partially inhibit the enzyme.

The lead-salt precipitation technique of Wachstein and Meisel (25) has been applied recently in attempts to localize the adenylate cyclase activity in rat pancreatic islets of Langerhans (5), in capillaries from rat epididymal fat pads (27), in isolated rod outer segment of the retina (26), and in the rat nephron (24).

The main arguments used to support the feasi-

bility of these methods is the use of a specific adenylate cyclase substrate, 5'-adenylyl-imidodiphosphate (AMP-PNP)[1] (17, 18), an increase in the amount of lead precipitate upon addition of NaF or hormones known to stimulate the enzyme activity (5, 24, 27), and a decrease in the quantity of lead deposit resulting from the addition of alloxan (27), a specific and potent inhibitor of the adenylate cyclase in many tissues (1).

However, the validity of this technique has been previously questioned for other nucleotide phosphatases, especially ATPase (4, 15). It is well known that lead is a potent inhibitor of enzymes and tissue nucleotide phosphatases. It has been demonstrated that lead in the Wachstein and Meisel medium catalyzes a nonenzymatic hydrolysis of nucleotide phosphates (13, 16, 18) and also acts as a chelator of ATP (23). In the presence or absence of tissue, nucleotides themselves can be a significant part of lead precipitate (19). These problems in the use of lead for histochemical localization of nucleotide phosphatase recently have been reviewed in detail (3).

Despite the problems associated with the lead histochemical technique for phosphatase reactions, none of the studies to date utilizing this approach for the electron histochemical demonstration of adenylate cyclase have investigated the effect of lead on this system. Using either AMP-PNP or ATP, the present paper reports biochemical and histochemical data showing that lead is a potent inhibitor of the adenylate cyclase, is a cause of artifacts in the histochemical localization of this enzyme due to the nonenzymatic hydrolysis of the substrate, and that the adenylate cyclase activity cannot be visualized by this lead-salt technique.

## MATERIALS AND METHODS

### *Preparation of Tissues*

FAT CELL PLASMA MEMBRANE: Highly purified fat cell plasma membranes were isolated from isolated fat cells of 125–150-g male Wistar rats by differential and discontinuous Ficoll gradient centrifugation as described previously (14). For histochemical experiments membranes were used immediately after preparation, and for biochemical assay were used fresh or were frozen in 10 mM Tris-HCl (pH 7.4) and 0.25 M sucrose for no longer than 1 wk before use which had previously been shown to be satisfactory for assaying this enzyme (7).

ISLETS OF LANGERHANS: Pancreatic tissue from male Wistar rats (250–350 g) was disrupted by the collagenase digestion method of Lacy and Kostianovsky (11) and islets were isolated by centrifugation on a discontinuous Ficoll gradient (21). 400–500 islets could be obtained from three rats. The islets were then homogenized in 80 mM Tris-maleate (pH 7.4) containing 2 mM theophylline and 0.25 M sucrose. A 20-$\mu$l aliquot of fresh or boiled tissue was used for adenylate cyclase assay.

CAPILLARIES: The distal portion of epidiymal fat pads from 250- to 300-g male Wistar rats were first treated with collagenase as described previously in the preparation of fat cell membranes. After incubation with the collagenase, capillaries were separated from large vessels and fat cells by the method described by Wagner et al. (27). A 10- or 20-$\mu$l aliquot of fresh or boiled tissue was used for adenylate cyclase assay.

The protein content of each tissue preparation was determined by the method of Lowry et al. (12) using bovine serum albumin as standard.

### *Adenylate Cyclase Assay*

Formation of cyclic AMP (cAMP) by the different tissues was measured under similar conditions by a highly sensitive adenylate cyclase assay as recently described by Salmon et al. (20). The assay conditions are specified in legends to Figs. 1–5 and Tables I–VI. The reaction was started by the addition of appropriate amounts of tissue to the medium preincubated at 30°C for 5–10 min and run for 5 min. The reaction was stopped by one of two different methods. For fat cell plasma membranes, 100 $\mu$l of "stopping solution" containing 2% sodium dodecyl sulfate (SDS), 1 mM cAMP, and 5 mM ATP were added at room temperature. 800 $\mu$l of a solution containing 1 mM ATP, 1 mm cAMP, and 10,000 cpm of [$^3$H]cAMP were added for estimation of the recovery in the subsequent chromatography on Dowex 50AG-WX4 and neutral alumina columns (ICN Pharmaceuticals, Inc., Life Sciences Group, Cleveland, Ohio) (20). For the assay of islet homogenate or fat pad capillaries, the reaction was stopped by adding 200 $\mu$l of the stopping solution without SDS. After the addition of 800 $\mu$l of the [$^3$H]cAMP recovery solution, tubes were boiled for 5 min followed by centrifugation at 2,500 rpm for 10 min. The supernates were processed through the columns and the final eluates were collected directly into scintillation vials containing 10 ml of Instagel. Duplicate or triplicate samples were counted for 10 min in a liquid scintillation counter set for separation of $^3$H and $^{32}$P. Results are expressed in picomoles cAMP formed per milligram protein per minute ± standard error of the mean.

[1] *Abbreviations used in this paper:* ADP-$NH_2$, adenylyl phosphoramidate; AMP-PNP, 5'-adenylyl-imidodiphosphate; cAMP, cyclic adenosine 3',5'-monophosphate; cGMP, cyclic guanosine 3',5'-monophosphate; PDE, cyclic nucleotide phosphodiesterase; PNP, diphosphoimide; SDS, sodium dodecyl sulfate.

### *Cyclic Nucleotide Phosphodiesterase (PDE) Assay*

The material contained in the neutral alumina column eluate of the adenylate cyclase assay system was treated with PDE as previously described (8). Briefly, the 4-ml eluates containing 100 mM imidazole-HCl buffer (pH 7.4) were made 5 mM $MgCl_2$, $1 \times 10^{-5}$ M AMP, 0.2% bovine serum albumin and incubated for 10 min at 30°C in the presence or absence of 60 mU of phosphodiesterase. The reaction was stopped by adding 0.2 ml of 0.3 N $ZnSO_4$ and 0.23 ml of 0.3 N BaOH. After centrifugation at 2,000 rpm for 5 min the zinc-barium precipitation procedure was repeated. 10 ml of Instagel were added to 4 ml of the second supernate for measurement of the remaining radioactive cAMP.

### *Nucleotide Paper Chromatography*

The 3-ml water eluate of the Dowex column of the adenylate cyclase assay system was evaporated to dryness by $N_2$ stream at 50°C and resuspended in 50 $\mu$l of $H_2O$. The nucleotides present were separated on Whatman 3 MM chromatography paper, using 95% ethanol-1.0 M ammonium acetate (7:3 and made pH 6.6 with 1 M acetic acid) as a solvent during 9 h of ascending migration. Quantities of 50 nmol standards of ATP, [$\gamma^{32}$P]AMP-PNP, ADP, [$^3$H]AMP, and [$^3$H]cAMP were located by ultraviolet light absorption and/or by radioactive determination. The respective $R_f$s of these compounds were 0.04, 0.04, 0.09, 0.24, and 0.42. The AMP-PNP also showed another spot detected by both ultraviolet light absorption and radioactivity measurement which migrated between ADP and AMP, having an $R_f$ of 0.13. The paper was cut into 1-cm pieces and eluted with 5 ml of water by gentle agitation for 30 min in scintillation vials. The paper was removed and 10 ml of Instagel were added for counting the radioactive nucleotides.

### *Measurement of Lead-Phosphate Interactions*

The interaction of $Pb(NO_3)_2$ with the incubation medium of the adenylate cyclase assay and with added phosphate was quantified by measuring the light scattering of the solution in a Farrand photoelectric fluorometer (Farrand Optical Co., Inc., Valhalla, N. Y.) using only a primary filter. The composition of the incubation mixture is described in legends to Tables I–VI. The optical density resulting from the refraction of light due to the particles in the solution was read relative to buffer alone before addition of lead and after addition and incubation with radioactive phosphate. Tubes were then centrifuged for 10 min at 3,000 rpm and an aliquot of the supernate was counted in 4 ml of water and 10 mg of Instagel for quantification of the nonprecipitated labeled phosphate.

### *Electron Histochemical Techniques*

The incubation conditions for the localization of the adenylate cyclase on purified fat cell membranes are described in legends to Figs. 1–5. The reaction was stopped by adding 1 ml of cold 3% glutaraldehyde in 25 mM Tris-HCl, pH 7.4, and 0.9% NaCl. After fixation for at least 30 min, membranes were centrifuged at 18,000 rpm (40,000 *g*) in an IEC centrifuge (model B-20, Damon/IEC Div., Damon Corp., Needham Heights, Mass.) for 20 min. Pellets were washed with 0.1 M cacodylate buffer, pH 7.4, and cut into small pieces with a razor blade. Tissue pieces were postfixed in 1% osmium tetroxide in 50 mM cacodylate buffer and stained en bloc with 1% uranyl acetate in 0.9% NaCl. The tissue was dehydrated in a graded ethanol series followed by toluene and embedded in Epon 812. Thin sections were cut on a Sorvall Porter-Blum MT-2 ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.). Unstained sections were photographed with a Philips 200 electron microscope operating at 60 kV.

### *Materials*

Male Wistar rats were bought from National Animal Laboratories, St. Louis, Mo. Biochemical products were purchased from the following sources: AMP-PNP, [$^{32}$P]AMP-PNP (1–5 Ci/mmol), [$^{32}$P]ATP (50–100 Ci/mmol), $^{32}$P (285 Ci/mg P): ICN Corp., Chemical & Radioisotopes Div., Irvine, Calif.; all other nucleotides, PDE, and dextran (mol wt 250,000): Sigma Chemical Co., St. Louis, Mo.

## RESULTS

### *Inhibition of Adenylate Cyclase Activity by Lead*

Since lead is a potent inhibitor of ATPase and of many other enzymes, it was first of interest to measure the adenylate cyclase activity of purified fat cell plasma membranes in the presence of increasing concentrations of lead. As shown in Fig. 1, the enzyme with AMP-PNP as substrate is very sensitive to low concentrations of lead. A significant 35% inhibition is already observed at $1 \times 10^{-5}$ M $Pb(NO_3)_2$ and there is less than 5% of the activity remaining at $1 \times 10^{-4}$ M $Pb(NO_3)_2$. The apparent $K_i$ is $4 \times 10^{-5}$ M. With ATP as substrate, a similar curve was observed (data not shown) and sometimes inhibition greater than 50% was observed at $1 \times 10^{-5}$ M $Pb(NO_3)_2$.

### *Adenylate Cyclase Histochemistry at High Lead Concentration*

In a first attempt to localize the adenylate cyclase activity, fat cell plasma membrane histo-

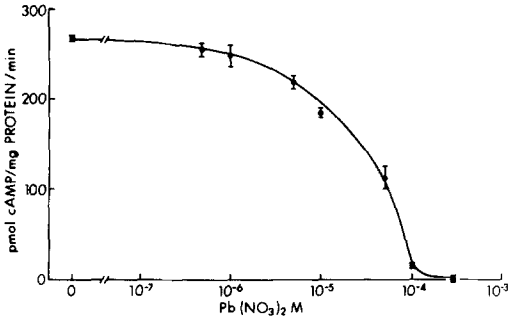



FIGURE 1 Effect of increasing concentrations of lead on the basal adenylate cyclase activity of fat cell plasma membrane. 25 μg of membrane protein were incubated in 100 μl of 25 mM Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$, 0.5 mM AMP-PNP, 130 mM NaCl, and increasing concentrations of $Pb(NO_3)_2$. The reaction was stopped by adding 100 μl of SDS stopping solution, and cAMP formed was measured as described in Materials and Methods.

chemistry was performed under Howell's and Whitfield's incubation conditions (5). After 30 min of incubation of purified plasma membranes in the adenylate cyclase medium containing $4 \times 10^{-3}$ M $Pb(NO_3)_2$ and $5 \times 10^{-4}$ M AMP-PNP as substrate, lead precipitate was found on almost every vesicle, as clearly shown in Fig. 2. A heavier concentration of precipitate was found on the outside surface of the vesicles than on the inside. The same phenomenon was observed with isolated liver cell plasma membranes (data not shown). Yet, as shown in Fig. 1, adenylate cyclase activity is completely inhibited at $5 \times 10^{-4}$ M lead.

Alloxan, $1 \times 10^{-2}$ M, completely inhibits the basal or NaF-stimulated activity of purified fat cell plasma membrane (data not shown). After incubation of the membranes in the presence of $1 \times 10^{-2}$ M alloxan, many vesicles appear to be free of coarse lead precipitate but still have very fine lead deposits (Fig. 3). However, in other parts of the preparation there are still coarse precipitates as well as large aggregates of lead deposits, mainly on the outer surface of the vesicles. Fig. 4 shows that under similar incubation conditions in the presence of buffer and lead but without substrate or $Mg^{++}$, one can still see a fine deposit localized on both sides of all plasma membrane vesicles.

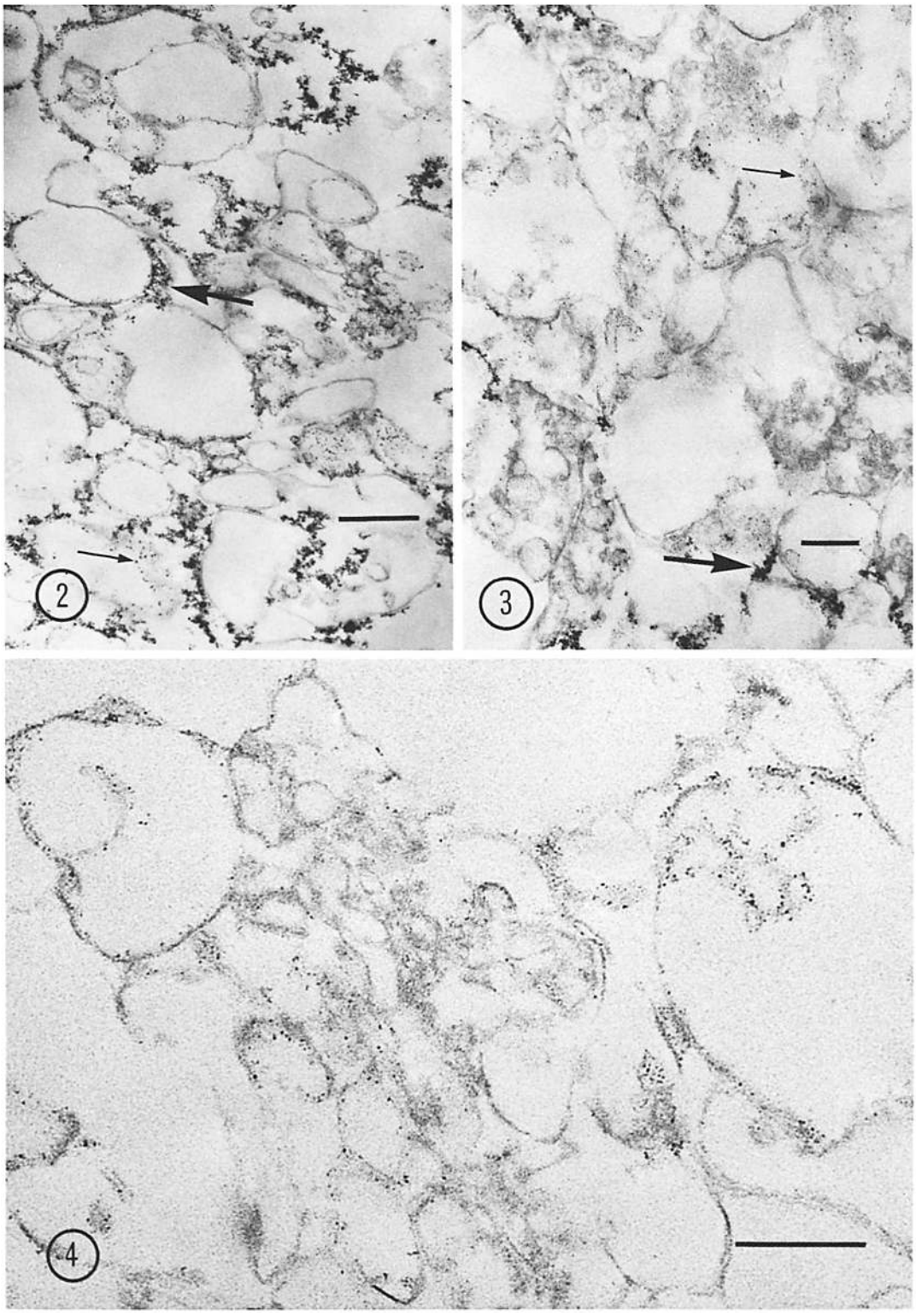
### *Phosphate Precipitation and Adenylate Cyclase Histochemistry at Low Lead Concentration*

The above data indicate that at high lead concentrations the adenylate cyclase activity is markedly inhibited, yet large quantities of lead precipitation were observed in the electron histochemical studies. Experiments were performed to test the feasibility of performing the electron histochemistry using lead concentrations low enough not to completely inhibit the enzyme. Using the light scattering and radioactive phosphate technique for measuring lead-phosphate precipitation, it was found that, by varying the lead and phosphate concentrations in standard incubations, the product of the lead and phosphate concentrations had to be equal to or exceed $1 \times 10^{-10}$ M before precipitation could be measured. Thus, a concentration of lead must be chosen to allow maximum enzyme activity so that the concentration of diphosphoimide (PNP) produced would be enough to exceed the solubility product. Theoretically, with lead at $2 \times 10^{-5}$ M, the enzyme activity of the fat cell plasma membrane should be at least half maximal and, based on data in Fig. 1,

FIGURE 2 Fat cell plasma membranes were incubated for 30 min at 30°C in 500 μl of medium containing 500 μg of membrane protein, 80 mM Tris-maleate, pH 7.4, 0.5 mM AMP-PNP, 2 mM $MgSO_4$, 2 mM theophylline, 5 mM NaF, 8% dextrose, and 4 mM $Pb(NO_3)_2$. The reaction was ended by fixation in cold glutaraldehyde, and the membranes were processed for electron microscopy as described in Materials and Methods. Large and small arrows indicate lead deposits on the outside and on the inside surfaces of the vesicles, respectively. Scale bar is 0.25 μm in all micrographs. × 47,150.

FIGURE 3 Fat cell plasma membrane incubated as in Fig. 2, but with added 10 mM alloxan. Large and small arrows indicate the lead deposits on the outside and inside surfaces of the vesicles, respectively. × 34,650.

FIGURE 4 Fat cell plasma membrane incubated as in Fig. 2, but without substrate and $Mg^{++}$. × 79,200.


2
3
4

this would be around 150 pmol/mg protein/min. This activity would yield, at best, about $1-5 \times 10^{-6}$ M PNP during the standard incubation for histochemistry. This would give a product of $1 \times 10^{-10}$ M and therefore be at the lowest concentration where precipitation occurred. Other considerations that would prevent attainment of the solubility product would include permeability of the membranes, that the PNP is being produced over an extended period and not all at once, etc. This experiment was actually performed with $2 \times 10^{-5}$ M $Pb(NO_3)_2$, $5 \times 10^{-4}$ M AMP-PNP as substrate, and 1.5 mg plasma membrane protein/ml. The electron micrographs of the plasma membranes failed to reveal any lead precipitate despite the fact that the enzyme activity assayed at the same time was 150 pmol/mg protein/min. These negative results stimulated further attempts to find a method which would protect the enzyme from lead inhibition at at the same time allow specific precipitation by lead.

### *Effect of ATP on the Inhibition of Adenylate Cyclase by Lead*

It has been shown by Tice (23) that lead chelates ATP and that ATPase activity, inhibited under conditions of high lead-ATP ratio, was overcome by increasing ATP concentrations. Fig. 1 illustrates that the adenylate cyclase activity is inhibited at concentrations of lead lower than concentrations of substrate used. However, one might postulate that higher concentrations of ATP would protect the enzyme and allow the use of higher amounts of lead. cAMP formation has been measured in the presence of $2 \times 10^{-5}$ M $Pb(NO_3)_2$ and increasing ATP concentrations from 0.25 to 2.5 mM. The mean control enzyme activity was 313 ± 24 pmol/mg of protein/min in contrast to the composite activity of 12 ± 2 from all the ATP concentrations tested in the presence of lead, showing that no protection of adenylate cyclase occurred.

### *Effect of Dextran on the Inhibition of Adenylate Cyclase by Lead*

Dextran has been shown to be useful in avoiding the precipitation of lead in a reaction medium (22). Wagner et al. (27) have used dextran in their studies on the localization of adenylate cyclase in fat pad capillaries. When fat cell plasma membranes were incubated in Wagner's medium containing 0.6 mM AMP-PNP, 6% dextran, and 2 mM $Pb(NO_3)_2$, subsequent electron microscope examination revealed virtually no reaction product (Fig. 5). Only a few small dots of lead can be seen. No significant change in this small amount of precipitate was noted when incubations were performed in the presence of $1 \times 10^{-5}$ M epinephrine, 5 mM alloxan, or in the absence of substrate. (Data not shown.)

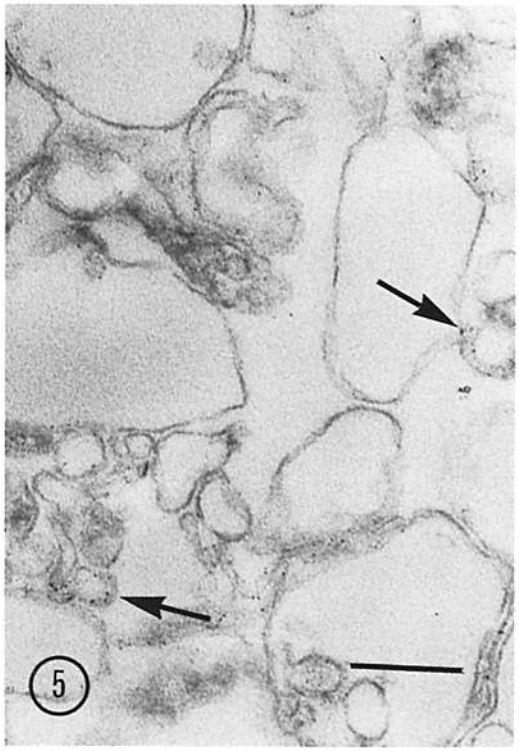



FIGURE 5 Fat cell plasma membranes were incubated for 30 min at 30°C in 500 μl of medium containing 400 μg of membrane protein, 80 mM Tris-maleate, pH 7.4, 0.6 mM AMP-PNP, 5 mM theophylline, 4 mM $MgSO_4$, 6% dextran, and 2 mM $Pb(NO_3)_2$. The reaction was ended by fixation in cold glutaraldehyde, and the membranes were processed for electron microscopy as described in Materials and Methods. Arrows indicate lead deposits. × 61,200.

The possibility existed that dextran, while preventing lead precipitation in the medium, could also protect adenylate cyclase from inhibition by $Pb(NO_3)_2$ and that the small quantity of precipitate seen in Fig. 5 was related to the location of the enzyme. The molar ratio of dextran to $Pb(NO_3)_2$ in Wagner's medium was 0.12. A set of experiments using 0.5 mM ATP and dextran (mol wt 250,000) in five concentrations varying from 0.01

to 1% yielded, from all the incubation conditions, a mean adenylate cyclase activity of fat cell plasma membranes of 193 ± 5 pmol/mg protein/min in the absence of $Pb(NO_3)_2$ and 102 ± 3 in the presence of $1 \times 10^{-5}$ M $Pb(NO_3)_2$. Thus, dextran itself had no effect on the adenylate cyclase activity nor did it alter the degree of inhibition of the enzyme by lead when the molar ratio of dextran to lead was increased from 0.01 to 4.0.

### *Effect of β-Mercaptoethanol on the Inhibition of Adenylate Cyclase and the Precipitation of Phosphate by Lead*

In another attempt to prevent the inhibition of the adenylate cyclase by $Pb(NO_3)_2$, the effect of β-mercaptoethanol was studied on the activity of the enzyme in the presence of $2 \times 10^{-5}$ M $Pb(NO_3)_2$. At that concentration, as reported in the experiment in Table I, basal adenylate cyclase is only 20% of the control and the NaF- and epinephrine-stimulated activity is only about one-third of the basal and one-tenth of the stimulated activity without lead. Increasing the concentrations of β-mercaptoethanol progressively restores the basal and stimulated state of the enzyme. At 10 mM of the reducing agent, there is 80% recovery of the basal activity and about 60% of the NaF and epinephrine stimulation. Full recovery is observed at $2 \times 10^{-2}$ M β-mercaptoethanol.

In view of this protecting effect of the β-mercaptoethanol on the adenylate cyclase activity, it was of interest to study the effect of the reducing agent on the precipitation of phosphate by lead. As shown in Table II, at $1 \times 10^{-2}$ M and higher concentrations of β-mercaptoethanol, there is no reaction product detected by light scattering upon addition of phosphate. The same concentrations of the reducing agent also prevent precipitation of $^{32}P$ which is almost completely recovered in the supernate. The increasing optical density observed in the presence of $Pb(NO_3)_2$ and $NaH_2PO_4$ with 1.0 and $5 \times 10^{-3}$ M β-mercaptoethanol is probably related

TABLE I

*Effect of Increasing Concentrations of β-Mercaptoethanol on the Basal and NaF- and Epinephrine-Stimulated Adenylate Cyclase Activity of Fat Cell Plasma Membrane in the Presence and Absence of Lead*

| β-Mercaptoethanol $1 \times 10^{-3}$ M | $Pb(NO_3)_2$ $2 \times 10^{-5}$ M | NaF $5 \times 10^{-3}$ M | L-Epinephrine $1 \times 10^{-5}$ M | Adenylate cyclase |
|---|---|---|---|---|
| | | | | *pmol cAMP/mg protein/ml* |
| 0 | − | | | 119 ± 11 |
| 0 | + | | | 26 ± 1 |
| 5 | − | | | 113 ± 8 |
| 5 | + | | | 48 ± 7 |
| 10 | − | | | 105 ± 4 |
| 10 | + | | | 80 ± 1 |
| 20 | − | | | 117 ± 3 |
| 20 | + | | | 119 ± 5 |
| 0 | − | + | | 343 ± 8 |
| 0 | + | + | | 38 ± 5 |
| 5 | − | + | | 351 ± 10 |
| 5 | + | + | | 218 ± 2 |
| 20 | − | + | | 390 ± 7 |
| 20 | + | + | | 384 ± 7 |
| 0 | − | | + | 434 ± 30 |
| 0 | + | | + | 35 ± 4 |
| 5 | − | | + | 466 ± 4 |
| 5 | + | | + | 291 ± 8 |
| 20 | − | | + | 509 ± 17 |
| 20 | + | | + | 504 ± 4 |

24 μg of membrane protein were incubated for 5 min at 30°C in 100 μl of 10 mM Tris-HCl buffer, pH 7.4, containing 5 mM $MgCl_2$, 0.25 M sucrose, 0.5 mM ATP, various concentrations of β-mercaptoethanol, with or without 5 mM NaF or $1 \times 10^{-5}$ M L-epinephrine, and in the presence and absence of $2 \times 10^{-5}$ M $Pb(NO_3)_2$. The reaction was stopped by adding 100 μl of SDS stopping solution, and cAMP formed was measured as described in Materials and Methods.

TABLE II

*Effect of Increasing Concentrations of β-Mercaptoethanol on the Precipitation Reaction in the Adenylate Cyclase Medium*

| β-Mercaptoethanol $1 \times 10^{-3}$ M | Optical density after addition of $Pb(NO_3)_2$ | Optical density after addition of $NaH_2PO_4$ | Supernate $^{32}P$ |
|---|---|---|---|
| | | | *cpm* |
| — | — | 0.10 | 9,200 |
| — | 0.11 | 0.13 | 4,250 |
| 1 | 0.13 | 0.18 | 4,550 |
| 5 | 0.10 | 0.22 | 6,060 |
| 10 | 0.10 | 0.10 | 8,950 |
| 15 | 0.10 | 0.10 | 8,900 |
| 25 | 0.10 | 0.10 | 8,910 |

1 ml of medium containing 10 mM Tris-HCl (pH 7.4), 0.5 mM ATP, 5 mM $MgCl_2$, various concentrations of β-mercaptoethanol, and $2 \times 10^{-4}$ M $Pb(NO_3)_2$ was incubated for 30 min at 30°C in the presence of $2 \times 10^{-4}$ M $^{32}P$. The optical density and counts in the supernate were measured as described in Materials and Methods.

TABLE III

*Effect of Lead on the Basal and NaF-Stimulated Adenylate Cyclase Activity of Pancreatic Islet Homogenate*

| | Control | NaF | $Pb(NO_3)_2$ | NaF + $Pb(NO_3)2$ |
|---|---|---|---|---|
| | *pmol cAMP/mg protein/min* | | | |
| Boiled tissue | 0.56 | 0.58 | 0.56 | 0.55 |
| Fresh tissue | 1.1 | 2.4 | 0.62 | 0.62 |
| Net activity | 0.54 | 1.72 | 0.06 | 0.07 |

100 μg of islet homogenate protein were incubated for 5 min at 30°C in 100 μl of 80 mM Tris-maleate buffer, pH 7.4, containing 0.5 mM ATP, 4 mM $MgCl_2$, 2 mM theophylline, 0.25 M sucrose with or without 10 mM NaF, and in the presence or absence of 0.5 mM $Pb(NO_3)_2$. As a control, part of the homogenate was boiled for 7 min before incubation. The reaction was stopped by adding 200 μl of stopping solution, and the cAMP formed was measured as described in Materials and Methods.

to the increasing ratio of $NaH_2PO_4$ to non-β-mercaptoethanol complexed $Pb(NO_3)_2$, resulting in an alteration of the light scattering. At $1 \times 10^{-2}$ M β-mercaptoethanol the quantity of noncomplexed lead is so low as not to allow any precipitation as shown by the $^{32}P$ and optical density data.

The effect of the sulfhydryl agent was studied on the histochemistry of $5 \times 10^{-4}$ M AMP-PNP, $1 \times 10^{-4}$ M $Pb(NO_3)_2$ and 5 or $10 \times 10^{-3}$ M β-mercaptoethanol. Simultaneous biochemical determination of the adenylate cyclase activity under similar conditions showed that the enzyme activity was 9% of the control $5 \times 10^{-3}$ M β-mercaptoethanol and more than 60% of the activity was recovered at $10 \times 10^{-3}$ M. (Data not shown.) However, under these conditions no precipitation was observed on electron micrographs.

## *Effect of Lead on Adenylate Cyclase Activity of Islets of Langerhans and Fat Pad Capillaries*

The inhibitory effect of lead on adenylate cyclase has not been reported in other tissues in which studies have been performed in an attempt to localize the enzyme by the lead-salt technique (1–5). It was felt imperative to verify the effect of lead on the adenylate cyclase in these tissues under similar conditions used for histochemical studies.

To study the effect of lead on adenylate cyclase activity of islets of Langerhans, fresh or boiled homogenates were incubated in the presence of $5 \times 10^{-4}$ M $Pb(NO_3)_2$ and $5 \times 10^{-4}$ M ATP. As shown in Table III, basal activity is 0.54 pmol/mg protein/min and goes up to 1.72 pmol/mg protein/min with $1 \times 10^{-2}$ M NaF. However, in the presence of lead, the basal activity is negligible and does not respond to fluoride stimultion.

When adenylate cyclase activity was measured on freshly prepared fat pad capillaries in the presence of $5 \times 10^{-4}$ M ATP, 6% dextran, and $2 \times 10^{-3}$ M $Pb(NO_3)_2$, there was a complete inhibition of the basal fluoride-stimulated activity (Table IV). Although the boiled tissue controls did not have any significant basal or stimulated activity, addition of $2 \times 10^{-3}$ M $Pb(NO_3)_2$ to the denatured enzyme repeatedly resulted in the formation of a cAMP-like compound. When this boiled tissue control value is subtracted from the fresh tissue activity, the net activity is virtually zero in the presence of lead.

## *Identification of cAMP and AMP Formed from AMP-PNP by Lead in the Absence of Tissue*

The finding of an elevated level of a cAMP-like compound in the presence of $2 \times 10^{-3}$ M $Pb(NO^3)_2$ in the boiled tissue controls (Table IV) suggested that this high concentration of lead might cause a nonenzymatic formation of cAMP. It has been shown previously that such concentrations of lead can cause the hydrolysis of ATP to other nucleotides resulting in the artifactual production of lead phosphate precipitates (13, 16, 18).

It seemed imperative to determine if lead in the absence of tissue could cause nonenzymatic hydrolysis of AMP-PNP to various other nucleotides including cAMP.

The first approach was to show that lead can cause the formation of a compound in the adenylate cyclase system which elutes from the neutral alumina as cAMP and is equally susceptible as standard cAMP to PDE. Table V (part A) shows that $2 \times 10^{-3}$ M lead with [$^{32}$P]AMP-PNP as substrate caused a significant increase in the counts per minute of a compound which chromatographed on columns as [$^{32}$P]cAMP. Neither 6% dextran nor $1 \times 10^{-2}$ M NaF affected the formation of this compound by lead, whereas, $1 \times 10^{-2}$ M alloxan almost completely abolished its production. Furthermore, this compound which was isolated along with a [$^{3}$H]cAMP recovery marker was degraded to the same extent as the [$^{3}$H]cAMP by PDE as shown in Table V (part B).

The next approach was to paper chromatograph the less pure material isolated in the Dowex water eluate after incubations performed as above, including [$^{3}$H]cAMP or [$^{3}$H]AMP as recovery markers. The quantity of material found in the cAMP region of the chromatograph of the control was truly negligible and was only demonstrable due to the calculation process (Table VI). Lead caused a significant formation of cAMP, accounting for 0.03% of the original AMP-PNP. In

TABLE IV

*Effect of Lead on the Basal and NaF-Stimulated Adenylate Cyclase Activity of Capillaries from Fat Pad Tissue*

| | Control | NaF | $Pb(NO_3)_2$ | NaF + $Pb(NO_3)_2$ |
|---|---|---|---|---|
| | | *pmol cAMP/mg protein/min* | | |
| Boiled tissue | 0.4 ± 0.03 | 0.4 ± 0.04 | 3.3 ± 0.13 | 2.9 ± 0.04 |
| Fresh tissue | 1.2 ± 0.16 | 6.0 ± 1.3 | 3.4 ± 0.14 | 2.8 ± 0.3 |
| Net activity | 0.8 | 5.6 | 0.1 | −0.1 |

180 μg of purified capillary protein were incubated for 5 min at 30°C in 100 μl of 80 mM Tris-maleate buffer, pH 7.4, containing 0.5 mM ATP, 4 mM $MgCl_2$, 7 mM theophylline, 6% dextran with or without 10 mM NaF, and in the presence or absence of 2 mM $Pb(NO_3)_2$. As a control, capillaries were boiled for 10 min before incubation. The reaction was stopped by adding 200 μl of stopping solution, and the cAMP formed was measured as described in Materials and Methods.

TABLE V

*Formation of cAMP in the Adenylate Cyclase Assay in the Presence and Absence of Lead and Its Degradation by Phosphodiesterase*

| Substances added | $Pb(NO_3)_2$ $2 \times 10^{-3}$ M | cAMP | | Degradation |
|---|---|---|---|---|
| | | −PDE | +PDE | |
| | | *cpm* | | |
| A. [$^{32}$P]AMP-PNP | | | | |
| None | − | 5 ± 2 | 2 ± 1 | — |
| None | + | 505 ± 33 | 186 ± 17 | 63.2 |
| 6% dextran | + | 567 ± 26 | 239 ± 12 | 57.9 |
| 10 mM NaF | + | 508 ± 11 | 225 ± 10 | 56.8 |
| 10 mM alloxan | + | 40 ± 9 | 18 ± 1 | — |
| B. [$^{3}$H]cAMP | ± | 9,558 ± 119 | 3,117 ± 174 | 67.4 |

100 μl of adenylate cyclase medium containing 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, and 0.5 mM [$\alpha$-$^{32}$P]AMP-PNP (4 μCi) were incubated for 30 min at 37°C with or without 2 mM $Pb(NO_3)_2$ and in the presence or absence of substances to be tested. The reaction was stopped by dilution with 1 ml of 5 mM ATP solution containing 20,000 cpm [$^{3}$H]cAMP for estimation of nucleotide recovery on subsequent column chromatography as described in Materials and Methods. The aluminum oxide column eluates were then incubated in the presence or absence of phosphodiesterase as described in Materials and Methods. Results are expressed as the mean ± SEM of triplicate counts of [$\alpha$-$^{32}$P]cAMP recovered in the supernate after the $ZnSO_4$-BaOH precipitation (part A). The [$^{3}$H]cAMP figures represent the composite recovery from all the 15 experimental samples (part B) and are the mean ± SEM.

TABLE VI

*Chromatographic Identification of cAMP and AMP Formed in the Adenylate Cyclase Assay in the Presence or Absence of Lead*

| Substances added | $Pb(NO_3)_2$ $2 \times 10^{-3}$ M | cAMP | AMP |
|---|---|---|---|
| | | *pmol* | |
| None | – | 4 | 348 |
| None | + | 28 | 2653 |
| Alloxan | – | 4 | 368 |
| Alloxan | + | 5 | 736 |

200 μl of the adenylate cyclase medium containing 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$ and 0.5 mM [$^{32}$P]AMP-PNP (9 μCi) were incubated for 30 min at 30°C with or without 10 mM alloxan and in the presence or absence of 2 mM $Pb(NO_3)_2$. The incubation was ended by adding 1 ml of either [$^3$H]cAMP (20,000 cpm) or [$^3$H]AMP (35,000 cpm) for estimation of the recovery on the subsequent chromatographic procedures. The nucleotides recovered in the Dowex eluates of the adenylate cyclase assay were then isolated by paper chromatography as described in Materials and Methods. $^{32}$P counts found in the corresponding [$^3$H]cAMP and [$^3$H]AMP regions were corrected for recovery and transformed to picomoles of nucleotides. Each number represents the mean of duplicate samples which did not differ by more than 5%.

contrast, the AMP found in the control accounts for over 0.3% of the original AMP-PNP and the addition of lead increased the nucleotide by eightfold or to 2.6% of the original material. Alloxan, in effect, completely inhibited the formation of cAMP by lead but blocked only 84% of the AMP formation attributable to the lead. The remaining AMP was still significantly above the control.

Experiments similar to the above were performed using [$^{32}$P]ATP as substrate. Lead caused a significant formation in the adenylate cyclase assay of a PDE-sensitive material which migrated as cAMP on paper chromatography. The quantities produced were similar to those found using [$^{32}$P]AMP-PNP as substrate. Alloxan also blocked this lead induced formation of cAMP from ATP. (Data not shown.)

## DISCUSSION

The present study demonstrates that lead is a potent inhibitor of adenylate cyclase. The concentration of lead, giving an almost complete inhibition of the enzyme in the fat cell plasma membrane, is 21–40 times less than the concentration used for the histochemical localization of adenylate cyclase. Indeed no residual enzyme activity was found in fat pad capillaries or pancreatic islet homogenates when assayed in media similar to those previously used for the electron histochemical localization of the adenylate cyclase in these tissues (5, 27).

In view of the inhibition of the adenylate cyclase activity by the lead, experiments performed to test the feasibility of doing the electron histochemistry using lead concentrations low enough to inhibit the enzyme only partially showed that at low concentration of lead a precipitate would not occur. It was demonstrated that the product of the lead and phosphate concentrations must exceed $1 \times 10^{-10}$ M in order to cause precipitation and that under the best of conditions for the enzyme this apparently did not occur since no precipitate was found on electron micrographs of the plasma membranes. The major reason is that the activity of adenylate cyclase is quite low compared to that of other enzymes being localized by histochemistry and would not produce enough PNP from the substrate to exceed the solubility product. For example, the adenylate cyclase activity of fat cell plasma membranes is less than 1/3,000th compared to the ATPase activity of the same membranes (14). When compared to that of islet homogenates and fat pad capillaries (Tables III and IV), the adenylate cyclase activity of the fat cell plasma membranes is easily 100 times greater than is found with these whole tissues making it all the more unlikely for enough PNP to be produced to exceed the solubility product coefficient.

Several attempts were made to protect the adenylate cyclase activity and at the same time to allow the use of higher concentrations of lead. ATP has been shown to chelate $Pb^{++}$ and to protect ATPase against inhibition by lead (23). However, even at a ratio of ATP to lead of 10, the inhibition of the adenylate cyclase by a small concentration of lead was not prevented, showing that the enzyme is sensitive to very low concentrations of free lead.

It seemed practical to determine if a sulfhydryl reducing agent could protect the adenylate cyclase from lead inhibition. Increasing concentrations of β-mercaptoethanol prevented the inactivation of the enzyme and restored the response to NaF and epinephrine. The sulfhydryl reagent similarly prevented the $^{32}$P precipitation. No lead precipitate was observed on micrographs of fat cell plasma membranes incubated in the presence of β-mercaptoethanol, although the enzyme activity was pro-

tected. The protection offered by the β-mercaptoethanol on the adenylate cyclase activity and the prevention of a precipitation reaction as shown by $^{32}P$ and electron microscope studies can probably be best explained by the sulfhydryl reagent reducing the free lead concentration.

Another important finding was that lead caused the nonenzymatic hydrolysis of AMP-PNP to AMP and cAMP and of ATP to cAMP. It has been shown previously that similar concentrations of lead can cause the production of various nucleotides from ATP (18). Cook et al. (2) have reported that barium hydroxide caused the formation of a compound from ATP which was characterized as cAMP. Kimura and Murad (10) have recently demonstrated the formation of cyclic GMP (cGMP) from GTP in the presence of various divalent cations excluding lead. It is possible that lead caused the formation of other nucleotides such as adenylyl phosphoramidate ($ADP\text{-}NH_2$) or ADP and of adenosine, but no attempts were made to document their production.

The electron microscope studies concerning adenylate cyclase histochemistry must be interpreted in the light of the above biochemical data. Since it has been clearly shown that the enzyme itself is inactive, the precipitate observed cannot be related to adenylate cyclase localization. The electron histochemical findings in this study can be explained in this manner. The quantity of precipitate seen on sections of fat cell plasma membranes incubated in the complete adenylate cyclase medium described by Howell and Whitefield (5) can be accounted for by the lead-phosphate compounds resulting from the nonenzymatic hydrolysis of AMP-PNP (Fig. 2). AMP would be of greater significance in this respect than cAMP since the production of the former was 100 times greater than the latter and represented a conversion of at least 2.6% of the total substrate. Previous studies in this laboratory (6, 9) have shown that most of these vesicles are right-side-out vesicles so that the location of the majority of precipitate on the outside of the fat cell plasma membrane vesicles was inconsistent with the supposed localization of the enzyme on the inner aspect of the membrane. When both substrate and $Mg^{++}$ were removed from the medium, a small quantity of fine precipitate remained which can be explained by the direct precipitation of lead on the membrane, which has been described by others (19).

Wagner et al. (27) in their studies on fat pad capillaries have used 6% dextran in an attempt to prevent or reduce nonspecific lead precipitation. The use of dextran did reduce the amount of precipitate associated with fat cell plasma membranes as well. Yet biochemical studies showed that dextran did not protect the plasma membrane adenylate cyclase from inhibition by lead even when present far in excess of the ratio of dextran to lead used in the histochemical technique. Thus, the precipitate visualized in the presence of the concentrations of lead used for histochemistry must be nonspecific.

Alloxan, a known inhibitor of adenylate cyclase (1), markedly reduced the lead precipitate in the studies of Wagner et al. (27). The same effect was found with fat cell plasma membranes (Fig. 3). Biochemical studies clearly showed that alloxan prevented the majority of nonenzymatic hydrolysis of AMP-PNP by lead. This would explain the reduced precipitate seen in the histochemical studies and is not related to the fact that alloxan can inhibit adenylate cyclase since the enzyme was already inactivated. The remaining precipitate could be accounted for by the remaining lead hydrolysis of AMP-PNP, which still represents over 0.3% of the total substrate, or possibly by other nonenzymatic hydrolysis products not documented in this study.

This study has shown that the lead histochemical approach to the localization of adenylate cyclase cannot be utilized. The enzymatic activity is completely inhibited at the lead concentrations usually employed for other enzyme histochemistry. At low concentration of lead allowing reasonable enzyme activity, the amount of PNP formed by a preparation of fat cell plasma membrane having a high specific activity as compared to whole tissue is too low to exceed the lead-phosphate solubility product coefficient. Moreover, at high concentrations, lead causes a significant nonenzymatic hydrolysis of AMP-PNP to AMP, cAMP, and possibly other nucleotides. The large quantities of precipitate reported by others (5, 24–27) as demonstrating adenylate cyclase activity must be artifact. It is essential that the biochemistry of any histochemical approach be thoroughly investigated with each tissue studied before the morphological observations are interpreted.

The authors wish to thank Mr. Robert Smith and Mrs. Judy Roy for their excellent technical assistance.

This work was supported by National Institutes of Health grant AM 11892. Dr. Lemay was a Postdoctoral Research Fellow of the Medical Research Council of Canada.

*Received for publication 7 November 1974, and in revised form 30 December 1974.*

## REFERENCES


1. COHEN, K. L., and M. W. BITENSKY. 1969. Inhibitory effects of alloxan on mammalian adenyl cyclase. *J. Pharmacol. Exp. Ther.* **169**:80–86.
2. COOK, W. H., D. LIPKIN, and R. MARKHAM. 1957. The formation of a cyclic dianhydrodiadenylic acid (I) by the alkaline degradation of adenosine-5′-triphosphoric acid (II). *J. Am. Chem. Soc.* **79**:3607–3608.
3. ESSNER, E. 1973. Phosphatases: In electron microscopy of enzymes. *In* Principles and Methods. Vol. 1. M. A. Hayat, editor. D. Van Nostrand Company Inc., New York. 44–76.
4. GANOTE, C. E., A. S. ROSENTHAL, H. L. MOSES, and L. W. TICE. 1969. Lead and phosphate as sources of artifact in nucleoside phosphatase histochemistry. *J. Histochem. Cytochem.* **17**:641–650.
5. HOWELL, S. L., and M. WHITFIELD. 1972. Cytochemical localization of adenyl cyclase activity in rat islets of Langerhans. *J. Histochem. Cytochem.* **20**:873–879.
6. JARETT, L. 1974. Subcellular fractionation of adipocytes. *Methods Enzymol.* **31**(Pt. A):60–71.
7. JARETT, L., M. REUTER, D. W. MCKEEL, and R. M. SMITH. 1971. Loss of adenyl cyclase hormone receptors during purification of fat cell plasma membranes. *Endocrinology.* **89**:1186–1190.
8. JARETT, L., and R. M. SMITH. 1974. Mode of action of $N^6$-$O^{2'}$-dibutyryl cyclic 3′,5′ AMP on fat cell metabolism. *Diabetes.* **23**:29–40.
9. JARETT, L., and R. M. SMITH. 1974. Electron microscopic demonstration of insulin receptors on adipocyte plasma membranes utilizing a ferritin-insulin conjugate. *J. Biol. Chem.* **249**:7024–7031.
10. KIMURA, H., and F. MURAD. 1974. Nonenzymatic formation of guanosine 3′:5′-monophosphate from guanosine triphosphate. *J. Biol. Chem.* **249**:329–331.
11. LACY, P. E., and M. KOSTIANOVSKY. 1967. A method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes.* **16**:35–39.
12. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
13. MIETKIEWSKI, K., F. DOMKA, L. MALENDOWICZ, and J. MALENDOWICZ. 1970. Studies on ATP hydrolysis in medium for histochemical demonstration of ATPase activity. *Histochimie.* **24**:343–353.
14. MCKEEL, D. W., and L. JARETT. 1970. Preparation and characterization of a plasma membrane fraction from isolated fat cells. *J. Cell Biol.* **44**:417–432.
15. MOSES, H. L., and A. S. ROSENTHAL. 1968. Pitfalls in the use of lead ion for histochemical localization of nucleoside phosphatases. *J. Histochem. Cytochem.* **16**:530–539.
16. MOSES, H. L., A. S. Rosenthal, D. L. Beaver, and S. S. Schuffam. 1966. Lead ion and phosphatase histochemistry. II. Effect of adenosine triphosphate hydrolysis by lead ion on the histochemical localization of adenosine triphosphatase activity. *J. Histochem. Cytochem.* **14**:702–710.
17. RODBELL, J., L. BIRNBAUMER, S. L. POHL, and H. M. J. KRANS. 1971. The glucagon-sensitive adenyl cyclase system in plasma membranes of rat liver. *J. Biol. Chem.* **246**:1877–1882.
18. ROSENTHAL, A. S., H. L. MOSES, D. L. BEAVER, and S. S. SCHUFFMAN. 1966. Lead ion and phosphatase histochemistry. I. Non-enzymatic hydrolysis of nucleoside phosphates by lead ion. *J. Histochem. Cytochem.* **14**:698–701.
19. ROSENTHAL, A. S., H. L. MOSES, C. E. GANOTE, and L. TICE. 1969. The participation of nucleotide in the formation of phosphatase reaction product: a chemical and electron microscope autoradiographic study. *J. Histochem. Cytochem.* **17**:839–847.
20. SALOMON, Y., C. LONDOS, and M. RODBELL. 1974. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548.
21. SCHARP, D. W., C. B. KEMP, M. J. KNIGHT, W. F. BALLINGER, and P. E. LACY. The use of Ficoll in the preparation of viable islets of Langerhans from the rat pancreas. *Transplantation (Baltimore).* **16**:686–689.
22. SZMIGIELSKI, S. 1971. The use of dextran in phosphatase techniques employing lead salts. *J. Histochem. Cytochem.* **19**:505–507.
23. TICE, L. W. 1969. Lead-adenosine triphosphate complexes in adenosine triphosphatase histochemistry. *J. Histochem. Cytochem.* **17**:85–94.
24. TOSKIHIDE, S., R. GRACIA-BUNNEL, and D. BRANDES. 1974. Ultrastructural cytochemical localization of adenylate cyclase in the rat nephron. *Lab. Invest.* **30**:222–229.
25. WACHSTEIN, M., and E. MEISEL. 1957. Histochemistry of hepatic phosphatases at a physiological pH with special reference to the demonstration of bile canaliculi. *Am. J. Pathol.* **27**:13–23.
26. WAGNER, R. C., and M. BITENSKY. 1974. Adenylate cyclase in electron microscopy of enzymes. *In* Principles and Methods. Vol. 2. M. A. Hayat, editor. D. Van Nostrand Company Inc., New York. 110–131.
27. WAGNER, R. C., P. KREINER, R. J. BARRNETT, and M. W. BITENSKY. 1972. Biochemical characterization and cytochemical localization of a catecholamine-sensitive adenylate cyclase in isolated capillary endothelium. *Proc. Natl. Acad. Sci. U. S. A.* **69**:3176–3179.
28. YOUNT, R. G., D. BABCOCK, W. BALLANTYNE, and D. OJALA. 1971. Adenylyl imidodiphosphate, an adenosine triphosphate analog containing a P-N-P linkage. *Biochemistry.* **10**:2484–2489.