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Experimental Differential Light-Scattering Correction to the Circular Dichroism of Bacteriophage T2

(light-detection geometry/C-form DNA/ordered asymmetry)

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ABSTRACT **Experimental techniques are presented that can be used to assay and correct for differential light scattering effects in circular dichroism spectra of biological macrostructures. The assay is based upon use of variable detector geometries that collect light over large solid angles. Disrupted T2 virus suspensions and purified T2 phage DNA exhibit geometry-independent spectra; the spectrum of intact T2 virus is highly sensitive to detection geometry. On the basis of spectra obtained after light-scattering correction, the structure of T2 DNA in the phage particle is assigned to the C form. We conclude that: (*i*) The measured circular dichroism of a light-scattering specimen may be highly sensitive to light-detection geometry of the instrument. This effect is indicative of differential scattering intensity for left and right circularly polarized light. (*ii*) Some optically active particles, although they scatter light intensely, exhibit circular dichroism that is independent of detection geometry and, therefore, apparently uninfluenced by differential light scattering. We infer that whether differential light scattering arises may depend upon the presence or absence of ordered asymmetry in the organization of the scattering particle. (*iii*) The circular dichroism of any light-scattering specimen should be measured again in apparatus designed for differential light-scattering correction as a prerequisite to meaningful structural conclusions. (*iv*) Differential scattering effects in circular dichroism may be potentially useful as a probe for large-order organization of the scattering particle.**

Circular dichroism (CD) studies, although at one time devoted to simple molecules (1, 2), have recently been focused upon a variety of complex biological macrostructures. Much promising CD work has been contributed on the molecular conformation of DNA in chromosomes (3), chromatin (4–12), and related material, such as reconstituted nucleohistone (13–19) or other model systems for DNA–protein interactions (20–23). The structure of virus particles has been studied with CD in this laboratory (24). The conformation of membrane-incorporated proteins has recently motivated considerable CD effort (25–34).

Due to their large size and particulate nature, biological macrostructures tend to form intensely light scattering suspensions. The effect, if any, of light scattering on CD measurements has not been well understood, although it has been discussed (25–33, 35–39). Several possible artifacts of CD measurement have been identified: absorption flattening (25–27, 29, 30, 32, 33, 35), other concentration-obscuring effects (30, 32, 33), and differential light scattering (25, 26, 29, 31–33). It is the last of these with which we are concerned.

Abbreviations: CD, circular dichroism; PMT, photomultiplier tube.

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Differential light scattering occurs when left and right circularly polarized light is scattered with different efficiency out of the measuring beam of a CD spectropolar-meter. The result is to modify the differential absorption measurement that constitutes normal CD. This result necessarily complicates the interpretation of CD data from scattering specimens and the comparison with spectra from molecularly dispersed samples of the same chemical compound. Several theoretical treatments of differential light scattering (35–39) have appeared, and some experimental work has been presented (25, 26, 29, 31, 32) or proposed (37, 38) for particular applications.

We now report a quite general experimental approach to assay the presence of differential light scattering and to correct for its effects on measurements in a circular dichrograph. The proposed assay involves determination of whether the observed CD spectrum varies with changes in the light-collection geometry of the spectropolarimeter. The collection geometry may be described in terms of the position, size, and shape of the light-detection element relative to the incident measuring beam and the sample cell.

As a basis for later discussion, we consider first the measurement of normal, unpolarized light absorption spectra. For a photomultiplier tube (PMT) detector located on the optical axis, and with a sufficiently collimated measuring beam, the absorption spectrum recorded for a nonscattering sample should be independent of the distance from sample cell to detector. In the presence of a scattering sample, on the other hand, some of the incident beam may be deviated from the beam axis through an angle great enough to miss the detector. Such scattered light will appear to have been absorbed and produce anomalously high optical densities.

Experimental remedies have frequently involved an increase of the solid angle of detection to enable capture of scattered photons missed by normal instruments. This may be done most simply by increasing the size of the detector, decreasing the distance from sample cell to detector, or both. Clearly, the scattered photons must penetrate the sample cell to reach a downbeam detector. When detected, the scattered photons are counted as if they were transmitted photons. Although considerable uncertainty may exist about the effective optical path through a light-scattering sample, it has been found, from work with mixtures of scattering and absorbing particles, that far more accurate absorption spectra result from detection of the scattered light and counting it as transmitted than from counting it as absorbed (*Methods in Enzymology*, in press*). The preceding generalization is not necessarily applicable to all samples or circumstances, e.g., absorption measurements on highly fluorescent specimens.

When large-angle detection is acceptable, one of the most convenient contemporary instrumental techniques involves the use of an end-window photomultiplier tube detector with a large area photocathode. In the Cary Instruments (model 1462) scattered transmission accessory, for example, a 2-inch (5.1-cm) diameter PMT may be translated along the optical axis over a range of several inches to a closest position just behind the sample cuvettes. A series of spectra recorded at various tube positions successively closer to the sample cell display a monotonic reduction, and even elimination, of spectral features due to scattering, such as the long-wavelength extinction tails extending outside the absorption band. Optical densities inside the band are also reduced. For coliphage, OD_{260} measurements are about 10% lower with scattered light correction in the model 1462 than in the standard Cary 14 or Cary 15 (40).

In principle, the large-angle detection approach should be equally applicable to CD. Ideally at least, the CD photomultiplier tube detector measures only the light intensity and is insensitive to its polarization. Therefore, if left and right circularly polarized light are scattered with equal efficiency, and if the scattered light from each polarization is equally distributed in space around an optically active sample, the scattered light contribution will subtract out from recorded CD spectra regardless of the solid angle of detection. But if the spectra should change with a modification in the solid angle of detection, the spectral change must imply that one circular polarization or the other is contributing more scattered intensity to the region of space that is accessible to the larger-angle geometry but inaccessible to the smaller. The difference between two such spectra provides a direct measure of the "differential light scattering" into that region of space where measuring photons are detectable in one case but not detectable in the other.

There is nothing in the preceding argument that limits its application to instruments using end-window photomultiplier tubes or other planar detectors. One device capable of larger solid-angle detection is the fluorescent scattering (fluorscat) cell (*Methods in Enzymology*, in press*). The use of the fluorscat scattering cell, as well as a movable, end-window photomultiplier, to assay for differential light scattering from T2 phage and to study the secondary conformation of T2 DNA *in vivo* forms the subject of this paper.

Materials and Methods. The three principal instrumental configurations used are:

(*i*) Unmodified Cary 6001, 6003, or 61 CD spectropolarimeters, whose data are termed "conventional spectra."

(*ii*) A modified Cary 6001 instrument containing a movable end-window photomultiplier with 2-inch (5.1-cm) diameter photocathode, whose data are termed "large-angle planar detector spectra."

(*iii*) A Cary 6001, either modified or unmodified, used in conjunction with special fluorescence cells that enlarge the collection angle beyond that obtained with a planar detector (*Methods in Enzymology*, in press*). Data from these fluorescent scattering cells are termed "fluorscat spectra."

The Cary 6001 modifications were as follows:

(*i*) The ORD modulator, focusing lens, side-window photomultiplier, and the light mask separating the sample elevator and ORD modulator compartments were removed. The opening between these two compartments was widened by 1 inch (2.54 cm). The photomultiplier preamplifier was remounted to the underside of the top plate of the elevator.

(*ii*) A Dumont no. KM2703 photomultiplier was installed on a variable-position roller-bearing mount in the ORD modulator compartment. The mount is supported by a plate that can be translated horizontally and that rests, in turn, on a facsimile of the ORD modulator's adjustable-height kinematic leveling table.

(*iii*) A positioning rod extending outside the instrument by way of a light lock allows the photomultiplier to be moved along the optical axis to locations 0.04–5.0 inches (0.10–12.7 cm) from the sample cell. The leveling provisions described above enable alignment such that the photocathode remains normal to the measuring beam and centered on the optical axis. The positioning rod is grooved at 1.5-inch (3.8-cm) intervals to allow several photomultiplier positions to be located reproducibly with negligible error. Whenever the photomultiplier extends into the sample space, the sample elevator is automatically locked in place to preclude tube damage or misalignment.

(*iv*) Shielded wiring connects the cathode, anode, and final dynode pin positions of the PMT socket to the preamplifier. The photomultiplier is magnetically shielded, and electrically shielded at cathode potential.

All sample cells were 1-cm path length. The zero ellipticity line in all spectrograms was obtained from solvent baselines, and no instrumental adjustments were made between solvent and sample runs. All spectra were run without multipots, but with the 14-Å spectral bandwidth automatic slit program, unless otherwise noted. Data were acquired with an instrumental time constant of 1 sec and digitally recorded by an on-line computer with available pen-averaging, baseline subtracting, and data smoothing programs (41). Subsequent processing to obtain difference curves and plotted output was also done on a CDC 6400 computer. Extinction per mol of phosphate at 260 nm was assumed to be 6440 for T2 DNA and 10,206 for intact T2 virus (24).

All CD instruments were calibrated with d-10 camphor sulfonic acid (Eastman Organic, 1 mg/ml in H_2O). Calibration readings of 0.304 ± 0.004 degrees ellipticity at 290 nm were obtained and checked periodically during several weeks of experimentation. Agreement between the various instruments was within 1.5%.

Calibration tests were also performed on optically inactive samples providing extinction due to: (*i*) Absorption: potassium dichromate solution (Mallinckrodt Chem.), 6 mg/liter in 0.01 N KOH; (*ii*) scattering: alumina suspensions (Vitro Labs.) of nearly spherical particles in the range of 0.01–0.2 μm diameter in 0.01 N KOH. All instruments gave readings, so-called inactive sample artifacts, of no greater than 1 millidegree/OD for the above samples. These readings varied from one instrument to another, but observed magnitudes were nearly independent of tube position in the modified Cary 6001. Inactive sample artifacts for the fluorescent cells were consistently lower than those observed with standard cuvettes.

T2 phage and T2 phage DNA preparation, purification, and all subsequent handling was by reported techniques (24). In particular, virus was purified by hydroxyapatite column chromatography, then dialyzed exhaustively against 0.5 M NaCl–1 mM $MgSO_4$–1 mM Tris·HCl (pH 6.8). Phage were disrupted by freeze-thawing, leading to release of DNA into

the surrounding solvent. Viral DNA was extracted with phenol and dialyzed extensively against 0.1 M NaCl–10 mM Na-phosphate (pH 7.2).

RESULTS

Conventional CD spectra of intact T2 bacteriophage, disrupted T2 bacteriophage, and T2 DNA have been reported (24). Above 250 nm, the observed CD of a disrupted phage suspension is essentially that of T2 DNA. The CD of T2 DNA exhibits a positive maximum at 280 nm ($\Delta\epsilon$ = 1.99), is zero at 265 nm, and exhibits a negative minimum at 250 nm ($\Delta\epsilon$ = −5.01). The spectrum of a shocked suspension, due to the presence of the protein phage coats, begins to diverge from that of DNA at 245 nm and below. The coats scatter sufficient light to register OD_{350} = 0.04 per OD_{260} in a Cary 14 absorption spectrophotometer (Dorman & Maestre, unpublished data). Such scattering leads to no detectable signal outside the absorption band when conventional CD spectra are measured.

The conventional CD spectrum of intact T2 phage differs from that of T2 DNA in several respects (24): the positive maximum is red-shifted to 286 nm and reduced in magnitude to $\Delta\epsilon$ = 1.6, crossover is shifted to 276.5 nm, the negative minimum is slightly red-shifted and enhanced to $\Delta\epsilon$ = −6.9. Below 240 nm, the intact T2 CD spectrum is intermediate between that of shocked T2 and purified T2 DNA. The intact virus scatters light at OD_{350} = 0.07 per OD_{260} in a Cary 14 (Dorman & Maestre, unpublished data) and exhibits a positive tail at long wavelength extending well into the visible spectrum. This tail admits no theoretical explanation in terms of a DNA structural modification. However, a nonzero CD signal outside the absorption band might conceivably be due to differential light scattering from a highly compacted DNA organization inside the phage head. To investigate this possibility, and the extent to which other features of the intact T2 CD spectrum might derive from differential light scattering, we performed the following experiments.

The large-angle CD spectrum of intact T2 phage was measured in a Cary 6001 spectropolarimeter modified to accommodate an end-window PMT with 2-inch (5.1-cm) diameter photocathode. The PMT may be positioned on the optical axis of the instrument at various distances from the sample cell. The spectra obtained from distant, intermediate, and close PMT positions may be compared with conventional Cary 60 data in Fig. 1. For the most part, the conventional T2 spectrum exhibits values that are intermediate between the large-angle data. However, as the solid angle is increased by moving the large-angle detector to the intermediate and close positions, the 286-nm peak and the long-wavelength tail are reduced. Indeed, the tail is no longer evident in the close PMT spectrum.

The shape of the differential light-scattering contribution detected with the large-angle modified Cary 6001 geometry was obtained by subtraction of the close PMT spectrum from the distant PMT spectrum. Such difference spectra are presented in Fig. 2 for intact T2 phage, disrupted T2 phage, and purified T2 DNA. The intact phage difference spectrum displays a long-wavelength tail, has a maximum at 290 nm, goes to zero at 265 nm, and has a larger maximum at 220 nm. In contrast, the T2 DNA and the disrupted phage suspension spectra exhibit essentially no dependence on PMT position down to 220 nm. Because these latter specimens

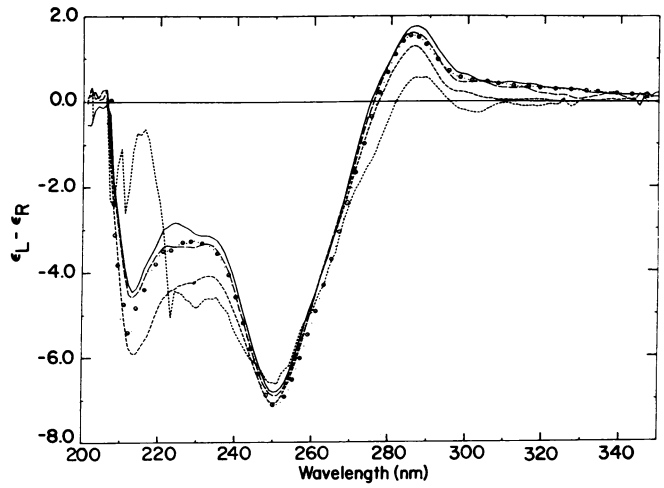

FIG. 1. Intact T2 phage CD spectra with four large-angle plus conventional detection geometries. Three curves represent large-angle planar detector data recorded in modified Cary 6001 CD spectropolarimeter with 2-inch (5.1-cm) diameter end-window photomultiplier (PMT) at distant (——), intermediate (— —), and close (– – –) positions relative to sample cuvette. Nominal clearance (inches) between cuvette and PMT was 5.00, 3.04, and 0.04, respectively (12.7, 7.72, and 0.10 cm). Solid angle of detection increases as PMT is moved closer to cuvette. The lowest curve (------) was recorded in unmodified Cary 6001 with a fluorescent scattering (fluorscat) cell that offers a larger solid detection angle than the close PMT geometry. Fluorscat spectrum represents the computer average of three runs with monochrometer slits set at 2.4 mm. The conventional spectrum (⊙· · · ·⊙) was obtained with a standard cuvette in an unmodified Cary 6003 CD instrument.

produce spectra that do not change with the solid angle of detection, we may conclude that differential light-scattering contributions, if any, are beneath the sensitivity level of the present measurements. Moreover, this result demonstrates the absence of a systematic position dependence to the large-angle planar detector CD spectra, even in the presence of a light-scattering specimen such as the disrupted phage.

The fluorescent scattering (fluorscat) cell CD spectrum of intact T2 was measured in an unmodified Cary 6001 (lowest curve of Fig. 1). The fluorscat spectrum is zero above 330 nm, assumes small negative values between 330 and 310 nm, and exhibits a negative minimum at 303 nm ($\Delta\epsilon$ = −0.27). The positive maximum, slightly red-shifted, is further reduced to about half the value obtained in the close PMT spectrum. Inasmuch as the fluorscat cell is capable of detecting light scattering through angles even larger than 90 degrees, the reported data demonstrate that the more scattered light detected, the greater is the depression of the positive 286-nm ellipticity band in the intact T2 phage spectrum. It may be concluded that differential light scattering is contributing significantly to the magnitude of this band observed in the conventional CD spectrum of intact T2.

DISCUSSION

T2 DNA conformation *in vivo*

The CD spectrum of DNA in aqueous solution at low salt concentration has been discussed above. In comparison, DNA spectra obtained in the presence of high salt (42), methanol (Maestre, unpublished results), ethylene glycol (43, 44),

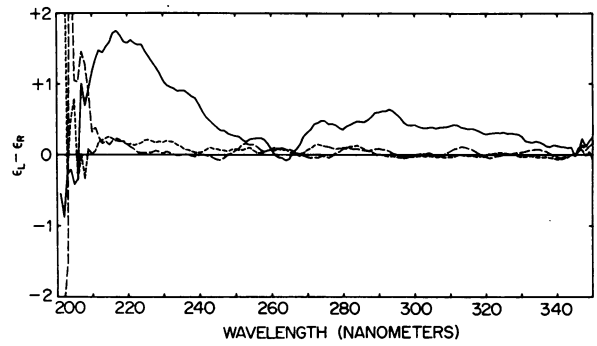

FIG. 2. CD difference spectra computed for large-angle planar detector data by subtracting close PMT spectrum from distant PMT spectrum of intact T2 bacteriophage (——), disrupted T2 bacteriophage (— —), and purified T2 DNA (- - -). Only the intact virus exhibits a nonzero difference spectrum indicative of differential light scattering. Values obtained at wavelengths below 210 nm are believed to be artifacts due to high sample absorbance and the resultant instrumental noise level.

and in Li-DNA films at low ($\leq$75%) relative humidity (42) exhibit depression of the positive ellipticity band with little or no effect on the adjacent negative band. X-ray structures determined for Li-DNA fibers formed at salt and humidity conditions thought to be comparable to those used for the cited Li-DNA film studies indicate that under such conditions DNA exists in the C conformation (45). We note the conclusion of Nelson and Johnson (43) that C-form DNA possesses the minimum specific volume of the DNA structures characterized to date by x-ray. Thus, a C-form helix might be a favorable conformation for DNA that is tightly compacted into a phage coat.

The CD spectrum of purified T2 phage DNA, the fluorscat spectrum of intact T2 phage, and the low-humidity (C-form) Li-DNA film spectrum for calf-thymus DNA are shown in Fig. 3. Comparison of the T2 DNA spectrum with the fluorscat, scatter-corrected phage spectrum reveals that compaction of the DNA into the T2 phage coat leads to substantial depression and red shift of the 280-nm DNA band in the manner characteristic of a transition to C-form geometry. The extensively glucosylated T2 DNA exhibits a solution-form CD that is highly nonconservative and whose prominent spectral features (e.g., extrema and crossover) are red-shifted about 5 nm relative to calf-thymus DNA (24). When allowances are made for these intrinsic differences, however, the correspondence of the CD spectrum for intact T2 virus and for low-humidity Li-salt DNA films is quite striking (Fig. 3). With decreasing wavelength, both spectra exhibit a perceptible negative tail, a small trough, a slightly larger positive maximum, crossover in the vicinity of 280 nm, a shoulder at about 270 nm, and a large negative trough between 240 and 250 nm. On the basis of this correspondence, and the related x-ray fiber structure, we conclude the secondary conformation of T2 DNA *in vivo* may be assigned to the C-form, or to a similar geometry.

General conclusions

The present work clearly demonstrates that CD spectra of a light-scattering suspension may be very sensitive to instrumental light-detection geometry. If scattered light were generated in equal intensities for both circular polarizations, we would expect the resultant spectra to be invariant with changes in the solid angle of detection. Thus, we interpret geometry-sensitive spectra as an indication that differential light scattering is present. Differential light scattering may be a general property of biological macrostructures in suspension. Where it is present, the observed CD of such suspensions will vary with the experimental geometry used, the optical path length, and the relative dimensions of beam cross section, cell diameter, and the position and size of the light-detection apparatus. Under these circumstances, structural conclusions based upon CD data obtained from particulate suspensions in conventional CD spectropolarimeters may be unreliable or meaningless.

It is important to emphasize from our work with disrupted phage suspensions that all particulate suspensions need not exhibit geometry-dependent CD spectra. Although the disrupted T2 samples contain essentially intact phage coats after the DNA is released (46), no significant spectral changes are observed upon going from conventional to large-angle planar to fluorscat detection geometries. Thus, if the phage coats do generate differential light scattering, the effect apparently is not large enough to influence conventional measurements. For practical purposes, we must conclude that differential light scattering will influence the CD of some, not all, particulate suspensions of optically active particles. Whether or not differential light scattering arises may depend then on the specific organization of the aggregated chromophores. We infer that where differential light scattering exists, the organization must exhibit ordered asymmetry. [Wrigglesworth and Packer (47) have made a related suggestion.] For intact T2 virus, we attribute the observed differential light scattering to asymmetry in the DNA packing organization.

Other plausible sources of differential light scattering have been proposed. In the treatment of Gordon (35), differential light scattering arises as a specific consequence of the par-

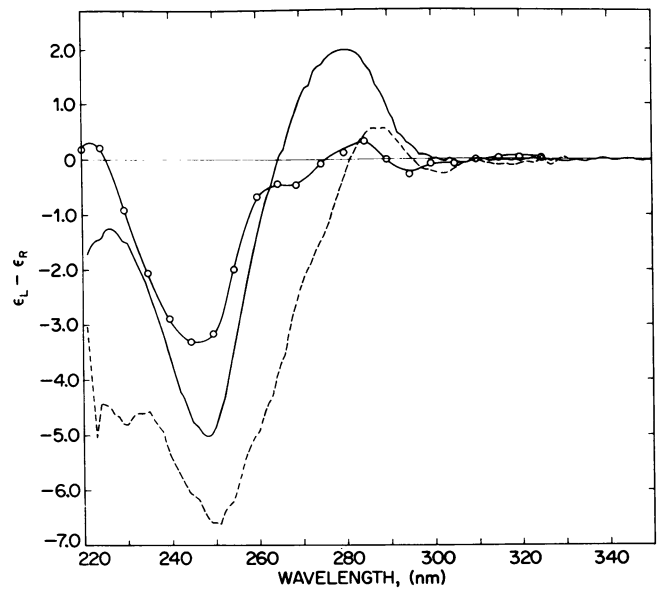

FIG. 3. CD spectra of T2 DNA recorded in a conventional Cary 6003 with a standard cuvette (——), and of intact T2 phage measured in an unmodified Cary 6001 with a fluorscat cell (- - -). The spectrum of a Li-salt DNA film at 75% relative humidity (O——O) is reproduced from earlier work (42).

ticulateness of the specimen rather than the organization of the particle. Using Mie theory for spherical particles and the optical properties of molecularly dispersed sample solutions, Gordon (35) and Gordon and Holzwarth (26) have obtained impressive agreement between the calculated and experimental CD spectra for two particulate suspensions—poly(L-glutamic acid) spheres and erythrocyte ghosts. Large spherical particles in suspension might conceivably represent a class of specimens that will yield to existing theory by the use of known scattering spatial distribution functions. However, such theory is presently limited to treatment of highly symmetric scattering arrays. It remains to be demonstrated that a similar approach can be applied to numerous important, but asymmetric, biological structures, e.g., coliphage. For asymmetric structures in particular, a generally applicable experimental approach is especially desirable. The large-angle detection techniques reported here promise to be useful. (But absorption flattening artifacts intrinsic to particulate suspensions should be independent of detection geometry, and will remain uncorrected by these large-angle techniques.)

Our work suggests that the CD spectra of any scattering specimen ought to be determined again under conditions permitting empirical analysis of possible differential light-scattering contributions. The data presented reveal a large light-scattering effect on the CD of intact T2 virus. We anticipate differential scattering contributions may be influencing the conventional CD of other viruses, chromosomes, chromatin, and membrane structures.

If the observed differential light scattering is in fact produced by an ordered asymmetry at the scattering center, which we believe to be the case, it is conceivable the shape of the scattering contribution curves may be used to assess details of the relevant ordered structure. As such, the differential light-scattering phenomenon might constitute a uniquely powerful probe for molecular organization at the level of tertiary or quaternary conformation.

* *Note Added in Proof.* A more detailed description of the fluorscat cell and related large-angle light detection techniques has been written; see Chap. 30 in *Enzyme Structure*, Vol. 27D of *Methods in Enzymology*, eds. Hirs, C. H. W. & Timasheff, S. N. (Academic Press, New York).

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