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Investigations into the relationship between structure and function of diphtheria toxin

(separation of A and B chains/labeling of A and B chains/modifications of specific amino acids/membrane binding of B chain)

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ABSTRACT **Studies on the structure–function relationship of diphtheria toxin are reported. New methods are described for the preparation of pure intact ("unnicked") toxin and for the preparation of the individual A and B chains. A biological assay method for the B chain is also presented, as well as a method for the labelling of "nicked" (one peptide bond broken) diphtheria toxin with ^{131}I such that the label is confined to only one of the two polypeptide chains.**

Alterations of diphtheria toxin with specific reagents reveal that modifications of the tryptophan, methionine, and arginine residues did not result in a significant loss in toxicity, whereas treatment of the toxin with *o*-phthalaldehyde or by photooxidation with rose bengal results in a complete loss of the toxic activity. Modification of tyrosine by iodination results in active toxin, whereas modification by tetranitromethane causes a loss in activity.

Preliminary results also indicate that the isolated A chain is about an order of magnitude more active in incorporating adenosine diphosphoribose into translocase (elongation factor 2) than whole or nicked toxin is under identical conditions.

The observed structural properties are discussed in view of the functional activity of diphtheria toxin in cell-free systems as well as in cell cultures. Evidence is presented indicating that the B chain binds to membranes: it inhibits the action of nicked toxin on HeLa cells.

The structural and kinetic properties of diphtheria toxin, as well as its mechanism of action, have been studied extensively in several laboratories during the past decade (for a review, see ref. 1). Diphtheria toxin inhibits protein synthesis by inactivating the translocase (elongation factor 2) in the cell (2–4). The reaction catalyzed by the toxin is

Translocase + NAD →
translocase-ribose-diphosphoadenosine + nicotinamide
(inactive)

and it appears to be irreversible under physiological conditions (1).

The toxin molecule consists of a single polypeptide chain of about 63,000 daltons (5, 6), containing two disulfide bridges (7). One of its peptide bonds is relatively weak and is readily hydrolyzed by a dilute trypsin solution. The product, "nicked" toxin, consists of two polypeptide chains, A and B, held together by a disulfide bond (8, 9). A second disulfide bond is located in the B chain. Reduction of the disulfide bonds results in a separation of the two chains, and a concomitant loss of most of the toxin activity in animals. The A chain, however, is fully active *in vitro* in inhibiting protein synthesis (9, 10). This observation has led to the hypothesis that the B chain is needed for the toxin molecule (or the A chain) to enter the cell (11). The complete amino acid sequence of the A chain has recently become available (12).

A recent report by Iglewski and Rittenberg (13) indicated that protein synthesis in Ehrlich–Lettré ascites carcinoma cells is much more sensitive to the action of diphtheria toxin than that in normal mouse spleen cells, and that Ehrlich–Lettré tumors regressed in mice following an injection of the affected mice with diphtheria toxin. A difference in sensitivity to diphtheria toxin was also found in human breast carcinoma cells as compared to normal human cells. These observations suggest that diphtheria toxin may prove to be a useful anti-cancer agent, although more recent data indicate that the difference in sensitivity of tumor cells to toxin as compared to normal cells may not be as large as originally thought (14, 15).

It is therefore of considerable interest to us to investigate the mechanism of action of diphtheria toxin in greater detail. More specifically, we are trying to gain further information concerning the amino acid residues of the toxin that are essential for its activity, and to investigate the mechanism by which diphtheria toxin enters the cell. Some of the results obtained during this investigation are presented in this paper.

MATERIALS AND METHODS

Materials. Diphtheria toxin was purchased from the Connaught Medical Research Laboratories, Toronto, Canada. Horse antiserum to diphtheria toxin was a gift of D. M. Gill, and glutathione–insulin transhydrogenase was generously supplied by P. T. Varandani. Tritiated NAD^+ and the ^{14}C-labeled amino-acid mixture were purchased from New England Nuclear Corp. All other chemicals were commercial preparations.

HeLa cells were from a stock strain and were maintained in Dulbecco's modified minimal essential medium (DME from Gibco) containing 2.5% fetal calf serum (FCS-Gibco) and 5.9% horse serum (HS-Gibco) on Falcon tissue culture dishes in a 5% CO_2 incubator at 37°. Experiments were carried out using Ham's F-12 medium (Gibco) containing 5% fetal calf serum.

Preparation of Pure Unnicked Diphtheria Toxin. Diphtheria toxin from Connaught Laboratories lot D-290 was purified according to the method of Goor and Pappenheimer (16). Sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis of purified toxin in the presence of mercaptoethanol revealed three bands corresponding to whole diphtheria toxin, A chain, and B chain. Toxin precipitated by 70% saturated ammonium sulfate was dissolved in a minimum volume of 10 mM sodium phosphate buffer, pH 7.2, and dialyzed overnight against 1 liter of the same buffer. $NaDodSO_4$ was then added to a final concentration of 0.1%, followed by dithioerythritol to 0.1 M. This solution was incubated for at least 1 hr at room temperature and was then applied to a Sepharose 4B column (4 × 95 cm) which

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

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had been previously equilibrated with 10 mM phosphate buffer, pH 7.2, 0.1% $NaDodSO_4$, and 1% mercaptoethanol. Protein was determined by the fluorescamine method (17). Fractions showing only whole toxin on $NaDodSO_4$ gel electrophoresis in the presence of mercaptoethanol were pooled and dialyzed extensively against 10 mM phosphate buffer, pH 7.2.

Preparation of Pure Nicked Diphtheria Toxin. Nicked diphtheria toxin was prepared from lot no. D-297, which contained approximately 10% whole toxin. Trypsin was bound to glass according to the procedure described by Lappi *et al.* (18), but all steps taken to exclude oxygen were omitted. Thirty-six milligrams of trypsin (Sigma Chemical Co.) was bound to 1 g of diazotized arylamine glass. The glass-bound trypsin had an activity of 2900 units/g of glass when assayed with *N*-benzoyl-L-arginine ethyl ester (19).

Ten milliliters of diphtheria toxin was dialyzed against 10 mM Tris·HCl, pH 7.5, overnight. $CaCl_2$ was then added to 20 mM. The toxin was added to 1 g of glass-bound trypsin and the mixture was shaken for 1 hr. The solution was then applied to a DE-52 DEAE-cellulose column (similar to the above) and washed with 10 mM Tris·HCl, pH 7.5, to remove the $CaCl_2$. The nicked toxin was then eluted as described above. $NaDodSO_4$ gel electrophoresis of the eluted toxin in the presence of mercaptoethanol revealed two bands corresponding to A chain and B chain.

Preparation of the *S*-sulfonated A and B Chains of Diphtheria Toxin. To 10 ml of a diphtheria toxin solution (9 mg/ml) was added 1 ml of a solution that contained 0.5 M sodium sulfite and 0.2 M cupric sulfate, and the mixture was adjusted to pH 9.5 with ammonium hydroxide (20). The reaction was allowed to proceed at room temperature for 5 min, and the mixture was then dialyzed against 20 mM Tris·HCl buffer, pH 7.5, to remove excess reagents. Any precipitate that had formed was removed by centrifugation. Four milliliters of the dialyzed solution was applied to a Sephadex G-150 column (98 × 2 cm), which had been previously equilibrated in 20 mM sodium phosphate buffer, pH 7.4, containing 36 mM sodium sulfite. The two sulfonated chains were eluted with the same buffer. Fractions of 3.5 ml each were collected, and the fractions were monitored for their protein content by measuring the absorbance at 276 nm. The pure chains were obtained in an overall yield of about 50%.

Preparation of the Reduced A and B Chains of Diphtheria Toxin. Reduced A and B chains were prepared by a method similar to that for pure unnicked toxin, but the starting material was nicked toxin purified as above.

Determination of Toxicity against HeLa Cells in Culture. HeLa cells were grown in 60 ml tissue culture dishes in Ham's F-12 medium supplemented with 5% fetal calf serum. Toxin or a toxin derivative was added to the medium to a final concentration of 0.25 μg/ml, and the cells were incubated at 37° for 24 hr. The medium was then removed and the cells were washed with phosphate-buffered saline without Mg^{2+} and Ca^{2+} to remove unattached cells. The attached cells were enzymatically removed from plates by incubation with 1 ml of 0.1% trypsin for 5 min at 37°, 9.0 ml of phosphate-buffered saline was added, and the cells were counted in a Coulter counter. The number of attached cells was used as a measure of cell viability.

Competition of Toxin Derivatives with Action of Diphtheria Toxin in Tissue Culture. Duplicate 35 mm tissue culture dishes of HeLa cells placed in Ham's F-12 medium supplemented with 5% fetal calf serum were incubated with desired concentrations of diphtheria toxin and diphtheria toxin derivatives. The cells were incubated at 37° for 3 hr, then 1 μCi of

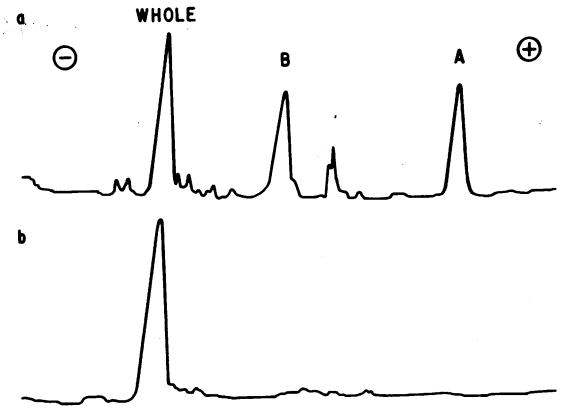

FIG. 1. $NaDodSO_4$-acrylamide gel electropherograms of (a) Lot D-298 diphtheria toxin from Connaught Laboratories; (b) whole toxin after purification as described in *Materials and Methods*.

^{14}C-labeled amino-acid mixture was added to each dish and the incubation was continued for 2 hr. The media were then removed and then the cells were fixed and solubilized and protein was precipitated (13).

Activity of Diphtheria Toxin *In Vitro*. Diphtheria toxin was assayed by the method of Honjo *et al.* (21).

Immunology. The toxin derivatives were qualitatively tested for antigenicity using the Ouchterlony microdiffusion technique.

RESULTS AND DISCUSSION

Properties of Pure Unnicked and Nicked Diphtheria Toxins. Pure unnicked diphtheria toxin has been prepared using a Sepharose-4B column. Purified toxin, containing unnicked as well as nicked toxin, is reduced with 0.1 M dithioerythritol in the presence of $NaDodSO_4$. Such a reduction causes the nicked toxin to separate into individual A and B chains, whereas the polypeptide chain of the unnicked toxin remains intact. The whole toxin is then separated from the individual A and B chains by chromatography over Sepharose-4B in the presence of the reducing agent. The reducing agent is removed from the collected toxin by extensive dialysis. Fig. 1b shows a gel electropherogram of unnicked toxin purified by this method. This gel was run in the presence of $NaDodSO_4$ and mercaptoethanol, and showed no bands corresponding to A or B chains.

The yield of pure unnicked toxin depends on the unnicked toxin content of the starting material. When the percentage of unnicked toxin in the starting material is high (80% or better), high yields of pure unnicked toxin are obtained. When this percentage is less than 50% in the starting material, the yields are poor due to overlap with the large amount of B chain, which elutes closely behind the unnicked toxin.

The prepared unnicked toxin does not react with *p*-hydroxymercuribenzoate even after 6 hr of incubation, and the toxin also does not react with 5,5′-dithiobis(2-nitrobenzoic acid), indicating the absence of any reactive —SH groups. Since the preparation is fully active *in vivo*, it appears that all disulfide bonds were reconstituted following the removal of the reducing agent. These data therefore suggest that reduced diphtheria toxin readily resumes its native conformation upon oxidation of the sulfhydryl groups, as long as the peptide chain has not been nicked. Reconstitution of intact toxin from the individual chains has thus far given only very poor yields in our laboratory as well as in that of others (22).

Fully nicked toxin is prepared by exposing the crude toxin

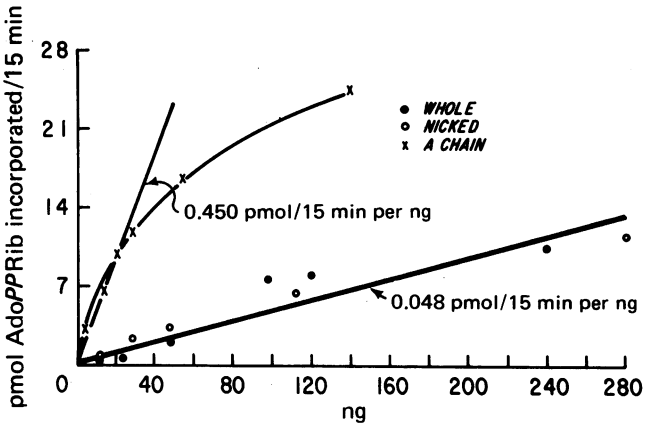

FIG. 2. *In vitro* activities of whole toxin, nicked toxin, and reduced A chain. Assays measuring incorporation of adenosine diphosphoribose were described in *Materials and Methods*.

to immobilized trypsin. The resulting material is then passed over a DEAE-cellulose column to remove impurities. The diphtheria toxin is quantitatively converted to fully nicked toxin by this method, and the fully nicked toxin is obtained in a quantitative yield from the column. Treatment of the toxin obtained with 2-mercaptoethanol results in a quantitative separation into the A and B chains.

It is of interest that the whole unnicked toxin has the same activity as the nicked toxin when administered to animals. In addition, we have found that unnicked toxin appears to be completely inactive on cultured HeLa cells under conditions where the nicked toxin is active as an inhibitor in protein synthesis and also as a cytotoxic agent.

The activity of nicked toxin in inhibiting protein synthesis *in vitro* is markedly less than that of the A chain. The data in Fig. 2 illustrate that whole toxin and nicked toxin display comparable activities. However, their rate of adenosine diphosphoribose incorporation is only 10% of the rate of the A chain. These data suggest either that the active site on the A chain is substantially masked in the whole and nicked toxin molecules, or that the A chain undergoes some structural rearrangement following separation from the B chain. The latter possibility has also been postulated by Pappenheimer and Gill (1) on the basis of the inability to regenerate nicked toxin from the separated A and B chains. There is also a possibility that noncovalent bonds hold the two chains together.

Cleavage of the Disulfide Bonds in Diphtheria Toxin. When reducing agents are added to diphtheria toxin, the disulfide bridge linking the A and B chain is broken (9). The B chain, however, is rather insoluble under these conditions and precipitates within a few minutes after the addition of reducing agents (11). We have developed two methods for the preparation of the two peptide chains of diphtheria toxin that may be used to convert large amounts of toxin and do not yield any insoluble products.

Almost quantitative conversion of nicked toxin into the two chains is obtained by converting the disulfide bridges to the *S*-sulfonated residues. The conversion occurs readily at room temperature.

The two chains of diphtheria toxin may also be prepared in the -SH form in larger quantities. Addition of $NaDodSO_4$ to a concentration of 0.1% before reduction breaks the hydrophobic forces joining the A and B chains (1). The two subunits can then be separated by a Sepharose-4B column equilibrated with 0.1% $NaDodSO_4$ and 1% 2-mercaptoethanol.

The elution profile of the reduced chains is shown in Fig. 3a,

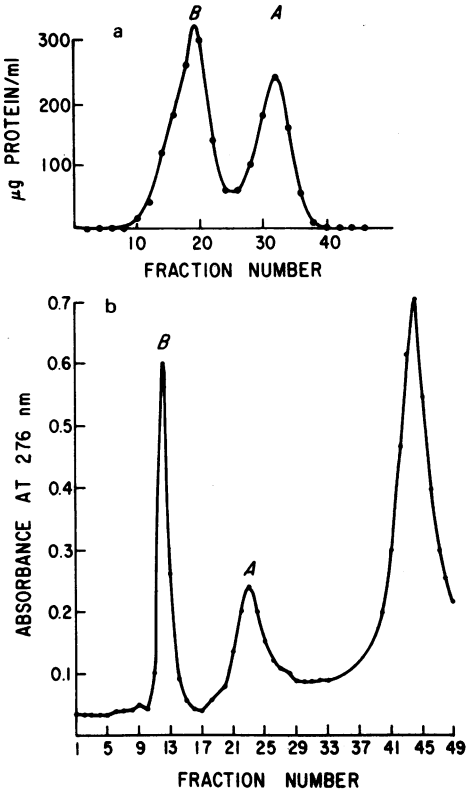

FIG. 3. Separation of the two chains of diphtheria toxin by gel chromatography. (a) Elution profile on the reduced A and B chains on Sepharose-4B; (b) elution profile of the *S*-sulfonated A and B chains on Sephadex G-150. See *Materials and Methods* for experimental details.

whereas that of the *S*-sulfonated chains is shown in Fig. 3b. The *S*-sulfonated material was prepared without prior purification and contains a third peak with absorbance at 276 nm. This contains smaller molecules from the culture medium.

The two *S*-sulfonated chains, either separate or mixed together, have no apparent toxic effects when injected into a guinea pig in doses of 80 μg/kg of body weight (0.8 μg of active toxin per kg of body weight kills a guinea pig in less than 48 hr). The *S*-sulfonated A chain is capable of inhibiting protein synthesis *in vitro* at the same rate as shown by the reduced A chain, suggesting that an intact sulfhydryl group is not essential for the adenosine-diphosphoribose-transferase activity. This finding is consistent with the fact that the carboxymethylated A chain is also fully active *in vitro* (11).

Biological Activity of the B Chain. Since the presence of the B chain appears to be a requirement for the toxic action of

Table 1. Effect of B chain on the inhibition of protein synthesis produced by diphtheria toxin

μg added to reaction mixture			cpm incorporated into protein	% inhibition of protein synthesis
Toxin	B chain	A chain		
0	0	0	23,348	0
0.025	0	0	6,899	72
0.025	0.025	0	12,164	42
0.025	0.25	0	15,590	33
0.025	1.30	0	18,616	19
0.025	0	1.30	6,605	72

Table 2. Properties of various toxin derivatives

Modifying reagent	Amino acid modified	Number present in		Activity *in vitro*	Toxicity in cell cultures	Reactivity with toxin antibody
		Native	Modified			
Koshland's reagent	Trp	6	0.5	+++	+++	+++
Photooxidation	His	18	0	None	None	None
Diacetyltrimer	Arg	16	12	++	++	
Cyclohexanedione	Arg	16	7	+++	+++	+++
o-Methylisourea	Lys	41	9	+++	+++	+++
o-Phthalaldehyde	Lys	41	6.5	None	None	None
Tetranitromethane	Tyr	19	5	None	None	
Iodine	Tyr			+++	+++	+++
Hydrogen peroxide	Met			+++	+++	+++

diphtheria toxin *in vivo* as well as in cell cultures, it has been proposed that the B chain is the part of the molecule that facilitates the entry of the toxin into the cell (11). No direct evidence for this hypothesis has as yet been reported. If this hypothesis is correct, one would expect that the presence of a certain amount of the isolated B chain (which is nontoxic) would inhibit the action of diphtheria toxin on whole cells.

We have carried out such experiments, and their results are shown in Table 1. The isolated B chain in the sulfhydryl form clearly exerts an inhibition on the action of diphtheria toxin in HeLa cells. No such inhibition is promoted by the reduced A chain. These results demonstrate that the B fragment of the toxin molecule probably exerts the initial interaction with the cell membrane.

The inhibition of the action of diphtheria toxin by the B chain thus provides for an easy method to assay directly for the interaction of various toxin derivatives and modified B chains with the cell wall. Using this method we found that the *S*-sulfonated B chain, even at concentrations as high as 1.3 μg/ml, is not able to inhibit the action of whole toxin on cultures of HeLa cells. This indicates that the sulfonyl groups may prevent the B chain from binding to the receptor site.

Reconstitution of Diphtheria Toxin. Reconstitution of nicked toxin from the isolated A and B chain has so far been rather unsuccessful; only very low yields of active reconstituted toxin have been obtained from a number of methods. Using the isolated A and B chains in the —SH form in the presence of 0.1 M 2-mercaptoethanol and 10% (vol/vol) glycerol, we obtained only a few percent of active toxin following the removal of the reducing agent by dialysis.‡ Similar results were obtained when we tried to recombine the A chain in the —SH form with the B chain in the —S—SO_3H form as well as with both chains in the —S—SO_3H form, whether or not 2-mercaptoethanol was added prior to dialysis. Yields of active toxin were equally low when the recombination experiments were done in 10% glycerol + 20% (vol/vol) dimethylsulfoxide. The addition of glutathione–insulin transhydrogenase to the reconstituted mixtures did not increase the yield of reassociated diphtheria toxin even in the presence of 1 mM oxidized glutathione or 100 μg/ml of bovine insulin.

When 5 mg of A chain (—SH form), labelled with ^{131}I, was mixed with 5 mg nicked toxin in 0.1 M phosphate buffer, pH 7.5, containing 0.1 M dithiothreitol and the mixture was incubated for 10 hr at 4°, followed by a separation of the products on Sephadex G-150, an exchange of the toxin chains occurred. In this experiment about 20% of the radioactivity was incorporated into the toxin as demonstrated by $NaDodSO_4$ gel electrophoresis. A similar result was obtained when 2.1 mg of B chain (—S—SO_3H form), labelled with ^{131}I, was mixed with 1 mg of nicked toxin under the same conditions, although the separation of the toxin from the excess B chain with Sephadex G-150 was incomplete and the chromatography was repeated. Using this technique we obtained radioactive toxin specifically labelled in either the A or the B chain.

Properties of Chemically Altered Diphtheria Toxin. Various derivatives of diphtheria toxin were made in order to obtain information about the nature of the amino acid residues that are essential for its biological activity. Using specific reagents, we modified the following residues: tryptophan, tyrosine, arginine, lysine, histidine, and methionine.

When nicked diphtheria toxin was iodinated with a 100-fold molar excess of iodine in the presence of 50 μCi of ^{125}I according to the procedure of DeZoeten *et al.* (23), 28 atoms of iodine were incorporated into the protein, as determined from the percentage of radioactive iodine that was incorporated. Since toxin contains 19 tyrosine residues, a total of more than one atom of iodine per tyrosine residue was incorporated.

Diphtheria toxin was also iodinated with 1 atom of iodine per mole of toxin to see if the iodination could be confined to a single chain. However, after separation of the A and B chains of the iodinated toxin by 2-mercaptoethanol and $NaDodSO_4$-gel electrophoresis, the results indicated that 80% of the radioactivity was incorporated into the B chain and 20% was incorporated into the A chain. Iodination with 1 mCi of carrier-free iodine resulted in the incorporation of 76% of the iodine into the B chain and 24% into the A chain. These results thus suggest that the reactivity and/or accessibility of one or more tyrosine residues in the A chain is only slightly less than that of one or more tyrosine residues in the B chain.

The prepared iodinated toxin was indistinguishable from the native toxin in its toxicity to cultured HeLa cells, suggesting that tyrosine residues do not play a major role in the mechanism of action of the toxin.

However, when we treated nicked toxin with tetranitromethane according to the procedure of Sokolovski *et al.* (24), 14 to 15 tyrosine residues were modified, and the obtained toxin was completely inactive.

Beugnier and Zanen (25) also reported that diphtheria toxin loses most of its toxicity upon the modification of tyrosine residues with tetranitromethane.

Treatment of nicked diphtheria toxin with 2-hydroxy-5-nitrobenzyl bromide (Koshland's reagent) (26) resulted in a modification of 5.5 of the six tryptophan residues. The toxin, however, did not show any loss in toxicity or activity. Fully active toxin was also obtained following treatment with cyclohexanedione (27), methylisourea (28), and hydrogen per-

‡ Preliminary results indicate only the sulfhydryl connecting the A and B chains is broken. The sulfhydryl in the B chain may not be reduced.

oxide (29). Activity also remained after treatment with diacetyl trimer (30). Treatment with *o*-phthalaldehyde (31) as well as photooxidation (32) of the toxin resulted in a complete loss of the toxic activity. A fluorodinitrobenzene derivative of the toxin could not be investigated due to its apparent insolubility. Table 2 summarizes the modifications and their effects. These results indicate that a modification of the lysine and histidine residues, as well as the terminal amino groups, may cause a loss in toxic activity.

The techniques described in this paper can be used to prepare modifications of the individual A and B chains, in order to study in more detail the mechanisms of adenosine diphosphoribose transfer by the toxin and the nature of its interaction with the cell membrane.

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