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Cholera Toxin-Catalysed ADP-Ribosylation of Erythrocyte Proteins: General Properties

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Upon incubation of lysed pigeon erythrocytes with NAD, adenosine diphosphate-ribose (ADP-ribose) is incorporated into nuclear poly ADP-ribose and into an unidentified acid-insoluble product of the cytosol. The properties of these incorporations have been examined and a method developed for reducing their amount whilst retaining the sensitivity of the lysate to cholera toxin. This method has allowed the detection and description of a set of cholera toxin-specific ADP-ribose transfers to membrane-bound and soluble proteins under conditions that lead to adenylate cyclase activation

Key words: **NAD, ADP-ribose, poly ADP-ribose, ADP-ribosyl protein, cholera toxin, adenylate cyclase, pigeon erythrocyte**

We have recently shown that cholera toxin's fragment A_1 catalyses the transfer of adenosine diphosphoribose from nicotinamide adenine dinucleotide (NAD) to a set of proteins present in pigeon erythrocytes. In erythrocytes lysates, transfer to a 42,000-Mr membrane protein, thought to be the guanyl nucleotide-binding protein that associates with adenylate cyclase, apparently results in an inhibition of guanosine triphosphatase (GTPase) and a consequent activation of cyclase [1]. With an amount of toxin and a length of incubation such that adenylate cyclase activation is incomplete or barely complete, the 42,000-Mr ADP-ribosyl protein is the major product. However, other ADP-ribosyl proteins are present and can predominate after extended incubations at high toxin and/or high NAD concentrations. It is not clear that any of these secondary targets are relevant to the activation of adenylate cyclase. Indeed several of them are soluble proteins that can be ADP-ribosylated in the absence of membranes and can have no direct relevance to adenylate cyclase. Nevertheless, they resemble the principal target in being ADP-ribosylated only under conditions that would result in cyclase activation were membranes present, namely in the presence of sufficient nucleoside triphosphate and with sufficient reduced fragment A_1.

I document here some properties of these reactions.

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METHODS

Erythrocyte lysates and fractions were prepared fresh for each experiment [1]. Endogenous NAD was removed from ghosts by washing and was hydrolysed in cytosol by incubation with an insoluble NAD glycohydrolase. Using 1.5-ml Eppendorf centrifuge tubes, 10-mg portions of pig brain NAD glycohydrolase were washed twice by centrifugation. Fresh cytosol (1-ml portions) were incubated with the enzyme for 30 min at 34° on a rotator and then cleared by centrifugation. The NAD concentration was thus reduced from about 100 μM to less than 1 μM, usually less than 0.3 μM. The system's ability to respond to cholera toxin was not otherwise impaired (Table I). This pretreatment was undertaken in order to maximize the specific activity of the radioactive NAD used next, whilst allowing the use of relatively little toxin. In general, the isolated cytosol, or lysates reconstituted from 1 part ghosts and 2 parts cytosol, were incubated with 10 mM thymidine, 10 mM nicotinamide, 10 μM [^{32}P]-NAD, and 10 μg/ml activated cholera toxin in a total volume of 100 μl for 30 min at 37°, conditions which result in more or less complete activation of adenylate cyclase (Table II). Controls contained the toxin activation mixture (final 0.025% SDS and 0.25 mM dithiothreitol). After incubation, lysate samples were diluted in 2 ml isotonic buffer and centrifuged to separate ghost and cytosol fractions. These were precipitated with 5% TCA, collected on glass fiber filters and counted.

RESULTS

Interfering Reaction 1: Poly ADP-Ribose Formation

Intact pigeon erythrocytes do not metabolize exogenous NAD appreciably. However, lysed erythrocytes form significant amounts of acid-insoluble ADP-ribose derivatives from NAD and consume thereby 0.5–1% of the NAD per hour. Two of these products are so abundant that they severely confused the demonstration of toxin-specific incorporation. The first was identified as poly ADP-ribose [3] by the following known properties: a) inhibition of its synthesis by nicotinamide, b) inhibition by thymidine, c) inhibition by ATP, d) its rapid turnover, e) stimulation by ADP-ribose, which probably inhibits degradation, f) stimulation by a brief pulse of DNase I, which increases the number of sites at which synthesis can start [4, 5], g) its inhibition by further nuclease digestion of DNA, h) its location in the nucleus, and i) its large size – it does not enter a 5% polyacrylamide gel. (No ADP-ribosylated histone was detected in this study.)

None of the following agents were found to reduce poly ADP-ribose synthesis by more than 15%: 1 mM AMP, 1 mM DTT, 10 mM l-arginine, 1 mM GTP, 1 mM EGTA, 1 mM Mg^{++}, or postincubation with Bacillus subtilis NAD glycohydrolase. Synthesis was reduced by 100 mM histamine or 1-methyl imidazole, which may act by stabilizing the nicotinamide-ribose bond [6] or by forming adducts with NAD and thereby reducing the NAD concentration [7]. More accumulates at 25° than at 37°. Synthesis is almost eliminated by heating ghosts at 49° for 10 min before adding NAD.

The amount made, although significant in the present context, is small in relation to the synthetic capacity of metabolically active nuclei. It is reduced by washing ghosts, during which they presumably lose much poly ADP-ribose polymerase. In a reconstituted lysate, supplemented with inhibitors, its overall synthesis is reduced 100-fold (Table III).

TABLE I. Reversible Elimination of Toxin Sensitivity by Hydrolysis of NAD

	Toxin, μg/ml		
	0.01	0.1	1
	adenylate cyclase, pmoles cyclic AMP/μl/h		
Sham-treated cytosol: endogenous NAD ~ 100 μM[a]	1.3	7.1	16.6
Sham-treated cytosol: + 5 mM NAD	8.1	18	30
NADase-treated cytosol: residual NAD < 1 μM[a]	0	1.2	4.2
NADase-treated cytosol: + 10 μM NAD	2.1	3.7	12.0
NADase-treated cytosol: + 5 mM NAD	7.3	15	20

Cytosol incubated 30 min at 34° alone or with brain NADase was recombined with washed ghosts, 5 mM ATP, activated cholera toxin, and NAD as indicated, and was incubated for 30 min at 37°. Ghosts were washed and assayed for cyclase activity.
[a]The amounts of endogenous and residual NAD were estimated by isotope dilution in the EF2 reaction [2].

TABLE II. Adenylate Cyclase Activation in Fresh and Reconstituted Lysates

	Fresh lysate		Reconstituted lysate		
	5 mM NAD		10 μM NAD		100 μM NAD
Activated toxin, (μg/ml): Additive	0.01	0.1	1	10	0.1
	Increase in Adenylate Cyclase Activity (pmoles cyclic AMP/μl ghosts/h)				
Control	8.1	20	12	29	27
+ 10 mM thymidine	13.7	23	13	–	32
+ 10 mM nicotinamide	9.8	18	9.2	–	30
+ thymidine + nicotinamide	–	–	11	–	22
no cytosol	–	–	–	1.0	

Fresh lysate, or lysate reconstituted as in Table I, was incubated with the amounts of NAD and toxin shown above the table, plus 5mM ATP and the additives shown on the left for 30 min at 37°.

TABLE III. Representative Amounts of Poly ADP-Ribose Synthesized (pmoles ADP-ribose/ 25 μl ghosts*)

	Fresh lysate	Reconstituted lysate
No addition	28	2.0
+ 10 mM thymidine	23	0.72
+ 10 mM nicotinamide	13	0.57
+ 5 mM ATP	11	1.3
+ all three inhibitors	7	0.24

*10 μM NAD, 37°, 30 min.

Interfering Reaction 2: A Cytosolic Product

An unknown acid-insoluble product is formed from NAD in the cytosol whether or not ghosts are present. Polyacrylamide gel analysis of the product reveals a smear of fast-running material(s), but no discrete bands are formed even if trichloroacetic acid replaces the usual methanol-acetic acid fixative.

Its synthesis is faster at 37° than at 25°, is unaffected by preheating (49°, 10 min), is little affected by ATP or by added DNA, and is only partially reduced by high levels of DNase, an effect that might be due to a contaminant rather than to the DNase itself. Synthesis is, however, quite sensitive to nicotinamide (Table IV).

Postincubation for 30 min at 37° with 10 mM nicotinamide results in about a 20% reduction in the amount of product, revealing that there is some turnover. The rate of loss is not altered by ADP-ribose.

Cholera Toxin-Dependent Incorporation of ADP-Ribose

Under conditions that result in adenylate cyclase activation, significant radioactivity is transferred to a number of erythrocyte proteins from NAD isotopically labeled in any part of the ADP-ribose but not from NAD labeled in the nicotinamide [1].

Figure 1 shows time courses of the overall incorporation at three toxin concentrations. Two unexpected aspects of the incorporation are illustrated in Figure 1b. First, the amount of incorporation is not tightly coupled to adenylate cyclase activity. Second, some of the products are soluble although incorporation into ghost proteins predominates in the standard incubation conditions. The amount of product per ghost depends on the cytosol concentration but is independent of the ghost concentration if the cytosol concentration is held constant (Fig. 2). The cytosol is known to provide a macromolecule that seems to be necessary for all toxin-catalysed incorporations into ghosts.

The most important difference in the incorporation of ADP-ribose into ghost and soluble fractions is, of course, the nature of the acceptor proteins and the consequent physiological changes. By and large, however, they have similar environmental requirements and results obtained with one fraction are usually applicable to the other.

TABLE IV. Toxin-Independent Formation of a Cytosolic ADP-Ribose Derivative (pmoles acid-insoluble ADP-ribose)

Addition \ NAD:	1 μM	10 μM	50 μM	1 mM
Temperature 37°				
None		8.7		244
Nicotinamide, 10 mM		0.9		
ADP-ribose, 5 mM		19.3		
DNase, 2,000 units/ml	3.5	3.5		
Temperature 25°				
None	0.60	5.5	23	
Nicotinamide, 10 mM		1.3	4.3	
ADP-ribose, 5 mM	1.07			
ATP, 5 mM	0.52	4.8		
ATPase, 1 mg/ml		5.2		

NAD glycohydrolase-treated cytosol (OD_{540} = 95), 50 μl, was incubated for 2 h with [^{32}P]-NAD, precipitated with 5% TCA, filtered, and counted.

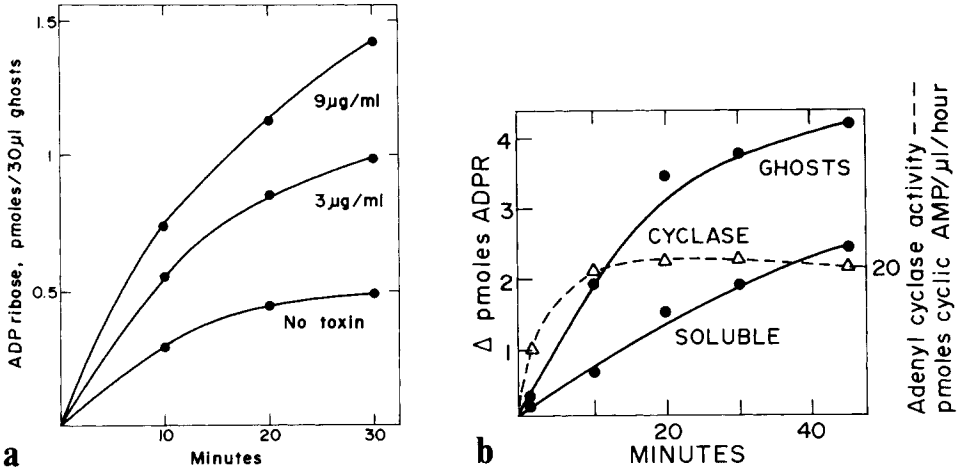

Fig. 1. Dependence of ADP-ribose incorporation on the length of incubation with cholera toxin: 100 µl reconstituted lysate, 10 mM thymidine, 10 mM nicotinamide, 5 mM ATP, 10 µM NAD, 37°. a) Total incorporation into ghosts at 0, 3, and 9 µg/ml toxin; b) 50 µg/ml toxin: net incorporation into ghosts at 0, 3, and 9 µg/ml toxin; b) 50 µg/ml toxin: net incorporation into ghost and soluble fractions (background incorporation subtracted) and adenylate cyclase activities of the ghosts.

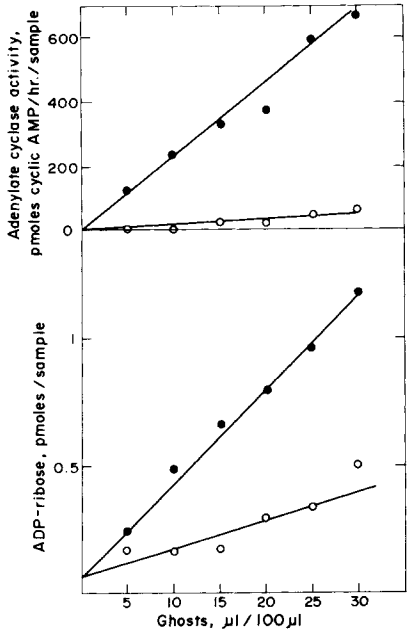

Fig. 2. Dependence of ADP-ribose incorporation on concentration of ghosts: 5- to 30-µl ghosts were incubated in 100 µl volume at constant cytosol concentration (OD_{540} = 45) and no (○) or 10 µg/ml (●) toxin.

Toxin-Dependent ADP-Ribosylation of Cytosolic Proteins

ADP-ribose incorporation increases in rate and extent with toxin concentration at least up to 200 μg/ml (9 μM) subunit A, with an apparent K_m of 0.75 μM. The measured K_m for NAD has usually approximated 400 μM. Approximate reciprocity between toxin and NAD concentrations is illustrated in Table Va, which also emphasizes that the toxin acts catalytically. SDS-polyacrylamide gel analysis shows that the same products are labeled at high toxin-low NAD levels as at low toxin-high NAD levels.

The incorporation varies linearly with cytosol concentration. It is often reduced by the presence of ghosts (Fig. 3).

A nucleoside triphosphate is required (Table Vb). Incorporation that is inhibited by ATPase is restored by ATP or another nucleoside triphosphate. In many cases it is probable that the true requirement is for GTP and that other triphosphates serve to generate GTP from residual GMP or GDP [8].

Temperature Dependency and Sensitivity to SDS

The initial rate of activation of adenylate cyclase by cholera toxin in vitro rises smoothly with temperature up to 37°–39° (and then drops off sharply) [9], but in certain circumstances the eventual ADP-ribose transfer and cyclase activation are considerably

TABLE V. Toxin-Catalysed ADP-Ribosylation (ΔADP-ribose incorporated, pmoles/sample) of Soluble Proteins

Va. Effect of Toxin and NAD concentration*

Toxin (pmoles/sample)	NAD 10 μM	30 μM	100 μM
0.44			30
1.3		28	94
4	24	76	200
12	62	172	488
37	110	282	656

Vb.†

ATP added	25°		37°	
Addition	0	5 mM	0	5 mM
No addition	12	62	1.8	10.2
Nicotinamide, 10 mM	9	44	0.6	6.0
ATPase, 1 mg/ml	2.9	–	0.3	–
ADP-ribose, 1 mM	–	60	–	9.8
Arginine, 20 mM	–	36	–	–
Lysine, 20 mM	–	56	–	–
Histidine, 20 mM	–	57	–	–

NAD-depleted cytosol, containing a little endogenous ATP, was incubated for 2 h at 25° in 100 μl, OD_{540} = 48, precipitated with 5% trichloroacetic acid, filtered, and counted. Values are corrected for the incorporations without toxin, namely 2, 5, and 24 pmoles at 10, 30, or 100 μM NAD.

*5 mM ATP, 10 mM nicotinamide.

†10 μM NAD, 10 μg/ml toxin (12 pmoles/sample).

reduced by temperatures above 30° (Fig. 3). A temperature inversion is always evident during extended (eg, 18-hour) incubations. The inversion is emphasized by SDS, which is used to preactivate cholera toxin and is therefore usually present during the incubation at a concentration of 0.025%. SDS also decreases the time before the temperature inversion is evident so that it is clearly seen at one hour. The proportional activation of toxin by SDS is consequently much greater at 25° than at 37° (Fig. 4). The reduction at higher temperatures applies to both membrane-bound and soluble proteins but has a slightly greater effect on the former. Transfer to soluble targets is especially temperature-sensitive in the absence of ghosts, perhaps only because ghosts absorb SDS and reduce its effective concentration. Below 25° the effect of temperature on incorporation into cytosolic proteins differs according to whether ghosts are present or not (Fig. 3).

Low-temperature optima appear to be characteristic of ADP-ribosyl transfer reactions. Reported examples include 20° for ADP-ribosylation of the alpha subunit of Escherichia coli RNA polymerase [10], about 25° for poly ADP-ribose synthesis [11], 25° for the ADP-ribosylation of EF2 by diphtheria toxin [12] and 15–20° for the self-modification of diphtheria toxin [13].

Other Modifiers of ADP-Ribosylation Rate

The following compounds are inhibitors (Table Vb):

1. Nicotinamide (10 mM), which apparently inhibits to a greater extent than can be accounted for by its participation in the reverse reaction.
2. Arginine (20 mM) – Moss and Vaughan [14] reported that subunit A catalyses a slow transfer of ADP-ribose to arginine and related compounds which suggests that arginine may resemble a natural substrate (inhibition by arginine supports this idea; it is more effective than other basic amino acids although higher concentrations of imidazole derivatives (eg, 100 mM histamine) are inhibitory for the reason given before).
3. Isobutylmethylxanthine (2 mg/ml), theophylline (2 mg/ml) and related compounds, possibly by competition with ATP.
4. Sulfhydryl reagents – both the ADP-ribosylation of ghosts and the activation of cyclase are inhibited by sulfhydryl reagents, for example, N-ethylmaleimide and

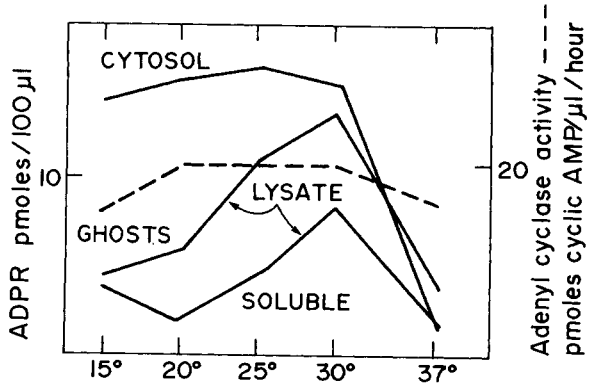

Fig. 3. Temperature dependence of the toxin-catalysed incorporation. Cytosol alone, or reconstituted lysate, 100 µl, was incubated with 50 µg/ml activated toxin for 1 h. The lysate was separated into ghost and soluble fractions. A portion of the ghost fraction was assayed for adenylate cyclase activity (dashed line): Above 20° the cyclase was fully activated but inactivation at 37° is evident.

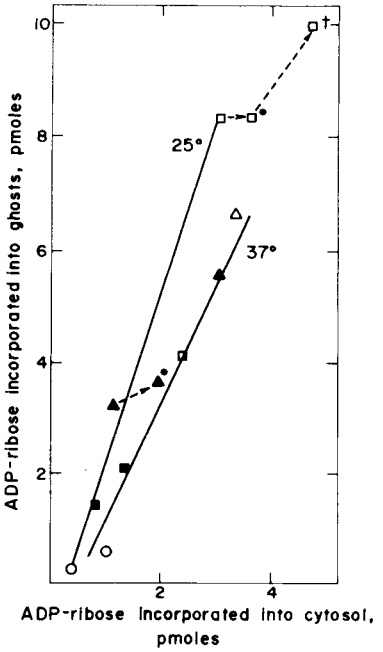

Fig. 4. Effects of temperature on toxin-catalysed incorporations of ADP-ribose into ghost and cytosol fractions: 30 μl packed ghosts, 60 μl NAD-depleted cytosol (OD_{540} = 98), 10 μM [^{32}P] NAD, 5 mM ATP, 10 mM nicotinamide, and 10 mM thymidine were incubated for 1 h at 25° or 37°. ○) no toxin; ■) 50 μg/ml toxin not pretreated; □) 50 μg/ml toxin preincubated with SDS and dithiothreitol (DTT) (0.015% SDS and 0.15 mM DTT during assay); ▲) 50 μg/ml subunit A, not pretreated, plus 0.25 mM DTT. The dashed lines represent temperature shift experiments *) 25° 1 h, then 37° 1 h; †) 25°, 37°, 25°, 1 h each.

dithiothreitol (1 mM or probably any amount over 0.2 mM). These probably interact with the target, not with the toxin, for carboxymethylated toxin is fully active.

5. More than 0.03% SDS.
6. Tris (see below).

The following compounds have little or no effect on the rate of ADP-ribosylation or on the rate of activation of adenylate cyclase.

1. Sodium fluoride (10 mM).
2. Epinephrine, isoproterenol, or propanolol (10^{-4} M).
3. α-NAD (1 mM).
4. Mg (up to 40 mM), Mn (5 mM), and Ca (1 mM) ions.
5. Porcine calcium-dependent regulator (see Moss and Vaughan [15]).
6. Inhibitors of proteolysis (phenylmethylsulfonyl fluoride, tosyllysylchloromethyl ketone, soy bean trypsin inhibitor, aprotinin), which are not inhibitory and may slightly increase the extent of ADP-ribosylation. Proteolysis certainly occurs in erythrocyte cytosol, as demonstrated by the fragmentation of iodine-labeled toxin that is discussed later.

For the ADP-ribosylation of ghosts and the activation of adenylate cyclase the optimum pH is about 6.6. In the region 6.4–7.0, acetate, phosphate, and MOPS buffers are satisfactory. There may be a slight preference for acetate but the great stimulation by

high concentrations of acetate and phosphate ions of the NAD-glycohydrolase activity of cholera toxin [16] is not reflected here. In the pH range 7–8, HEPES buffers are satisfactory but tris buffers are clearly inhibitory.

These observations may be related to some aspects of the nonenzymic hydrolysis of NAD. At 100°, hydrolysis is stimulated by phosphate ions and tris inhibits this phosphate stimulation [6, 17].

Analysis of the Products

Polyacrylamide gel analysis of the ADP-ribosylated cytosol reveals that there are several radioactive peptides, the principal ones being 24,000 Mr, 22,000 Mr, and smaller. They are mostly retained by a PM30 Amicon ultrafilter whose nominal cutoff is 30,000 Mr. Sepharose 6B resolves them into two principal peaks. The breakthrough peak is visibly turbid and probably contains mainly aggregated material; the amount depends on the temperature of the incubation, its length, and the SDS concentration. It contains labeled 22,000 Mr, 15,000 Mr, part of the 24,000 Mr, and some others. Most of the protein (especially 24,000 Mr) runs in a broad peak centered at about 150,000 Mr, which is clearly separated from the bulk of the protein (mainly hemoglobin.).

This behaviour is unexpected, for it is not obvious why a heterogeneous group of fairly small peptides should all migrate on Sepharose as if they were of much the same large size. Furthermore, when cytosol is fractionated *before* ADP-ribosylation, the majority of the acceptor proteins migrate as if they are *smaller* than hemoglobin. It is possible that aggregation or large changes in shape may accompany ADP-ribosylation.

The ghost-bound product is also heterogeneous. Many of the same proteins are present as in the cytosol (see below) and in addition there are several peptides exclusive to the ghosts. Apart from the major protein of 42,000 Mr that we believe to be the physiologically significant acceptor, there are ghost-specific peptides of 150,000, 98,000, 35,000, 20,000, 18,000 Mr, and others. On Sepharose 6B, detergent-extracted ghost products migrate as if of 100,000-600,000 Mr although the peptide sizes are much smaller. They cannot all be linked together for only certain of them pass through ultrafilters.

Significance of the ADP-Ribosyl Cytosolic Proteins

At one time it appeared that radioactive cytosolic products could be transferred to ghosts if ghosts were present. Thus 1) the elution profiles obtained for ADP-ribosylated cytosol and ADP-ribosylated ghosts (after solubilization in detergents) are apparently complementary (Fig. 5B, C); 2) some of the same radioactive peptides are present in ghosts and cytosol fractions; 3) by incubating radioactive cytosol with untreated ghosts under conditions that preclude new synthesis, we could detect the apparent transfer of some radioactive material. Such transfer did not require toxin, ATP, or NAD. It was very slow at 0°, faster at 37° than 25°, and was stimulated at 37° by 0.025% SDS.

We are now persuaded that such transfer is adventitious and irrelevant. It may represent precipitation of, or nonspecific adsorption of, certain proteins or trapping of a portion of cytosol within resealed ghosts. It can be decreased by measures that reduce such events. Furthermore, we have been consistently unable to demonstrate adenylate cyclase activation accompanying the transfer of cytosolic proteins to ghosts. Adenylate cyclase activity starts to rise as soon as all ingredients (activated toxin, nucleoside triphosphate, NAD, cytosol, and ghosts) are mixed, without the delay that would suggest a long-lived intermediate [8, 9]. Preincubating the other ingredients before the addition of ghosts is of no benefit. Likewise we have tested for the accumulation of a cytosolic intermediate in-

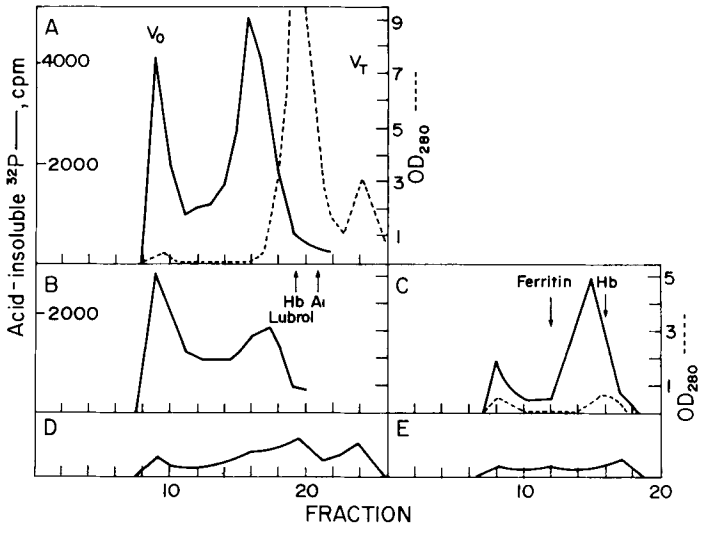

Fig. 5. Sepharose 6B elution profiles of the toxin-specific product: A) Cytosol incubated with [^{32}P] NAD (90 cpm/pmole), toxin 10 μg/ml, nicotinamide, thymidine, and ATP; B) cytosol from lysate incubated with NAD and toxin; C) ghosts from lysate incubated with NAD and toxin, Lubrol extract; D) as B, without toxin; E) as C with toxin. Indicated markers were hemoglobin (Hb), Lubrol PX micelles, cholera toxin fragment A_1 (monomeric, as upper panel of Fig. 6), and ferritin.

volved in the activation process by preincubating cytosol with ATP, NAD, and toxin, then adding either antitoxin or NAD glycohydrolase and continuing the incubation in the presence of ghosts. No activation ensues. The only experiments that appeared to indicate an intermediate consisted of uninterrupted incubations of complete lysate with NAD, ATP, and toxin during which samples were removed at intervals for assay. Cyclase activity appeared to continue to rise (at a reduced rate) after addition of antitoxin or NAD glycohydrolase. The effect was eliminated by adding theta toxin (an oxygen-labile hemolysin from Clostridium perfringens) to prevent resealing. Thus, there is no evidence for a long-lived soluble intermediate in the activation of adenylate cyclase, but rather the activation of adenylate cyclase appears to stem from the ADP-ribosylation of a protein that is at all times associated with ghosts. The unidentified cytosolic protein that is involved in this event is probably not ADP-ribosylated.

Behaviour of Cholera Toxin Fragment A_1

We had previously noticed that cholera toxin lost activity if incubated with an erythrocyte lysate. Several reasons for this loss were uncovered by observation of the changes to ^{125}I-labeled toxin (Fig. 6). During iodination of subunit A its disulfide bond was reduced and enzymically active [^{125}I] fragment A_1 was liberated. Fragment A_2 was not iodinated [18]. Fragment A_1 suffered several changes during incubation with cytosol. That which remained monomeric retained its original activity, but much was converted to large- and intermediate-sized aggregates that had low abilities to activate adenylate cyclase although they yielded monomeric A_1 when fractionated on SDS gels. Also, much A_1 was hydrolysed to small material that gives no identifiable ^{125}I protein bands and could not activate adenylate cyclase. In contrast to the results reported by others [19], we have never found active material substantially smaller than fragment A_1.

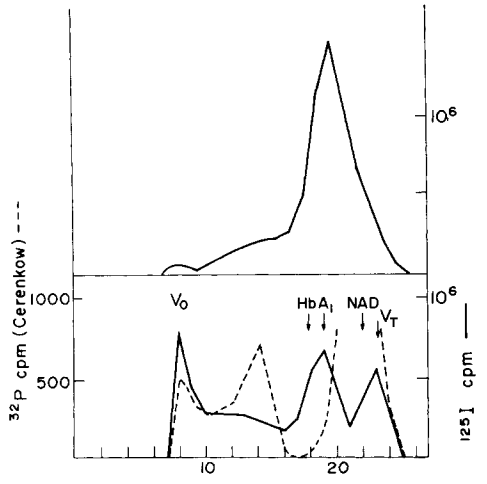

Fig. 6. Fractionation of iodinated fragment A_1. (Upper panel) As control, 5 μg of ^{125}I-fragment A_1 (100 μl, 1.4 cpm/pg) was mixed with 800 μl NAD-free 35% cytosol, thymidine, nicotinamide, and ATP and immediately fractionated on a column of Sepharose 6B. (Lower panel) The same, but supplemented with 10 μM [^{32}P] NAD (15 cpm/pmole by Cerenkow radiation) and incubated for 20 h at 25° before fractionation.

In lysates, as in cytosol, part of the A_1 associated into less-active aggregates. The part that remained monomeric and fully active showed no tendency to bind to ghosts. However, much of the ^{125}I also became firmly fixed to the ghosts and was not redissolved by Lubrol. It could be liberated by DNase I digestion, but then it bound to Sepharose columns and gave no peaks in the eluate. The reason for this behaviour is not clear but it may account for the impression that fragment A_1 is a hydrophobic protein.

ADP-Ribosylation of Turkey Erythrocyte Membrane Proteins

Less-convenient data were obtained using turkey rather than pigeon blood. The inhibitor mix is less effective at reducing the background incorporation in reconstituted turkey lysates, and the toxin-specific increase in incorporation was consequently hard to gauge (Table VI).

We have reported before that turkey lysates contain a moderately active NAD glycohydrolase that limits the useful "working life" of such lysates to a few minutes. The results with individual inhibitors (Table VI) suggest that both thymidine and nicotinamide, by reducing the hydrolysis of NAD, prolong the working life of the lysate and enable a higher adenylate cyclase activity to be obtained in the presence of cholera toxin.

DISCUSSION

The experiments described here were performed during our attempts to identify the role of NAD in the activation by cholera toxin of adenylate cyclase. We supposed that the toxin might ADP-ribosylate the cyclase itself or an associated protein and that to demonstrate this we would need to detect ADP-ribosylation occurring at about 1,000 sites per erythrocyte. Among the problems we encountered were the formation of acid-insoluble ADP-ribosyl compounds in the absence of cholera toxin and the toxin-specific incorpora-

TABLE VI. ADP-Ribosylation of Turkey Erythrocyte Membranes

Incubation[a]	Ghost ADPR		Increase in adenylate cyclase activity pmoles cyclic AMP/μl ghosts/h
	No toxin ADPR	10 μg/ml toxin ADPR	
Pigeon			
30 min	131 cpm	221 cpm	33
Turkey			
30 min	2,776	3,189	2.9
10 min	2,201	2,153	7.3
10 min with:			
no nicotinamide	1,448	1,561	4.5
no thymidine	6,142	6,169	4.5
no ATP	1,606	1,720	0.6
no cytosol	860	886	0.4
no NAD	–	–	0.0

Adenylate cyclase was measured under "basal" conditions. As reported by Field [20] the turkey lysate values were uniformly five times higher if assayed with 10 μM epinephrine.

[a]Incubation: 100 μl reconstituted lysate with 10 μM NAD (310 cpm/pmole), 10 mM thymidine, 10 mM nicotinamide, 5 mM ATP.

tion of ADP-ribose into proteins other than the primary target. As described in this paper, both of these processes can occur to levels much in excess of 1,000 per erythrocyte. However, with measures to minimize such processes we were able to show that the toxin-specific target that is most readily ADP-ribosylated is a protein located on the inner surface of the plasma membrane whose molecular weight is the same as that reported for a GTP-binding component of adenylate cyclase, namely 42,000 Mr. The effects of GTP on adenylate cyclase are much altered by cholera toxin. The 42,000-Mr protein appears to be present in excess on adenylate cyclase but, up to the point at which adenylate cyclase is fully activated by cholera toxin its ADP-ribosylation parallels the enzyme activity. At this point about 1,500 copies of the protein are ADP-ribosylated – reasonably close to the estimated number of adenylate cyclase molecules present – while many fewer copies of any other protein are yet ADP-ribosylated. Adenylate cyclase activity falls again when the ADP-ribose is removed by the reverse enzymic reaction. (The fluoride-stimulated cyclase activity that falls during incubation of membranes with cholera toxin rises again, upon such reversal.) Guanine nucleotides stimulate both the ADP-ribosylation and the rise in adenylate cyclase activity. These were the principal lines of evidence upon which we based our proposal that the major effect of cholera toxin is to ADP-ribosylate the GTP-binding protein and thereby to change its interaction with the catalytic component of adenylate cyclase [1].

The difficulties encountered in detecting physiologically significant ADP-ribosylation with cholera toxin contrasts with the relative ease of demonstrating the analogous reaction catalysed by diphtheria toxin. For the latter, not only is the target EF2 two orders of magnitude more abundant than adenylate cyclase but also ADP-ribosylation of other cell proteins is much less evident. In the case of cholera toxin it is not totally clear that its physiologic effects are limited to modification of the adenylate cyclase system and that the ADP-ribosylation of other proteins should be dismissed as side-reactions. It is conceivable that

ADP-ribosylation might change the activities of some enzymes to a significant extent. To determine whether this is so, it will be necessary, of course, to identify some of these "secondary" target proteins.

We must also inquire about the relationship between the various target proteins. Some structural similarity surely exists but we do not known whether, for example, all the target proteins bind GTP, it being some feature of the nucleotide-binding site that the toxin recognizes, or whether they are related merely by having accessible arginine residues.

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