Biochimica et Biophysica Acta, 514 (1978) 128—136
© Elsevier/North-Holland Biomedical Press

BBA 78193

ISOLATION OF THE MEMBRANES OF AN ENTEROTOXIGENIC STRAIN OF *ESCHERICHIA COLI* AND DISTRIBUTION OF ENTEROTOXIN ACTIVITY IN DIFFERENT SUBCELLULAR FRACTIONS

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(Received March 20th, 1978)

Summary

The intracellular localization of enterotoxin in *Escherichia coli* AP1, a strain of porcine origin which produces high levels of heat-labile, but no heat-stable enterotoxin, has been examined. The cytoplasmic and outer membranes of this strain both contained enterotoxin activity, while the membranes isolated from a serologically related non-enterotoxigenic strain (*E. coli* AP2) also of porcine origin, did not show enterotoxin activity.

The periplasmic fraction isolated from the enterotoxigenic strain contained considerable enterotoxin activity, but this activity was associated with outer membrane fragments present in the periplasmic fraction. Thus, of the total cellular enterotoxin activity, about 55%, 15% and 30% were present in the outer membrane, cytoplasmic membrane and the cell cytoplasm, respectively. The specific activity of enterotoxin was 20 units per mg protein in the cytoplasm and 90 and 150 units per mg protein in the cytoplasmic and outer membranes, respectively.

Introduction

In recent years it has become clear that a considerable fraction of the acute diarrhea which occurs in man and other mammals is due to various enteropathogenic strains of *Escherichia coli* [1,2] which, when cultured in the laboratory, produce heat-labile and heat-stable extracellular enterotoxins [3]. The heat-stable enterotoxin appears to be a small protein or peptide [4,5], while heat-labile enterotoxins have been isolated as large protein-polysaccharide complexes [6,7] and as proteins of various molecular weights [8—10].

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The appearance of extracellular enterotoxin is not due to cell lysis (Wensink, J., unpublished observations) and newly synthesized enterotoxin must, therefore, be transferred through the cytoplasmic membrane, the murein layer and the outer membrane before appearing in the medium. In addition, since one of the *E. coli* enterotoxins has been isolated from the periplasmic fraction [11] and the extracellular enterotoxin of *Salmonella enteriditis* may originate in the cell wall [12], enterotoxins may well be stored in one or more of the various cell envelope layers prior to excretion or release.

As a first step in gaining some insight into the synthesis, storage, and excretion of enterotoxin by *E. coli*, we have studied its subcellular distribution *.

Materials and Methods

Bacteria and growth conditions. Two specific *E. coli* strains were selected from a series of porcine strains characterized previously [13]. The first, arbitrarily designated *E. coli* AP1, belongs to a class of isolates with serotype 08 : K "200" : H31, where K "200" refers to a provisional and as yet uncharacterized K antigen [13]. The second strain, designated *E. coli* AP2, belongs to a class of isolates with serotype 08 : K "200" : NM ** [13]. The first strain produces a heat-labile toxin while the second strain does not. Neither strain produced heat-stable enterotoxin [14]. Both strains were grown in a medium containing 3% proteose peptone (Oxoid Ltd., London) and 0.5% NaCl (pH 7.5). The medium was ultrafiltered with an artificial kidney (C-DAK, model 4, Cordis Corp., Miami, Florida 33137, U.S.A., see ref. 15) which filtered out proteins with molecular weights larger than 10 000. The kidney also removed Mg^{2+} which may have bound to the kidney fibers or to some of the proteins in the retentate, and the medium was therefore supplemented with 100 μM $MgCl_2$ to prevent Mg^{2+} starvation. After supplementation of the medium with 0.2% glucose the bacteria were grown to the stationary phase (16 h) at 37°C in a G25 rotary shaker (New Brunswick Scientific, 200 rev./min) to a final density of 2.0 mg/ml. Cell densities are expressed as mg dry mass/ml [16].

Isolation of membranes, cytoplasm and periplasm. Cells were harvested by centrifugation for 10 min at 5000 $\times g$. Spheroplasts were made at 0—4°C [17]; the Tris/EDTA/sucrose buffer contained 4 mM EDTA. The spheroplasts were centrifuged for 10 min at 5000 $\times g$ (0—4°C). The supernatant, which contained the periplasmic fraction [18], was concentrated about 3—4-fold with an artificial kidney (see above), followed by a further 10-fold concentration with a PM-10 ultrafilter (Amicon Corporation, Lexington, Massachusetts 02173, U.S.A.) and stored at —80°C. The spheroplast pellet was resuspended in 10 mM Tris-HCl (pH 8.0), containing 10 μg/ml RNAase (EC 3.1.4.22, Miles-Seravac Ltd., Berkshire, U.K.) and 10 μg/ml DNAase (EC 3.1.4.5, Sigma, St. Louis, U.S.A.), and disrupted at 0—4°C in a French press at 5000—6000 lbs/inch^2

* Some of these results have been presented previously (Wensink, J., Gankema, H. and Witholt, B., (1977) 11th FEBS Meet., Copenhagen, 1977, Abstr. B7-1-363).

** NM, non-motile.

[19]. After removal of unlysed cells (5000 $\times g$, 10 min) the total membrane fraction was centrifuged at 176000 $\times g$ for 2 h. The supernatant, containing the cytoplasmic fraction, was stored at −80°C. The membrane pellet was washed by resuspension in 10 mM Tris-HCl/1 mM EDTA (pH 8.0) and centrifuged as above. The washed membranes were resuspended in 20% (w/w) sucrose, 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) and separated on analytical sucrose gradients [17]. Large scale preparations were centrifuged for 72 h at 25 000 rev./min in a SW25-2 rotor (Beckman Instruments Inc.). Gradients were fractionated [17], membranes were collected by centrifugation for 2 h at 176 000 $\times g$, resuspended in 10 mM Tris-HCl, 1 mM $MgCl_2$ (pH 8.0), and stored at −80°C.

Chemical analyses, enzyme assays, and electron microscopy. Sodium dodecyl sulfate-polyacrylamide (12.5%) slab-gel electrophoresis [20], staining procedures [20] and computer plotting [15] have been described. Analyses for protein, total phosphorus and 2-keto-3-deoxyoctulosonic acid and enzyme assays have been described or referred to previously [15]. Total carbohydrate was estimated by the phenolsulfuric acid technique [21] with glucose as standard.

Assays for enterotoxin activity. Enterotoxin activity was initially measured by following the stimulation of cyclic AMP synthesis in Baby Hamster Kidney cells (BHK 21, catalog number 0-26200, Flow Laboratories Inc., Rockville, Md. 20852, U.S.A.). This procedure will be described (Guinée, P.A.M. and Jansen, W.H., manuscript in preparation). Briefly, 25 μl of an enterotoxin sample was transferred to a monolayer of about 10^5 BHK cells, in a 25-well tissue culture plate (Sterilin 306V). 30 min before the transfer the tissue-culture medium was replaced by 1 ml of fresh medium (Wistar) [22] containing 10 units penicillin, 10 μg streptomycin and 0.05 mM methyl-isobutyl-xanthine as phosphodiesterase inhibitor. After incubation for 24 h at 37°C in air with 5% CO_2, the monolayer was lysed with 100 μl perchloric acid (35%). After neutralisation with 2 M KOH and freezing to remove $KClO_4$, the cyclic AMP concentration was measured by a radioisotope dilution test (Radiochemical Centre, Amersham, U.K.). The sterile proteose peptone medium and the culture supernatant of a non-enterotoxigenic strain (*E. coli* 02:K-:NM **, isolated from a healthy person [13]) were used as negative controls. One unit of enterotoxin activity is defined as the amount necessary to give a half-maximal response.

Enterotoxin activity was also determined based on morphological changes of Vero cells exposed to enterotoxin preparations. The procedure described by Speirs et al. [23] was modified as follows. Vero cells (ATCC CCL 81, obtained from Flow Laboratories Inc.) were suspended to $0.5 \cdot 10^4$–$2 \cdot 10^4$ cells/ml in medium 199 (Gibco Bio-Cult Ltd., Paisley, U.K.) containing 2.5 μg/ml amphotericin B/100 units/ml penicillin/0.5 mg/ml streptomycin/10% newborn calf serum (Gibco). The cells were added to 24-well tissue culture plates (1 ml/well), and incubated overnight at 37°C in an atmosphere of 5% CO_2. The Vero monolayers were washed twice with 1 ml of the above medium, except that serum was omitted, after which 1 ml medium (no serum) was added to each monolayer. Enterotoxin samples were diluted serially in phosphate-buffered saline (no metals), and 50 μl of each dilution was added to a monolayer. After incubation overnight as above, cell morphology was scored from zero (no change in the monolayer, compared to controls to which only phosphate-

buffered saline had been added) to four (all cells in the monolayer were altered [23]). One unit of enterotoxin activity (Vero cell assay) is defined as the amount of enterotoxin which causes morphological changes in about 50% of the cells in a monolayer.

Results

A number of *E. coli* strains which have been isolated from newly born piglets suspected of *E. coli* enterotoxicosis, have been found to be enterotoxigenic as determined with the ligated gut test in 6-week-old pigs [13]. Among these strains, those with serotype 08:K "200":H31 were found to be particularly enterotoxigenic [13]; *E. coli* AP1 is the most active representative of this class. When *E. coli* AP1 is grown in proteose-peptone, most enterotoxin remains intracellular; as determined with the Vero cell assay, only 2—3% of the total enterotoxin activity is found extracellularly. To determine the localization of the intracellular, heat-labile enterotoxin of *E. coli* AP1, cells were fractionated and analysed as described below.

Characteristics of the cytoplasmic and outer membranes of E. coli AP1. The outer and cytoplasmic membranes of *E. coli* AP1 have densities of 1.252 ± 0.002 g/cm^3 and 1.183 ± 0.003 g/cm^3, respectively. These densities are higher than those found for typical laboratory strains [17,19,20].

The isolated membranes were assayed for three cytoplasmic membrane markers; the cytoplasmic membrane contained 20 times more NADH oxidase, 7 times more D-lactate dehydrogenase and 9 times more succinate dehydrogenase than the outer membrane.

As expected, 2-keto-3-deoxyoctulosonic acid, which occurs only in lipopolysaccharides, was found predominantly in the outer membrane. The ratio of total carbohydrate (measured as glucose) to 2-keto-3-deoxyoctulosonic acid corresponds to about 20 μmol hexose/μmol 2-keto-3-deoxyoctulosonic acid. Given the basic structure of 08 lipopolysaccharides [24], and the fact that of the three 2-keto-3-deoxyoctulosonic acid moieties in the lipopolysaccharide core only two are detected [25], the average O-antigen chain of this strain must contain about 8 O-antigen subunits of three mannose moieties each [24].

The protein composition of the membranes was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis: as usual, the cytoplasmic membrane (Fig. 1a) contains many, while the outer membrane (Fig. 1b) contains only a few proteins. Based on the nomenclature of Henning [26], the major outer membrane proteins probably correspond to or are very similar to proteins I (36 500 daltons; the matrix protein described by Rosenbusch [27]) and II* (33 000 daltons). These proteins show the solubility characteristics and trypsin susceptibilities typical of the corresponding proteins isolated from K-12 and other laboratory strains [26—28]: when cell envelopes obtained by sonication of whole cells are treated with 2% sodium dodecyl sulfate/5 mM EDTA/50 mM Tris-HCl (pH 7.4) at 37°C for 30 min, the faster moving protein is solubilized. The slower moving protein fails to be solubilized by the same treatment at 60 or 80°C, but is solubilized by extraction at 100°C for 10 min. Similarly, the faster moving protein is degraded by trypsin, while the slower moving protein is not (Dekker, J., unpublished data).

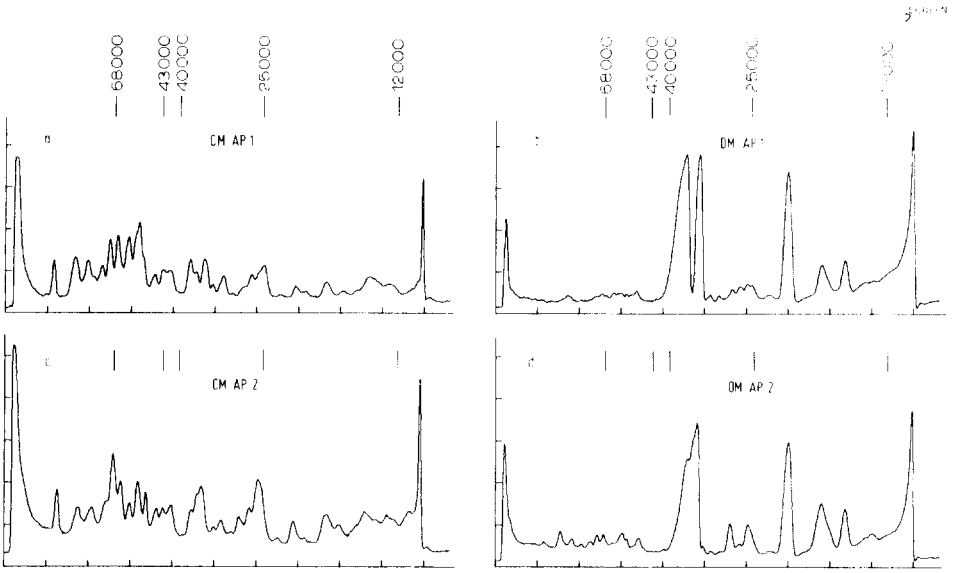

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the outer membrane proteins of *E. coli* AP1 (upper panels) and *E. coli* AP2 (lower panels). a and c, cytoplasmic membranes (CM); b and d, outer membranes (OM). The position of molecular weight standards are indicated by vertical lines above each profile.

In addition to these typical outer membrane proteins, the outer membrane also contains a major protein with an apparent molecular weight of 21 500; this protein is not found in the outer membrane of various laboratory strains [19,20,29—31].

Enterotoxin activity of the membranes of E. coli AP1. The dose response curves of Fig. 2 show that the membranes of *E. coli* AP1 possess enterotoxin activity as measured with the cyclic AMP test. The cytoplasmic membrane elicits a greater response than the outer membrane at saturating enterotoxin concentrations, but the outer membrane is more active than the cytoplasmic membrane in causing a half-maximal response.

The observed activities might be a general, possibly artifactual property of *E. coli* membranes (and especially the outer membrane; see below), for instance due to the presence of O-antigens or contaminating K-antigens. To exclude this possibility, the membranes of a nonenterotoxigenic strain with the same O- and K-antigens (*E. coli* AP2) were also isolated and characterized. These membranes, which resemble the corresponding *E. coli* AP1 membranes with respect to density, marker enzymes, and chemical composition (data not shown), exhibit little or no enterotoxin activity (Fig. 2). Only at 50—60-fold higher concentrations does the outer membrane of the non-enterotoxigenic strain evoke the same response obtained with the outer membrane from the enterotoxigenic strain. Thus, the enterotoxin activity of the membranes of *E. coli* AP1 is not a general membrane property, nor can it be related to O- and K-antigens which may be present as intrinsic membrane components (outer membrane) or as contaminants (cytoplasmic membrane).

In an attempt to determine whether membrane enterotoxin activities might

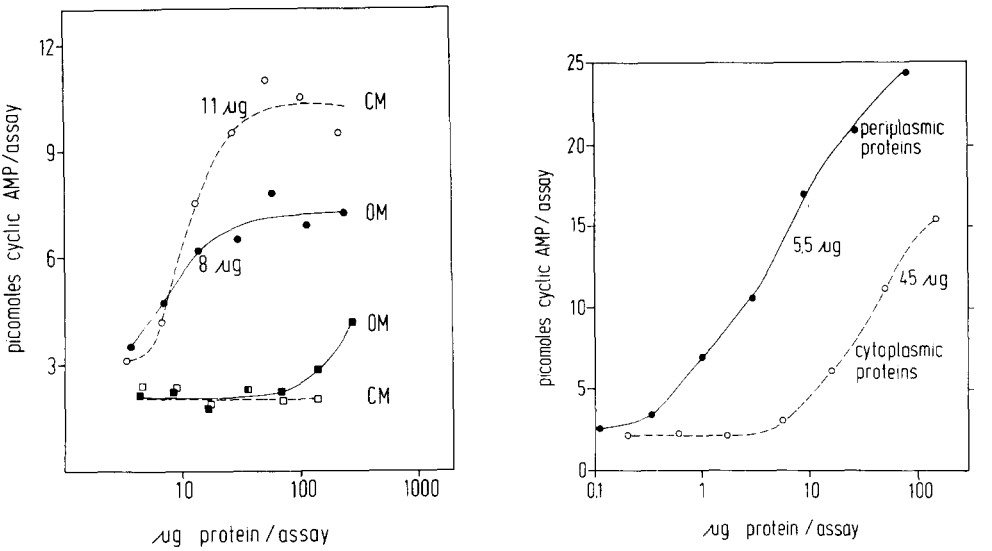

Fig. 2. Enterotoxin dose response curves for the membranes isolated from the enterotoxigenic strain *E. coli* AP1 (circles) and the non-enterotoxigenic strain *E. coli* AP2 (squares). CM, cytoplasmic membrane; OM, outer membrane.

Fig. 3. Enterotoxin dose response curves for the periplasmic and cytoplasmic proteins isolated from *E. coli* AP1.

be related to specific membrane proteins, *E. coli* AP2 membrane proteins were analysed (Fig. 1c, d). Little can be concluded from the cytoplasmic membrane protein profiles of *E. coli* AP1 and *E. coli* AP2 (Fig. 1a, c), since these profiles and their mutual differences are quite complex. In contrast, the outer membrane profiles (Fig. 1b, d) are simple and resemble each other; in addition, the major outer membrane proteins of *E. coli* AP2 show the same solubility characteristics and trypsin sensitivities as the corresponding proteins of *E. coli* AP1. The major difference between the two outer membranes concerns the mobility of one protein. Examination of numerous slab gels shows that while the matrix proteins derived from *E. coli* AP1 and *E. coli* AP2 have the same mobility, the protein which resembles protein II* migrates with a higher apparent molecular weight when derived from *E. coli* AP2 outer membranes than when derived from *E. coli* AP1 outer membranes. Thus, either the enterotoxin activity of *E. coli* AP1 is related to a specific form of protein II*, or it is due to proteins which are present in the outer membrane in such small amounts that they are not detected in these slab gels.

Enterotoxin activity of periplasmic and cytoplasmic proteins. The periplasmic and cytoplasmic proteins also contain enterotoxin activity, as shown in Fig. 3. Since the maximum amounts of protein used in these assays are close to saturating, as is suggested by other experiments (data not shown), half-maximal activities in the cyclic AMP test are obtained with 5.5 µg periplasmic protein and 45 µg cytoplasmic protein, respectively. Similar relative activities were obtained when Vero cell morphology was used as a criterion for enterotoxin activity.

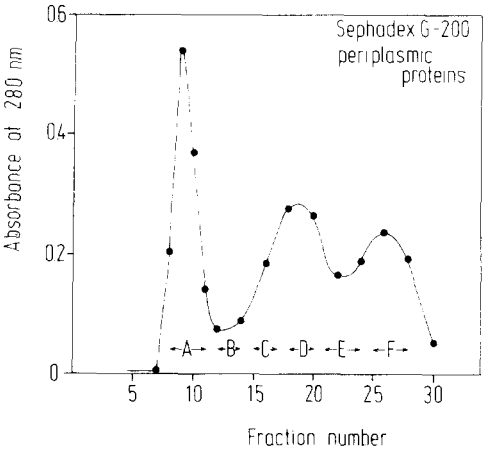

Fig. 4. Fractionation of the periplasmic material released from *E. coli* AP1. The concentrated periplasmic fraction was eluted from a Sephadex G-200 column (2.1 × 45 cm) with 0.1 M NH_4-acetate (pH 8.0) at 0—4°C.

To determine which periplasmic proteins contain enterotoxin activity, they were fractionated over a Sephadex G-200 column and pooled. Both enterotoxin assays revealed activity only in peak A, which showed a half-maximal activity with 3 μg protein in the cyclic AMP test (Fig. 4).

Peak A, which eluted with the void volume, was analysed in some detail. While peaks B—F contained a variety of successively smaller proteins on sodium dodecyl sulfate polyacrylamide slab-gels, the protein composition of peak A was similar to that of outer membrane (data not shown). Peak A was very similar or identical to the outer membrane fragments which are released from the outer membrane of growing *E. coli* K-12 cells [15,34], and which are also released during growth of *E. coli* AP1 and AP2 (unpublished observations); peak A not only contained typical outer membrane proteins, but also carbohydrate (twice as much as the outer membrane) and phosphorus (15% more

TABLE I

ENTEROTOXIN ACTIVITY OF THE SUBCELLULAR FRACTIONS ISOLATED FROM *E. COLI* AP1

Fraction	Protein recovered in fraction (mg protein/g cell dry mass)	Enterotoxin (cyclic AMP assay) *		Enterotoxin (Vero morphology assay) ** Total activity (units/g cell dry mass)
		Specific activity (units/mg protein)	Total activity (units/g cell dry mass)	
Cytoplasmic membrane	25	90	2200 (14%)	$11 \cdot 10^5$ (37%)
Outer membrane	25	125	3100 (20%)	
Periplasm (outer membrane fragments)	30	180	5400 (34%)	$12 \cdot 10^5$ (40%)
Cytoplasm	250	20	5000 (32%)	$7 \cdot 10^5$ (23%)

* Average results from 2—4 dose—response curves for each fraction.

** Fractions obtained in an experiment in which the membranes were not separated; average results from 3—6 dilution series for each fraction.

than the outer membrane). Thus, it is likely that the material found in peak A did not originate in the cellular periplasmatic space as such, but consisted of outer membrane fragments which were released from the outer membrane during the spheroplasting procedure. As a result, these fragments were found in the "periplasmic fraction".

Table I summarizes the results presented above and indicates how much enterotoxin activity is present in each cell fraction derived from 1 g cell dry mass.

Discussion

E. coli AP1, a strain of porcine origin, was chosen for studies of the localization of heat-labile enterotoxin because it is one of the most active producers of heat-labile enterotoxin isolated so far in the Netherlands [13], because it does not produce heat-stable enterotoxin, and because it could be compared to another strain of porcine origin (*E. coli* AP2) which has the same O- and K-antigens but does not produce enterotoxin.

Table I shows that about 70% of the enterotoxin activity found in *E. coli* AP1 is localized in the cell envelope. Although the cytoplasm of 1 g cells (dry mass) contains 5000 units (cyclic AMP assay) of enterotoxin, the specific activity of this enterotoxin is substantially lower than that present in the various envelope layers. Half of the cell envelope enterotoxin was recovered in the "periplasmic fraction"; this activity was not derived from the periplasmic space, but was associated with outer membrane fragments which were released together with soluble periplasmic proteins.

The heat-labile enterotoxins isolated so far are monomeric proteins with molecular weights ranging from 20 000 to 102 000 [8,11,32]. It is not clear from our results which, if any, of these enterotoxins we are dealing with; enterotoxin activity may be related to a major outer membrane protein similar to protein II* [26], but it is more likely to be associated with an outer membrane protein which is present in such small amounts that it is not detected on slab gels.

Recent research on enterotoxin structure has focused on individual proteins [8—10,32]. Nevertheless, enterotoxin activity in the supernatant of enterotoxigenic strains is usually associated with large polysaccharide-protein complexes [6,7]. Similar complexes are released by a variety of pathogenic and non-pathogenic gram-negative organisms (for a review, see ref. 33), and we have recently shown that in the case of normally growing *E. coli* K12 they consist of outer membrane fragments [15], which are specifically released from those areas where newly synthesized proteins are incorporated into the outer membrane [34].

It has been argued that polysaccharide-protein complexes with enterotoxin activity are artifacts [8]. The points raised above suggest however that such complexes may well have physiological significance. Specifically, Gram-negative bacteria may use the outer membrane as an offensive weapon by inserting protein toxins into it, and under appropriate conditions outer membrane fragments containing high specific activities of toxin might be released before they

have been thoroughly fixed in the cell wall [34]. Such a process could explain why enterotoxin usually appears in culture supernatants in the form of large complexes [6,7] and we are, therefore, comparing the structural characteristics of extracellular enterotoxin to that found in various subcellular fractions.

Acknowledgements

We thank Drs. A.J. Scheffer and S. Welling for their hospitality and help with the Vero cell morphology assay, Mr. N. Panman for drafting, Mr. K. Gilissen for photography and Ms. A. van Rijsbergen for typing the manuscript.

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