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MOLECULAR AND STRUCTURAL CHARACTERISTICS OF HOUSE FLY BRAIN ACETYLCHOLINESTERASE

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Summary

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) from the brains of house flies (*Musca domestica* L., tetrachlorvinphos-resistant strain) was examined for molecular and structural features, including molecular weight, Stokes radii, partial specific volumes, sedimentation coefficients and frictional ratios.

Acetylcholinesterase purified by affinity chromatography was examined in the electron microscope by negative staining and three molecular forms were clearly observed (monomers, dimers and tetramers). Several tetrameric configurations were observed as well as structures of similar size showing tails. In the preparations of acetylcholinesterase so far examined, no globular structures having more than four monomeric units were observed.

Introduction

We have studied the properties of a mutant acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) derived from house flies resistant to the organophosphate, tetrachlorvinphos [1,2]. The gene producing the mutant acetylcholinesterase in a tetrachlorvinphos-resistant strain of house fly has been introduced into the genome of a susceptible marker strain; genetic studies showed that mutant acetylcholinesterase was controlled by a gene on chromosome II, about five crossover units from the marker stubby wing [3]. The enzyme has been prepared to 94% purity and shown to behave like the wild enzyme in sucrose density gradients and polyacrylamide gel electrophoresis [4]. It offers

* These calculations utilize the original authors' molecular weight estimates, which are probably too low because they were derived from s values calculated from comparisons with globular proteins, just as in our own earlier work.

some advantages over the wild enzyme in that it is more stable at 4°C and shows better resolved peaks in isoelectric focusing techniques.

A good deal of information on molecular and structural features is available for certain vertebrate acetylcholinesterase from the peripheral nervous system [5—9]. However, such information for invertebrates is limited to data on sedimentation coefficients, molecular weights and subunit compositions [4,10,11]. We report the molecular and structural features from an invertebrate, specifically from its central nervous system.

Materials and Methods

Materials. Sepharose 6B, α-chymotrypsin and thyroglobulin were obtained from Sigma Chemical Co. (St. Louis, Mo.); cytochrome *c* from Schwarz/Mann (Orangeburg, N.Y.); aldolase, catalase and β-galactosidase from Worthington Biochemical Corp. (Freehold, N.J.); acetylthiocholine chloride and 5′,5′-dithiobis(2-nitrobenzoic acid) from Nutritional Biochemicals (Cleveland, Ohio); uranyl formate from K and K Laboratories (Plainview, N.Y.); ^2H_2O (99%) from Aldrich Chemical Co. (Milwaukee, Wisc.). The chemicals used in polyacrylamide gel electrophoresis were obtained from Bio Rad Laboratories (Richmond, Calif.). All reagents used were of analytical grade.

Extraction. A 20% homogenate (w/v) of heads of house flies (*Musca domestica* L., tetrachlorvinphos-resistant strain) was prepared as described earlier [1]. The homogenate was centrifuged at 100000 × *g* for 1 h at 4°C and the resulting supernatant preparation was used as the enzyme source.

Enzyme assay. A Varian Techtron double-beam recording spectrophotometer with temperature-controlled compartment at 25°C was used and acetylcholinesterase activity in the fractions was estimated using four different substrates. When acetylthiocholine or butyrylthiocholine was the substrate, the method of Ellman et al. [12] was used. The rate of *p*-nitrophenyl acetate hydrolysis was measured at 402 nm in 50 mM sodium phosphate buffer (pH 7.4). For indophenyl acetate the method of Kramer and Gamson [13] was followed except that 50 mM sodium phosphate buffer (pH 7.4) was used. The total reaction mixture was 0.3 ml and the final concentration of substrates were as follows: 1.21 mM acetylthiocholine, 4.84 mM butyrylthiocholine, 1 mM *p*-nitrophenyl acetate and 0.5 mM indophenyl acetate. Stock solutions of *p*-nitrophenyl acetate and indophenyl acetate were prepared in 95% ethanol and then were diluted by the reaction mixture to a final concentration of 1%. For *p*-nitrophenyl acetate and indophenyl acetate separate runs were performed in the absence of enzyme to measure the non-enzymic hydrolysis; the reported enzymic values were corrected appropriately.

Sepharose 6B chromatography. A 1.6 × 100 cm column (K16/100 Pharmacia Fine Chemicals, Uppsala, Sweden) of Sepharose 6B was equilibrated with 0.05 M sodium phosphate (pH 7.4). The following standard proteins were used (Stokes radius in parentheses): cytochrome *c* (1.64 nm), α-chymotrypsin (2.09 nm), aldolase (4.5 nm), β-galactosidase (6.9 nm), thyroglobulin (8.5 nm) [14] and catalase (5.22 nm) [15]. Cytochrome *c* and thyroglobulin were estimated as described by Andrews [14]. α-Chymotrypsin activity was assayed according to the method of Klyosov et al. [16]. Aldolase, catalase and β-galactosidase

activity was determined as described in the Worthington enzyme manual [17]. Crude acetylcholinesterase (1.6 ml) was applied to the column. The experiments were carried out in triplicate.

Sucrose density gradients. Linear 5—20% sucrose density gradients in 50 mM sodium phosphate buffer (pH 7.4) were prepared as described earlier [4]. The ^2H_2O gradients were made up in 99% ^2H_2O containing 50 mM anhydrous Na_2HPO_4. The pH was adjusted to 7.4 with 12 M HCl. Aldolase added as a marker protein, and acetylcholinesterase, both prepared in ^2H_2O, were centrifuged at 39000 rev./min for 20 h at 4°C using an SW 40 swing-out bucket rotor in a L2-65B Beckman ultracentrifuge. Aldolase and acetylcholinesterase peaks were assayed as described earlier [4].

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed on 7% polyacrylamide gels in Tris/glycine buffer (pH 8.3) and acetylcholinesterase activity was located on the gels using an acetylthiocholine method [4].

Electron microscopy of acetylcholinesterase. Acetylcholinesterase purified by affinity chromatography [4] was used for electron micrograph studies. The enzyme was negatively stained with 0.5% uranyl formate for 1 min. Uranyl formate was preferred over other stains, e.g. uranyl acetate and sodium phosphotungstic acid, because of its greater penetrating ability into narrow particle irregularities [18], thereby aiding in the differentiation of particles (size and shape) in a heterogeneous preparation. All electron microscope examinations were performed on an AEI EM6B operated at an accelerated voltage of 80 kV.

Results

Fig. 1 shows the results of Sepharose 6B chromatography of the crude mutant enzyme, assayed using four different substrates. There are three major peaks of acetylcholinesterase activity, which are termed A, B and C, in order of decreasing Stokes radius. These peaks react readily with acetylthiocholine and butyrylthiocholine; to a lesser extent with *p*-nitrophenyl acetate, and least with indophenyl acetate. When material from these three peaks was electrophoresed and stained with acetylthiocholine (Fig. 2) it was found that peak A (which emerged with the void volume) did not enter the gel (Fig. 2A); peak B, was identified electrophoretically as isozyme I [4] (Fig. 2B); and peak C as isozyme III [4] (Fig. 2C). The Stokes radius, a, of isozyme I was 9.35 nm and of isozyme III was 5.65 nm (Table I).

The partial specific volume, $\bar{v}$, was then calculated by comparing the sedimentation velocity in sucrose-H_2O and sucrose-^2H_2O gradients, using aldolase as a marker. Whereas in sucrose-H_2O the small 11.5-S and large 6.9-S peaks were well resolved [4]; in the more dense sucrose-^2H_2O they were not, and the 6.9 S peak was therefore selected for study. The $\bar{v}$ was calculated from:

$$\bar{v}_A = \frac{(1 - \bar{v}_B \rho_D^B) - a(1 - \bar{v}_B \rho_H^B)}{\rho_H^A(1 - \bar{v}_B \rho_D^B - a\rho_D^A(1 - \bar{v}_B \rho_H^B)} \quad (1)$$

where the superscript A refers to acetylcholinesterase, B to aldolase, and subscript H to H_2O and D to ^2H_2O; ρ is the density at the point in the gradient which is at the mean position of the migrating molecule; and $a = (y_H^B/y_D^B)(y_D^A/y_H^A)$ where y is the distance travelled from the meniscus. Eqn. 1 is essen-

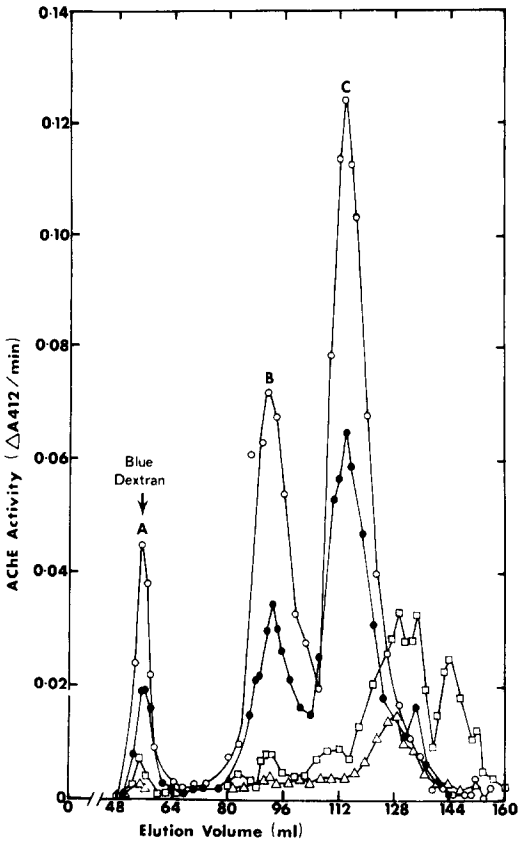
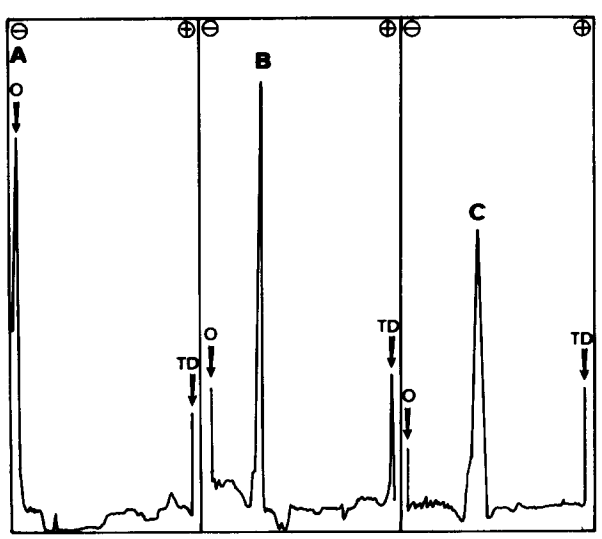


Fig. 1. Gel filtration of crude acetylcholinesterase on Sepharose 6B. The activity was assayed as described in Materials and Methods with four different substrates: ○———○, acetylthiocholine; ●———●, butyrylthiocholine; □———□, *p*-nitrophenyl acetate; △———△, indophenyl acetate.



Fig, 2. Polyacrylamide gel scans of crude acetylcholinesterase of three Sepharose 6B fractions stained with acetylthiocholine. T.D. denotes the portion of tracking dye. The gels were scanned at 410 nm with a Beckman Acta III spectrophotometer.

TABLE I

MOLECULAR PARAMETERS OF PRINCIPAL ACETYLCHOLINESTERASE FORMS

Isozymes	Stokes radius (nm)	Sedimentation coefficient * (s_{20w})	Molecular weight ($\times 10^{-3}$)		Frictional ratio (f/f_0)
			Gel filtration	Electron micrograph	
I	9.35	11.50	501	540	1.76
III	5.65	6.9	182	212	1.49

* Taken from ref. 4.

tially that of Meunier et al. [19]; we have compared it with other approaches and found it optimal [20]. The value of $\bar{v}$ was found to be 0.752 ± 0.022 for crude mutant enzyme (six determinations) and 0.756 ± 0.041 for the purified (five determinations). Brief experiments with the crude wild-type enzyme indicated a similar value.

Combination of this $\bar{v}$ value with the values for Stokes radius and sedimentation coefficient, s, permits the calculation of M, the apparent molecular weight, by the method of Seigel and Monty [21]. This calculation yields values of M of 501000 for I and 182000 for III (Table I). These values were then used in the other Siegel-Monty equation [21] for f/f_o, the frictional ratio. This calculation yielded values for f/f_o of 1.76 for I and 1.49 for III (Table I). If these high frictional ratios were due exclusively to the non-spherical shape of the isozymes, they would imply axial ratios for I and III of about 14 and 9, respectively, for a prolate ellipsoid, or 19 and 11, respectively, for an oblate ellipsoid [22].

Fig. 3a shows a field view of acetylcholineasterase particles as observed in the electron microscope after negative staining and appears to be rather homogeneous with respect to the size of the particles in solution. Fig. 3b shows several basic monomeric units (arrows) of the acetylcholinesterase. This basic unit appears to be globular, having a diameter of about 5—7 nm and is separated into two parts by electron opaque region. This monomer resembles very closely the subunit configurations described by Rieger et al. [7] in their reconstruction of the tetramer molecule of electric eel. Fig. 3c shows several forms identified as dimers consisting of two monomers each measuring approx. 5—7 nm across. The dimer varied in length (11—14 nm), probably due to the stain surrounding the dimers or due to relative association of the monomeric units making up the dimer. Figs 3d—3f represent the more frequently observed form. These forms appear to be in a tetrameric configuration and had an overall diameter of 12.5—15 nm. In some cases (Fig. 3d) the particle had a clearly distinguishable tetrameric form. where the basic monomeric units were orientated in a planar view. In other examples (Figs. 3e and 3f) the tetrameric form was not clearly visible.

We have observed the tail-like appendage previously described in eel acetylcholinesterase preparations [7,8]. In our preparations the tail is not observed as frequently, although scattered throughout the field one can observe what might be isolated tail pieces (Fig. 3h). Figs. 3g and 3h illustrate the extent of the tail structures. Tails measuring up to 45 nm in length have been observed.

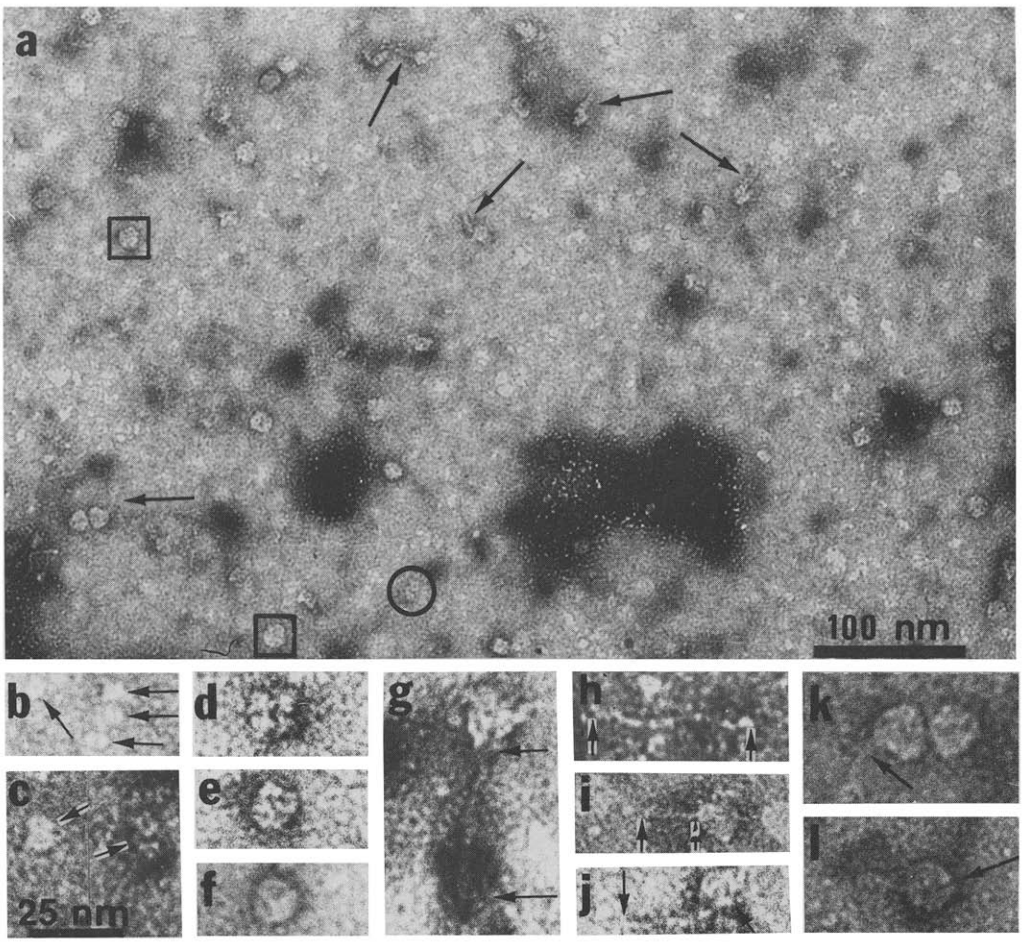


Fig. 3. Acetylcholinesterase isolated from house fly brain and negatively stained with 0.5% uranium formate. (a) A field view illustrating the major components making up the sample. Arrows indicate particles having a tail-like appendage. Most of the particles observed did not exhibit a tail. The basic monomeric unit is not clearly visible at this magnification. The dimer (circle) and the tetramer form (square) are observed in this picture as well as the particle in the lower left corner of the figure identified as being two tetramers apparently attached (see k). (b) A higher magnified view of several single monomeric units (arrows) each having an overall diameter of 5—7 nm. Each monomer is separated into two smaller parts by an electron dense line. (c) Several forms identified as dimers, each dimer consisting of two monomers, each measuring 5—7 nm across. (d, e, f) Several forms of the acetylcholinesterase showing four monomeric units (tetramer); (d) in a separated, planar array, (e) in a more closely packed form and (f) in a form where the individual monomeric units are less clearly observed. (g) The extent of the tail-like appendage is demonstrated (distance between arrows). Tails measuring up to 45 nm in length have been observed. (h) Occasionally, isolated tail pieces have been seen in the various preparations under study. (i, j) Two particles exhibiting tail appendages less than 45 nm in length. The width of the tails measured was 2 nm. (k) Two particles (tetramers) appear to be joined together. One of the particles exhibits a tail (arrow) while the other particle appears to have an electron dense groove or cleft. (l) A similar size particle as in k exhibiting a distinct slit at the outer edge of the particle (arrow). The significance of the cleft or slit is not known at this time and appears to be unique for the house fly brain acetylcholinesterase. No subunit structure can be seen in the larger particles. Magnification of Figs. b—l are the same.

The width of the tail measures approx. 2 nm, in good agreement with the measurements obtained for the *Electrophorus* acetylcholinesterase. In addition, a few thicker tails (3.5—5 nm) have been seen.

Some of the tailed forms appear to be somewhat smaller than the principal (tetrameric) component, with a head size of 10.0—12.0 nm (Figs. 3i and 3j). This might correspond to a minor component, not seen with sucrose density gradients, but previously observed with polyacrylamide gel electrophresis [23] as migrating between the 11.5- and 6.9-S forms.

Several cases were observed in which two tetramers appeared to be joined together (Fig. 3k). These particles exhibited shorter tails (<45 nm). In Fig. 3k, one particle exhibits a tail (arrow), the other exhibits a dark staining groove (or cleft) down the center. This cleft has been noted in a number of particles and depending upon the orientation, a slit can be seen at the outer edge of the particle (Fig. 3l, arrow). The significance of the cleft or the slit is not known at this time and it has not been described for *Electrophorus* acetylcholinesterase. Where one sees a groove or where there is a tail present, the subunit structure is not seen. It would appear that the particles can exhibit a tightly packed configuration which does not permit penetration of the stain.

In none of the house fly preparations examined has large globular aggregates been observed which could be compared to the poly-tetramers observed in the eel acetylcholinesterase [7,8].

Discussion

In an earlier report [4] we estimated M for isozymes I and III from sucrose density sedimentation data, making the usual simplifying assumptions [24] that the proteins were globular and had a $\bar{v}$ essentially equal to that of the standard, aldolase ($\bar{v} = 0.73$). The values of M we calculated were 306000 and 143000 for I and III. These contrast with the values of 501000 and 182000 in the present paper. The error in the early estimates caused by assuming $\bar{v} = 0.73$ instead of the value reported in this paper of 0.75, is quite small. From Eqn. 1 one can calculate in the case of I (knowing that its value of y_H was 0.678) that s was under-estimated by about 1%, and thus M by a little more than this. But the large frictional ratios reported herin show that this enzyme departs far from being a simple rigid unhydrated sphere: it is either very irregular, very hydrated or both, and thus the values calculated in the present paper are more precise, though still imperfect.

Previous estimates of $\bar{v}$ for acetylcholinesterase have usually been calculated from amino acid compositions; for eel, Leuzinger et al. [25] calculated 0.731; Dudai et al. [8] calculated 0.72; for *Torpedo*, Taylor et al. [26] calculated 0.716. But the measured values for eel are rather different: Bon et al. [27] found 0.752, very close to our value for house fly of 0.756.

Comparisons of molecular weight and f/f_o are difficult because of the numerous isozymes reported. The most extensive work [9] is with eel, and shows three unsymmetrical tailed forms of high Stokes radius (12—15 nm) and M values of 430000—1100000. In addition, there are the untailed, symmetrical forms, of small Stokes radius (6—8 nm) and M values of 180000—370000. The unsymmetrical forms have very high f/f_o values, averaging 2.33 calculated * from Massoulié et al. [9], the symmetrical forms average 1.67, very like the value of 1.65 reported for purified "lytic" *Torpedo* enzyme by Taylor et al. [26].

We thus conclude that our 11.5-S form is distinctly unsymmetrical, as shown by its high f/f_0 of 1.76, and it is plausible that it corresponds to the tailed tetramer. The molecular weight of the tetramer was calculated by assuming it to be made of four spheres in planar array, each with diameter 6.88 nm (half of the average tetramer diameter) and $\overline{v} = 0.756$. This calculation gives a molecular weight of 540000 for the tetramer (Table I), in good agreement with our value of 501000 for the 11.5-S component. The tailed tetramer had a 12—15 nm head, and a tail 45 nm long and 2 nm wide. Such drastic asymmetry would explain the high f/f_0 of 1.76. These dimensions are also compatible with the Stokes diameter for the 11.5-S form of 19 nm (i.e. calculated as double the Stokes radius), since the Stokes diameter of a sphere-plus-tail must of course be larger than the diameter of its spherical portion.

Our findings are roughly compatible with those of Steele and Smallman [10] for another house fly strain. Their major isozyme was 11.8-S, close to our 11.5-S value. They estimated M for it as 260000 based on an assumption of globularity, a value close to our early value [4] of 306000 based on a similar false premise. Their second major component was 7.4-S, like ours of 6.9-S, and their calculated M was 164000 based on this same premise, close to our early value of 143000 which we now reestimate at 182000. The molecular weight of the dimer (Fig. 3) was calculated by assuming it to be made of two spheres, each with an average diameter of 6.3 nm and $\overline{v} = 0.756$. This calculation gives a molecular weight of 212000 for the dimer (Table I), in good agreement with our value of 182000 for the 6.9-S isozyme. Steele and Smallman [10] also found small amounts of a 5.3-S form, which we do not see on gel filtration, but may well be isozyme IV seen in our polyacrylamide gels and on sucrose density gradient containing polyacrylamide gel as a 5.0-S form [28]. The molecular weight of the monomeric unit was calculated by assuming the average diameter of 6 nm sphere and $\overline{v} = 0.756$. This calculation gives a molecular weight of 90000, which corresponds to a single subunit of 82000 molecular weight on sodium dodecyl sulphate gel electrophoresis (Tripathi, R.K., unpublished).

It appears that a major portion of our acetylcholinesterase is like that of the eel, in being highly unsymmetrical, probably tailed, with a repeat unit of about 500000 daltons, and having a fairly high $\overline{v}$. In the comparable case of *Torpedo* enzyme, there is persuasive evidence that the tail is collagenous, and serves to anchor the enzyme into the basement membrane, rather than the enzyme being a component of the post-synaptic membrane, as previously thought [29]. The apparent similarity of organization and properties is all the more surprising when one considers that the house fly enzyme is central, probably dendro-dendritic, does not cross-react immunologically with eel or *Torpedo* enzyme (Tripathi, R.K., unpublished) and has a different set of substrate preferences [30].

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