Plasma Membrane Cyclic Adenosine 3':5'-Monophosphate Phosphodiesterase of Cultured Cells and Its Modification after Trypsin Treatment of Intact Cells

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SUMMARY

Treatment of intact chicken embryo fibroblasts in tissue culture with trypsin leads to an alteration of the kinetic properties of a particulate cyclic adenosine 3':5'-monophosphate (cyclic AMP) phosphodiesterase. Distribution studies show that this phosphodiesterase is principally located in the plasma membrane. Little activity is found in nuclear, mitochondrial, and microsomal fractions. The trypsin-sensitive phosphodiesterase activity does not function on the exterior of the cell membrane. The K_m and V_max of the enzyme are altered by trypsin treatment of intact cells and this effect is quantitatively reproduced by trypsin treatment of the isolated plasma membrane fraction. The supernatant fraction of chicken embryo fibroblasts contains phosphodiesterases capable of hydrolyzing both cyclic AMP and cyclic GMP. DEAE-cellulose chromatography of the supernatant fraction resolves phosphodiesterase activity into two peaks. One fraction is specific for cyclic AMP and is kinetically similar to the plasma membrane enzyme, whereas the other fraction hydrolyzes both cyclic nucleotides in a Michaelis-Menten fashion. The cyclic AMP-specific phosphodiesterase appears to be under negative cooperative regulation. These studies show that chicken embryo fibroblasts contain a cyclic AMP phosphodiesterase in the plasma membrane, the kinetic properties of which can be altered by an enzyme that acts on the outer cell membrane.

EXPERIMENTAL PROCEDURE

The secondary CEF were grown in Eagle’s modified essential medium supplemented with fetal bovine serum, sodium glutamate (2 mM), pyruvate (1 mM), dextrose (2 g per liter), tylosine (1%), penicillin (1%), streptomycin (1%), and tryptose phosphate broth (10%). The cells were plated in medium containing 10% fetal bovine serum and the medium was then changed daily with 5% serum. Secondary CEF (2 to 3 mg of protein per 50-cm² dish) were used throughout. The cells were rinsed with cold phosphate-buffered 0.9% NaCl solution three times followed by rinsing twice with homogenization buffer (50 mM Tris-acetate (pH 6.0)-3.75 mM mercaptoethanol). Cells were homogenized (0.7 ml of buffer per dish) with a Dounce homogenizer (“B” pestle, 15 strokes). All steps were carried out at 4°C. Various fractions prepared by centrifugation and DEAE-cellulose chromatography were used. Protein was determined by the method of Lowry et al. (5) using bovine serum albumin as a standard.

Preparation of Two-Phase System—The polyethylene-glycol dextran 500 two-phase aqueous system for isolating plasma membranes was prepared by the method of Brunette and Till (6) with slight modification. Fifty milliliters of 16.7% (w/v) dextran 500 in deionized water, 25.75 ml of 23% (w/v) polyethylene
glycol (Carbowax 6000) in deionized water, 72 ml of 0.22 M sodium phosphate, pH 6.5, and 12 ml of deionized water were mixed and allowed to settle in a separatory funnel. After the two phases separated, the top and bottom phase were collected and stored at 4° until used.

Isolation of Plasma Membrane Fraction—For the isolation of plasma membranes, cells were allowed to swell for 10 min in a hypotonic buffer (10 mM Tris-aceate (pH 6.0)-3 mM MgCl₂) and then homogenized in this buffer rather than in the standard homogenization buffer. Following homogenization the homogenate was centrifuged at 800 × g for 10 min. The pellet was dissolved in 2.5 ml of the top phase of the two-phase separation system. An equal volume of bottom phase was mixed with the top phase solution and centrifuged at 8500 rpm in a Sorvall RC-2R centrifuge for 10 min (HB-4 swinging bucket rotor). The plasma membrane fraction migrated to the interface, whereas nuclei and cell debris sedimented to the bottom. The supernatant containing the plasma membrane at the interface was decanted gently, mixed, and recentrifuged twice. The final interface was removed by a Pasteur pipette and diluted 1:4 with the standard homogenization buffer. Under these conditions only one phase remained. The solution was centrifuged at 1000 × g for 10 min in an International centrifuge to sediment the plasma membrane and then it was resuspended in homogenization buffer.

DEAE-cellulose Chromatography—Chromatography was performed in columns (0.9 × 20 cm) with a volume of 10 ml. At least 2 column volumes of buffer passed through the column prior to application of the sample. Phosphodiesterase was eluted with a linear gradient from 0 M to 1 M sodium acetate as previously described. For stepwise elution with 0.3 M and 0.7 M sodium acetate, 2.5-ml columns were employed.

Phosphodiesterase Assay—In order to be able to detect high and low Kₘ phosphodiesterase activity, a sensitive radioactive assay was used (8, 9). [H]-Labeled cyclic AMP was assayed to [3H]-labeled 5'-AMP by cyclic nucleotide phosphodiesterase. The 5'-AMP was then converted to [3H]adenosine by a snake venom nucleotidase (Ophiophagus hannah). AG1-X2 resin was added directly to the reaction mix to bind all unreacted [3H]-labeled cyclic AMP. The [3H]adenosine remaining in solution was counted in a liquid scintillation spectrometer. The reaction volume was 0.200 ml contained 0.05 ml of assay buffer (40 mM Tris-Cl (pH 8.0)-5.75 mM mercaptoethanol), 0.05 ml of cyclic AMP (25 pmoles, 430,000 cpm), and 0.100 ml of enzyme solution. When Kₘ studies were performed the assay buffer was replaced by 0.05 ml of the appropriate concentration of unlabeled cyclic AMP. The cyclic AMP concentrations ranged from 0.5 μM to 20 μM. Cyclic GMP phosphodiesterase activity was assayed in a similar manner.

Trypsin Treatment of Intact Cells—Confluent secondary CEF were fed (medium changed) with 2% fetal bovine serum 12 hours prior to the experiment. Trypsin (final concentration 3.5 to 140 μg per ml) was added to each plate of confluent cells for the given period of time (0 to 60 min). Then the medium containing the trypsin was removed and the cells were washed as before in homogenization buffer containing 1.25 mg per ml of soybean trypsin inhibitor. Total protein and soluble cyclic GMP phosphodiesterase activity were assayed to determine the degree of cell destruction by trypsin.

Trypsin Treatment of Homogenized Cells—Trypsin at a final concentration 5 μg per ml was added to a given enzyme preparation and incubated for 30 min at room temperature. The reaction was stopped by the addition of soybean trypsin inhibitor at 50 μg per ml. The control solution was incubated as above without trypsin and stopped with soybean trypsin inhibitor.

DNA Synthesis—DNA synthesis was measured by labeling growing cells for 2 hours with [3H]thymidine (6 Ci per μM). The medium was then removed and the nuclear and plasma membrane fractions were isolated as described above. The fractions were precipitated with cold 10% trichloroacetic acid and the pellet was collected by centrifugation. The trichloroacetic precipitation was repeated three times. Finally, 0.5 ml of Nuclear Chicago Solubilizer was added to the pellet and the solution was heated to 55° for 10 min. The solution was then mixed with 10 ml of a toluene-Liquifluor mixture and counted.

Computer Analysis—The partition functions for the models used were generated by the matrix method (10). The experimental parameters (velocity and free cyclic AMP concentration) are related to the partition function for a given model by the following equation: 

\[ \phi = \left( \frac{1}{n} \right) \left( \frac{d \ln Q}{d \ln (mK_0)} \right) \]

where Q is the partition function of a given model, m is the free cyclic AMP or free magnesium concentration, K₀ is the intrinsic association constant, and φ is the reaction velocity normalized by the maximum velocity. The experimental data are fit to the theoretical model by a nonlinear least square error program (11). An IBM Magnetic Card Selectric Typerwriter-data phone remote terminal system was used to submit the data to the central NIH computer facility.

Materials—[H]-Labeled cyclic AMP (22.1 Ci per mm) and [3H]-labeled cyclic GMP (4.47 Ci per mm) were obtained from New England Nuclear. Cyclic AMP and cyclic GMP were acquired from Schwarz-Mann and Calbiochem, respectively, and were not further purified. Polyethylene glycol (Carbowax 6000) was obtained from Union Carbide, dextran 500 from Pharmacia, Inc., and anion exchange resins (AG1-X2 200-400 mesh and Cellex D) from Bio-Rad. Both resins were extensively washed in 0.5 N NaOH, 0.5 N HCl, and deionized water prior to use. Soybean trypsin inhibitor and snake venom (Ophiophagus hannah) were obtained from Sigma and trypsin from Microbiological Associates, Inc.

RESULTS

Preparation of Plasma Membrane—Cell homogenates were initially centrifuged at 800 × g for 10 min to sediment nuclei, cell debris, and membranes. The supernatant fraction was then subjected to three more centrifugations to obtain a mitochondrial, microsomal, and soluble fraction (Fig. 1). The 800 × g pellet was subjected to further fractionation in the two-phase aqueous separation system (6) to obtain a nuclear and plasma membrane fraction (Fig. 1). The layer at the interface is the plasma membrane fraction and is greatly enriched in the activity of adenylate cyclase, an enzyme associated with the plasma membrane. By electron microscopic analysis, the interface fraction mainly contained plasma membrane vesicles with some contamination with rough endoplasmic reticulum (Fig. 2). No nuclei or mitochondria were observed. However, to quantitate the amount of possible nuclear contamination, the cells were labeled with [3H] thymidine. The nuclear fraction contained 36,000 cpm of labeled DNA, whereas the plasma membrane fraction contained less than 1,000 cpm.

Distribution of Cyclic Nucleotide Phosphodiesterase—The distribution of cyclic nucleotide phosphodiesterase in the various fractions and the effect of Dounce homogenization on the distribution following homogenization for 15 and 75 strokes in a manner.

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Dounce homogenizer are listed in Table 1. When homogenized for 15 strokes, phosphodiesterase activity was found in the plasma membrane and soluble fractions. Very little activity was found in the microsomal fraction indicating that the small amount of microsomal material in the plasma membrane fraction was not contributing significant amounts of phosphodiesterase activity. The phosphodiesterase in the plasma membrane fraction appears to be specific for cyclic AMP as it does not hydrolyze the other naturally occurring cyclic nucleotide, cyclic GMP.

With more extensive homogenization, less of the particulate enzyme was found in the plasma membrane fraction and more in other fractions (Table 1). After 75 strokes considerable phosphodiesterase was reproducibly found in the nuclear fraction and the small amount found in the mitochondrial fraction after a shorter homogenization was also increased. This redistribution could be due to fragmentation of the plasma membrane or to solubilization of the phosphodiesterase with subsequent adsorption to other particulate fractions. The $K_m$ of the cyclic nucleotide phosphodiesterase in the nuclear fraction (Table 1) after homogenization with 75 strokes was identical with that of the enzyme in the plasma membrane fraction (data not shown).

The soluble fraction ($S_1$) contained phosphodiesterase activity that hydrolyzed both cyclic AMP and cyclic GMP. The cyclic GMP $K_m$ was 3 $\mu$M. When the $S_1$ fraction was subjected to chromatographic analysis on DEAE-cellulose columns, two active cyclic AMP phosphodiesterase peaks were found (Fig. 3). The first peak, which elutes with 0.3 M sodium acetate, hydrolyzed cyclic AMP and cyclic GMP. Cyclic AMP hydrolysis by this fraction followed normal Michaelis-Menten kinetics (Fig. 4b). The enzyme in the second peak was specific for cyclic AMP and had kinetic characteristics similar to those of the plasma membrane enzyme (Fig. 4b) (see below).

**Table I**

| Fraction $^a$ | Dounce 15X | Dounce 75X |
|---------------|------------|------------|
|               | Total units $b$ | Units per mg of protein | Total units | Units per mg of protein |
| $P_1$ (mitochondria) | 2.0 | 1.9 | 4.0 | 3.3 |
| $P_2$ (microsomes) | <1.0 | <1.0 | <1.0 | <1.0 |
| $S_1$ (soluble) | 15.2 | 10.0 | 15.0 | 8.7 |
| $P_4$ (nuclei, cell debris) | <1.0 | <1.0 | 5.2 | 4.6 |
| Interface (plasma membrane) | 14.8 | 5.0 | 8.2 | 4.6 |
| Recovery | 58% | 59% |

$^a$ See Fig. 1 for description of the various fractions.

$^b$ A unit is defined as picomoles of cyclic AMP hydrolyzed per min per assay volume.

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**Fig. 2.** Electron micrograph of the isolated plasma membrane fraction. The bar represents 1 $\mu$m. Magnification, $\times$ 31,900. Courtesy of Dr. Mark C. Willingham.
Fig. 3. DEAE-cellulose chromatography of a 30,000 × g supernatant preparation. The linear gradient was 0 to 1 M sodium acetate. The gradient was started at Fraction 40. ○--○, cyclic AMP hydrolysis (0.12 μM); •—•, optical density at 280 nm.

Fig. 4. a, Lineweaver-Burk plot of cyclic AMP hydrolysis by the 800 × g pellet fraction-Dounce 15x. The 800 × g pellet fraction was routinely used for most kinetic studies as the cyclic nucleotide phosphodiesterase in this fraction is bound to the plasma membrane (Table I). Velocity expressed as picomoles of cyclic AMP hydrolyzed per min per mg of protein per assay volume. b, Lineweaver-Burk plot of cyclic AMP hydrolysis by a 0.3 M sodium acetate cut (○—○) and a 0.7 M sodium acetate cut (O—O) obtained from chromatographing a 30,000 × g supernatant preparation on DEAE-cellulose. Velocity expressed as picomoles of cyclic AMP hydrolyzed per min per assay volume. The kinetic data from a and from b (0.7 M sodium acetate) yielded identical binding constants when fit to a two-enzyme model.

Kinetic Analysis of Plasma Membrane Cyclic Nucleotide Phosphodiesterase—The hydrolysis of cyclic AMP by the plasma membrane enzyme does not follow normal Michaelis-Menten kinetics (Fig. 4b). These data can be fit to a negative cooperative model (11) and yield a cooperativity constant of 0.2, suggesting that this enzyme can function to hydrolyze cyclic AMP in a negatively cooperative fashion. However, steady state kinetic data cannot be used to distinguish between one enzyme undergoing site-site interactions or a membrane-bound enzyme containing two different binding sites for cyclic AMP. Magnesium ion is required for catalytic activity of the enzyme. Basal activity in the absence of magnesium can be removed by EDTA treatment and activity is restored by the addition of magnesium after the enzyme preparation is dialyzed to remove the EDTA (data not shown). The hydrolysis of a fixed concentration of cyclic AMP (1.0 μM) as a function of Mg²⁺ concentration is presented in Fig. 5a. Magnesium continues to stimulate enzyme activity up to 30 mM. The Mg²⁺ kinetics like that of cyclic AMP is suggestive of complex hydrolysis (Fig. 5b). The data can be fit to a two-enzyme model and yield a low Kₘ of 0.06 mM and a high Kₘ of 1 mM for magnesium.

Trypsin Treatment of Intact Cells—Mild trypsin treatment of intact cells can alter the properties of the external cell membrane (12, 13) even when trypsin is covalently linked to insoluble polymers (14). Could mild trypsin treatment of the cell exterior alter the properties of the plasma membrane cyclic nucleotide phosphodiesterase? Confluent CEF were treated with 3.5 μg per ml of trypsin in medium containing 2% fetal bovine serum. Within 5 min plasma membrane-bound cyclic nucleotide phosphodiesterase activity was reduced and was decreased further after 30 min (Fig. 6). The presence of soybean trypsin inhibitor in the incubation medium blocked the trypsin effect on the plasma membrane enzyme. Hydrolysis of cyclic GMP by the 30,000 × g supernatant fraction was simultaneously measured to assess any possible effect of trypsin on a soluble enzyme. The activity of the enzyme hydrolyzing cyclic GMP was not affected by the trypsin treatment (Fig. 6). As total cell protein and the membrane pellet protein were unchanged by trypsin treatment, the specific activity of the cyclic nucleotide phosphodiesterase decreased.

The effect of trypsin treatment of intact cells on the kinetic properties of the membrane cyclic nucleotide phosphodiesterase was determined as a function of time. At each time point indicated (Fig. 7b) the plasma membrane fraction was isolated and enzyme activity was measured. There was a noticeable change in the Kₘ of the plasma membrane enzyme within 10 min of treatment and a change in Vₘₙₙₑₓ was observed after 1
Cyclic AMP hydrolysis by exterior of intact CEF

| Time (min) | Cyclic AMP metabolised | Total cpm/10 μl medium |
|-----------|------------------------|------------------------|
| 0         | 0.0                    | 52,180                 |
| 10        | 0.4                    | 48,620                 |
| 30        | 1.2                    | 48,620                 |
| 60        | 3.0                    | 49,033                 |
| 120       | 5.2                    | 49,033                 |
| 210       | 7.3                    | 47,572                 |

a Cyclic AMP, 3 × 10⁻² M.

Computer model study of membrane-bound cyclic nucleotide phosphodiesterase

| Fraction | $K_1$ | $K_1'$ | $V_1$ | $V_1'$ | $V_1/V_1'$ | Decrease in $V_1$ % | Decrease in $V_1'$ % |
|----------|-------|--------|-------|--------|------------|---------------------|---------------------|
| P₀ (800 x g pellet) | 0.38  | 4.6    | 2.62  | 18.0   | 7.2        | 15                  | 37                  |
| P₁ treated with 5 μg/ml of trypsin for 30 min | 0.62  | 4.7    | 2.24  | 11.9   | 5.3        | 15                  | 37                  |

a The data were fit to a two independent enzyme model.
b $K_1$ defines the dissociation constant to the high affinity site.
c $K_1'$ defines the dissociation constant to the low affinity site.
d $V_1$ defines the maximum velocity of the high affinity form in picomoles per min per assay volume.
e $V_1'$ defines the maximum velocity of the low affinity form; units as in Footnote d above.
f $V_1/V_1'$ is the ratio between the low and high affinity forms in terms of $V_{max}$.
g The per cent difference represents the difference between the high affinity form $V_{max}$ before and after trypsin treatment.
h Same as Footnote g above for the low affinity form.

Discussion

Chicken embryo fibroblasts contain cyclic nucleotide phosphodiesterases capable of hydrolyzing both of the naturally occurring cyclic nucleotides. Upon fractionation of the homogenate we find that the particulate fraction contains a cyclic AMP-specific phosphodiesterase, whereas the soluble fraction hydrolyzes both cyclic AMP and cyclic GMP. DEAE-cellulose chromatography resolves the soluble material into two peaks of cyclic AMP-phosphodiesterases. The soluble enzyme is kinetically similar to the particulate enzyme, and the amount of particulate enzyme present in the supernatant fraction can be increased by further homogenization of the membrane preparation. Thus the soluble cyclic AMP-specific phosphodiesterase and the particulate phosphodiesterase are most likely identical.

The particulate phosphodiesterase is greatly enriched in the plasma membrane fraction. The plasma membrane was identified by electron microscopy and activity of adenylyl cyclase, which is a marker enzyme for plasma membrane (15). Very little activity is detectable in nuclear, mitochondrial, or micro-

Fig. 7. a, time course of the effect of trypsin (140 μg per ml in medium with 5% fetal bovine serum) on cyclic nucleotide phosphodiesterase as in Fig. 6. b, Lineweaver-Burk plot of cyclic AMP hydrolysis by the 800 x g pellet at time: 0 min, ΔΔΔΔ; 10 min, ••••; and 60 min, O---O. At each time point indicated the cells were rinsed with buffer containing soybean trypsin inhibitor and homogenized as in Fig. 6.

Fig. 8. Isolated plasma membrane was treated with trypsin (5 μg per ml) and the cyclic nucleotide phosphodiesterase activity was compared to that of untreated membranes. O---O, trypsin treated; ••••, untreated. The effect of trypsin treatment on the isolated plasma membrane cyclic nucleotide phosphodiesterase is similar to the effect on cyclic nucleotide phosphodiesterases isolated from intact cells treated with trypsin (Fig. 7b). Detailed kinetic analysis of control (••••) and trypsin-treated (O---O) isolated plasma membrane cyclic nucleotide phosphodiesterase. Trypsin alters the biphasic nature of the curve.

The effect of trypsin treatment on intact cells (Fig. 7b) can be quantitatively reproduced by trypsin treatment of the isolated plasma membrane (Fig. 8a). The results of detailed kinetic analysis show that trypsin treatment of isolated membranes alters the biphasic characteristic of the Lineweaver-Burk plot (Fig. 8b). A computer analysis of these data (Table II) reveals that trypsin alters the $K_m$ of the high affinity site but not of the low affinity site. Trypsin also decreases the $V_{max}$ of both sites.

To test whether the plasma membrane phosphodiesterase might function on the exterior of the cell, 3H-labeled cyclic AMP was incubated in medium layered over cells with and without 5% serum. Over a 3½-hour incubation period no hydrolysis was observed in the medium containing serum. In the cells
sonal fractions. Thus we conclude the cyclic AMP-specific enzyme is a normal component of the plasma membrane of chicken embryo fibroblasts. The plasma membrane enzyme exhibits anomalous kinetics for both cyclic AMP and magnesium. Similar kinetics have been reported for cyclic AMP hydrolysis for a cyclic AMP phosphodiesterase found in a number of other tissues (7, 9, 16–21). The biphasic plot we observe with the chick cell enzyme is not due to contamination by the soluble cyclic AMP-cyclic GMP phosphodiesterase as the plasma membrane enzyme does not hydrolyze cyclic GMP and has different kinetic constants from the soluble cyclic AMP-cyclic GMP enzyme.

Our study does not reveal whether the plasma membrane enzyme is two separate enzymes or one enzyme that is regulated by site-site interactions. Analysis of intact completely purified cyclic nucleotide phosphodiesterase is required to resolve this point. Nevertheless, cyclic AMP is hydrolyzed in a negatively cooperative fashion as described by Koshland and co-workers (22, 23). Computer model studies yielded a cooperativity constant ranging between 0.1 and 0.2 (11) for various enzyme preparations. Negative cooperativity allows the phosphodiesterase to amplify increases in cyclic AMP levels while effectively repressing decreases.

Since the low $K_m$ negative cooperative phosphodiesterase copurifies with the plasma membrane, it was of particular interest to determine whether substances known to act on the exterior of the cell could affect this enzyme. For this study we chose trypsin which affects the growth and cyclic AMP levels of cultured cells (25, 26). Trypsin covalently linked to insoluble polymers is able to stimulate growth (14). Treatment of intact chicken embryo fibroblasts with trypsin did alter the kinetic properties of the plasma membrane phosphodiesterase without affecting the soluble cyclic GMP enzyme. The trypsin action was shown to be time-dependent. Kinetic analysis revealed that the membrane phosphodiesterase prepared from cells treated with trypsin for 10 min showed an increase in $K_m$. When cells were treated for 1 hour, there was no further increase in $K_m$, but a decrease in $V_{max}$ was observed. The phosphodiesterase catalytic activity does not function on the cell exterior, and, therefore, it is apparent that proteolytic modification of the outer cell membrane can alter the kinetic properties of a membrane-bound phosphodiesterase. Whether this is a direct effect on a portion of the enzyme protruding through the membrane or an indirect effect is not yet established. The trypsin effect on intact cells is quantitatively reproduced by trypsin treatment of the isolated plasma membrane. The results of computer model analysis of the kinetic properties of the enzyme showed that the $K_m$ of the high affinity site and the $V_{max}$ of both sites were altered by trypsin treatment.

When treated with trypsin, contact-inhibited 3T3 cells show a marked decrease in cyclic AMP levels followed by resumption of growth (26). Under the growth conditions employed in our investigations, the chicken embryo fibroblasts never attain density-dependent inhibition of growth as defined by Selton and Rubin (25), and we do not know whether or not trypsin affects their growth. Thus no comparison can be made between our results with trypsin and results of investigations on the effect of trypsin on contact-inhibited cells. In our view, the important finding in the trypsin experiments is that a cyclic AMP phosphodiesterase which is in the plasma membrane and appears to exhibit its catalytic function inside the cell can have its activity changed by an agent that acts on the outside of the cell. Possible effects of other agents which affect growth, such as insulin, serum, or viral transformation, are currently being investigated.

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REFERENCES

1. Johnson, G. S., Friedman, R. M., and Pastan, I. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 425
2. Eise, A. W., and Puck, T. T. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 358
3. Otten, J., Johnson, G. S., and Pastan, I. (1971) Biochem. Biophys. Res. Commun. 44, 1192
4. Anderson, W. B., Johnson, G. S., and Pastan, I. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 1055
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265
6. Brunette, D. M., and Till, J. E. (1971) J. Membrane Biol. 5, 215
7. Russell, T. R., Terasaki, W. L., and Appleman, M. M. (1973) J. Biol. Chem. 248, 1334
8. Brooker, G., Thomas, L. J., and Appleman, M. M. (1968) Biochemistry 7, 4177
9. Thompson, W. J., and Appleman, M. M. (1971) Biochemistry 10, 311
10. Schneider, F. W., Russell, T. R., and Rawlings, P. K. (1970) J. Mol. Biol. 48, 103
11. Russell, T. R., Thompson, W. J., Schneider, F. W., and Appleman, M. M. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1791
12. Burger, M. M. (1969) Proc. Natl. Acad. Sci. U. S. A. 62, 994
13. Kappeler, M., and Dajanski, E. (1972) Nature New Biol. 235, 184
14. Burger, M. M. (1971) Biomembranes 2, 247
15. Del Pierre, J. W., and Kannoussy, M. L. (1971) J. Cell Biol. 56, 275
16. Rosen, O. M. (1970) Arch. Biochem. Biophys. 137, 435
17. Thompson, W. J., and Appleman, M. M. (1971) J. Biol. Chem. 246, 3145
18. Beavo, J. A., Hardman, J. G., and Sutherland, E. W. (1970) J. Biol. Chem. 245, 5049
19. Jard, S., and Burnard, M. (1970) Biochem. Biophys. Res. Commun. 41, 781
20. Kakiuchi, S., Yamazaki, R., and Teshima, Y. (1971) Biochem. Biophys. Res. Commun. 42, 968
21. D’Armsito, M., Johnson, G. S., and Pastan, I. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 450
22. Conway, A., and Koshtani, D. E., Jr. (1968) Biochemistry 7, 4011
23. Levyite, A., and Koshtani, D. E., Jr. (1969) Proc. Natl. Acad. Sci. U. S. A. 62, 1121
24. Appleman, M. M., Thompson, W. J., and Russell, T. R. (1973) in Advances in Cyclic Nucleotide Research (Greenwald P., and Robison, G. A., eds) Vol. 3, Ch. 2. Raven Press New York
25. Sefton, B., and Rubin, H. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 3154
26. Otten, J., Johnson, G. S., and Pastan, I. (1972) J. Biol. Chem. 247, 7082
