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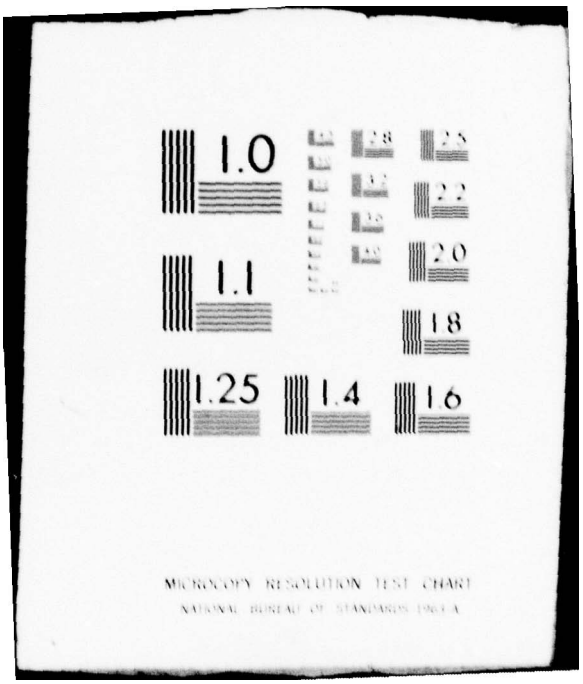
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RECEPTOR-MEDIATED INTERNALIZATION AND DEGRADATION OF DIPHTHERIA TOXIN BY MONKEY KIDNEY CELLS

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The receptor-mediated internalization and degradation of radiolabeled diphtheria toxin by cultured monkey kidney cells was studied. The ability of a number of enzymes and chemicals to remove cell surface-bound toxin was tested; the combination of pronase and inositol hexaphosphate (PIHP) proved most effective. Using PIHP, the kinetics of toxin-cell association at 37°C was resolved into two components: surface binding and internalization. The PIHP assay also allowed estimation of the half-time of toxin internalization (about 25 min). An assay involving precipitation of culture supernatants with trichloracetic

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acid was developed and used to measure the rate of degradation and excretion of cell-associated toxin. Agents which markedly inhibited toxin internalization similarly prevented degradation, implying an intracellular location for the degradative process. The primary radioactive product excreted by Vero cells was monoiodotyrosine. The extent and rate of toxin degradation indicated lysosomal involvement. Finally, agents which blocked internalization and/or degradation (e.g., antibody and concanavalin A) protected cells from the cytotoxic action of diphtheria toxin, suggesting that these processes are necessary for expression of biological effect.

RECEPTOR-MEDIATED INTERNALIZATION AND DEGRADATION OF DIPHTHERIA TOXIN BY MONKEY KIDNEY CELLS

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Running Title: Internalization and Degradation of Diphtheria Toxin

SUMMARY

The receptor-mediated internalization and degradation of radiolabeled diphtheria toxin by cultured monkey kidney cells was studied. The ability of a number of enzymes and chemicals to remove cell surface-bound toxin was tested; the combination of pronase and inositol hexaphosphate (PIHP) proved most effective. Using PIHP, the kinetics of toxin-cell association at 37°C was resolved into two components: surface binding and internalization. The PIHP assay also allowed estimation of the half-time of toxin internalization (about 25 min). An assay involving precipitation of culture supernatants with trichloracetic acid was developed and used to measure the rate of degradation and excretion of cell-associated toxin. Agents which markedly inhibited toxin internalization similarly prevented degradation, implying an intracellular location for the degradative process. The primary radioactive product excreted by Vero cells was monoiodotyrosine. The extent and rate of toxin degradation indicated lysosomal involvement. Finally, agents which blocked internalization and/or degradation (e.g., antibody and concanavalin A) protected cells from the cytotoxic action of diphtheria toxin, suggesting that these processes are necessary for expression of biological effect.

It has been recently shown that diphtheria intoxication of highly sensitive mammalian cell lines is a receptor-mediated process (1); that is, the intracellular events culminating in cytotoxicity are preceded by the binding of toxin to specific cell-surface receptors. The intracellular mode of action of diphtheria toxin has been extensively characterized (2, 3): an enzymatically active portion of the toxin, fragment A, catalyzes the transfer of the ADPR[1] moiety of NAD to EF-2, an essential translocase involved in eukaryotic protein synthesis. ADPR-EF-2 is metabolically inactive and subsequent cell death is directly attributable to inhibition of intracellular protein synthesis. The means by which the toxin or toxin-receptor complex is internalized by the cell is as yet unclear.

Recent studies have shown that many macromolecules, including insulin (4), human choriogonadotropin (5), and EGF (6), are rapidly internalized by cells. However, there is little direct evidence that the biological function of these molecules requires internalization. On the other hand, it is obvious that at least the active fragment of diphtheria toxin must enter the cell in order to effect the ADP-ribosylation of EF-2. Thus internalization in this case is a prerequisite for expression of biological activity.

In this sense, diphtheria toxin is more analogous to LDL, a cholesterol-carrying serum macromolecule whose metabolic effects are exerted through a receptor-mediated internalization and degradation process (7). Degradation of LDL occurs in secondary lysosomes and results in the release of free cholesterol into the cytoplasm. Similarly, diphtheria toxin must also undergo some form of degradation (or fragmentation) to express its biological activity. Toxin which is

either intact or has a cryptic peptide bond cleavage ("nicked" toxin) is enzymatically inactive *in vitro* (3), but is cytotoxic to cells and animals *in vivo*. Enzymatically active fragment A must therefore be released from whole toxin during cellular intoxication subsequent to the binding of toxin to receptor.

As shown by our previous studies, the kinetic response of sensitive cells to ^{125}I-labeled diphtheria toxin at 37°C is biphasic, reaching a peak at 1-2 h and decreasing thereafter to a steady state approximately 50% of the maximum (1). It seemed probable that this pattern resulted from sequential cell-surface binding, internalization, and intracellular processing of the ^{125}I-labeled toxin. At physiological temperature, however, it is difficult to quantitate the degree of cell-surface binding since total cell-associated radioactivity presumably represents both surface-bound and internalized material. In this investigation, we have developed a technique which permits such quantitative studies and have defined the relationship between surface binding and subsequent intracellular uptake and processing.

MATERIALS AND METHODS

Cells and Cell Culture--Seed stock for the Vero and BS-C-1 cell lines was obtained from the American Type Culture Collection (ATCC). The lines were maintained in 75-cm^2 T-flasks (Costar #3075) or 700-cm^2 roller bottles (Costar #1234) with the medium and serum supplement recommended by ATCC.

Media and Sera--All media, vitamins, antibiotics, and amino acids were obtained from Grand Island. Fetal calf serum was purchased from Reheis and heat-inactivated for 30 min at 56°C before use in cell culture experiments.

Materials--"Low pH" carrier-free sodium iodide-125 was obtained from New England Nuclear. Pronase (B grade, 89,000 P.V.F./g), heparin, and methyl-α-D-mannopyranoside were purchased from Calbiochem; trypsin (183 U/g) and collagenase (Type IV, 150 to 250 U/mg) were obtained from Worthington Biochemical Corp. Inositol hexaphosphate was obtained from P-L Biochemicals, dextran sulfate and concanavalin A from Pharmacia. Adenosine-5'-triphosphate (ATP) and 3-iodo-L-tyrosine (mono-iodotyrosine, MIT) were purchased from Sigma. All other chemicals were reagent grade and were used without further purification.

Toxin--Diphtheria toxin was obtained from Connaught Laboratories and purified by chromatography over DEAE-cellulose (DE-52). The final product ran as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was indistinguishable in cell culture experiments from purified diphtheria toxin (23 MLD/mg) supplied by Dr. A. M. Pappenheimer, Jr., Harvard University. The toxin was radiolabeled as previously described by the method of Roth (8) to a specific activity of

1 to 2 x 10^7 cpm/µg (0.1 to 0.2 mol iodine/mol toxin). The labeling procedure had no detectable effect on the biological activity of the toxin as measured by cytotoxicity assay. Diphtheria toxin-specific antiserum, obtained by hyperimmunization of a goat, was prepared using purified diphtheria toxoid obtained from Dr. R. Holmes, Uniformed Services University of Health Sciences.

Binding Assay--Details of the binding assay have been previously described (1). Cells were seeded in 24-well tissue culture plates (Costar #3524) and grown to a concentration of approximately 1 x 10^5 cells/well. On the day of the experimentation, the medium was replaced with 1 ml of Hanks' 199, supplemented with 10% heat-inactivated FCS and 25 mM Hepes, pH 7.4 (complete Hanks' 199). ^{125}I-diphtheria toxin (0.03 µg/ml) or ^{125}I-toxin plus a 100-fold excess of unlabeled toxin were added in 10-µl aliquots to the wells and incubations were carried out at the temperatures and for the times indicated. The monolayers were then rinsed four times with Hanks' balanced salt solution (HBSS), solubilized in 1.0 ml of 0.1 M NAOH and counted in a Model 1185 automatic gamma counting system (Searle Analytic, Inc.).

The difference between binding of labeled toxin in the presence and absence of excess unlabeled toxin represents specific binding; the data presented in this paper are shown in the form of specific counts. The level of nonspecific binding in all cases represented less than 10% of the total.

Internalization Assay--Cell monolayers were incubated with ^{125}I-toxin with or without a 100-fold excess of unlabeled toxin as described above. The monolayers were then rinsed three times with cold HBSS and incubated 60 min at 4°C with 0.5 ml of pronase (0.25 mg/ml)

plus inositol hexaphosphate (IHP) (10 mg/ml) in HBSS. At the end of the incubation period, 0.5 ml of 100% heat-inactivated FCS was added to each well and the detached cells were immediately transfered to 1.5 ml polypropylene centrifuge tubes and spun for 1 min in a microfuge (Eppendorf Model 5412). Both pellets and supernatants were counted as above.

Assay of Trichloroacetic Acid-Soluble Fragment Release--Cell monolayers were incubated with ^{125}I-diphtheria toxin with or without a 100-fold excess of unlabeled toxin at 4°C for the times indicated. The monolayers were then rinsed three times with cold HBSS, the medium replaced with 1 ml/well complete Hanks' 199 and incubation continued under the conditions indicated. At timed intervals, the medium was removed from each sample well and added to an equal volume of cold 10% trichloroacetic acid (TCA) in 12 x 75 mm glass culture tubes. Samples were mixed thoroughly and centrifuged at 4°C for 10 min at 1200 x g, after which both pellets and supernatants were counted as above.

Characterization of TCA-Soluble Degradation Products--Cell monolayers in 75-cm^2 T-flasks were incubated for 1 h at 37°C in 5 ml complete Hanks' 199 containing 0.1 µg/ml ^{125}I-diphtheria toxin. The monolayers were rinsed three times with HBSS and incubated an additional 4 h at 37°C in Hanks' 199 supplemented with 1% FCS, 25 mM Hepes buffer, pH 7.4. At this point, the culture supernatant was collected, centrifuged to remove any detached cells, and MIT (100 µmol in 50% acetone) was added as carrier. Samples were lyophilized, redissolved in 1.0 ml of water, and precipitated by addition of TCA to 10%. Approximately 50% of the original cell-associated radioactivity was

found in the supernatant (15,000 cpm). TCA was removed by three ether extractions. The supernatant was diluted to 10 ml in 0.02 M HCl and adsorbed to an 0.8-ml bed of Dowex 50 X-2. The column was washed with 0.01 M acetic acid and eluted with 0.15 M NH_4OH. The radioactive fractions (13,000 cpm) were pooled and lyophilized. One-tenth of the redissolved sample was spotted on a silica gel thin-layer plate and the chromatograms developed with n-butanol/acetic acid/H_2O (10:1:1).

RESULTS

Release of Diphtheria Toxin from the Cell Surface--Studies of receptor-mediated internalization in other systems (9, 10) have been facilitated by the development of chemical or enzymatic treatments which release bound ligand from the cell surface. Initially, we compared the effects of a large number of putative releasing agents in our system under conditions where all cell-associated toxin was retained on the cell surface. Cells were prebound to equilibrium with ^{125}I-labeled diphtheria toxin at 4°C, at which temperature endocytic uptake is considered to be minimal. At the start of the experiment, cells were rinsed thoroughly to remove unbound toxin, releasing agent was added, and incubation continued for 1 h at 4°C. At the end of the incubation period, the treated cells were centrifuged and radioactivity in supernatants and pellets determined. Supernatant activity represents the amount of the total cell-associated radioactivity released from the cell surface by the agent tested.

Results of those experiments are shown in Table I. Incubation with pronase plus inositol hexaphosphate (PIHP) was reproducibly most effective in removing receptor-bound ^{125}I-diphtheria toxin from the cell surface.

Trypsin plus IHP, pronase alone, or pronase + 0.2 g/liter EDTA were found to be nearly as effective as PIHP in removing surface-bound radioactivity; IHP alone (10 mg/ml) removed about half the receptor-bound toxin. All other chemicals tested were ineffective. We found PIHP, at the concentrations and incubation periods routinely used, preferable to trypsin plus IHP since it elicits complete detachment of the treated cell monolayers, thus facilitating subsequent transfer steps in the analytical procedure.

The kinetics of ^{125}I-diphtheria toxin removal from the cell surface by PIHP treatment are shown in Fig. 1. Vero cells were incubated with ^{125}I-labeled toxin for 18 h at 4°C. Then the cell monolayers were washed rapidly with HBSS and incubated with PIHP for varying periods of time. Fig. 1 demonstrates that in the absence of PIHP treatment total cell-associated radioactivity remains essentially constant over the 1-h time period of the experiment. In the presence of PIHP, however, increasing amounts of the cell-associated radioactivity were released into the medium until, at 60 min, over 85% of the total was removed from the cells. Control experiments using an established procedure (11) showed no apparent plasma membrane damage as assessed by leakage of [^{14}C] aminoisobutyric acid from cells treated with PIHP in the manner described. The results shown in Table I and Fig. 1 led us to adopt a 1-h incubation in PIHP as a standard assay for cell surface-bound toxin.

Kinetics of Toxin Internalization--Development of the PIHP method allows detailed examinations of the nature of the association of diphtheria toxin with Vero cells. The kinetics of toxin internalization as assayed by the PIHP technique were followed at 4 and 37°C. Results

are shown in Fig. 2. Cells were equilibrated to either 4 or 37°C and ^{125}I-toxin added at zero time. Samples were harvested either using PIHP or by solubilization in NaOH as described for the binding assay in Materials & Methods. At 4°C, PIHP-releasable radioactivity continued to increase in the presence of ^{125}I-labeled toxin and at all time points represented approximately 85% of the total cell-associated radioactivity. This indicates that essentially all of the ^{125}I-labeled toxin remained on the cell surface. Very little radioactivity was associated with the cell pellet. At 37°C, however, the increase in PIHP-releasable radioactivity was accompanied by an increase in PIHP-resistant (pellet) radioactivity. Both reached a maximum level around 1 to 2 h after toxin addition and decreased thereafter; PIHP-releasable (surface-bound) counts decreased more rapidly than PIHP-resistant (internalized) counts. After 1 to 2 h, approximately 50% of the total cell-associated radioactivity was resistant to release by PIHP. This experiment demonstrates that the biphasic kinetic curve exhibited by Vero cells at 37°C can be resolved into two components using the PIHP technique: total cell-associated radioactivity is the sum of PIHP-releasable (surface-bound) and PIHP-resistant (internalized) radioactivity.

Half-Time of Toxin Internalization--Since it is difficult to determine the half-time of toxin internalization from experiments of the type shown in Fig. 2, additional experiments were performed using cells prebound with ^{125}I-labeled diphtheria toxin at 4°C (Fig. 3). At zero time, following a 12-h incubation at 4°C, essentially all of the cell-associated radioactivity represents surface-bound diphtheria toxin and is fully releasable by PIHP. At this point the cells were

rinsed three times to remove unbound toxin and the incubation was continued at 4 or 37°C. At 4°C, the slight decrease in PIHP-releasable radioactivity was due to dissociation of surface-bound toxin from its receptor. There was no detectable increase in PIHP-resistant radioactivity. With continued incubation at 37°C, PIHP-releasable radioactivity decreased markedly over the period of the experiment with a concomitant increase in PIHP-resistant activity, representing internalized toxin. From these data, the half-time of internalization of diphtheria toxin by Vero cells was estimated to be 25 min.

The concentration of ^{125}I-labeled toxin used in these studies (0.03 ug/ml) has been shown to saturate approximately 10 to 20% of the available surface receptor sites yielding 10,000 to 20,000 toxin molecules bound per cell (1). Calculations based on the data shown in Fig. 3 show that approximately 10,000 toxin molecules were internalized per cell.

Degradation of Internalized Toxin--As shown in Fig. 3, when cells were prebound with ^{125}I-diphtheria toxin at 4°C and subsequently rinsed and warmed to 37°C, total cell-associated radioactivity decreased rapidly to about 20% of the initial level. The decrease was hypothesized to result from the internalization, intracellular degradation, and subsequent excretion of toxin fragments by the cells. This hypothesis was supported by the data shown in Fig. 4. When cells were prebound with ^{125}I-diphtheria toxin at 4°C, rinsed, and transferred to 37°C, the ^{125}I-toxin was rapidly degraded into TCA-soluble fragments. The amount of TCA-soluble radioactivity in the culture supernatant increased with time of incubation at 37°C to a plateau level around 3 to 4 h, at which point approximately 60 to 70% of the original cell-associated radioactivity

had been excreted in degraded form. About 10% of the original cell-associated radioactivity was detectable in the culture supernatants in TCA-precipitable form.

Degradation experiments of this type were performed using prebound, rinsed cells because the ^{125}I-diphtheria toxin preparation contained a persistent contaminating amount of TCA-soluble radioactivity (approximately 3 to 5%) which complicated the detection of cell-dependent fragment excretion. The rinse procedure effectively eliminated this problem. Chloroform extraction of culture supernatants incubated in this manner did not demonstrate measurable free iodine (12).

Characterization of TCA-Soluble Fragments--The nature of the TCA-soluble material released by the cells at 37°C was investigated using thin-layer chromatography. The chromatogram in Fig. 5 demonstrates that the majority of the TCA-soluble radioactivity (approximately 85%) is in the form of ^{125}I-monoiodotyrosine. The extent and rapidity of cellular degradation of toxin suggest lysosomal proteolysis.

Effect of Concanavalin A on Toxin Degradation--The effect of concanavalin A (Con A) on the generation of TCA-soluble radioactivity is shown in Fig. 6. Con A was recently shown to inhibit the cellular internalization of diphtheria toxin without affecting toxin-receptor binding (13). In this system, it was shown that the Con A-induced inhibition of toxin internalization is accompanied by a block of the release of TCA-soluble radioactivity. By 180 min, nearly 60% of the original control cell-associated radioactivity was found in the form of TCA-soluble fragments in the culture medium, while less than 25% was degraded to TCA-soluble form by the cells treated with Con A. This

demonstrates that receptor-mediated internalization is a prerequisite for degradation.

Effect of Specific Antibody on Internalization and Degradation--Internalization and degradation of diphtheria toxin by cells treated with antibody specific for diphtheria toxoid was studied and the results (Table II) were analogous to those obtained with Con A. Here the cells were prebound with radiolabeled diphtheria toxin for 12 h at 4°C, rinsed, and incubated with specific antibody for 30 min. Following this incubation, cells were rinsed again, fresh medium added, and the monolayers were transferred to 37°C. At various times after transfer, samples were harvested and assayed for toxin internalization and degradation.

Antibody at this concentration reproducibly interfered somewhat with the efficiency of the PIHP assay; though initial levels of total cell-associated radioactivity as calculated from the sum of PIHP-releasable and PIHP-resistant counts were essentially the same (control = 8820 cpm; antibody-treated = 8980 cpm), PIHP treatment removed only 82% of the receptor-bound toxin from the antibody-treated cells, as opposed to 94% from the controls. Therefore in the antibody-treated samples the extent of internalization is probably overestimated. In experiments of this type it was found that internalization was 35-45% retarded in the antibody-treated cells relative to the controls, as determined by comparisons of the amount of PIHP-releasable radioactivity remaining after a 90 min incubation at 37°C. Concomitantly, toxin degradation, determined by measurement of TCA-soluble radioactivity in the culture medium, was 60-70% inhibited by antibody. This inhibition of degradation led to an accummulation of intracellular radioactivity; after a 90 min

incubation at 37°C, there were over 2-fold more PIHP-resistant counts associated with the antibody-treated cells than with the controls.

These data strongly imply that cellular internalization of toxin is required for degradation. Also, since toxin-specific antibody (14) and Con A (15) effectively block the cytotoxic effects of diphtheria toxin at the concentrations used in these experiments (data not shown), the data support the hypothesis that diphtheria intoxication of mammalian cells requires sequential receptor binding, internalization, and intracellular processing and degradation.

DISCUSSION

The studies presented demonstrate that receptor-bound diphtheria toxin is rapidly internalized and degraded by sensitive mammalian cells. Since our previous work (1) showed that binding of toxin to specific receptor is a necessary step in diphtheria intoxication, it appears likely that the internalization process described here is responsible for the delivery of activated toxin (or toxin fragment) to the cytoplasm.

Certain other studies of diphtheria toxin uptake have indicated that both sensitive and resistant cells adsorb and degrade toxin by an apparently nonspecific pinocytotic mechanism (16, 17). It was hypothesized that this nonspecific process accounts for the vast majority of cellular toxin uptake, while a second undefined mechanism of entry accounts for the small number of toxin molecules capable of exerting the biological (cytotoxic) effect. A number of proposals have been put forward in attempts to define this second (specific) mechanism (17-19);

most postulate some form of direct traversal of the plasma membrane by the toxin molecule (or A fragment).

Our system, while certainly not ruling out the possibility of multiple mechanisms of toxin entry, measures only uptake mediated by toxin-specific cell-surface receptors. The fate of receptor-bound toxin was studied initially using a technique which effectively differentiates between surface-bound and internalized toxin. At 4°C, under conditions in which endocytic uptake is minimal, over 85% of the specific cell-associated ^{125}I-toxin was releasable by treatment with pronase plus IHP (PIHP) (Table I, Figs. 1 and 2). With increasing periods of incubation at 37°C, however, the cell-associated radioactivity became increasingly resistant to PIHP release, presumably due to progressive toxin internalization (Fig. 2). This susceptibility of cell surface-bound ^{125}I-diphtheria toxin to PIHP treatment is analogous to the release of surface-bound ^{125}I-labeled EGF by proteolytic treatment with 0.25% trypsin (9). At 37°C, ^{125}I-EGF was shown to become increasingly resistant to proteolytic release, suggesting progressive cellular internalization. Continued incubation at 37°C in the presence of ^{125}I-EGF results in a loss of total radioactivity from the cells. These data were used by Carpenter and Cohen (6) to support the hypothesis that surface-bound EGF is sequentially internalized, degraded, and excreted by mammalian cells.

The value of biochemical techniques capable of releasing receptor-bound macromolecules from the cell surface is further exemplified by uptake studies utilizing the effect of sulfated glycosaminoglycans on cell-associated LDL (16). Heparin has been shown to elicit the dissociation of ^{125}I-LDL from its cell surface receptor

in a manner analogous to the release of receptor-bound concanavalin A by α-methyl-D-mannopyranoside. Heparin-mediated release was attributed to negative charges on the heparin molecule, which may interact with positively charged regions on LDL to form soluble complexes. A similar mechanism may account for the partial release of cell surface-bound diphtheria toxin by the polyanion inositol hexaphosphate. At 10 mg/ml, IHP was observed to effectively block the binding of ^{125}I-diphtheria toxin to its surface receptor (unpublished observation). When cells were pre-bound at 4°C with ^{125}I-toxin, a subsequent 60-min incubation with IHP induced the release of approximately half of the cell-associated radioactivity. Neither heparin nor dextran sulfate elicited ^{125}I-diphtheria toxin release, however; and IHP-mediated release was substantially enhanced by concomitant treatment with proteolytic enzyme, indicating that the observed detachment of surface-bound radioactivity is not solely attributable to receptor site competition by polyanion.

Receptor-mediated internalization of diphtheria toxin was accompanied by extensive degradation, as evidenced by the appearance of TCA-soluble radioactivity in the culture supernatants after periods of incubation at 37°C (Fig. 4). The TCA-soluble radioactivity was characterized by thin-layer chromatography and found to consist primarily of ^{125}I-moniodotyrosine (Fig. 5). The rapidity and extent of the degradation process strongly imply the involvement of lysosomes. Since 60 to 70% of the cell-associated ^{125}I-toxin is degraded in this manner, it appears that the majority of the cell-associated toxin is internalized, processed in lysosomes and finally excreted in the form of single amino acids. This does not, however, preclude the possibility that a biologically active toxin fragment escapes lysosomal proteolysis. There

is ample precedent in the literature for proteins which resist lysosomal digestion (20, 21); it is conceivable that such is the case with fragment A. A lysosomal processing or degradation step has been indicated in the cellular uptake of numerous polypeptide hormones, growth factors, and essential serum macromolecules, including human choriogonadotropin (5), LDL (7), EGF (6), and α_2-macroglobulin (22); it appears that the majority of the cell-associated diphtheria toxin may undergo a similar processing or degradation step. Additional support for this hypothesis is derived from the finding that the cytotoxic effects of diphtheria toxin are blocked by certain lysosomotropic agents, notably chloroquine (S. H. Leppla, R. B. Dorland, and J. L. Middlebrook. Submitted for publication).

The apparent close coupling of internalization with degradation in our system makes it unlikely that we are merely observing penetration of the membrane by toxin. It seems more consistent with our results to suggest that internalization occurs by adsorptive endocytosis, followed by release of fragment A from whole toxin in lysosomes. The internalization and degradation of receptor-bound diphtheria toxin appear to be directly related to expression of biological effect. When internalization and degradation are blocked by Con A (Fig. 6) or specific antibody (Table II), cytotoxicity is inhibited. We propose that, like certain polypeptide hormones, growth factors, and serum macromolecules, diphtheria toxin exerts it biological activity by sequential surface receptor binding, internalization via adsorptive endocytosis, and intracellular processing or degradation, probably in lysosomes.

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FOOTNOTES

[1]The abbreviations used are: ADPR, adenosine diphosphoribose; EF-2, elongation factor 2; EGF, epidermal growth factor; LDL, low-density lipoprotein; FCS, fetal calf serum; MIT, monoiodotyrosine; HBSS, Hanks' balanced salt solution; IHP, inositol hexaphosphate; Hepes, N-2-hydroxyethylpipirazine-N'-ethanesulfonic acid; Con A, concanavalin A.

TABLE I

Release of diphtheria toxin from the cell surface by various compounds

Vero cells were bound for 18 h at 4°C with 0.03 μg/ml ^{125}I-diphtheria toxin. The monolayers were rinsed three times with HBSS and incubated for 1 h at 4°C in the presence of releasing agent at the concentrations listed. FCS was then added to a final concentration of 50%, the wells were scraped with a small Teflon scraper, and triplicate samples were processed as described in Materials and Methods.

Compound	Radioactivity released
	% of initial cell-bound specific radioactivity
HBSS (control)	10
Trypsin (2.5%)	
Alone	35
+ EDTA (0.2 g/liter)	26
+ IHP (10 mg/ml)	76
Collagenase (1.0%)	
Alone	14
+ EDTA (0.2 g/liter)	16
+ IHP (10 mg/ml)	60
Pronase (0.25%)	
Alone	88
+ EDTA (0.2 g/liter)	84
+ IHP (10 mg/ml)	89
IHP (10 mg/ml)	
Alone	43
+ EDTA (0.2 g/liter)	39
EDTA (0.2 g/liter)	11
Heparin (10 mg/ml)	14
Dextran sulfate (10 mg/ml)	19
Sodium sulfate (10 mg/ml)	12
Concanavalin A (100 μg/ml)	9
Methyl-α-D-mannopyranoside (0.2 M)	9
ATP (1 mM)	12
Diphtheria toxin (3 μg/ml)	12

TABLE II

Effect of antibody on internalization and degradation of diphtheria toxin by Vero cells

Cells were bound for 12 h at 4°C with 0.03 µg/ml ^{125}I-toxin. The monolayers were then rinsed three times with HBSS and incubated a further 30 min at 4°C in the presence or absence of 1:100 specific antibody. Cells were rinsed two times with HBSS, fresh complete medium added, and cells were transferred to 37°C for the times listed below. Internalization and degradation were assayed in triplicate samples as described in Materials and Methods. Standard error of the mean was in all cases less than 5%.

	Radioactivity	
	0 min	90 min[a]
	cpm	cpm
PIHP-releasable		
Control	8,260	2,060
+ Antibody	7,360	3,350
PIHP-resistant		
Control	560	2,210
+ Antibody	1,620	5,220
TCA-soluble		
Control	0	4,580
+ Antibody	10	1,820

[a]Incubation time.

FIG. 1. Kinetics of diphtheria toxin removal from the cell surface by PIHP. ^{125}I-toxin (0.03 µg/ml) was added to the cells for 18 h at 4°C. Cells were then rinsed three times with HBSS, PIHP (0.25 mg/ml pronase + 10 mg/ml IHP) was added, and incubations were carried out for the times indicated at 4°C. FCS was then added to a final concentration of 50%; the wells were scraped with a small Teflon scraper, and triplicate samples processed as described in Materials and Methods. Total cell-associated radioactivity (▲); PIHP-releasable radioactivity (□); PIHP-resistant radioactivity (○).

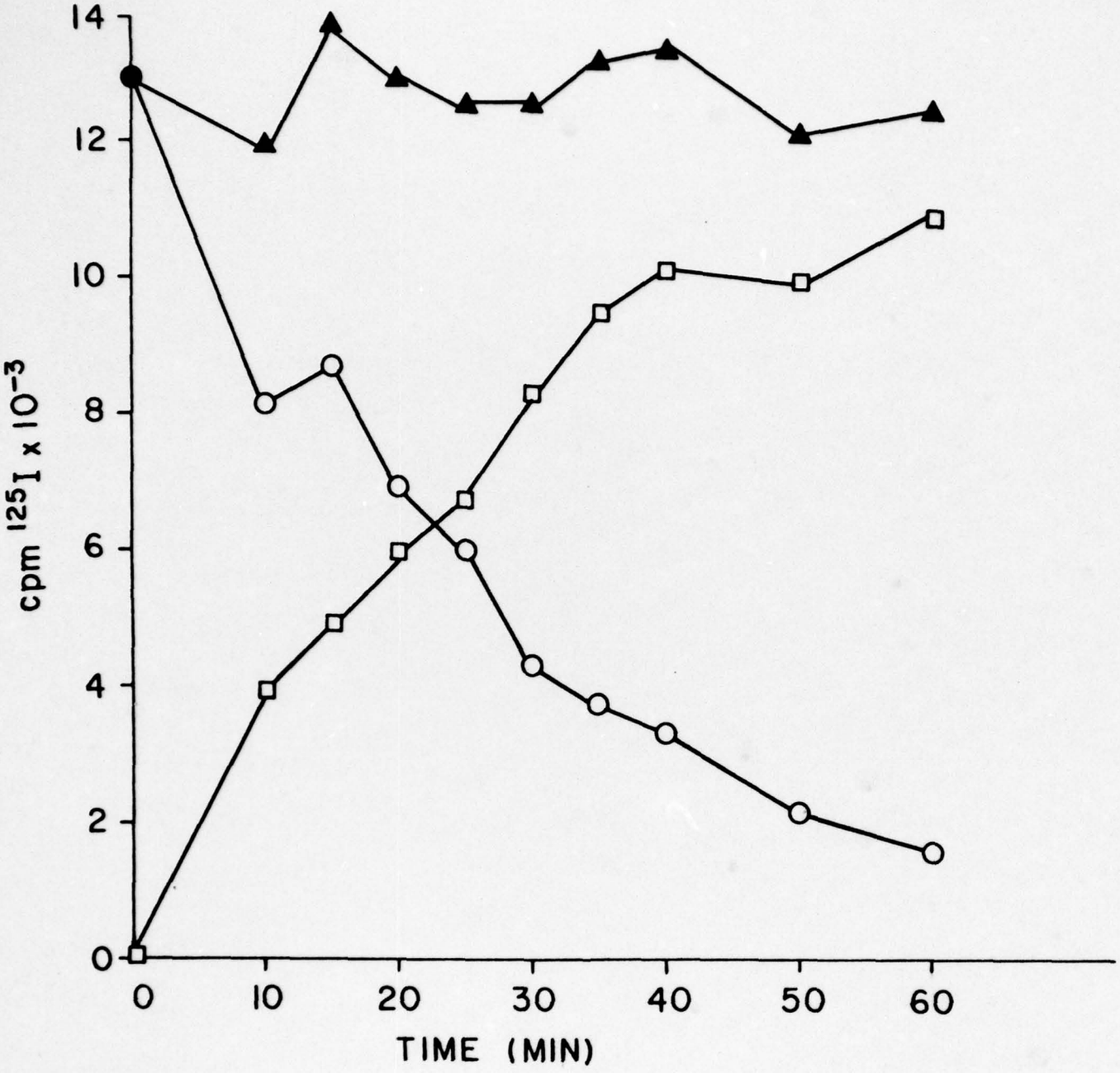
FIG. 2. Kinetics of diphtheria toxin internalization. ^{125}I-toxin (0.03 µg/ml) was added to the cells at either 37°C (A) or 4°C (B). At the times indicated, triplicate samples were assayed for total cell-associated radioactivity or for internalization as described in Materials and Methods. Total cell-associated radioactivity (●); PIHP-releasable radioactivity (□); PIHP-resistant radioactivity (△).

FIG. 3. Half-time of internalization of diphtheria toxin. ^{125}I-toxin (0.03 µg/ml) was added to cells for 18 h at 4°C. Cells were then rinsed three times with HBSS, fresh complete medium was added, and incubation was continued at either 4 or 37°C. At the times indicated, triplicate samples were processed as described in Materials and Methods. 4°C, PIHP-releasable radioactivity (○); PIHP-resistant radioactivity (□). 37°C, PIHP-releasable radioactivity (●); PIHP-resistant radioactivity (■).

FIG. 4. Kinetics of diphtheria toxin degradation. ^{125}I-toxin (0.03 μg/ml) was added to the cells for 18 h at 4°C. Cells were then rinsed three times with HBSS, fresh complete medium added, and incubation continued at either 4 or 37°C. At the times indicated, triplicate samples were processed as described in Materials and Methods. 4°C, Total cell-associated radioactivity (□); TCA-soluble radioactivity (■). 37°C, Total cell-associated radioactivity (○); TCA-soluble radioactivity (●).

FIG. 5. Characterization of TCA-soluble radioactivity. ^{125}I-toxin (0.1 μg/ml) was added to cell monolayers in 75-cm^2 T-flasks for 1 h at 37°C. The cells were then rinsed three times with HBSS, fresh medium was added, and incubation was continued for 4 h at 37°C. The culture supernatant was collected and processed as described in Materials and Methods. The position of the carrier 3-iodo-L-tyrosine (MIT), visualized by ninhydrin spray, is shown for comparison.

FIG. 6. Effect of concanavalin A on toxin degradation. ^{125}I-toxin (0.03 μg/ml) was added to cells for 12 h at 4°C in the presence or absence of Con A (100 μg/ml). Cells were rinsed three times with HBSS, complete Hanks' 199 with or without Con A (100 μg/ml) was added and incubation was continued at 37°C. At the times indicated, triplicate samples were processed as described in Materials and Methods. TCA-soluble radioactivity, control (●); Con A (■).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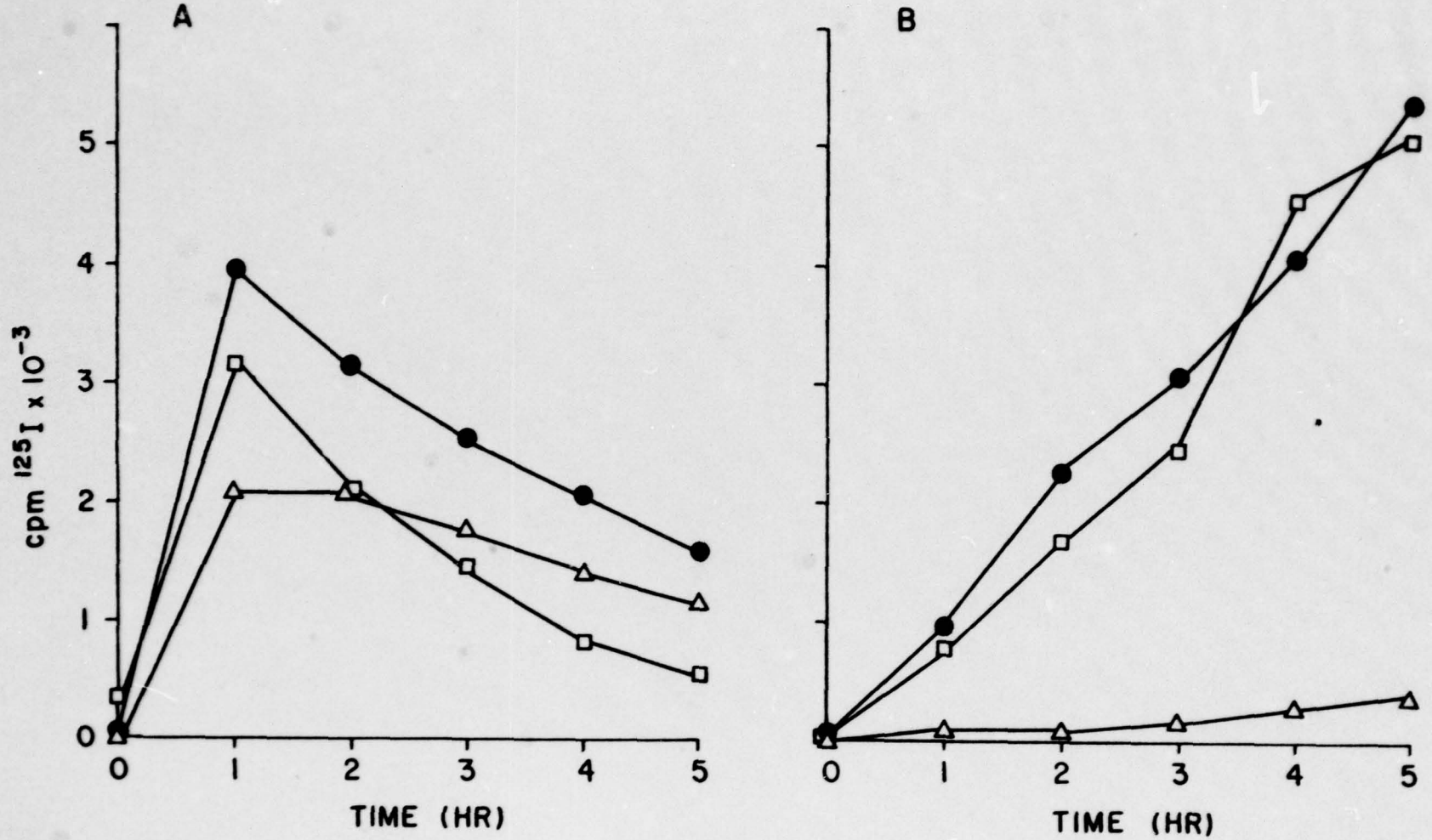
TIME (MIN)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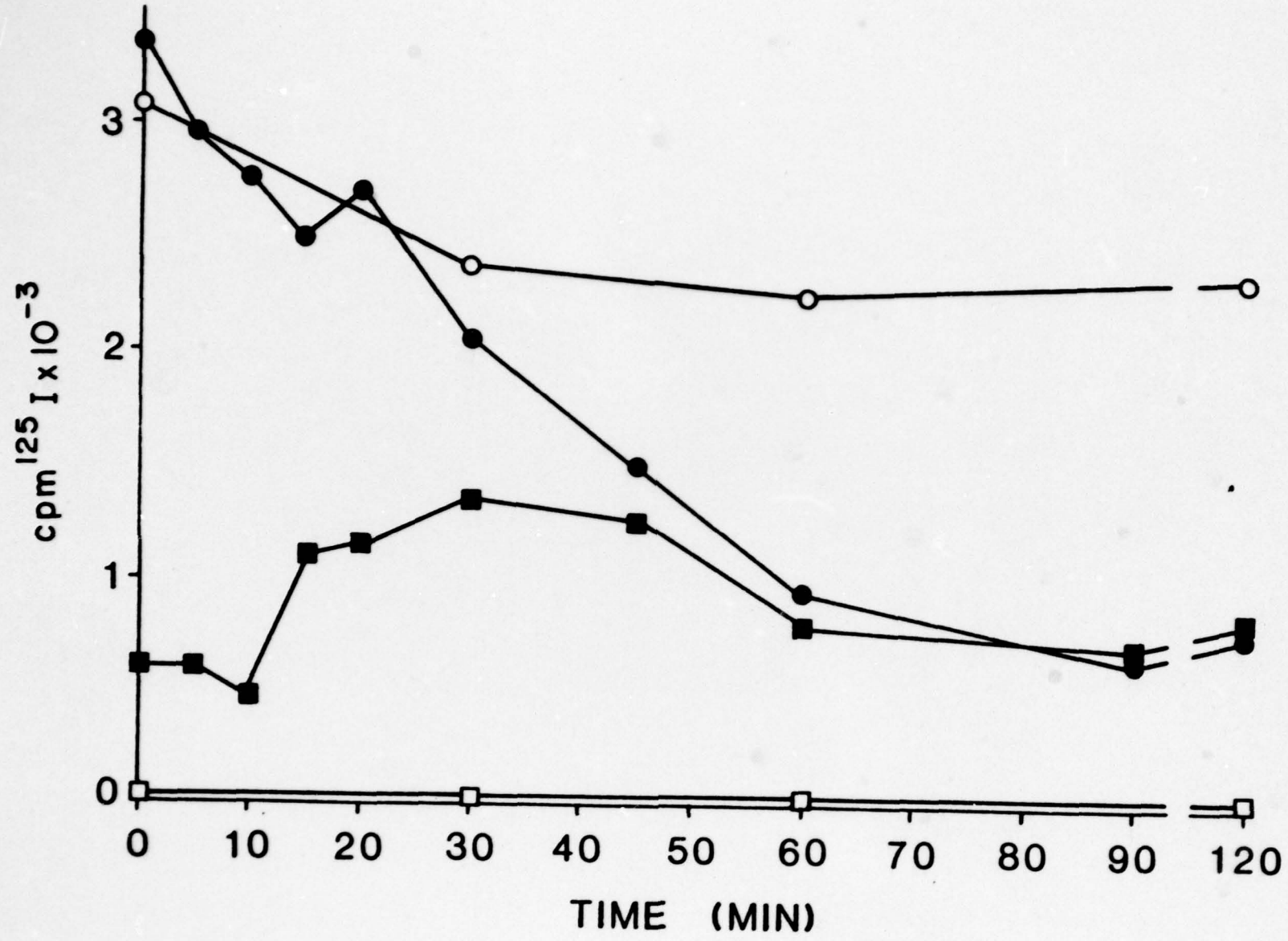
TIME (HR)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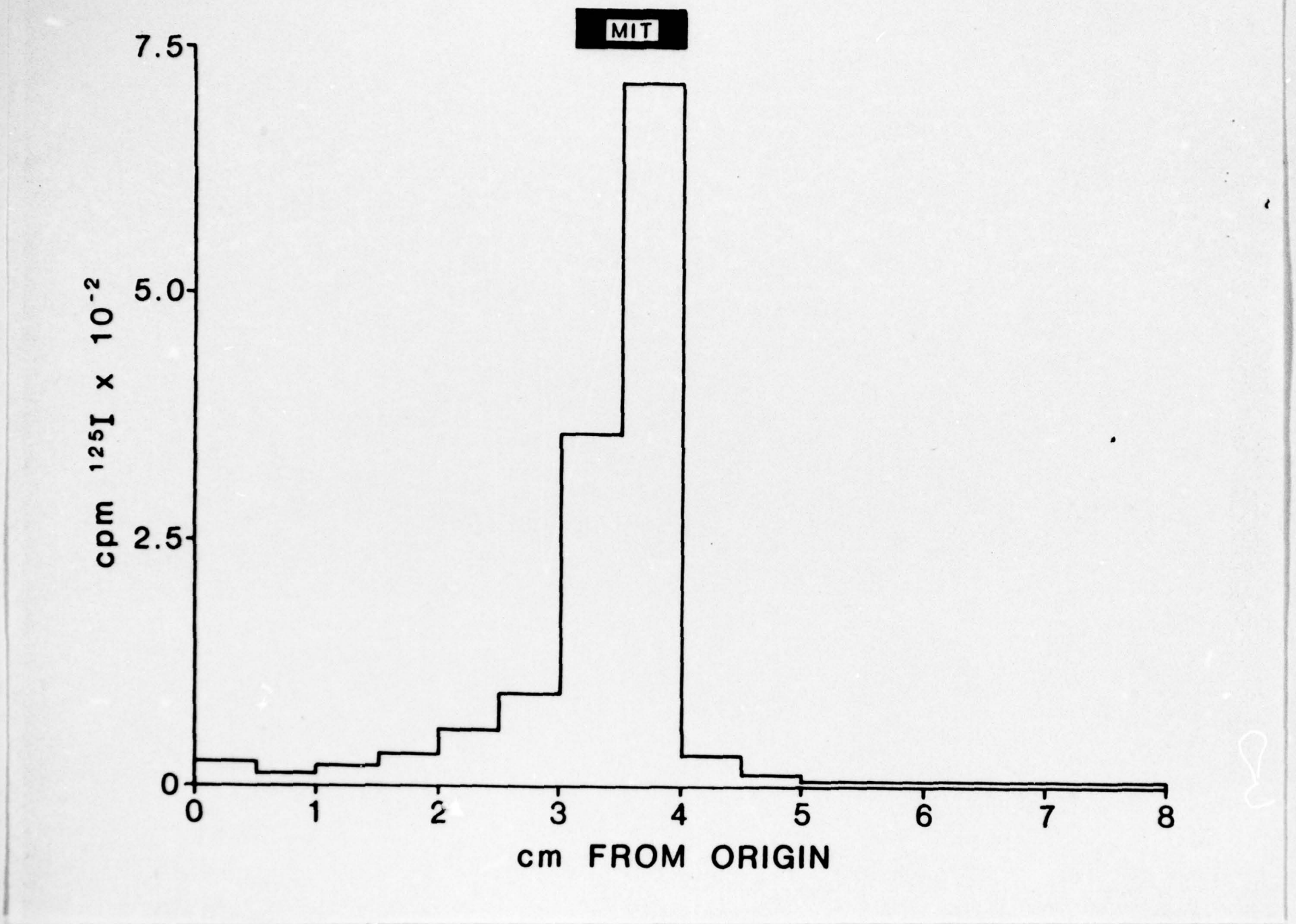
TIME (MIN)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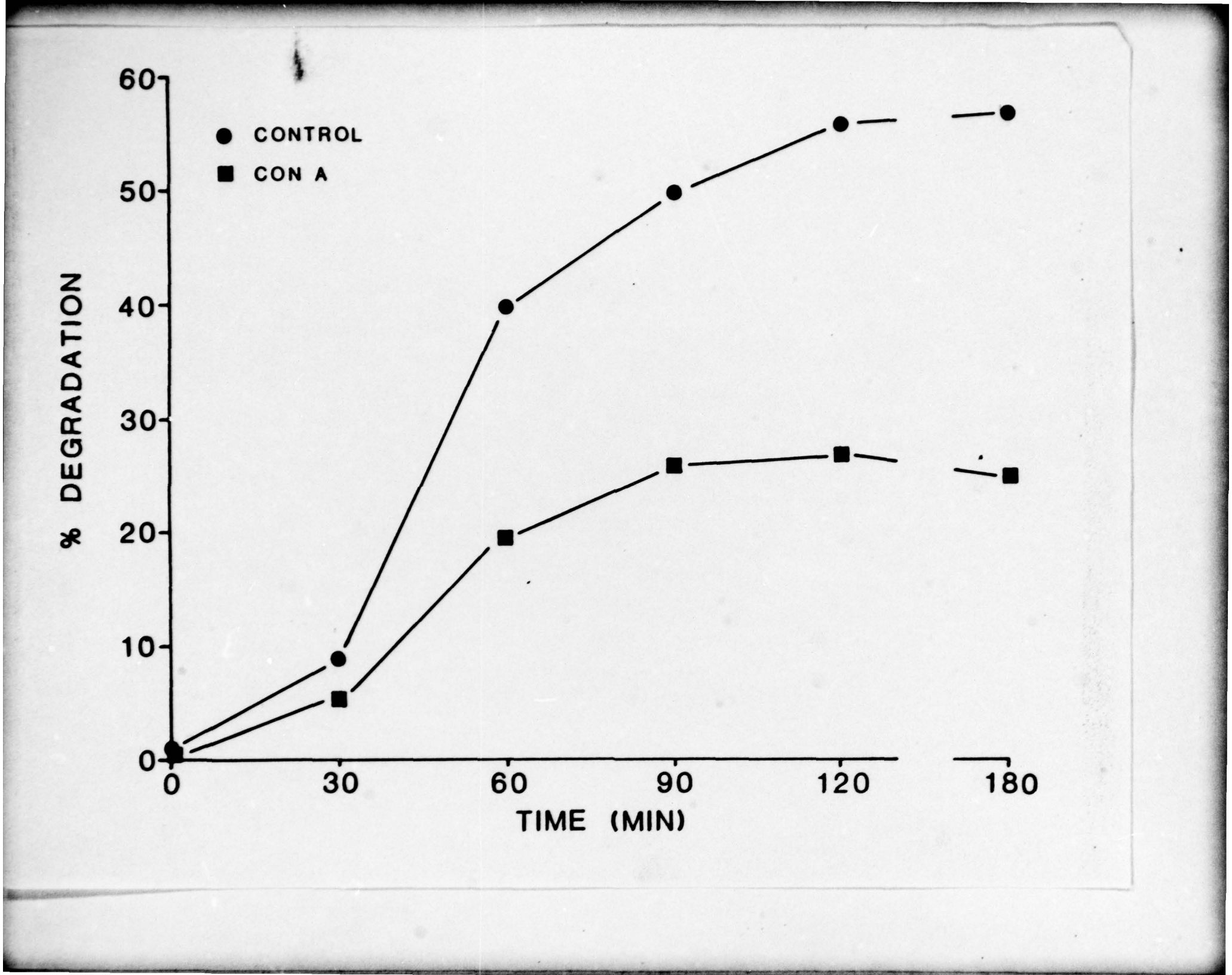
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% DEGRADATION
CONTROL
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