The requirements for cholera toxin-catalyzed ADP ribosylation of the purified regulatory component of adenylate cyclase are described. In addition to the toxin, this reaction is dependent on or is facilitated by NAD, GTP, phospholipid, and a factor found associated with plasma membranes from several sources. Factor activity is heat-labile and protease-sensitive but is unaffected by treatment with N-ethylmaleimide. Gel filtration indicates that the factor behaves as a monodisperse species with a Stokes radius of 3.2 nm. The factor thus appears to be a protein that is distinct from any of the known components of adenylate cyclase.

Factor activity was also detected in the cytoplasm of S49 cells. The cytoplasmic factor was smaller (Stokes radius = 2.0 nm) than the membrane-derived factor, and it was inactivated in the presence of sodium chloride.

The initial rate of activation of the regulatory component of adenylate cyclase by toxin was found to be linearly related to the amount of factor present in the reaction. This has allowed the quantitation and partial purification (33-fold from detergent extracts) of the factor from turkey erythrocyte membranes.

Cholera toxin activates adenylate cyclase by catalyzing the ADP ribosylation of the GTP-binding regulatory component of the enzyme complex (for review, see Ref. 1). The consequences of this toxin-dependent, covalent modification include inhibition of a hormone-stimulated GTPase activity (2) and enhancement of activation of adenylate cyclase by GTP. If [32P]NAD is included as a substrate for the toxin, putative subunits of G/F are radio-labeled (3). Optimal ADP ribosylation of G/F in membranes requires activated cholera toxin.

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The abbreviations used are: G/F, the regulatory component of adenylate cyclase; C, the catalytic component of adenylate cyclase; cyc-*, a variant of the S49 lymphoma line that is devoid of G/F activity; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

NAD (which serves as the ADP-ribosyl donor), and GTP (4). GTP may serve several functions, as described by Nakaya et al. (5), including acting as a cofactor in the toxin-dependent activation reaction and stabilizing the ADP-ribosylated product. In addition, assay of G/F-dependent adenylate cyclase activity serves as a sensitive index of the extent of covalent modification of G/F. It is not known if the occupation of a single GTP binding site is sufficient for observation of all of these effects of guanine nucleotides.

This laboratory has purified the regulatory component of adenylate cyclase from membranes of rabbit liver (3, 6) and turkey erythrocytes (7). Rabbit liver G/F consists of three peptides with apparent Mr = 36,000, 45,000, and 52,000. Turkey erythrocyte G/F lacks the 52,000-dalton peptide. Only the 52,000- and 45,000-dalton peptides are substrates for toxin-dependent ADP ribosylation (3, 6, 7). The requirements for toxin-dependent ADP ribosylation of pure G/F are described below; these include the presence of a membrane-derived or a soluble protein factor for optimal modification of the regulatory protein.

**Experimental Procedures**

**Materials**—Cholera toxin and L-a-dimyristoyl phosphatidylethanolamine were purchased from Calbiochem. L-a-dimyristoyl phosphatidylcholine, L-a-dipalmitoyl phosphatidylethanolamine, phosphatidylserine, phosphatidycholine, and cholesterol were purchased from Sigma. Lipid suspensions were sonicated in 20 mM sodium HEPES (pH 8) and 1 mM EDTA for 15 to 20 min prior to use. Trypsin, chymotrypsin, N-ethylmaleimide, and ATP were also obtained from Sigma. [α-32P]ATP was synthesized according to the procedure of Johnson and Walsch (8). This radio-labeled nucleotide was used both for adenylate cyclase assays and for the synthesis of [32P]NAD. NAD labeled with [32P] in the AMP moiety was synthesized by the method of Casell and Pfeiffer (9). G/F was purified from rabbit liver and turkey erythrocytes as described by Sternweis et al. (6) and Hanski et al. (7). All protein determinations were performed by the method of Schaffner and Weissmann (10).

**S49 Lymphoma Membranes and Extracts**—Clones of S49 lymphoma cells that were utilized and the methods for their culture have been described previously (11). The cyc- variant of the S49 cell, which is essentially devoid of Mg-ATP-dependent adenylate cyclase activity, contains the catalytic subunit of the enzyme but is deficient in the activity of G/F (12). Plasma membranes from these cells were purified according to the method of Ross et al. (11). Cholate extracts of S49 plasma membranes were prepared as described by Sternweis and Gilman (13), except that solubilization was performed at an initial protein concentration of 10 mg/ml in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 1% sodium cholate.

G/F and Adenylate Cyclase Assay—G/F was assayed by its ability to reconstitute adenylate cyclase activity in cyc- membranes. Samples of G/F (15 µl) were added to 25 µl (50 µg of protein) of cyc- membranes. Reconstitution and assay then proceeded as described by Sternweis and Gilman (13) in the presence of the activators indicated in the text. Conditions were chosen such that the rate of production of cyclic AMP in the assay was constant and linearly related to the concentration of G/F.

**Turkey Erythrocyte Membranes and Extracts**—Fresh turkey blood was collected and stored at 4 °C for up to 3 days in the presence of 4.75 mM EDTA and 0.25 mM ethylene glycol bis[2-aminoethyl ether]-N,N,N',N'-tetraacetic acid to prevent coagulation. Membranes were purified as described by Hanski et al. (7), and membranes proteins were solubilized with 1.25% sodium cholate in 25 mM Tris-HCl (pH 8), 1 mM EDTA, 0.25 M sucrose, 1 mM diithiothreitol, 10 µM phenylmethylsulfonyl fluoride, 0.7 M NaCl at an initial protein concentration of 10 mg/ml.

**Partial Purification of Factor from Turkey Erythrocyte Membranes**—The membrane-bound factor (see below) from turkey eryth-
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...membranes was used in the complete reaction mixture, only G/F and the A1 subunit of choler toxin were significantly labeled with 32P. The requirement for the factor can be seen in the experiment shown in Fig. 1, in which purified rabbit liver G/F served as the substrate for the toxin. Similar results were obtained when purified turkey erythrocyte G/F was used (Fig. 1). When either the factor or dimeristoyl phosphatidylcholine was omitted, the incorporation of 32P into G/F was almost completely abolished. Choler toxin is an absolute requirement for the incorporation of 32P into any macromolecule under these conditions. Omission of GTP from the reaction mixture also resulted in decreased incorporation of label into G/F. A similar pattern of requirements is observed if covalent modification of G/F is monitored by assay of its ability to reconstitute GTP-dependent adenylate cyclase activity in cyc- membranes (Fig. 1).

The activation of G/F as a function of time is shown in Fig. 2A. It is clear that the activation is absolutely dependent on cholera toxin and markedly accelerated by the factor. The initial rate of activation of G/F in the presence of the factor is constant for about 30 min. When initial rates are plotted as a function of the concentration of the factor in the activation reaction, a linear relationship was obtained (Fig. 2B). This can thus be used as a quantitative assay for the factor.

Membrane Sources of the Factor—The experiments shown above were performed with factor from turkey erythrocyte plasma membranes. Cyc- membranes are also a useful source of factor that is free of G/F activity (see below). Attempts to demonstrate factor activity in crude cholate extracts of rabbit liver membranes were unsatisfactory. This was likely the result of very high levels of NADase activity in such preparations (data not shown). However, rabbit liver factor activity eluted after G/F activity on the ACA-34 gel filtration column (the second step in the purification of G/F from rabbit liver) in the same way that turkey erythrocyte...
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G/F and factor are separated. It is at this stage of purification that the G/F from both rabbit liver and turkey erythrocytes loses the ability to serve as substrates for cholera toxin without the addition of factor. Thus, a membrane-bound factor required for cholera toxin-dependent ADP ribosylation of purified G/F is present in each of the three membrane preparations studied.

Characterization of the Factor—The factor from cyc− appears to be firmly associated with membranes, since complete solubilization requires at least 0.8% chololate. (Solubilization of factor or G/F from turkey erythrocyte membranes requires similar concentrations of cholate and high concentrations of salt, e.g. 0.7 M NaCl.) No activity is extracted from cyc− membranes with either 0.5 M NaCl or 3 mM EDTA in the absence of detergent. Initial characterization was performed using factor from a 1% cholate extract of purified cyc− membranes. The activity in this extract is heat labile and has a t1/2 of about 3 min at 50 °C (not shown). However, there was no loss of activity when extracts were incubated at 30 °C for 1 h. Factor activity is destroyed by treatment with chymotrypsin or trypsin (the former is consistently more effective), and it thus appears to be a protein or have a protein component necessary for activity (Table I). The factor does not contain a crucial sulfhydryl group, based on the results of treatment with N-ethylmaleimide (Table I). Lipids alone (soybean lipids, dimyristoyl phosphatidylcholine, or phospholipids extracted from rabbit liver plasma membranes) failed to replace the requirement for factor in the labeling reaction (not shown).

Because cyc− and other membranes contain the catalytic unit of adenylyl cyclase, we hypothesized that the ADP ribosylation of G/F by cholera toxin may require an interaction of G/F with C. This hypothesis is not supported by the data obtained. Complete thermal inactivation of C activity in cyc− membranes does not affect their ability to supply factor activity. In addition, C activity is destroyed by N-ethylmaleimide, while factor activity is unaltered by similar treatment.

Gel filtration of the factor from wild type membranes in the presence of 1% sodium cholate indicates that the activity behaves as a monodisperse species (Fig. 3A). The Stokes radius of the factor in cholate is approximately 3.2 nm, equivalent to a globular protein with Mr ≈ 50,000. Results of gel filtration chromatography and determination of the sensitivity of the factor to heat and N-ethylmaleimide thus suggest that the membrane-derived factor is distinct from the known components of adenylyl cyclase.

Cytoplasmic Factors that Enhance ADP Ribosylation—A factor(s) that supports the activation of G/F by cholera toxin and NAD was also detected in the 200,000 x g supernatant fraction of cyc− or wild type S49 cell homogenates. Based on

![Figure 2](http://www.jbc.org/) Time course of toxin-dependent activation of purified G/F and the effect of factor concentration on the initial rate of activation. A, G/F (10 μg/ml) was activated with cholera toxin (40 μg/ml) in a final volume of 60 μl either in the presence of factor (●) or in the absence of added factor (○). Also shown is the activity when cholera toxin was omitted from the reaction (■). Details of the assay are described under "Experimental Procedures." B, G/F (3.4 μg/ml) was activated with cholera toxin (40 μg/ml) in the presence of various concentrations of partially purified factor from turkey erythrocytes. Activation was at 30 °C for 20 min, as described under "Experimental Procedures." The highest concentration of factor shown (1.0) represents 5.5 μg/ml of protein. The factor was diluted with buffer containing cholate (0.85%) such that the amount of detergent in the reactions was constant.

![Figure 3](http://www.jbc.org/) Gel exclusion chromatography of factor from membranes, factor from cytoplasm, and G/F from S49 lymphoma cells. A, a cholate extract of wild type S49 membranes was prepared and analyzed on Ultrogel AcA-44 as described under "Experimental Procedures." G/F activity (●) or factor activity (○) were assayed as described under "Experimental Procedures." B, a cytoplasmic factor from cyc− S49 cells was prepared and analyzed on Ultrogel AcA-44 as described under "Experimental Procedures." Samples were applied in 0.5 ml and fractions of 0.3 ml were collected. The flow rate was 3.5 ml/h. Arrows denote the elution volume of blue dextran (left) and 2,4-dinitrophenylglycine (right). At the top is a calibration curve used for determination of the Stokes radius using the standards (●) lactate dehydrogenase (4.75 nm), malate dehydrogenase (3.68 nm), and cytochrome c (1.87 nm). Open circles at the top represent the peaks of G/F or factor activity from A and B.

**Table I**

Sensitivity of factor to proteases or N-ethylmaleimide

Extracts of cyc− membranes (2 mg of protein/ml) were treated as indicated and the appropriate reagent was then added to stop the treatment. In the control samples, the protease inhibitor or dithiothreitol was mixed with the protease or N-ethylmaleimide prior to exposure to the factor. The extracts were then used as a source of factor for activation of G/F by cholera toxin as described under "Experimental Procedures." G/F activity was assessed by reconstitution with cyc− membranes, and adenylyl cyclase activity was assayed in the presence of 100 μM GTP or 10 mM NaF. Protease (1 mg/ml) treatments were at 30 °C for 40 min. Treatment with trypsin and chymotrypsin was stopped by the addition of 3 mg/ml soybean trypsin inhibitor or 0.2 mM phenylmethylsulfonyl fluoride, respectively. N-Ethylmaleimide (10 mM) treatment was at 0 °C for 40 min and was stopped by the addition of 1 mM dithiothreitol.

| Treatment               | Reconstituted adenylyl cyclase activity remaining |
|-------------------------|-------------------------------------------------|
|                         | GTP     | Fluoride |
| Trypsin                 | 65      | 107      |
| Trypsin inhibitor + trypsin | 106    | 111      |
| Chymotrypsin            | 112     | 98       |
| Phenylmethylsulfonyl fluoride + chymotrypsin | 104     | 102      |
| N-Ethylmaleimide        | 112     | 98       |
| Dithiothreitol + N-ethylmaleimide | 115     | 34       |
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gel filtration in detergent-free solution, this activity behaves as a single species with an apparent \( M_r \approx 16,000 \) (Fig. 3B). The activity of cytoplasmic factor was destroyed by sodium cholate (1%).

It seems possible or probable that this activity is the same as that detected by Enomoto and Gill (17), who described a cytoplasmic factor from pigeon erythrocytes that lowered the concentration of cholera toxin required to activate adenylate cyclase. The factor from cytoplasm of pigeon erythrocytes also appeared to have \( M_r = 15,000 \) to 20,000 as determined by gel exclusion chromatography (17). Enomoto and Gill detected factor activity only in the presence of limiting concentrations of toxin; this may be due to the presence of membrane-bound factor in the pigeon erythrocyte preparations that were studied.

It is possible that the smaller, cytoplasmic factor represents a fragment of the membrane-bound species. Alternatively, the membrane-bound and cytoplasmic factors could represent distinct proteins with similar activities. Purification of membrane-bound and cytoplasmic factors and subsequent comparison will be required to establish their relationship.

**Effect of Lipids**—The presence of detergent (Lubrol PX and cholate are added with the G/F and factor, respectively) in the activation reaction was found to inhibit the toxin-dependent ADP ribosylation. Pure lipids were added in an attempt to lower the concentration of detergent in solution. Although factor activity cannot be supplied solely by the addition of lipid to purified G/F, the addition of certain phospholipids or cholesterol to reaction mixtures that contain the factor increases the rate of the reaction (Fig. 1). This effect of added lipid can be met equally well by dimyristoyl phosphatidylcholine, dimyristoyl phosphatidylethanolamine, dipalmitoyl phosphatidylethanolamine, or cholesterol. Dioctanoyl lecithin was inhibitory at all concentrations tested. It is not known whether the lipid acts by providing a hydrophobic environment, by sequestering detergent, or by some other mechanism. The finding that dioctanoyl lecithin inhibits the reaction may be explained by its detergent properties.

**Effect of Guanine Nucleotides**—Omission of GTP from the G/F labeling reaction results in decreased incorporation of \(^{32}P\) into G/F (Fig. 1). The magnitude of the decrease is somewhat variable (usually 50-80%) and much less than that seen when either the factor or the lipid was omitted. It is not known why a guanine nucleotide enhances covalent modification of purified G/F by cholera toxin, but GTP does appear to be the preferred nucleotide (ATP is without effect). We agree with Nakaya et al. (5) that there are three distinct functional effects of GTP. The nucleotide facilitates covalent modification of G/F, stabilizes the ADP-ribosylated regulatory protein, and activates adenylate cyclase (particularly when G/F has been ADP-ribosylated). Guanine nucleotides have been reported to increase the cholera toxin-dependent labeling of all membrane proteins to the same extent (18, 19). This suggests a site of action for guanine nucleotides other than on each individual substrate for cholera toxin. If this is the case, the toxin itself or the factor described above would seem to be likely candidates.

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