Biochem. J. (1987) 243, 153–158 (Printed in Great Britain)

The invasive adenylate cyclase of *Bordetella pertussis*

Intracellular localization and kinetics of penetration into various cells

Zvi FARFEL,* Eitan FRIEDMAN† and Emanuel HANSKI†‡

*Clinical Pharmacology Unit and The Department of Medicine, Sheba Medical Center, Tel Aviv University, Tel Hashomer 52621, and †Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel

The penetration of *Bordetella pertussis* adenylate cyclase into various mammalian cells exhibits similar kinetics; the accumulation of both intracellular cyclase activity and cyclic AMP is rapid, reaching constant levels after 15–60 min of incubation. The kinetics of enzyme penetration into turkey erythrocytes is different; cyclase activity and cyclic AMP accumulate linearly and do not reach constant levels even after 6 h of incubation. In the preceding paper [Friedman, Farfel & Hanski (1987) Biochem. J. **243**, 145–151] we have suggested that the constant level of intracellular cyclase activity reflects a steady state formed by continuous penetration and intracellular inactivation of the enzyme. In contrast with other mammalian cells, no inactivation of cyclase is observed in turkey erythrocytes. These results further support the notion that there is continuous penetration and deactivation of the invasive enzyme in mammalian cells. A 5–6-fold increase in specific activity of the invasive cyclase is detected in a pellet fraction of human lymphocytes in which a similar increase in specific activity of the plasma-membrane marker 5′-nucleotidase is observed. A similar increase in the invasive-cyclase specific activity is detected in a membrane fraction of human erythrocytes. Cyclase activity in a membrane-enriched fraction of human lymphocytes reached a constant level after 20 min of cell exposure to the enzyme. Similar time courses were observed for accumulation of cyclase activity and cyclic AMP in whole lymphocytes [Friedman, Farfel & Hanski (1987) Biochem. J. **243**, 145–151]. We suggest therefore that cyclic AMP generation by the invasive enzyme as well as the intracellular inactivation process occur while it is associated with a membrane fraction identical, or closely associated, with the plasma membrane.

INTRODUCTION

Confer & Eaton (1982) have shown that an extract from *Bordetella pertussis* containing calmodulin (CaM)-sensitive adenylate cyclase increased cyclic AMP levels in human neutrophils and macrophages. Those authors proposed that the bacterial adenylate cyclase penetrates host cells and utilizes intracellular ATP pools to produce high levels of cyclic AMP. Since this original observation was made, several laboratories have provided supporting evidence for this hypothesis; *B. pertussis* mutants deficient of adenylate cyclase were incapable of cyclic AMP generation in cells (Weiss *et al.*, 1985); the addition of CaM to a partially purified preparation of *B. pertussis* adenylate cyclase prevented cyclic AMP accumulation in cells (Shattuck & Storm, 1985); adenylate cyclase activity in lymphocytes exposed to a partially purified invasive enzyme had different sensitivity to various reagents as compared with the intrinsic membrane-bound enzyme [the preceding paper (Friedman *et al.*, 1987)]. Taken together, these findings strongly support the notion that *B. pertussis* adenylate cyclase penetrates cells.

In the preceding paper (Friedman *et al.*, 1987) we proposed that there is continuous penetration and intracellular deactivation of the invasive enzyme in human lymphocytes. This results in the formation of an intracellular steady-state level of cyclase activity. In the present study we examined the penetration kinetics of partially purified *B. pertussis* adenylate cyclase into various cells and its subcellular distribution.

MATERIALS AND METHODS

Materials

[α-^{32}P]ATP (80 Ci/mmol), cyclic [^{3}H]AMP (27 Ci/mmol) and [2-^{3}H]adenosine 5-monophosphate (16 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. ATP (A-2383), phosphenolpyruvate, pyruvate kinase, soybean trypsin inhibitor, acetylated trypsin, PMSF, 4-methylumbelliferyl β-D-glucoside, pepstatin, leupeptin and haemoglobin were obtained from Sigma. Percoll and Ficoll–Hypaque were purchased from Pharmacia. GTPγS was purchased from Boehringer-Mannheim, and forskolin from Calbiochem. Ultrogel ACA34 was obtained from LKB and Dowex AG 50 W-X4 resin from Bio-Rad Laboratories. All other reagents were purchased from standard commercial suppliers and were of the best grade available.

Methods

Culture of organism and preparation of partially purified enzyme. The methods for culture of *B. pertussis* strain 165 and preparation of partially purified invasive

Abbreviations used: PBS, phosphate-buffered saline (5 mM-sodium phosphate/140 mM-NaCl, pH 7.4); PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; CaM, calmodulin; LDH, lactate dehydrogenase; GTPγS, guanosine 5′-[γ-thio]triphosphate; FIC, fractions inducing cyclic AMP accumulation.

‡ To whom correspondence and reprint requests should be addressed.

adenylate cyclase (FIC) were detailed in the preceding paper. (Friedman *et al.*, 1987).

Cells. Human lymphocytes were isolated from whole blood as described by us previously (Hanski & Farfel, 1985). Human and turkey erythrocytes were isolated by centrifugation of whole blood at 2000 rev./min (*r* 20.0 cm) (IEC centrifuge) for 10 min. After removing the buffy coat by aspiration, the erythrocytes were washed twice in PBS as described above. Human erythrocytes were further centrifuged through a Ficoll–Hypaque gradient for 20 min at 1400 rev./min (*r* 20.0 cm), and the obtained packed cells were washed twice in PBS and used as target cells for the invasive enzyme. The procedure detailed above allowed almost complete separation of erythrocytes from other blood constituents.

Determination of cyclic AMP and adenylate cyclase. Determinations of total and intracellular cyclic AMP were performed as detailed in the preceding paper (Friedman *et al.*, 1987). Cells were incubated with FIC and then subjected to trypsin treatment as described by us previously (Hanski & Farfel, 1985). Cell lysates were prepared as described in the preceding paper (Friedman *et al.*, 1987), and adenylate cyclase assays were performed as previously described (Hanski & Farfel, 1985).

Subcellular fractionation. Cells were incubated with FIC in 5 ml of PBS at 36 °C. At the end of the incubation periods, cells were transferred to incubation at 4 °C, washed twice by centrifugation at 482 $g_{max.}$ (IEC centrifuge) for 10 min at 4 °C and subjected to the trypsin treatment described by us previously (Hanski & Farfel, 1985). Trypsin-treated cells were resuspended in 10 ml of cavitation solution containing 20 mM-Hepes, 2 mM-$MgCl_2$, 1 mM-EDTA (HME buffer) supplemented with 0.5 mM-DTT, 0.15 mM-NaCl, leupeptin (10 μg/ml), pepstatin (10 μg/ml), 25 mM-6-aminohexanoic acid and 10 μM-PMSF, at pH 7.4. Cells were disrupted by using the nitrogen-cavitation technique detailed by Ross *et al.* (1977). The homogenate obtained was centrifuged at 157 $g_{max.}$ (IEC centrifuge) for 5 min at 4 °C. The supernatant was aspirated and saved. The pellet containing nuclei and unbroken cells was resuspended in about 3 ml of cavitation solution and washed again as described above. The two supernatants were combined and the pellet of nuclei and unbroken cells was resuspended in HME buffer and then frozen and thawed twice in liquid N_2. The combined postnuclear supernatants were centrifuged in 60 T_i Beckman fixed-angle rotor at 40000 rev./min (160000 $g_{max.}$) for 60 min. The supernatant and the pellet obtained were separated and the latter was resuspended in cavitation solution. For density-gradient centrifugation, the 160000 $g_{max.}$ pellet was resuspended in HME buffer supplemented with 0.25 M-sucrose, pH 7.4 (resuspension solution). The pellet suspension was layered on top of an 8 ml Percoll gradient adjusted to a density of 1.07 g/ml by adding one-tenth the final volume of 10-times-concentrated resuspension solution, and then centrifuged in a Beckman 65T_i rotor for 30 min at 20000 rev./min (35000 $g_{max.}$). The gradient was drained from the top and separated into 22 fractions of 0.4 ml each. From each fraction, 30 μl samples were assayed for adenylate cyclase activity, 20 μl samples for β-D-glucosidase activity and 50 μl samples for 5′-nucleotidase activity.

Marker-enzyme assays. 5′-nucleotidase was measured essentially by the method of Avruch & Wallach (1971); the reaction mixture contained, in addition to the reagents described, 0.1% Triton X-100. β-D-Glucosidase was assayed by the procedure of Avila *et al.* (1979) using 4-methylumbelliferyl β-D-glucoside as a substrate. LDH activity was determined as described by Decker *et al.* (1977).

The intrinsic membrane-bound adenylate cyclase served as a marker enzyme for plasma membranes of human erythrocytes. The assay was conducted at 35 °C in a final volume of 200 μl and the assay mixture contained: 20 mM-Hepes, pH 7.4, 1 mM-[α-^{32}P]ATP (140 c.p.m./pmol), 10 mM-$MgCl_2$, 0.1 mM-GTPγS, 0.1 mM-forskolin and protein samples. After 40 min of incubation the assay was terminated and cyclic [^{32}P]AMP was isolated by the method of Salomon *et al.* (1974).

Haemoglobin concentration was determined by measuring the A_{418} of the fractions diluted in HME buffer. The absorbance of a standard solution of haemoglobin (1 mg/ml) under similar conditions was 3.54.

Protein. Protein was determined by the method of Bradford (1976).

RESULTS

Kinetics of penetration of *B. pertussis* adenylate cyclase into various cells

We have previously shown that the penetration of *B. pertussis* adenylate cyclase is cell-selective; the enzyme penetrates human lymphocytes, but apparently does not penetrate human erythrocytes (Hanski & Farfel, 1985). These observations were based on measurements of cyclic AMP and cyclase accumulation using (0.5–1.0) × 10^6 cells per determination. Shattuck & Storm (1985), using 3×10^9 human erythrocytes per determination, demonstrated an increase in intracellular cyclic AMP in response to a partially purified preparation of the invasive enzyme. The increase in cyclic AMP was rapid and after 15 min reached a maximal concentration, which was about 1000-fold lower than that observed in lymphocytes (Hanski & Farfel, 1985). Using 1×10^9 human erythrocytes per determination we were able to demonstrate penetration of the invasive enzyme into these cells. The time courses of intracellular cyclase activity accumulation and total cyclic AMP generation in human erythrocytes exposed to partially purified enzyme (FIC) are shown in Fig. 1(*a*). Both cyclase accumulation and total cyclic AMP generation proceeded without a lag and reached a constant level after about 60 min of cell exposure. The accumulation of intracellular cyclic AMP, however, appeared to be more rapid; it reached a constant level after 10 min (results not shown), similar to the finding of Shattuck & Storm (1985). The difference in the rates of accumulation of total and intracellular cyclic AMP may be caused by a rapid extrusion of cyclic AMP into the cell medium.

Human lymphocytes, neutrophils and erythrocytes, and murine S49 lymphoma cells all responded to the invasive *B. pertussis* adenylate cyclase in accumulation of both cyclase activity and cyclic AMP, which reached a constant level within 15–60 min. The response of turkey

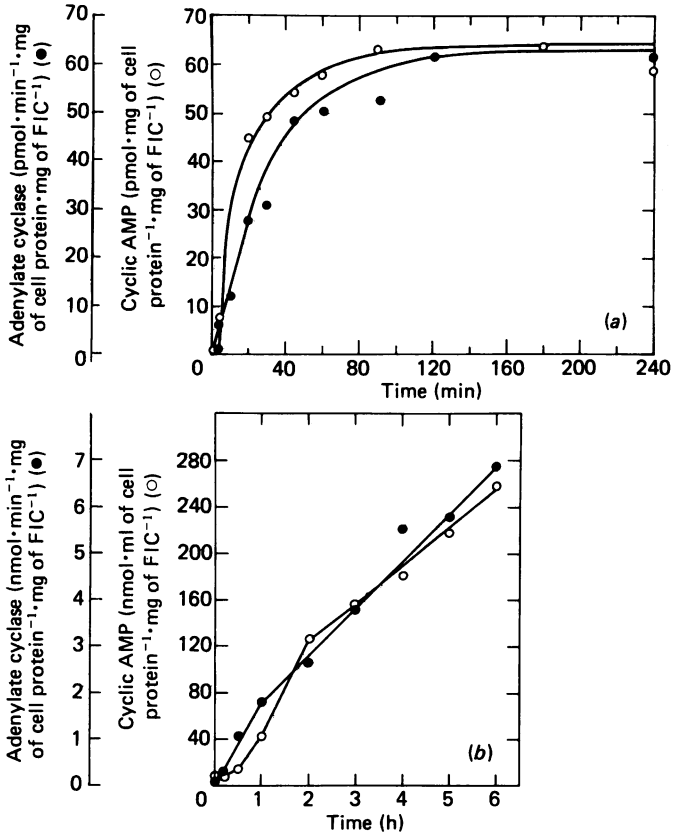

Fig. 1. Kinetics of enzyme penetration into human erythrocytes (*a*) and turkey erythrocytes (*b*)

Human erythrocytes (1×10^9 cells) and turkey erythrocytes (1×10^7 cells) were incubated at 36 °C in 0.5 ml of PBS (final vol.) with 100 μg of FIC. At the indicated times, intracellular cyclase activity and total cyclic AMP were determined as described under 'Methods'.

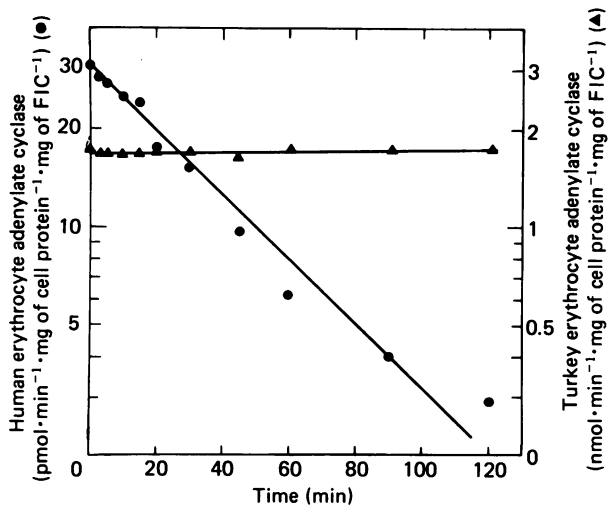

Fig. 2. Absence of decrease of intracellular *B. pertussis* cyclase activity in turkey erythrocytes

Human erythrocytes [1×10^{10} cells (●)] or turkey erythrocytes [1×10^8 cells (▲)] were incubated at 36 °C (in 5 ml of PBS) for 30 min and 2 h respectively with 1.0 mg of FIC. Cells were washed and trypsin-treated as described under 'Methods'. Treated cells were resuspended in 6 ml of PBS and reincubated at 36 °C. At the indicated times, samples (0.5 ml) were withdrawn and the amount of intracellular cyclase activity was determined.

erythrocytes was completely different; both intracellular cyclase activity and total cyclic AMP accumulation increased linearly and did not reach a constant level even after 6 h of incubation (Fig. 1*b*). In the preceding paper (Friedman *et al.*, 1987) we have shown that removal of the invasive enzyme from the cell medium resulted in a decrease in the intracellular level of cyclase activity in lymphocytes, and suggested that there is continuous penetration and deactivation of the enzyme. To characterize further the processes leading to steady-state attainment, we performed the experiment shown in Fig. 2. Human and turkey erythrocytes were exposed to FIC for 30 and 120 min respectively, and cells were washed and treated with trypsin as described under 'Methods' and reincubated at 36 °C. As Fig. 2 shows, removal of the enzyme from the cell medium resulted in a rapid decrease ($t_{\frac{1}{2}} \approx 30$ min) of intracellular cyclase activity in human erythrocytes, whereas no decrease was observed in turkey erythrocytes. The apparent absence of intracellular inactivation of the enzyme in turkey erythrocytes was not dependent on the duration of cell exposure to FIC; no decay was observed after cells were incubated with the enzyme for either 20 min or 6 h (results not shown). The turkey erythrocytes' integrity was preserved during 6 h exposure, as judged by the absence of haemoglobin in the cell medium. In contrast with human lymphocytes, where a small amount of catalytic activity was released back to the medium [the preceding paper (Friedman *et al.*, 1987], no catalytic activity was detected in the medium of human or turkey erythrocytes.

Subcellular localization of *B. pertussis* adenylate cyclase

Localization of the invasive enzyme throughout the process of its penetration was studied by subcellular fractionation of lymphocytes exposed to FIC. The fractionation protocol, which is detailed under 'Methods', involved exposure of lymphocytes for 2 or 60 min to FIC, washing of cells, treating with trypsin, disruption by nitrogen cavitation, removal of nuclei and unbroken cells by low-speed centrifugation and separation of cytoplasm and cell pellet by centrifugation at 160000 $g_{max.}$. Table 1 summarizes the specific activities and recoveries of adenylate cyclase and marker enzymes obtained in a representative fractionation experiment. About 3% of LDH activity (a marker enzyme for cytoplasm) and about 12% of 5'-nucleotidase activity (a marker enzyme for plasma membranes) remained associated with the post-cavitation low-speed pellet (P_1). This showed that the disruption of lymphocytes by the nitrogen-cavitation technique, under the conditions described under 'Methods', was quite efficient. High-speed centrifugation of the postnuclear supernatant (S_1) allowed satisfactory separation between particulate (P_2) and cytoplasmic (S_1) fractions. Table 1 demonstrates that, after 2 and 60 min of exposure of cells to the invasive enzyme, there was an identical increase of 5–6-fold in its specific activity in the particulate fraction (P_2). A similar increase in the specific activity was observed for the plasma-membrane marker 5'-nucleotidase. We have employed an identical fractionation protocol for human erythrocytes exposed to FIC. The intrinsic membrane-bound adenylate cyclase of un-

Table 1. Fractionation of human lymphocytes exposed to *B. pertussis* adenylate cyclase

Cells (2×10^7) were incubated with 0.75 mg of FIC at 36 °C in 5 ml of PBS. After 2 and 60 min incubation, cells were treated, disrupted by nitrogen cavitation, fractionated, and the assays of adenylate cyclase and marker enzyme were performed as described under 'Methods'. Results are means of three determinations, and the values in parentheses indicate the percentage recoveries. The distribution of LDH and 5'-nucleotidase were similar after 2 or 60 min of FIC exposure, so only the 60 min-exposure data are shown. Two additional experiments which were performed with different preparations of FIC and cells resulted in similar distribution of adenylate cyclase and enzyme markers. One unit of LDH activity is defined as the reduction of 1 μmol of pyruvate to L-lactate at 36 °C and pH 7.5.

Fraction	Volume (ml)	Protein (mg)	Specific activity (nmol/min per mg or, for LDH, unit/min per mg)			
			Adenylate cyclase			
			2 min	60 min	LDH	5'-Nucleotidase
Cavitate	10.1	1.14	0.88 (100)	4.65 (100)	199.1 (100)	224.1 (100)
Low-speed pellet (P_1)	0.7	0.20	0.50 (10)	1.50 (5.7)	37.5 (3.3)	150.0 (11.7)
Low-speed supernatant (S_1)	12.4	0.88	0.94 (83)	4.56 (75.5)	195.5 (75.7)	202.3 (70)
High-speed pellet (P_2)	1.2	0.12	5.17 (62)	25.83 (58.5)	18.3 (1.0)	919.2 (43.2)
High-speed supernatant (S_2)	12.0	0.70	0.10 (7)	0.60 (7.9)	200.0 (61.7)	17.9 (4.9)

exposed cells served as a marker enzyme for plasma membranes. Although the enzyme activity in human erythrocytes is quite low, it can be reproducibly determined when the enzyme is activated by forskolin and GTPγS (Hanski & Garty, 1983). 5'-Nucleotidase and ouabain-inhibitable (Na^+/K^+)-ATPase were abandoned as plasma-membrane markers, since their activities were too low to be accurately determined in the fractions obtained. Table 2 shows an increase in the specific activity of the invasive cyclase in the pellet fraction P_2 of human erythrocytes similar to that found for human lymphocytes. Further fractionation of lymphocyte P_2 fractions on Percoll density gradients (1.07 mg/ml) is shown in Fig. 3. As shown in Fig. 3(*a*), this gradient centrifugation removed most lysosomes; lysosomal activity was distributed between two peaks; 80% migrated in a single, less buoyant, peak. Similar distribution of lysosomal activity has been shown by density-gradient fractionation of human diploid fibroblasts (Rome *et al.*, 1979). In Fig. 3(*b*), human lymphocytes were incubated with FIC for 3, 6, 20 and 60 min, P_2 fractions were prepared as described under 'Methods' and then subjected to Percoll (1.07 g/ml)-density-gradient centrifugation. As shown, adenylate cyclase activity was confined to a single peak coinciding with 5'-nucleotidase activity. The cyclase activity in this peak progressively increased with incubation time, reaching a constant level after 20 min, similar to cyclase activity accumulation and cyclic AMP generation in whole cells [the preceding paper (Friedman *et al.*, 1987)].

DISCUSSION

The kinetics of *B. pertussis* adenylate cyclase penetration into various mammalian cells was similar; constant levels of both intracellular cyclase and cyclic AMP were reached after 15–60 min of cell incubation with the bacterial enzyme. Howevever, the kinetics of enzyme penetration into turkey erythrocytes exhibited different features; both cyclase activity and cyclic AMP accumu-

Table 2. Fractionation of human erythrocytes exposed to *B. pertussis* adenylate cyclase

Cells (7×10^{10}) were incubated with 1.5 mg of FIC at 36 °C in 10 ml of PBS. After 90 min, cells were treated with trypsin, disrupted by nitrogen cavitation, fractionated, and adenylate cyclase activity and haemoglobin was determined as described under 'Methods'. For determination of adenylate cyclase activity of the intrinsic membrane-bound enzyme, 7×10^{10} cells were incubated in the absence of FIC and treated as described above. The adenylate cyclase activity was determined in the presence of forskolin and GTPγS as described under 'Methods'. The results shown are mean of three determinations, and the numbers in parentheses indicate the percentage recoveries. Similar results were obtained in an additional experiment.

Fraction	Volume (ml)	Protein (mg)	Specific activity		
			Adenylate cyclase of exposed cells (pmol/min per mg)	Haemoglobin* (mg/mg of protein)	Intrinsic adenylate cyclase (pmol/min per mg)
Cavitate	11.0	2408	3.5 (100)	1.0 (100)	0.14 (100)
Low-speed pellet (P_1)	1.4	388	2.7 (12.5)	1.1 (17.8)	0.11 (13.2)
Low-speed supernatant (S_1)	9.0	2106	3.4 (86)	0.9 (79.9)	0.11 (69.7)
High-speed pellet (P_2)	0.8	418	13.5 (67)	0.2 (3.4)	0.40 (49)
High-speed supernatant (S_2)	8.2	1620	0.8 (15.9)	1.0 (71.5)	0.02 (9.6)

* Haemoglobin content is expressed as mg of haemoglobin per mg of protein in the fractions.

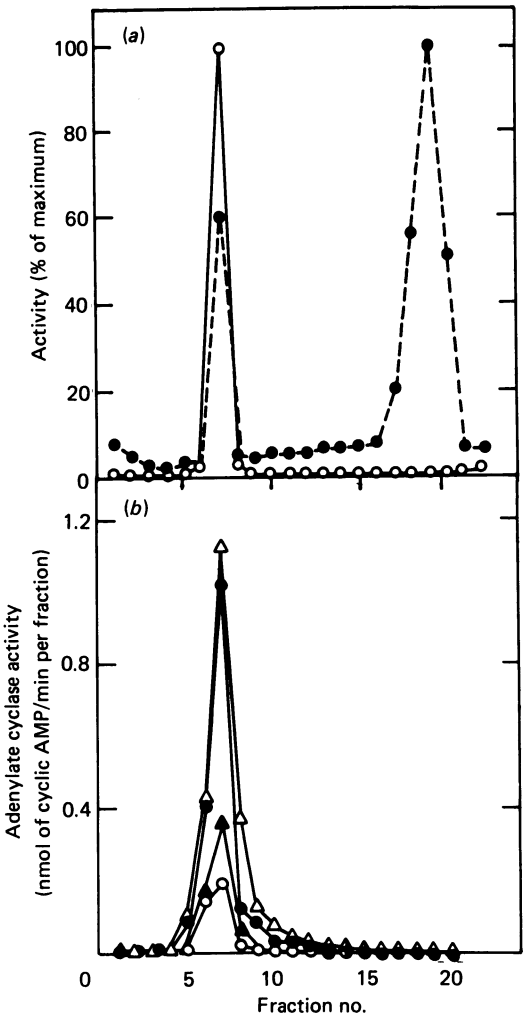

Fig. 3. Fractionation of lymphocyte postnuclear pellet by Percoll-density-gradient centrifugation

(*a*) Distribution of marker enzymes. The postnuclear (160000 $g_{max.}$) pellets obtained as described in the text were resuspended in 0.8 ml of HME buffer supplemented with 0.25 M-sucrose and applied on top of an 8 ml Percoll gradient (1.07 g/ml). The gradients were centrifuged and samples were collected as described under 'Methods'. A representative distribution of 5'-nucleotidase activity (○) and β--glucosidase activity (●) is shown. These activities were determined in triplicates as described under 'Methods'. Activities recovered on the gradients were 70–85% and 55–65% for 5'-nucleotidase and β-glucosidase respectively. (*b*) Distribution of cyclase activity. Human lymphocytes (3×10^6 cells) were incubated with 1 mg of FIC for 3 min (○), 6 min (▲), 20 min (●) and 60 min (△). After incubation, cells were washed, trypsin-treated, disrupted by nitrogen cavitation and 160000 $g_{max.}$ postnuclear pellets were prepared as described under 'Methods'. The percentages of cyclase activity recovered in these pellets were 58, 54, 61 and 55 respectively. The pellets were layered on the Percoll gradients and the gradients were centrifuged as described in (*a*). The recovery of cyclase activity ranged from 68 to 84%. The cyclase activity values shown were normalized to the amount of protein added to each gradient.

lation were linear and did not reach constant levels, even after 6 h of incubation. This fact allowed us to examine the hypothesis which we raised in the preceding paper (Friedman *et al.*, 1987), that the observed constant level results from continuous penetration and intracellular inactivation of the enzyme. Removal of the enzyme from the cell medium resulted in a rapid decay of intracellular activity in human lymphocytes (Friedman *et al.*, 1987) and human erythrocytes. However, no decay of cyclase activity was observed in turkey erythrocytes under similar conditions. These results suggest that the inactivation process is essential for the attainment of a constant intracellular level; in its absence, a linear accumulation of the enzyme is observed. Whether or not these different kinetics reflect a cell-specific or a species-specific phenomenon remains to be established.

The kinetics of intracellular cyclic AMP production by the invasive enzyme may not necessarily reflect the kinetics of enzyme penetration. In human lymphocytes exposed to the invasive enzyme for 2 h, more than 90% of cyclic AMP was found within the cells (Hanski & Farfel, 1985). However, in turkey erythrocytes, intracellular cyclic AMP reached a constant level after 1 h of incubation (results not shown), although total cyclic AMP continued to accumulate in a similar manner to the intracellular catalytic activity. Shattuck & Storm (1985) have shown that a constant level of intracellular cyclic AMP was reached in human erythrocytes after 15 min of incubation with a partially purified enzyme. By using our preparation of the invasive enzyme, we demonstrated a constant level of cyclase and total cyclic AMP in human erythrocytes only after 60 min of incubation and constant level of intracellular cyclic AMP after 10 min. The differences between the kinetics of intracellular and total cyclic AMP production may result from the use of different enzyme preparations, or alternatively from rapid extrusion of cyclic AMP into the cell medium. Cyclic AMP production by the invasive enzyme in turkey erythrocytes was more efficient than in human erythrocytes and lymphocytes (Friedman *et al.*, 1987), by 40- and 10-fold respectively. This may be related to the absence of enzyme-inactivation process in turkey erythrocytes, which allows a prolonged exposure of the invasive enzyme to the intracellular ATP pool. The different kinetics of enzyme accumulation and inactivation in the various cells studied provides additional evidence for enzyme entry into cells.

Disruption of human lymphocytes by the nitrogen-cavitation technique allowed us to separate cytoplasmic and particulate fractions in a manner which minimized cross-contamination. Irrespective of the period of cell exposure to the invasive enzyme, about 90% of the recovered cyclase activity was detected in the particulate fraction; 5'-nucleotide was similarly distributed. To further clarify the subcellular distribution of the invasive enzyme, we have used human erythrocytes as target cells for the enzyme. Although the efficiency of enzyme penetration into human erythrocytes is low, its kinetics of penetration is similar to that observed in other cells. Human erythrocytes can serve as a useful model for subcellular-localization studies because of their relative simplicity. The distribution of cyclase activity in human erythrocytes was found to be similar to the distribution in human lymphocytes. Thus the invasive enzyme is most probably associated with a membrane fraction that could be either the plasma membrane or a closely associated

fraction. Cyclase accumulation in this membrane-enriched fraction, obtained by centrifugation of a high-speed lymphocyte pellet (P_2) on Percoll density gradients reached a constant level after 20 min of cell exposure to the enzyme, a time similar to that required to obtain constant levels of cyclase and cyclic AMP in whole cells (Friedman *et al.*, 1987). These observations, taken together with the inactivation process of the enzyme, imply that cyclic AMP generation as well as the inactivation occur while the enzyme is associated with this membrane fraction. The exact nature of this association and a better understanding of the inactivation process must await further purification and radiolabelling of the invasive enzyme.

Although the understanding of how bacterial toxins enter cells is still fragmentary, it appears that toxins enter cells from various kinds of intracellular vesicles rather than by direct penetration of the plasma membranes (Olsnes & Sandvig, 1985). In contrast with other bacterial toxins, the penetration of *B. pertussis* adenylate cyclase appears to occur directly from the cell surface; the appearance of cyclase and generation of cyclic AMP in the cell are rapid and proceed without any noticeable lag [chloroquin and NH_4Cl do not prevent enzyme entry (Confer *et al.*, 1984)], and the enzyme penetrates human erythrocytes, which do not contain organelles required for endocytosis such as Golgi apparatus and lysosomes.

Only a minority of the internalized polypeptide-toxin molecules are actually responsible for exertion of their biological effect (Olsnes & Pihl, 1982; Tsuzuki & Wu, 1982). Such a low number of molecules is difficult to trace. Therefore the current most reliable method for studying toxin entry is to monitor alterations in the host cells. These alterations, however, occur only subsequent to toxin entry. In contrast, the increase in host cyclic AMP levels in response to *B. pertussis* adenylate cyclase is immediate and correlates well with enzyme entry, which can be easily and accurately monitored. These properties of *B. pertussis* adenylate cyclase make it a useful tool for studying the entry of bacterial polypeptides into animal cells.

Received 1 September 1986/17 November 1986; accepted 1 December 1986

We thank Mrs. R. Levin for excellent secretarial assistance. This research was supported in part by grants from the Fund for Basic Research administered by the Israeli Academy of Science and Humanities, the Minerva Foundation (to E. H.), and a grant for Basic Research administered by the Israeli Academy of Sciences and Humanities (to Z. F.).

REFERENCES

Avila, J. L., Casanova, M. A., Avila, A. & Bretanna, A. (1979) J. Protozool. **26**, 304–311

Avruch, J. & Wallach, D. F. H. (1971) Biochim. Biophys. Acta **599**, 689–698

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Confer, D. L. & Eaton, J. W. (1982) Science **217**, 948–950

Confer, D. L., Slungaard, A. S., Graf, E., Panter, S. S. & Eaton, J. W. (1984) Adv. Cyclic Nucleotide Protein Phosphorylation Res. **17**, 183–187

Decker, L. A. (ed.) (1977) Worthington Enzyme Manual, Worthington Biochemical Corporation, Freehold, NJ

Friedman, E., Farfel, Z. & Hanski, E. (1987) Biochem. J. **243**, 145–151

Hanski, E. & Farfel, Z. (1985) J. Biol. Chem. **260**, 5526–5532

Hanski, E. & Garty, N. (1983) FEBS Lett. **162**, 447–452

Olsnes, S. & Pihl, A. (1982) Pharmacol. Ther. **15**, 355–381

Olsnes, S. & Sandvig, K. (1985) in Endocytosis (Pastan, I. & Willingham, M. C., eds.), pp. 195–234, Plenum Publishing Co., New York

Rome, L. H., Garvin, A. J., Allietta, M. M. & Neufeld, E. F. (1979) Cell (Cambridge, Mass.) **17**, 143–153

Ross, E. M., Maguire, M. E., Sturgill, T. W., Biltonen, R. L. & Gilman, A. G. (1977) J. Biol. Chem. **252**, 5761–5775

Salomon, Y., Londos, C. & Rodbell, M. (1974) Anal. Biochem. **58**, 541–548

Shattuck, R. L. & Storm, D. R. (1985) Biochemistry **24**, 6323–6328

Tsuzuki, J. & Wu, H. C. (1982) Biochim. Biophys. Acta **720**, 390–399

Weiss, A. A., Hewlett, E. L., Mayers, G. A. & Falkow, S. (1985) J. Infect. Dis. **150**, 219–223