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Comparison of the Binding of Cholera and *Escherichia coli* Enterotoxins to Y1 Adrenal Cells†

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ABSTRACT: The binding of iodinated cholera and *Escherichia coli* (LT) enterotoxins to Y1 mouse adrenal cells was studied by using saturation analysis (Scatchard). Each toxin bound to Y1 cells with similar affinity [$K_A = (1.5\text{-}2.0) \times 10^9\ M^{-1}$], but there appeared to be twice as many receptor sites per cell for *E. coli* toxin ($\sim 4 \times 10^5$). Despite the increased binding of *E. coli* toxin, Y1 cells respond sooner to, and to smaller concentrations of, cholera toxin. The binding of each toxin was inhibited competitively by both toxins, although twice as much *E. coli* toxin was required to inhibit 50% of the binding of cholera toxin as was needed for either homologous inhibition or the inhibition of *E. coli* toxin binding by cholera toxin. The B subunits of both toxins were equally effective in competing for the binding of both iodinated toxins. Whereas the A subunits of both toxins had little or no effect on the binding of *E. coli* toxin, they consistently inhibited 20-40% of the binding of cholera toxin to cells. These results suggest that there are receptor loci on cells for the A subunit and that conformational differences exist between the two toxins that might explain the greater sensitivity of Y1 cells to cholera toxin. A model is suggested in which cholera toxin exhibits a greater degree of multivalent ligand binding than does the *E. coli* toxin, resulting in a more favorable situation for apposition of the A subunit to its receptor or for its insertion into the membrane.

The heat-labile enterotoxins of *Vibrio cholerae* and *Escherichia coli* resemble each other immunochemically and in their mechanisms of action. Both toxins are NAD glycohydrolases (Moss et al., 1979c; Moss et al., 1979b), effect ADP-ribosylation of membrane proteins (Moss et al., 1979b; Gill & Meren, 1978), and activate adenylate cyclase. They share antigenic determinants but possess unique determinants as well (Clements et al., 1980; Kunkel & Robertson, 1979). Their subunit structures and amino acid sequences show similarities, especially between the "binding" (B) subunits (Clements & Finkelstein, 1979; Robertson et al., 1979).

There is good evidence that cholera enterotoxin (CT) binds to GM_1 gangliosides present in cell membranes (Holmgren et al., 1973; King & van Heyningen, 1975; Moss et al., 1976; Fishman et al., 1979). The evidence that this ganglioside also serves as a receptor for the *E. coli* toxin (LT) has been indirect (Donta & Viner, 1975; Moss et al., 1979a), and there are conflicting reports regarding the similarity of the receptors for the two toxins (Holmgren, 1973; Pierce, 1973; Guerrant & Brunton, 1977). With the successful purification of LT, it has become possible to directly analyze and compare their binding properties.

Experimental Procedures

Tissue Culture. Y1 mouse adrenal cells were derived from those used in previous studies (Donta & Viner, 1975). The cells were maintained and propagated in Ham's nutrient mixture F-10 (GiBCO) supplemented with 15% horse serum and 2.5% fetal calf serum in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Assessment of biologic activity (changes in morphology; cyclic AMP levels) utilized previously described methods (Donta & Haddow, 1978).

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Toxins. Purified cholera toxin was purchased from Sigma; its purity was confirmed with $NaDodSO_4$–polyacrylamide gel electrophoresis. Purified preparations of *E. coli* enterotoxin (LT) were the generous gifts of Drs. J. Clements (Clements & Finkelstein, 1979) and D. Robertson (Kunkel & Robertson, 1979). Purified cholera toxin subunits A and B were the generous gift of Dr. N. Ohtomo of the Chemo-Therapeutic Research Institute, Kumanoto, Japan (Ohtomo et al., 1976). Subunit A_1 of CT was prepared and donated by Dr. C. Lai, Hoffmann-La Roche (Lai et al., 1979). Subunits A and B of *E. coli* LT were also the gift of Dr. Robertson. All toxin preparations were stored at 4 °C in 0.05 M Tris-HCl–0.001 M EDTA, pH 7.5 buffer. Dilutions of the toxin were made in 0.05 M phosphate–0.15 M NaCl, pH 7.4 buffer (PBS). Both CT-B and LT-B subunits were active on Y1 cells only at concentrations exceeding 1 μg/mL. All CT-A and LT-A preparations induced activity in Y1 cells at concentrations greater than 1 μg/mL.

Toxin Iodination. The cholera and *E. coli* toxins were radiolabeled with iodine-125 by using the Chloramine T method (Cuatrecasas, 1973a). For each iodination, 5 μg of toxin was incubated with 25 μg of Chloramine T and 1 mCi of carrier-free iodine-125 (New England Nuclear) in a total volume of 40 μL of 0.05 M phosphate buffer, pH 7.4, for 15 s at room temperature. The reaction was terminated by the addition of 100 μg (0.1 mL) of sodium metabisulfite. The mixture was then applied to a Sephadex G-100 column at 4 °C equilibrated and eluted with 0.05 M barbital buffer, pH 8.0, containing 0.25% bovine serum albumin (BSA). The iodinated toxin was collected in 0.75-mL fractions and tested for biologic (morphologic) and binding activities. The active fractions (specific activity 10–30 μCi/μg) were pooled for further use and stored at 4 °C for no longer than 10 days. The iodination procedure resulted in an average incorporation of one iodine atom per molecule of toxin, and losses in biologic activity did not exceed 15–20%. When analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and autoradiography, 55% of the iodine was localized in the A subunit and 45% in the B subunit. Both iodinated A and B subunits had the same mobility as the native preparation, and no other iodinated products were visualized. Ninety-five percent of the iodinated toxin was precipitated in 5% trichloroacetic acid.

Binding Assays. Y1 cells used in binding assays were removed from culture vessels by trypsin treatment (0.1% trypsin for 3–5 min), followed by centrifugation, washing in PBS, and resuspension in culture medium. The cell concentration was determined, with a hemocytometer, and adjusted to 1×10^5 cells/mL by appropriate dilution. One milliliter of cell suspension was then incubated in borosilicate glass tubes with 5 ng of [^{125}I]toxin at 37 °C for 30 min (length of time to reach equilibrium) in the presence or absence of unlabeled toxin or subunit. Following incubation, the mixture was diluted with cold medium and poured over EHWP (Millipore) filters under vacuum, and the filters were rapidly washed with 3 volumes of cold PBS containing 0.1% BSA. The radioactivity bound on filters was determined with a Nuclear-Chicago γ detector with a counting efficiency of 75%. Total binding and nonspecific binding (i.e., binding in the presence of 1000 ng/mL unlabeled toxin) were determined and the data analyzed according to the method of Scatchard (1949).

Results

Both ^{125}I-radiolabeled cholera and *E. coli* enterotoxins bound rapidly to Y1 adrenal cells, reaching maximum binding and saturation by 30 min (Figure 1). With [^{125}I]CT, 3–5% of the toxin was bound, 60–80% of which was specific; the specific

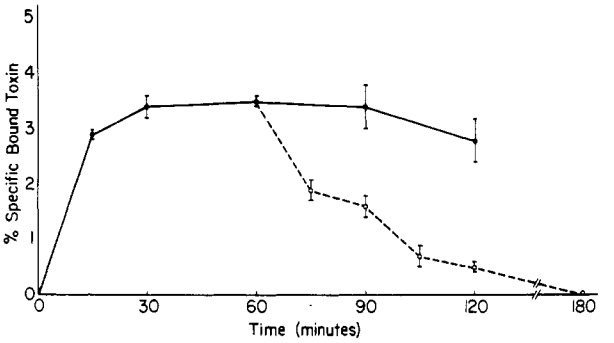

FIGURE 1: Binding of ^{125}I-labeled cholera toxin to Y1 cells over time. Y1 cells were incubated with [^{125}I]CT ± excess unlabeled toxin at 37 °C for the time periods indicated prior to filtration and determination of specifically bound counts (see Experimental Procedures for details). Reversibility of the binding process is shown by the decrease in specific toxin binding (○) when excess unlabeled toxin was added for the time periods indicated following prior incubation of cells for 60 min with [^{125}I]CT.

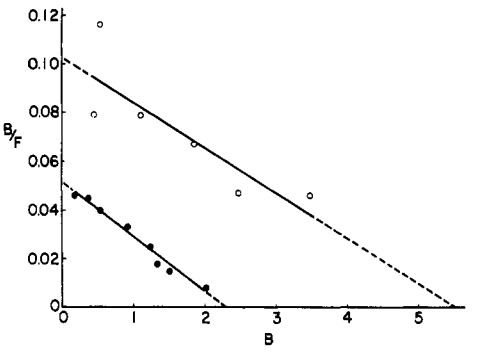

FIGURE 2: Scatchard plots of the binding of [^{125}I]CT (●) and [^{125}I]LT (○) to Y1 cells. B = bound (in nanograms per milliliter); B/F = bound/free. In these experiments, there were 1.6×10^5 receptors/cell for CT, with a K_A of 1.9×10^9 M^{-1}, and 3.7×10^5 receptors/cell for LT, with a K_A of 1.7×10^9 M^{-1}.

binding of [^{125}I]LT was ~90% of the 6–9% total bound. Toxin binding was reversible, even after 60 min of preexposure to [^{125}I]toxin (Figure 1), thus justifying the use of Scatchard analysis for study of the binding process. Analyses of toxin binding data revealed a single class of receptors for each toxin (Figure 2). Similar results were found when varying amounts of [^{125}I]toxin were used to develop Scatchard plots (Roth, 1973), thus confirming the identities of labeled and unlabeled toxins. The two labeled toxins appeared to have a similar binding affinity (K_A = ~2×10^9 M^{-1}), but analyses of receptor numbers revealed 2–3 times as many receptors for LT (~4×10^5/cell) as that for CT. The use of LT purified by two different methods did not alter these results. Similar results were also obtained whether cells were harvested by mild trypsin treatment or mechanical removal.

Although Y1 cells appear to possess greater numbers of receptors for LT, they respond to 10–100-fold lower concentrations of CT, and the onset of activity, when supramaximal concentrations of both toxins are used, occurs 30 min earlier with CT (Figure 3). A comparison of the molar quantities needed to achieve biologic activity with that required for specific binding is shown in Figure 4. For CT, half-maximal responses were achieved when <1% of available receptors were bound; for LT, there appears to be a direct relationship between the number of occupied receptor sites and biologic activity.

Similarities in the receptor sites for the two toxins were demonstrated by the finding that either toxin could compete for the binding of the heterologous labeled toxin. The quantity

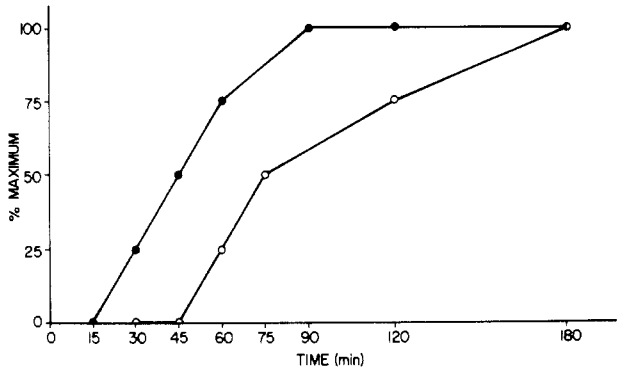

FIGURE 3: Onset of biologic activity (morphologic changes) following exposure of Y1 cells to supramaximal concentrations (1 μg/mL) of cholera toxin (●) or *E. coli* LT toxins (○).

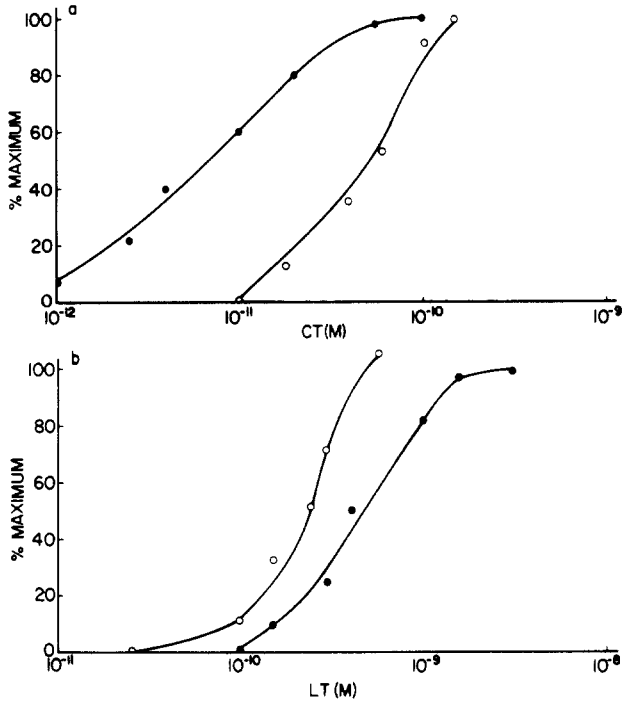

FIGURE 4: Relationships between the concentrations of cholera toxin (a) and *E. coli* toxin (b) and biologic activity (●) or binding of [^{125}I]toxin (○). Biologic activities were assessed at 24 h post toxin exposure (evaluation of morphologic changes and increases in extracellular cyclic AMP levels yielded equivalent results).

Table I: Inhibition of the Binding of [^{125}I]CT or [^{125}I]LT to Y1 Cells by CT and LT

	[^{125}I]CT	[^{125}I]LT
CT	50[a]	50
LT	100	30–50

[a] The amount (ng) of unlabeled toxin required to inhibit 50% of the specific binding of [^{125}I]toxin.

of LT needed to inhibit 50% of the binding of [^{125}I]CT, however, appeared to be twice that needed for either homologous inhibition or for the inhibition of [^{125}I]LT binding by CT (Table I).

Both CT-B and LT-B subunits were able to compete for the binding of either iodinated holotoxin. In contrast to the differences observed by using holotoxins, comparable concentrations of B subunits (20–40 ng/mL) resulted in a 50% inhibition of [^{125}I]toxin binding. When the A subunits of CT and LT were used as competitors of the binding process, somewhat surprising results were obtained. All CT-A and LT-A preparations were consistently able to compete for the binding of [^{125}I]CT (Table II). Complete inhibition could not be achieved, and the dose–response curves were shallow (Figure 5). In contrast, the A subunit preparations competed for little, if any, of the binding of [^{125}I]LT to cells (Table II). The differences observed between the inhibition of the binding of [^{125}I]CT and [^{125}I]LT by the A subunit preparations, especially that by the CT-A_1 preparation, would seem to preclude the possibility that the A preparations were contaminated with B subunit.

Table II: Inhibition of [^{125}I]Toxin Binding by A Subunits

	[^{125}I]CT	[^{125}I]LT
CT-A	76 ± 3[a]	93 ± 3
LT-A	80 ± 3	89 ± 3
CT-A	61 ± 4	106 ± 3

[a] The results are expressed as the percentage of [^{125}I]toxin specifically bound (means ± SE) to Y1 cells in the presence of subunit (10–1000 ng/mL); control binding (in the absence of subunit) = 100%.

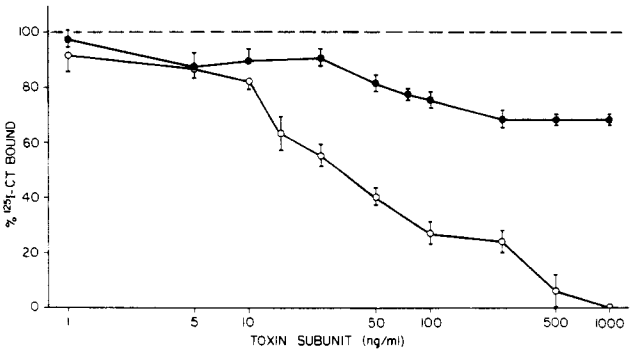

FIGURE 5: Inhibition of the binding of [^{125}I]CT to Y1 cells by cholera toxin subunits A (●) and B (○). The results are expressed as a percentage (mean ± SE) of the specific binding of [^{125}I]toxin in the absence of subunit.

For evaluation of the possibility that the differences observed were due to differences in the "nicked" status of the two toxins (Clements & Finkelstein, 1979), the radiolabeled Clements LT was further subjected to trypsin treatment prior to evaluation of its binding in the presence of A subunit. No changes were observed, either in the binding characteristics of the [^{125}I]LT or in the inability of the CT-A subunit to inhibit the binding of the trypsin-treated holotoxin.

Discussion

The results of these studies provide more direct evidence for similarities between the receptor sites for cholera and *E. coli* enterotoxins. Prior evidence was based on analyses of the comparative abilities of the two toxins to bind to GM_1 ganglioside (Donta & Viner, 1975; Moss et al., 1979a), the putative receptor material. Some investigators had proposed that the receptor sites may differ, based on the differing abilities of choleragenoid (the naturally occurring equivalent of cholera toxin B subunit) and ganglioside to inhibit the biologic activities of the two toxins (Holmgren, 1973; Pierce, 1973; Guerrant & Brunton, 1977). Some of these differences may have been due to the use of unpurified LT preparations, although differences in receptor binding properties of the two toxins might better explain the results (see further discussion below).

The two toxins bind to Y1 adrenal cells with similar affinities, but there appear to be more receptors for LT than for CT. Y1 cells, however, are more sensitive to the effects of CT. As both toxins bind with equal rapidity, this greater sensitivity of Y1 cells to CT implies the existence of a means for faci-

litation of the cell's response to CT. It is possible that the A subunit of cholera toxin is more active than the A subunit of LT, but this possibility seems unlikely (Moss et al., 1979b). Both toxins' A subunits compete for a significant fraction of the binding of [^{125}I]CT and little or none of the binding of [^{125}I]LT, findings which suggest that (a) there are receptors for the A subunit and (b) differences exist in the conformational states of the A subunits within the two toxins. If the A subunit of LT is not in as optimal a position to bind to its receptor locus, then the cell's sensitivity and onset of responses to the toxin might be affected.

Conformational differences between the two toxins, especially at the subunit level, need to be confirmed by more direct means (e.g., circular dichroism and X-ray crystallography). It may be that the binding of LT to target cell membranes induces membrane-associated events that subsequently result in the better positioning of LT-A ligands to receptor loci. Alternatively, the A subunit of LT may be internalized by mechanisms that differ from those involved with the CT-A subunit. Our in vitro attempts to modify the LT preparations did not yield toxins whose binding could be readily inhibited by subunit A. The Robertson LT is minimally affected by trypsin, while the Clements preparation gains biologic effectiveness on Y1 cells. Neither LT, however, becomes as active as CT on Y1 cells, nor is the onset of activation of Y1 cells shortened.

Our experimental results are compatible with a model in which both toxins initially bind to the same receptor for the B subunit. With CT, this is rapidly followed by the binding of its A subunit to an adjacent receptor, but this process either requires a longer period of time for LT or is not applicable to it. It may be that cholera toxin exhibits a greater degree of multivalent binding than does LT, thus creating a more favorable situation for apposition of the A subunit to its receptor or for its insertion into the membrane; conversely, the binding of LT to fewer receptor sites per holotoxin could result in a less productive situation. As a result, the number of available binding sites for LT would be greater than that for CT. The requirement for greater quantities of LT to inhibit the binding of CT might then be a result of the decreased ability of LT to interfere with the combined B and A subunit binding process.

Although the majority of toxin binding seems to be mediated by the B subunit, at smaller concentrations (e.g., 1–10 ng/mL), differences between the abilities of the A and B subunits to inhibit the binding of [^{125}I]CT are marginal. Hence, the existence of separate receptor loci for the A subunit might facilitate the toxin's effects, especially at submaximal concentrations.

The evidence presented here for the existence of receptor sites for the A subunit is not the first such evidence. In previous reports, we (Donta, 1979), as well as others (Sahyoun & Cuatrecasas, 1975), noted that the A subunit competed for the binding of CT. The latter group surmised that this inhibition was "nonspecific" because of the failure to demonstrate specificity of binding of the [^{125}I]A subunit. This failure of specificity, however, may have been due to differences between the A subunit in its native configuration and that in its iodinated state. The A subunit contains 5 times as many tyrosine residues per mole as does subunit B (Clements et al., 1980; Lai et al., 1976), and the iodination procedure may have resulted in alteration of the subunit's binding and biologic activities. Alternatively, Y1 adrenal tumor cells may possess unique, A containing, receptor sites. The presence of such sites was also suggested by Knoop, who used a milder iodination procedure (Knoop, 1978). Knoop, however, did not attempt to determine the specificity of the binding process. In preliminary studies, we have found that the A subunit can similarly compete for the binding of [^{125}I]CT, and not that of [^{125}I]LT, to cell types other than Y1 (e.g., neuroblastoma, rat hepatoma cells, and porcine intestine). More direct evidence for the existence of A receptor sites must await the successful iodination and demonstration of specific binding of the A subunit to cells.

In Y1 cells, despite being able to inhibit the binding of CT, the B subunit is without effect on CT's or LT's morphologic or steroidogenic actions. These results are probably best explained by the presence of large numbers of toxin receptors (>100 000/cell) on these cells and the need to occupy less than 1% of the receptors to achieve a response. Alternatively, the B subunit cannot block the binding of the A subunit to its receptor site. In other cell systems (Pierce, 1973; Guerrant & Brunton, 1977), the B subunit (choleragenoid) had been observed to inhibit the actions of CT but not LT. In the intestinal systems, the numbers of receptors for CT are 1 order of magnitude less than that found on Y1 cells (Holmgren et al., 1975). The numbers of receptors on intestinal cells for LT has not been determined, but the receptor numbers on CHO cells appear to be several times that for CT (S. T. Donta et al., unpublished observations). The existence of fewer receptors for CT on these other cell types and perhaps even more stringent steric requirements for the binding of CT might then account for the apparent discrepant observations.

The Scatchard analyses suggest the existence of only one class of receptors. Other investigators (Sattler et al., 1977; Fishman & Atikkan, 1980) have reported curvilinear Scatchard plots for the binding of cholera toxin. Sattler used equilibrium displacement dialysis, conditions which did not allow for the free interaction of ganglioside and toxin, and found an unusually shaped curve that may indicate positive cooperativity. Fishman and Atikkan did not display their data but stated that curvilinear plots indicating apparent positive cooperativity were obtained in studying the binding of cholera toxin to several different cell types. Greater concentrations (4×) of [^{125}I]CT were used in the latter studies than were used in ours; otherwise, the reasons for the discrepancies between their and our observations are not readily apparent. The amount of labeled material used may influence the ability to detect binding sites of differing affinities (Cuatrecasas, 1969; Ginsberg, 1977; Kono, 1969), but such use of varying amounts of [^{125}I]CT did not alter our findings of linear plots.

The observation of ready reversibility of the binding of [^{125}I]CT to Y1 cells is somewhat at variance with previous observations using other cell systems (Cuatrecasas, 1973b; Fishman et al., 1979; Holmgren & Lonnroth, 1976). In these other studies, reversibility of toxin binding was demonstrable but was found to be temperature dependent, with a maximum of 50% reversibility at 37 °C. Our observations with Y1 cells indicate that >90% of bound toxin can be rapidly displaced at 37 °C. Further studies, using additional cell types, are under way in order to determine whether these findings are unique to Y1 cells.

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Unfolding of 175-Base-Pair Nucleosomes†

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ABSTRACT: Calf thymus nucleosomes containing 175 base pairs of DNA unfold in two steps as the salt concentration is lowered, as detected by electric dichroism measurements. The transition midpoints are at 2.9 and 1.1 mM ionic strength at 7 °C with at most a small dependence on temperature. We identify the product of the 2.9 mM transition as an expanded disklike structure similar to the product of the 1.0-1.3 mM unfolding transition of 146-base-pair nucleosomes. The product of the 1.1 mM transition in 175-base-pair nucleosomes is elongated into a more asymmetric particle.

The structure of transcriptionally active chromatin is a subject of intense current investigation, whose general objective is to clarify how DNA which normally is bound to histones becomes accessible to RNA polymerase and regulatory proteins in general. Recent evidence has shown that active genes are highly susceptible to DNase I digestion (Gottesfeld et al., 1975; Weintraub & Groudine, 1976; Garel & Axel, 1976; Weisbrod et al., 1980; Giri & Gorovsky, 1980) although it is not yet certain whether the primary sensitive sites are intra- or internucleosomal. Weintraub & Groudine (1976) and Groudine et al. (1978) have observed that in globin and integrated viral genes the enhanced nuclease sensitivity could be maintained in isolated core nucleosomes, possibly implying a different conformation for active and inactive nucleosomes. Along these lines, Giri & Gorovsky (1980) concluded that the DNase I sensitivity of activated ribosomal genes arises from a reversible alteration of the core structure during activation. On the other hand, Garel & Axel (1976) did not find retention of DNase I sensitivity in isolated nucleosomes from active ovalbumin genes.

One way to characterize and compare the ability of nucleosomes to unfold is to examine their response to perturbing agents, of which low salt concentration is the most studied. Reduced ionic strength causes a reversible unfolding of core particles without loss of proteins; the transition has been characterized by hydrodynamic (Gordon et al., 1978; Harrington, 1981), electron microscopic (Oudet et al., 1977), electrooptical (Wu et al., 1979), and fluorescence (Dieterich et al., 1977, 1979; Dieterich & Cantor, 1981) techniques. While there has been some variability in the results, the emerging consensus is that there is a single unfolding transition, whose mechanism is complex, centered at about 1 mM mo-

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