Inhibitors of Cyclic Nucleotide Phosphodiesterases Inhibit Protein Carboxyl Methylation in Intact Blood Platelets*

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The cycle of protein-carboxyl methylation and de-
methylation was studied in intact blood platelets. Platelets rapidly incorporated L-[methyl-3H]methionine and after a delay of about 20 min, they evolved [3H]methanol. This evolution, and the amount of [3H] methanol liberated by treatment with base, was inhibited in a dose-dependent fashion by the cyclic nucleo-
tide phosphodiesterase inhibitors 3-isobutyl-1-meth-
yloxanthine, papaverine, diprydamole, and RA233 (2,6-bis(dietanolamino)-4-piperidinopyrimido[5,4-d] pyrimidine). Each of these compounds increased the incorporation of [3H]methionine into platelets. The ef-
efects of RA233 were studied in more detail. Inhibition of [3H]methanoh production was not potentiated by stimulators of the adenylate cyclase or the guanylate cyclase. The majority of the base-labile radioactivity was trichloroacetic acid precipitable. Thin layer chromatography of extracts of platelets incubated with L-
[35S]methionine showed that RA233 did not induce a cellular accumulation of [35S]-S-adenosylhomocysteine, and that it actually increased the amount of cellular [35S]-S-adenosylmethionine. Discontinuous polyacyr-
lamide gel electrophoresis at acid pH using the cationic detergent benzylidimethyl-n-hexadecylaminom monium chloride of platelets incubated with [3H]methionine showed incorporation of radioactivity into more than 30 protein bands, including one which co-migrates with calmodulin. The incorporation into the majority of these bands was inhibited by RA233 in a dose-
dependent fashion. It is suggested that caution should be used in ascribing the pharmacological effects of known phosphodiesterase inhibitors to increases in cyclic nucleotides, because some of these effects could be due to inhibition of protein carboxyl methylation.

The methyl esterification of free carboxyl groups of proteins occurs in all of the eukaryotic cells that have been examined (1), and a substantial body of evidence suggests that this reaction may be involved in the regulation of such cellular processes as exocytotic secretion and chemotaxis (2, 3). The investigation of such involvement is complicated by the finding that several combinations of reagents used to inhibit transmethylation reactions also induced an accumulation of cyclic AMP within the target cell, in part by the inhibition of 3',5'-cyclic nucleotide phosphodiesterases (4). In this paper, we report that several compounds which are known to inhibit cyclic nucleotide phosphodiesterases also inhibit protein-carboxyl methyl esterification in intact platelets, apparently by a mechanism not involving the accumulation of cyclic nucleotides.

MATERIALS AND METHODS

Human blood platelets were prepared from normal donors after they had ingested 700 mg of aspirin. The blood was anticoagulated with 1/3 volume acid-citrate-dextrose (1.25 g/100 ml citric acid, 3.8 g/ 100 ml trisodium citrate and 290 ml glucose), and centrifuged to prepare platelet-rich plasma. About 10 ml of this platelet-rich plasma (containing 2-3 × 10^6 platelets) was cooled to 15 °C and centrifuged at 2000 rpm for 10 min. The platelet pellet was gently resuspended in 10-15 ml of HEPES buffer (140 mM NaCl, 5 mM KCl, 15 mM HEPES, 1 mg/ml glucose, pH 7.5), and centrifuged again. The platelets were then resuspended in the required volume of the same buffer and used immediately.

The incorporation of radioactivity into the platelets was deter-
menced by centrifuging them through silicone oil (6) in micromed,
tination tubes (Sarstedt No. 702). The pellet was harvested by centrifugation and solubilized in Scintiverse liquid scintillation mixture (Fisher Scientific) and its radioactivity and that of the supernatant were determined.

The formation of radioactive methanol was determined as follows. Platelets were incubated with [methyl-3H]methionine, and [35S]methionol was added as an internal recovery standard. A sample was taken to determine the [3H]/[14C] ratio, and at the appropriate times, 50 μl of the incubation mixture was withdrawn and added to 50 μl of methanol containing 2% (v/v) acetic acid. After mixing and centrifugation, 50 μl of the supernatant was pipetted onto a twist of filter paper lodged in the neck of a scintillation counting vial containing 10 ml of Scintiverse, and the vial was immediately capped. Care was taken to ensure that the filter paper did not come into contact with the scintillation mixture; such contact was easily discovered by removing the filter paper and examining it under ultraviolet light. The vial was allowed to stand for at least 2 h and then the [3H]/[14C] ratio of the material that had evaporated into the scintillation mixture was determined. Preliminary experiments established that volatile [3H] production ceases in the methanol/acetic acid mixture (which can conveniently be stored overnight at -10 °C), that the filter paper is rapidly dried by the desiccating nature of Scintiverse (under actual experimental conditions, the half-time for [35S]methionol to transfer from the filter paper to the mixture is about 10 min), and that after drying none of the [3H] retained on the filter paper becomes volatile. Fractional distillation of the volatile [3H] released from platelets established its identity as methanol (Fig. 1).

Separation of methionine metabolites was carried out by thin layer chromatography. Samples of the platelet suspension were digested with ice-cold EDTA/saline (140 mM NaCl, 10 mM Na EDTA, pH 7.0) and centrifuged. The supernatant was discarded and the pellet was dispersed in 100 μl of 0.1 M acetic acid under nitrogen and the tube was briefly immersed in a boiling water bath and then cooled on ice and centrifuged. 5 μl of the supernatant was spotted on the preabsorbent zone of the thin layer plate, together with chromatography standards (about 50 nmol each of S-adenosylmethionine, methionine, methionine sulfoxide, methionine sulfone, S-adenosylhomocysteine, homocysteine, and 5'-deoxy-5'-methylthioadenosine). The plate (250 μl silica GF Univplate, channeled; Analtech, Newark, DE) was developed in an equilibrated tank with butanol/acetic acid/water.

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1 The abbreviations used are: HEPES, 4-[2-hydroxyethyl]-1-piper azine-ethanesulfonic acid; 16-BAC, benzylidimethyl-n-hexadecylam monium chloride.
Polyacrylamide gel electrophoresis was carried out at acid pH with the cationic detergent 16-BAC, using slight modifications of our published method (6). The incubation of platelets with [3H]methionine was terminated by the addition of an equal volume of a solution containing 18-BAC (5%, w/v), urea (5 M), glycerol (10%, v/v), acetic acid (50 mM), dithiothreitol (0.1%, v/v), EDTA (2 mM) made to volume with the electrode buffer. The sample (generally 100 μl) was applied to one of 15 wells cast in the stacking gel of a 32 cm × 14 cm × 1.5 mm Protean slab gel (Bio-Rad Laboratories, Richmond, CA). The composition of the stacking gel was: acrylamide (4%, w/v), N,N'-methylenebisacrylamide (0.35%), urea (10%, w/v), potassium phosphate (125 mM, pH 4.0), ferrous sulfate (4.3 μM), and ascorbic acid (0.43 mM). After bubbling with nitrogen gas, hydrogen peroxide was added to a final concentration of 0.45 mM. The running gel contained acrylamide (7.5%, w/v), N,N'-methylenebisacrylamide (0.26%, w/v), urea (15% w/v), ferrous sulfate (7.7 μM), and ascorbic acid (0.43 mM) in potassium phosphate buffer (150 mM), pH 2.0. After bubbling with nitrogen gas, polymerization was initiated by adding hydrogen peroxide 0.34 mM. The electrode buffer was glycine (150 mM) and phosphoric acid (27.5 mM). 16-BAC (0.1%, w/v) was added to the upper electrode buffer. Electrophoresis was carried out overnight at a constant current of 50 mA. The gel was fixed in two changes of methanol/acetic acid/water (4:1:5) stained with Coomassie blue, destained, hydrated with two changes of distilled water, and exposed to Kodak x-ray film at -80 °C for 1 week. Freeze-thawed platelets was unaffected by the addition of RA233 (2,6-bis(diethanolamino)-4-piperidinopyrimidino[5,4-d]pyrimidine) and dipyridamole were gifts from Dr. Karl Thomae GmbH, Biberach, West Germany. 16-BAC was purchased from Gallard-Schlesinger, Carle Place, NY. Prosta
glandin E, was purchased from Upjohn Co., Kalamazo, MI, and prostacyclin was a gift from the same company.

RESULTS

In preliminary experiments, we confirmed that washed platelets rapidly concentrate radioactivity when they are incubated with [methyl-3H]methionine, and this radioactivity subsequently appears in S-adenosylmethionine, protein, and a volatile product with a distillation profile identical with that of authentic [3H]methanol (Fig. 1). The generation of methanol by platelets labeled with [3H]methionine and then freeze-thawed was a function of the pH of the solution in which they are thawed (Fig. 2). At pH below 5, the rate of production of [3H]methanol by hydrolysis was negligible, but at high pH values, a rapid evolution of volatile radioactivity occurred. At pH 10, this amounted to about 10% of the platelet-associated radioactivity and was complete in about 2 min. In a series of experiments, we established that the rate of [3H]methanol production at pH 7.4 studied in freeze-thawed platelets was unaffected by the addition of EDTA, Ca**, Mg**, tosylarginine methyl ester, disopropyl fluorophosphonate, N-ethylmaleimide, or formaldehyde, suggesting that under these circumstances the majority of the hydrolysis of methyl esters was not enzymatic.

Methanol production in intact platelets was studied by sampling platelets during their incubation with [3H]methanol and estimating the radioactivity in the volatile fraction. After a delay of 10–20 min, [3H]methanol production accelerated and reached a stable rate in about 1 h. This stable rate was unaffected by agents which induce platelet aggregation and exocytotic secretion (thrombin and the calcium ionophore, A23187) (data not shown).

Compound RA233, a phosphodiesterase inhibitor structurally similar to dipyridamole (Fig. 3), inhibited the rate of production of volatile 3H without extending the delay (Fig. 4). This effect was potentiated neither by stimulating the adenylate cyclase with prostacyclin nor by stimulating the guanylate cyclase with sodium nitroprusside (Fig. 4).

This effect of RA233 was not due to inhibition of the uptake of radioactivity by platelet; indeed, the addition of RA233 increased this accumulation by about 250% in a dose-related fashion (Fig. 5, top). This figure also shows that RA233 inhibited the incorporation of 3H into base-labile methyl

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**FIG. 1.** Fractional distillation of volatile 3H. Washed platelets (500 μl, ~108 platelets) were incubated with 20 μCi of [3H]methionine and 0.1 μCi of [14C]methanol for 60 min. Then 500 μl of methanol containing 2% (v/v) acetic acid was added and the mixture was centrifuged. The supernatant was applied to filter paper lodged in a tube, and evaporated with a stream of dry nitrogen. The volatile material was collected in a trap cooled with solid CO2/methanol, and was diluted with 20 ml of 1:1 (v/v) methanol/water. This was subjected to fractional distillation, and the 3H and 14C of each fraction, and of the residue left in the boiling flask (about 1 ml) were determined. The figure shows the cumulative percentage of this 3H (●) and 14C (○) in the fractions.

**FIG. 2.** pH dependence of the hydrolysis of methyl esters. Washed platelets were incubated with 30 μCi of [3H]methionine for 90 min, and then sedimented. They were resuspended in 140 mM NaCl, 10 mM Na-EDTA, pH 7.0, and frozen in 100-μl aliquots containing about 4000 cpm of [3H]methanol and about 108 platelets. They were thawed by the addition of 300 μl of buffer having the indicated pH, and the mixture was incubated at 37 °C. At the times indicated thereafter, the volatile 3H was determined as described under "Materials and Methods" and is expressed as a fraction of the 3H in the platelets. The buffers used were 100 mM citric acid, pH 4 with NaOH, 100 mM citric acid, pH 5 with NaOH, 100 mM NaH2PO4, pH 6 with NaOH, 100 mM NaH2PO4, pH 7 with Tris base; 100 mM Tris base, pH 8 with H3PO4, 100 mM NaH2PO4, pH 9 with NaOH, and 100 mM H3BO3, pH 10 with NaOH.
esters, revealed by the increase in volatile "H after base-catalyzed hydrolysis. The majority of these methyl esters were precipitated by trichloroacetic acid, and RA233 reduced both the total incorporation of "H into trichloroacetic acid precipitate and the fraction made volatile by base treatment (Fig. 6). In these figures, the "H methanol production is expressed as a percentage of the "H originally added to the platelets. The inhibitory effect of RA233 would have appeared much greater had we expressed the "H methanol as a percentage of total "H taken up by the platelets.

In order to determine if RA233 inhibited the synthesis within platelets of the methyl donor, S-adenosylmethionine, we incubated platelets with [35S]methionine and with or without RA233 and washed them. 5 μl of the lysates of these platelets were chromatographed on a thin layer system which separated S-adenosylmethionine from methionine and its oxidation products (Fig. 7). The radioactivity in the spots was determined by liquid scintillation counting. In the control samples (total applied, ~5.0 x 10⁶ cpm), about 6% was found as S-adenosylmethionine and 16% as S-adenosylhomocysteine. In the RA233 samples, about 7 and 7.5%, respectively. We confirmed the identity of the radioactivity migrating with S-adenosylmethionine by subjecting the extracts to acid hydrolysis at 100 °C for 10 min before chromatography. This is known to hydrolyze S-adenosylmethionine to 5'-deoxy-5'-methylthioadenosine (7). As can be seen in Fig. 7, this treatment substantially reduced the radioactivity found with S-adenosylmethionine with a corresponding increase in the radioactivity recovered with 5'-deoxy-5'-methylthioadenosine.

Fig. 5. Influence of RA233 on [3H]methionine incorporation into platelets and their production of volatile and base-labile "H. Platelets from 30 ml of blood were resuspended in 6.7 ml of HEPES buffer (5 x 10⁸/ml) with 50 μCi of [3H]methionine and about 0.2 μCi of [14C]MeOH and incubated with the indicated concentration of RA233 for 60 min. The volatile "H/"C ratio before and after the platelet suspension was incubated at 37 °C with 125 mM Na₂CO₃, was determined. Additional samples were centrifuged and the percentage of the total "H that was found in the platelet pellet was determined. In samples from which platelets were omitted but which were otherwise processed in parallel, 0.26% of the "H was volatile, and this was not affected by the addition of RA233 or Na₂CO₃. This figure was subtracted from the data, which were then reexpressed as the percentage of total "H.

Table I shows that compounds commonly used as phosphodiesterase inhibitors have the same effect as RA233. Papaverine, dipyridamole, and isobutylmethylxanthine increased the incorporation of radioactive methionine into platelets, and inhibited both the production of methanol and base-labile methyl esters expressed in terms of the "H originally added. In this regard, papaverine was about 3 times as potent as...
RA233. Theophylline significantly increased the rate of production of methanol.

**DISCUSSION**

Blood platelets, like nucleated cells (1), actively incorporate methionine, from which they synthesize S-adenosylmethionine and the methyl donor for a variety of reactions including the methyl esterification of free carboxyl groups of proteins (8). This esterification nullifies the negative charge and hydrophilic nature of the carboxyl groups and can be reasonably predicted to have a substantial effect on the properties and function of proteins. The esters are unstable and hydrolyze spontaneously at neutral or alkaline pH, with the formation of methanol.

In the course of our investigation of the involvement of protein methylation in the regulation of platelet function, we examined the effects of a series of compounds known to inhibit platelet aggregation in vitro. These included dipyridamole (Persantine), which is used clinically as a coronary vasodilator and an inhibitor of platelet aggregation, and papaverine, a vasodilator and a spasmolytic. We found it convenient to do most of our studies with compound RA233, an analogue of dipyridamole which is more soluble than are the others in physiological salt solutions. Each is known to inhibit the cyclic AMP phosphodiesterases of platelets (9).

The addition of RA233 to platelets inhibited their production of methanol in a dose-related fashion and this inhibition was paralleled by a reduction in the base-labile methylation (detected either in whole platelets or in their proteins precipitated with trichloroacetic acid), and by a reduction in the amount of radioactivity in individual protein bands resolved on 16-BAC polyacrylamide gels, a procedure we have recently described for detecting base-labile protein methylation (6). There can thus be no doubt that RA233 inhibits protein methylation. Dipyridamole was about equiactive with RA233, and papaverine, which bears no structural relation to these two compounds, was about 3 times as potent. Isobutylmethylxanthine also inhibited protein methylation

We investigated the mechanism of this inhibition. It was not due simply to the inhibition of the uptake of the isotopically labeled methionine; indeed, each of these compounds substantially increased the incorporation of the methionine. It was not due to the accumulation within the platelets of cyclic nucleotides, since neither prostaglandin E1, a stimulator of the adenylate cyclase) nor sodium nitroprusside (a stimulator of the guanylate cyclase (10) potentiated the effect of the compounds. It was not due to an acceleration in the rate of hydrolysis of methyl esters, because this would have caused an increase rather than a decrease in the rate of [3H] methanol production, and it was not due to the accumulation of S-adenosylhomocysteine which might be brought about by the inhibition of S-adenosylhomocysteine hydrolase.

It would appear therefore that these drugs act (directly or indirectly)
Washed platelets (4 x 10⁹) in 50 μl of Hepes buffer were incubated with 30 μCi of [methyl-³H]methionine for 40 min with the indicated concentration of RA233. 50 μl of 16-BAC extraction buffer was then added and the sample was electrophoresed on a polyacrylamide gel at acid pH as described under "Materials and Methods." A photograph of the gel is shown on the right. Lane 0 is authentic calmodulin. Lane 2, 4, and 6 were no RA233 controls. Lanes 1, 3, 5, and 7 contained 0.1, 0.3, 1.0, and 3.0 mM RA233, respectively. The migration of molecular weight markers is shown in the center; they were (from the top down) β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and calmodulin (CDR).

Fig. 8. Effect of RA233 on protein methylation. Samples of washed platelets (4 x 10⁹) in 50 μl of Hepes buffer were incubated with 30 μCi of [methyl-³H]methionine for 40 min with the indicated concentration of RA233. 50 μl of 16-BAC extraction buffer was then added and the sample was electrophoresed on a polyacrylamide gel at acid pH as described under "Materials and Methods." A photograph of the Coomassie-stained gel is shown on the left, and an autoradiograph of the gel is shown on the right. Lane 0 is authentic calmodulin. Lane 2, 4, and 6 were no RA233 controls. Lanes 1, 3, 5, and 7 contained 0.1, 0.3, 1.0, and 3.0 mM RA233, respectively. The migration of molecular weight markers is shown in the center; they were (from the top down) β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and calmodulin (CDR).

TABLE 1

Effect of drugs on [³H]methionine metabolism

Washed platelets (3 x 10⁹/ml) were incubated with [³H]methionine (50 μCi/ml) and the indicated drug at 37 °C for 90 min. Duplicate samples were centrifuged and the radioactivity in the pellet was determined, and triplicate samples were taken for the determination of volatile radioactivity before and after treatment with 166 mM Na₂CO₃ for 10 min at 37 °C. The volatile radioactivity found in a parallel incubation to which no platelets were added has been subtracted from the results (0.94% ± 0.02%). * significance at p < 0.01 by unpaired two-tailed Student’s t test. In the absence of added compounds, platelets incorporated 7.5% of the radioactivity and the duplicate determinations of uptake did not differ from each other by more than 1% of total; these results were not analyzed statistically. Note that the volatile radioactivity is expressed as a percentage of the total radioactivity added to the platelets, not as a percentage of intracellular [³H].

| Concentration of addition | % H in pellet (control) | Volatile [³H] (% total ± 1 S.D.) | Free | % control | After Na₂CO₃ | % control |
|---------------------------|------------------------|---------------------------------|------|-----------|-------------|-----------|
| mM                        |                        |                                 |      |           |             |           |
| None                      |                        |                                 |      | 1.95 ± 0.04 | 1.95 ± 0.04 | 4.47 ± 0.09 | 4.47 ± 0.09 |
| Papaverine                |                        |                                 |      | 1.45 ± 0.01 | 1.45 ± 0.01 | 3.60 ± 0.06 | 3.60 ± 0.06 |
| 0.1                       |                        |                                 |      | 0.72 ± 0.08 | 0.72 ± 0.08 | 2.47 ± 0.02 | 2.47 ± 0.02 |
| 0.3                       |                        |                                 |      | 1.20 ± 0.11 | 1.20 ± 0.11 | 61*        | 61*        |
| RA233                     |                        |                                 |      | 0.75 ± 0.03 | 0.75 ± 0.03 | 38*        | 38*        |
| 0.3                       |                        |                                 |      | 1.20 ± 0.11 | 1.20 ± 0.11 | 61*        | 61*        |
| 1.0                       |                        |                                 |      | 1.20 ± 0.03 | 1.20 ± 0.03 | 61*        | 61*        |
| Dipyridamole              |                        |                                 |      | 1.93 ± 0.02 | 1.93 ± 0.02 | 3.78 ± 0.16 | 3.78 ± 0.16 |
| 0.1                       |                        |                                 |      | 2.20 ± 0.06 | 2.20 ± 0.06 | 67*        | 67*        |
| 0.3                       |                        |                                 |      | 2.20 ± 0.06 | 2.20 ± 0.06 | 67*        | 67*        |
| Theophylline              |                        |                                 |      | 2.44 ± 0.05 | 2.44 ± 0.05 | 3.96 ± 0.09 | 3.96 ± 0.09 |
| 1.5                       |                        |                                 |      | 2.58 ± 0.02 | 2.58 ± 0.02 | 4.84 ± 0.20 | 4.84 ± 0.20 |
| 5.0                       |                        |                                 |      | 4.96 ± 0.09 | 4.96 ± 0.09 | 111        | 111        |
| Isobutylmethoxyxanthine   |                        |                                 |      | 4.96 ± 0.09 | 4.96 ± 0.09 | 111        | 111        |
| 1.5                       |                        |                                 |      | 5.05 ± 0.08 | 5.05 ± 0.08 | 3.69 ± 0.04 | 3.69 ± 0.04 |
| 5.0                       |                        |                                 |      | 5.16 ± 0.01 | 5.16 ± 0.01 | 3.39 ± 0.09 | 3.39 ± 0.09 |

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Our demonstration that phosphodiesterase inhibitors inhibit protein methylation is of particular significance when compared with the finding that a variety of maneuvers used to inhibit the methylation of proteins and other substrates (often by causing the accumulation of S-adenosylhomocysteine or one of its analogues) greatly enhance the accumulation of cyclic AMP in intact cells, in part by inhibiting its hydrolysis (4, 11). It is not unreasonable, therefore, to raise the possibility that the activities of cyclic nucleotide phosphodiesterases in the intact cell might be influenced by protein methylation. Calmodulin is known to be both an excellent substrate for protein methylation (12), and to regulate the activity of cyclic nucleotide phosphodiesterases (13). We have detected the methylation of a protein in platelets co-migrating with authentic calmodulin, and this methylation is decreased by RA233. The available evidence suggests, however, that carboxymethylation of calmodulin decreases, rather than increases, its ability to potentiate the hydrolysis of cyclic GMP by phosphodiesterases (12).

That the experimental inhibition of methylation reactions is usually accompanied by increases in cellular cyclic AMP has cast a doubt over the ability of such experiments of establish a role for methylation reactions in the regulation of cellular processes (4). Our results admit to uncertainty in the opposite direction: it is possible that some of the effects of compounds traditionally attributed to their ability to inhibit cyclic nucleotide phosphodiesterases may in fact be due to their ability to inhibit protein methylation.

Macroscopic platelets were incubated with [³H]methionine (50 μCi/ml) and the indicated drug at 37 °C for 90 min. Duplicate samples were centrifuged and the radioactivity in the pellet was determined, and triplicate samples were taken for the determination of volatile radioactivity before and after treatment with 166 mM Na₂CO₃ for 10 min at 37 °C. The volatile radioactivity found in a parallel incubation to which no platelets were added has been subtracted from the results (0.94% ± 0.02%). * significance at p < 0.01 by unpaired two-tailed Student’s t test. In the absence of added compounds, platelets incorporated 7.5% of the radioactivity and the duplicate determinations of uptake did not differ from each other by more than 1% of total; these results were not analyzed statistically. Note that the volatile radioactivity is expressed as a percentage of the total radioactivity added to the platelets, not as a percentage of intracellular [³H].
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