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SOME PROPERTIES OF ISOLATED PIG PLASMA ESTERASES SENSITIVE TO 3,5-DI-*t*-BUTYLPHENYL METHYLCARBAMATE (BUTACARB)

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Abstract—Some properties of pig plasma esterases were determined using as substrates thiophenyl acetate, phenylacetate, acetylthiocholine iodide and butyrylthiocholine iodide. Under similar conditions, butacarb (3,5-di-*t*-butylphenyl methylcarbamate) was found to be a much more potent inhibitor than carbaryl (1-naphthyl methylcarbamate). High specific activity fractions were obtained by ammonium sulfate precipitation followed by chromatography on Sepharose 6B. The butacarb-sensitive esterases were adequately separated from albumin by Sepharose 6B. The active fractions were inhibited by mercuric, cobalt and manganese ions, and not by calcium and potassium ions or by Mipafox, an organophosphorus compound. Also, they were free of any cholinesterase activity. The properties observed indicated an arylesterase type of enzyme.

Pig plasma contains mostly arylesterases and low concentrations of other related enzymes. The presence of carboxylesterases is questionable. Arylesterases are genetically controlled by a set of multiple alleles [1, 2] and are 'specific' toward aromatic and aliphatic esters having a double bond (—C=C—O—) adjacent to the ester linkage [3]. They do not hydrolyze choline and other aliphatic esters. However, esterases such as carboxylesterases and cholinesterases readily hydrolyze aromatic esters.

Arylesterases, which are the characteristic type of esterase in mammalian blood plasma [4–8], are highly sensitive to heavy metallic ions such as mercuric ions [7–10] and are activated by calcium [9–11]. They are resistant to organophosphorus compounds and carbamates, including those used as selective cholinesterase inhibitors.

However, our preliminary work showed that pig plasma esterases hydrolyzing thiophenyl acetate (TPhAc) were readily and markedly inhibited by butacarb (3,5-di-*t*-butylphenyl methylcarbamate), but were less sensitive to carbaryl (1-naphthyl methylcarbamate). This difference in the reactivity toward similar compounds may be of importance in toxicological evaluations necessary for setting up tolerance levels of carbamate insecticides in foods. The present investigation deals with the partial isolation and characterization of butacarb-sensitive esterases from other plasma proteins.

MATERIALS AND METHODS

Enzyme assays. The esterase activity was determined spectrophotometrically according to a modified method of Ellman, Augustinsson *et al.* [12, 13], using TPhAc (Polyscience, Warrington, Pa.), acetylthiocholine iodide (ASChI) and butyrylthiocholine iodide (BSChI) (both from Sigma Chemical Co., St. Louis, Mo.) as substrates. The reaction rate was determined, at 30° in 0·05 M Tris–HCl buffer, pH 7·2. An LKB Reaction Rate Analyzer 8600 (Bromma, Sweden) or Beckman DB-G (Great Britain) spectrophotometer was used. Butacarb, 100 per cent purity (Boots Pure Drug Co., Ltd., Nottingham, England), and carbaryl, 99·9 per cent purity (Union Carbide Corp., W. Va., U.S.A.), were added individually or on after the other to the reaction solution to determine their effects on the enzymatic hydrolysis of TPhAc. Unless specified, the general procedure was as follows: (1) preincubation of enzyme solution for 0·5 min at 30°, (2) TPhAc added and the initial enzymatic rate observed for 1 min, (3) carbaryl added and the enzymatic rate observed for another 1 min and (4) butacarb added and the rate also observed for at least 1 min.

The enzyme activity was also determined gasometrically at 25° in a Warburg apparatus (B. Braun, Melsungen, West Germany) using phenyl acetate (Labkemi AB, Stockholm, Sweden) as substrate.

Determination of protein concentration. The protein concentration was determined by measuring the extinction at 260 and 280 nm [14]. Protein concentration (mg/ml) = $1{\cdot}45\ E_{280} - 0{\cdot}74\ E_{260}$; where E is the extinction at 260 and 280 nm respectively.

Separation methods. Chromatographic separation on Sephadex G-100 and Sepharose 6B (both obtained from Pharmacia Fine Chemicals, Uppsala, Sweden) was performed at 4°. Phosphate buffer (0·04 M, pH 7) was used in a Sephadex G-100 column, and either phosphate buffer (0·04 M, pH 7) or Tris–HCl buffer (0·05 M, pH 7·2) in a Sepharose 6B column. The Tris buffer was used with or without 1 mM $CaCl_2$ and 5 μM EDTA. The flow rate (0·25 to 0·30 ml/min) was maintained by using a peristaltic pump (Varioperpex, LKB-Produkter AB, Bromma, Sweden) after introducing the sample at the bottom of the gel column (1·5 cm i.d. × 85 cm ht). The void volume obtained was ~45 ml.

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The enzyme was also isolated by precipitation in an $(NH_4)_2SO_4$ solution [4, 11] before chromatography on Sepharose 6B. The precipitate obtained from a 50% saturated $(NH_4)_2SO_4$ solution was discarded. The supernatant was further saturated to 75% and the resulting supernatant discarded. The precipitate was dissolved in 0·05 M Tris–HCl buffer at pH 7·2, dialyzed for 3 days with several changes of the same buffer, and concentrated in an Amicon Diaflo cell (Instrument AB Lambda, Stockholm, Sweden).

Electrophoresis was performed always in duplicate, at 4° in polyacrylamide gels (pH 7·5, 7·5%, medium size pore) [15]. The developed gels were incubated with Coomassie Brilliant Blue R-250 (Serva, Heidelberg, West Germany), 5-bromoindoxyl acetate (5-BIA) (Sigma Chemical Co., So. Louis, Mo), 1-naphthyl acetate (NA) (Eastman Organic Chem., Rochester, N.Y.), or TPhAc. In addition, isoelectric focusing in polyacrylamide gels containing Ampholine (LKB-Produkter AB, Bromma, Sweden) was also performed in duplicate according to the previously reported methods [16–19].* After focusing, each gel was cut into 5-mm sections [19].* Each section was squashed in 1 ml of distilled water and was kept overnight at 4° before determining the pH at this temperature. Other gels, which were incubated with enzyme substrate or protein stain, were observed at 620 nm for Coomassie Brilliant Blue R-250, indophenyl acetate (IPA), and TPhAc-copper-dithioxamide complex [20]; at 610 nm for 5-BIA; at 390 nm for NA; and at 284 nm for protein with or without stains. The spectrophotometer (Unicam SP 1800, Pye Unicam, Great Britain) used was equipped with an automatic cuvette slider.

RESULTS

Effects of butacarb on enzyme activity. The enzyme activity of a Sepharose 6B fraction was inhibited to about 50 per cent by $3·2 \times 10^{-2}$ mM butacarb after 1–5 min of incubation. Likewise, with whole plasma, 40–50 per cent inhibition was observed with 3 to 6×10^{-3} mM butacarb after 5–20 min of incubation. With 2 to 5×10^{-2} mM carbaryl, only 3–8 per cent of enzyme activity was inhibited after 5–10 min of incubation. Prolonged incubation to 15 min with 5 to 10×10^{-2} mM carbaryl increased the inhibition to only 28 per cent.

Based on the general procedure used (see Materials and Methods), 0·25 mM carbaryl had no effect on the enzyme activity in Sepharose 6B fractions. Further tests showed that carbaryl added before or after TPhAc did not alter the inhibitory effect of 3×10^{-2} mM butacarb.

Gel chromatography. Protein analysis of Sepharose 6B fractions obtained from whole plasma showed two protein peaks. One appeared before the esterase region and the other was in the region where the bovine crystalline albumin standard was eluted. The esterase activity was observed in both the esterase and the albumin regions. The inhibition of esterase activity in the esterase region by 3×10^{-2} mM butacarb was greater than that in the albumin region.

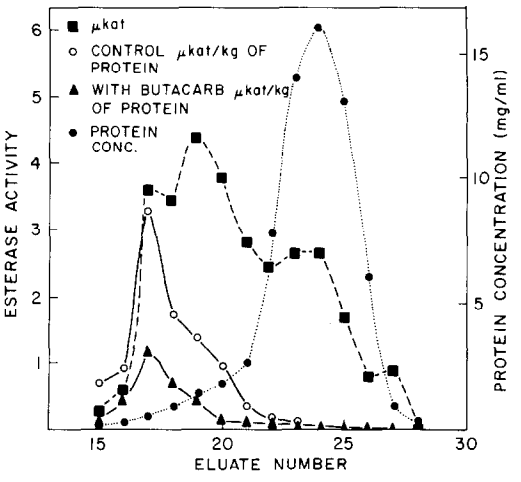

Fig. 1. A typical chromatogram of pig plasma eluted in Sepharose 6B. The plasma was precipitated by $(NH_4)_2SO_4$ and was dialyzed before chromatography. The enzyme activity (■) is in μkat; specific activities in μkat/kg of protein in the absence (○) or presence (▲) of $3·2 \times 10^{-2}$ mM butacarb; protein concentration (●) in mg/ml of eluate. The reaction was performed using 0·05 M Tris-HCl buffer, pH 7·2, containing 10^{-3} M TPhAc at 30°.

Figure 1 shows a typical Sepharose 6B chromatogram, of pig plasma, which was precipitated with $(NH_4)_2SO_4$. On the basis of enzyme activity (μkat), there are two peaks shown, one at the region of eluate 17 to 19 and the other on the albumin region, eluate 23 to 24. (The decrease in activity in eluate 18 was considered an artifact, since it was not observed in previous or subsequent elutions.) The activity in eluate 23 to 24 may be due to albumin esterases. Contrary to the preceding observation, the activity (μkat/kg) peak (eluate 17) was observed in the absence or presence of butacarb. This peak was distinctly separated from the protein peak (eluate 24 to 25), which comprised mostly serum albumin. The specific activity in eluate 17 to 19 was 30 times that observed for whole plasma.

Based on a gasometric method the enzyme activity in pig whole plasma at dilutions between 1:5 and 1:10 was 49–55 per cent inhibited after 50 min of incubation with 10^{-2} mM butacarb at 25°. Only 9–14 per cent inhibition was observed with 10^{-3} mM butacarb. Using the same conditions, 10^{-4} to 10^{-2} mM carbaryl and 10^{-3} to 10^{-1} mM Mipafox (bis-monoisopropylamino fluorophosphine oxide) did not inhibit the enzymatic hydrolysis of phenyl acetate. However, the esterase activity of Sepharose 6B fractions in the albumin region was found sensitive to carbamates. Butacarb and carbaryl each at 10^{-4} to 10^{-2} mM gave 53–63 and ~37 per cent inhibition respectively. The gasometric data for butacarb agreed with the spectrophotometric (TPhAc) data.

The similarity of enzyme activities in the three consecutive Sepharose 6B fractions in the presence of butacarb at various concentrations is shown in Fig. 2. The range of I_{50} values was narrow, from 1 to 2×10^{-2} mM. (I_{50} values were obtained by plotting the per cent inhibition on a probit scale against molar concentration on a logarithmic scale.)

* L. Lindqvist and K.-B. Augustinsson, manuscript submitted for publication.

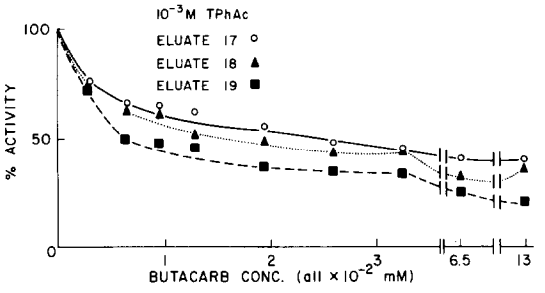

Fig. 2. Effects of butacarb concentration on TPhAc hydrolysis. Butacarb was added 0·5 min after TPhAc to the reaction solution containing Sepharose 6B eluate 17 (○), 18 (▲) or 19 (■) as presented in Fig. 1. The reaction was performed under the conditions stated in Fig. 1.

Effects of TPhAc concentration on enzyme activity. The constants calculated for Sepharose 6B fractions 17–19 were $V_{max} = 5{\cdot}6\ \mu$kat/l. and $K_m = 0{\cdot}5$ mM. Figure 3 shows the hydrolysis of TPhAc at various concentrations in the absence or presence of 3×10^{-2} mM butacarb, which was added 1 min after the addition of TPhAc. The degree of inhibition decreased as the TPhAc concentration increased, and it remained constant when TPhAc was increased from 0·7 to 1 mM.

Effects of ions on enzyme activity. Figure 4 shows the effects of K^+, F^-, Mg^{2+}, Mn^{2+} and Co^{2+} (each at 5 mM) on the TPhAc hydrolysis by esterases in an active fraction after Sepharose 6B chromatography. All tests were performed in the presence of 1 mM Ca^{2+}. Under this condition, Co^{2+} gave about 95 per cent inhibition; Mn^{2+}, 40 per cent; Mg^{2+}, 10 per cent; F^-, 5 per cent; and K^+, 0 per cent.

Likewise, the gasometric method showed that 10^{-2} mM $HgCl_2$ gave ~65 per cent inhibition of the esterase activity in pig whole plasma diluted 1:5 and incubated for 50 min at 25°. No inhibition was observed with $<10^{-3}$ mM $HgCl_2$. The esterase in the Sepharose 6B fractions with a high sp. act. was ~14 and 58% per cent inhibited by 10^{-3} mM and 10^{-2} mM $HgCl_2$ respectively. The esterase in the albumin region was 13 per cent inhibited after incubation with 10^{-4} to 10^{-2} mM $HgCl_2$ for 50 min.

ASChI and BSChI as esterase substrates. Table 1 shows that ASChI and BSChI were hydrolyzed at a very low rate. Consequently, we regard the cholinesterase activity present in our fractions as negligible.

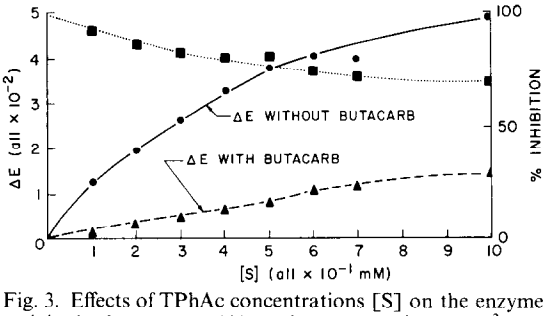

Fig. 3. Effects of TPhAc concentrations [S] on the enzyme activity in the presence (▲) or absence (●) of 3×10^{-2} mM butacarb. ΔE = change in absorbance/min; % inhibition (■) was based on the initial activity. The reaction was performed under the conditions stated in Fig. 1.

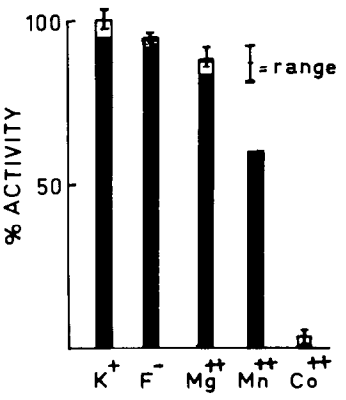

Fig. 4. Effects of ions, each at 5 mM, on the esterase activity of Sepharose 6B eluate 17 to 19. The per cent activity left after the addition of each ion was based on the control. The reaction was performed under the condition stated in Fig. 1.

Polyacrylamide gel electrophoretic studies. The protein patterns for the whole plasma Sepharose 6B eluates 17 and 25 (Fig. 5A) and $(NH_4)_2SO_4$ precipitate (Fig. 5B) were observed at 620 nm and were confirmed at 280 nm. As expected, band I indicates that the albumin concentration in the precipitated fraction was greatly reduced. The electropherogram of whole plasma contained five protein bands not readily differentiated by the spectrophotometer used. Bands II and III contained at least two protein bands each. Fraction 17 had no band I, whereas fraction 25 contained mostly bands I and II. Band III for fraction 25 was considered an artifact, since no stain was visible in the gel. All proteins in bands I–III hydrolyzed NA and TPhAc, but only that in band III was capable of hydrolyzing 5-BIA.

Figure 6 shows that the protein hydrolyzing 5-BIA was further separated by isoelectrofocusing into at least three major bands, two of which were detectable in the gels incubated with TPhAc; the first band (at the cathodic side) was not detectable with TPhAc. The second and third bands, which reacted with 5-BIA and TPhAc, focused at ~pH 6 and at pH 5–5·2. However, gels stained with Coomassie Brilliant Blue R-250 showed a much wider third band that focused at pH 4·2–5·3. The section of this band that focused at pH 4·2–5 was not detected enzymatically. The bottom part of Fig. 6 shows the corresponding

Table 1. Esterase activities in Sepharose 6B eluate 18 in the absence or presence of carbaryl or butacarb

	Activities* (μkat/kg protein ± S. E.)		
		After addition of:	
Substrate	Initial	Carbaryl (3×10^{-1} mM)	Butacarb (3×10^{-2} mM)
TPhAc	5·34 ± 0·18	4·84 ± 0·19	2·22 ± 0·11
ASChI	0·22 ± 0·03	0·22 ± 0·02	0·12 ± 0·01
BSChI	0·34 ± 0·02	0·25 ± 0·02	0·11 ± 0·02

* N = 3 except for BSChI, where N = 6.

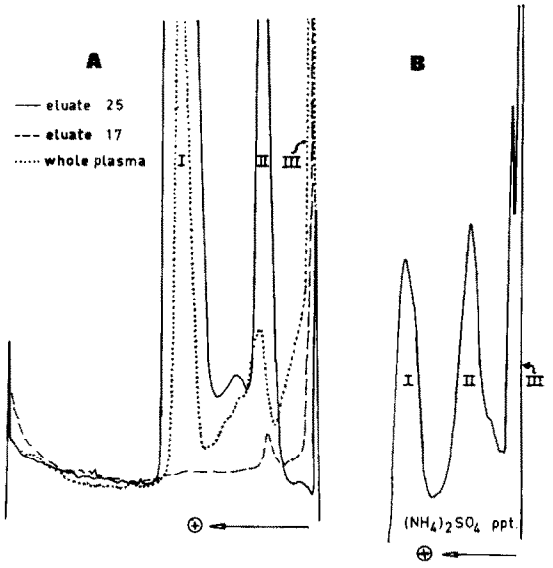

Fig. 5. (A) A comparison of protein absorbance at 620 nm after electrophoresis in polyacrylamide gels of whole plasma (.....); eluate 17 (----) and 25 (——). Refer to Fig. 1. (B) An electrophoretic pattern for the $(NH_4)_2SO_4$ precipitate. I, II and III indicate major absorbance peaks.

spectrophotometric patterns. The unusually high absorption at the right half of the gel incubated with 5-BIA was due only to opaqueness of the gel.

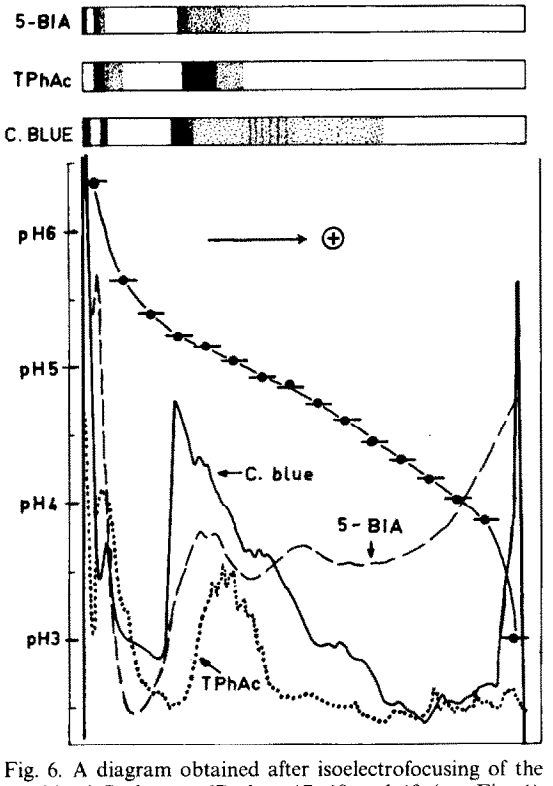

Fig. 6. A diagram obtained after isoelectrofocusing of the combined Sepharose 6B elute 17, 18 and 19 (see Fig. 1). The top diagram represents the gels; the bottom represents the absorbance of the gels specified in the text. Each 5-mm section of the polyacrylamide gel rod is indicated by a dash (——) and the pH of each section by a dot (●). The pH is the average of two readings.

DISCUSSION

It is of significance that pig plasma esterase hydrolyzing TPhAc is very sensitive to butacarb but not to carbaryl. The affinity of the esterase to butacarb may be due to the similarity of the butacarb and TPhAc structures (Fig. 7) and to the particular substituent group in the aromatic ring. The results obtained from Lineweaver–Burk type plots suggested that butacarb competes with TPhAc for the enzymes. However, further studues should be done to ascertain the nature of butacarb interaction with the pig plasma esterases.

Under identical conditions carbaryl, which belongs to the same group of esters as butacarb, did not readily inhibit the pig plasma esterases hydrolyzing TPhAc. This finding parallelled the previous observation on NA, which was slightly hydrolyzed by arylesterase at the rate of only $<0{\cdot}3$ μmole/hr/mg of enzyme [3]. It should be noted that NA is structurally similar to carbaryl. On the contrary, TPhAc was hydrolyzed at the rate of 180 μmoles/hr/mg of enzyme [3].

Both NA and 5-BIA appeared to be readily hydrolyzed by esterases in band III on acrylamide gels, although previous studies [3] indicated that these were not good substrates for arylesterases. However, arylesterases in humans may be different from those in the pig. The difference may also be due to the methodology applied. The work with NA [3] was done at pH 7·4 and this study with 5-BIA at pH 7·8, in which the indigo product is readily formed.

The inhibition of esterase activity by $HgCl_2$ suggests that arylesterases [9] are involved. In addition, the effects observed with Mn^{2+}, Mg^{2+} and Co^{2+} on the esterase activity in the Sepharose 6B fractions were consistent with those reported for human serum arylesterases [10–21].* Although Ca^{2+} is regarded as an activator [9–11], it did not markedly affect the activity when it was omitted in the solution. The inability of Mipafox to inhibit the esterase activity toward phenyl acetate indicates that carboxylesterase and cholinesterase were not responsible for the enzymatic reaction. Moreover, the inability of the esterase in the Sepharose 6B fractions to hydrolyze ASChI or BSChI also suggests the absence of cholinesterases.

To a certain extent, the pig esterase activity is satisfactorily separated from plasma albumin by chromatography on Sepharose 6B but not on Sephadex G-100. Sepharose 6B was previously used to isolate arylesterases (A-type esterases [10, 23]) in sera of different mammalian species [9]. Furthermore, the $(NH_4)_2SO_4$ precipitation method is also useful in obtaining from pig plasma an arylesterase fraction

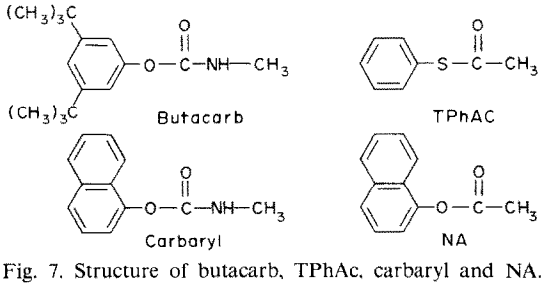

Fig. 7. Structure of butacarb, TPhAc, carbaryl and NA.

with a high sp. act. The high sp. act. of the precipitated samples and their corresponding Sepharose 6B fractions may be attributed to the precipitation of a large amount of protein particularly albumin and gamma globulin with $(NH_4)_2SO_4$ at 50% saturation. Cholinesterases co-precipitate with albumin [23] and, therefore, are not expected in the arylesterase fraction.

Gel electrophoretic results indicated that further purification is needed since a trace of protein, perhaps gamma globulins, is still present in the Sepharose 6B eluates containing the arylesterase activity. In addition, preliminary work using DEAE-cellulose indicates two arylesterase activity peaks in these fractions.

Unlike carbaryl [24], TPhAc, NA and IPA, 5-BIA was not hydrolyzed in the albumin region of the electrophoretic gels. This property of 5-BIA may be useful in isolation schemes to monitor the presence of albumin in the fractions.

In conclusion, this report supported the concept that the pig plasma esterases are basically that of an arylesterase type. More importantly, the sensitivity to inhibition by butacarb, and not by carbaryl, of both the plasma and isolated esterases suggests that this type of esterase should be evaluated along with other parameters in toxicological evaluations of carbamate or related pesticides.

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