ESTROGEN BINDING BY LEUKOCYTES DURING PHAGOCYTOSIS*

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Phagocytosis by neutrophilic polymorphonuclear leukocytes (PMNs) is associated with a burst of metabolic activity. Oxygen consumption is increased many fold (1), and much of the extra oxygen consumed is converted to H$_2$O$_2$ (2), largely through a superoxide anion (O$_2^-$) intermediate (3). Glucose oxidation via the hexosemonophosphate shunt (1), the reduction of nitroblue tetrazolium (4), the emission of light (chemiluminescence) (5), the conversion of iodide to a TCA-precipitable form (iodination) (6), and the degradation of the thyroid hormones (7, 8) by PMNs are all markedly elevated during phagocytosis. This burst of metabolic activity appears to be required for the destruction of ingested organisms by normal cells. The leukocytes of patients with chronic granulomatous disease (CGD) ingest particles normally; however, the associated metabolic burst is not seen (9) and these leukocytes do not kill certain microorganisms normally (10). As a result, repeated and severe infections are the hallmark of CGD.

This paper will describe an additional parameter of the phagocytosis-induced metabolic burst, the binding of estradiol. Binding is detected either by the conversion of estradiol to an alcohol-precipitable form or by autoradiographic localization. As with the other parameters of the metabolic burst, it is markedly impaired in CGD. The mechanisms involved in the binding of estradiol were investigated through the use of myeloperoxidase (MPO)-deficient PMNs, various inhibitors, and cell-free model systems.

Materials and Methods

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Abbreviations used in this paper: CGD, chronic granulomatous disease; G6PD, glucose 6-phosphate dehydrogenase; LPO, lactoperoxidase; MPO, myeloperoxidase; PMNs, polymorphonuclear leukocytes.
MPO was prepared from canine granulocytes (11) and lactoperoxidase (LPO) from bovine milk (12). Peroxidase activity was determined on the day of each experiment by the o-dianisidine method (13). Xanthine oxidase (10 mg/ml bovine milk, approximately 0.4 U/mg, suspended in 2.0 M ammonium sulfate-0.01 M EDTA) and catalase (bovine liver, 6.7 mg/ml; 62,900 U/mg) were obtained from Worthington Biochemical Corp., Freehold, N. J. Catalase was dialyzed against at least 1,000 volumes of water before use. Superoxide dismutase (bovine erythrocyte, 11,500 U/mg) was obtained from Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind. and glucose oxidase (type V, Aspergillus niger, 1,230 U/ml) from Sigma Chemical Co., St. Louis, Mo. Amino-1,2,4-triazole was obtained from Schwarz/Mann Div. Becton, Dickinson & Co., Orangeburg, N. J.

Preparation of Leukocytes. Venous blood was collected from healthy adult volunteers, five male patients (D. G., B. B., T. P., R. C., and D. C.) with CGD, the mother (L. C.) of two of these patients, one patient (B. W.) with a variant of CGD, familial lipochrome histiocytosis (14), two male siblings (D. J., T. J.) with severe leukocytic glucose 6-phosphate dehydrogenase (G6PD) deficiency (15) and their mother (E. J.), and one female (J. F.) and one male (B. F.) sibling with hereditary MPO deficiency. The criteria for diagnosis in these patients were considered in a previous paper (16). Blood drawn in syringes moistened with heparin (final concentration 20 U/ml) was used without further treatment in the autoradiographic studies. For other studies, the leukocytes (65-95% PMNs) were isolated by dextran sedimentation and hypotonic lysis of erythrocytes (17) and suspended in 0.154 M sodium chloride to a final concentration of 5 x 10⁶ PMNs/ml.

Conversion of Estradiol to an Alcohol-Precipitable Form. Unless otherwise indicated a mixture of radiolabeled and carrier estradiol (6 nmol; 0.05 μCi) dissolved in ethanol (0.055 ml) was evaporated to dryness under nitrogen in polystyrene 12 × 75 mm test tubes and the components indicated in the legends added. In whole cell experiments, the tubes were tumbled end over end 25 times a minute at 37°C in a Fisher Rotorack (Fisher Scientific Co., Chicago, Ill.), whereas in experiments in which isolated enzymes were employed, the tubes were incubated at 37°C in a water-bath shaker oscillating 80 times a minute. The reaction was stopped by the addition of 1.0 ml of absolute ethanol, and the tubes were placed in an ice bath until filtration. The precipitate was collected on a Whatman no. 115 filter paper (2.4 cm diameter) in an E-SB Precipitation Apparatus (Tracerlab Div., LFE Electronics, Richmond, Calif.) and washed with 10 ml of absolute ethanol. The filter paper was placed in a liquid scintillation vial and 0.5 ml of Nuclear Chicago Solubilizer (Nuclear-Chicago Corp., Des Plaines, Ill.) was added. The vials were kept at room temperature overnight, 20 ml of a 2,5-diphenyloxazole-1,4-bis-(4-methyl-5-phenyloxazolyl)-benzene-ethanol fluor (18) added, and the vials counted in a liquid scintillation counter.

Autoradiographic Localization of Estradiol. Tritiated estradiol (5-10 μCi) was evaporated to dryness under nitrogen in a 10 × 75 mm siliconized test tube. Heparinized whole blood (0.25 ml) and the other components indicated in the text were added (total vol 0.26 ml) and the mixture was incubated at 37°C in a water-bath shaker oscillating 80 times/min. Cover slip smears prepared at intervals were fixed in methanol for 4 min and washed in water for 1 h to remove water-soluble components. The cover slips were mounted with methacrylate on subbed slides with the smear exposed. Subbing was performed by dipping the slides in a filtered subbing solution consisting of 5 g of gelatin and 0.5 g of chromium potassium sulfate per liter. The smears were coated with Kodak NTB2 emulsion (one part emulsion plus two parts of 1% Kodak Photo-Flo 200 in water; Eastman Kodak Co., Rochester, N. Y.) using the dipping machine of Kopriwa (19). After appropriate exposure at 6°C, the autoradiograms were developed and the smears stained with a mixture of methylene blue and azure II (20).

Statistical Analyses. Statistical differences were determined using Student's t-test (not significant, P > 0.05). Instances in which experimental values were compared to paired controls are indicated (paired analysis); otherwise, comparisons were of independent mean values. Each n is the mean of duplicate values obtained in the same experiment.

Results

Intact Cells. Estradiol was converted to an alcohol-precipitable form on incubation with intact PMNs and preopsonized zymosan under the conditions employed in Table I. Little or no conversion occurred in the absence of either preopsonized zymosan or PMNs or when the leukocytes were preheated at 100°C for 15 min. Replacement of preopsonized with unopsonized zymosan resulted in a
 TABLE I

| Supplements                              | Estradiol conversion (pmol) |
|------------------------------------------|-----------------------------|
| PMNs + preopsonized zymosan             | 753 ± 38 (22)*              |
| PMNs                                     | 6 ± 2 (4)                   |
| Preopsonized zymosan                    | 22 ± 8 (3)                  |
| PMNs (heated) + preopsonized zymosan    | 20 ± 2 (3)                  |
| PMNs + zymosan (unopsonized)            | 31 ± 8 (3)                  |
| PMNs + zymosan + serum                  | 644 ± 46 (7)                |
| PMNs + serum                             | 28 ± 4 (3)                  |
| PMNs + preopsonized *L. plantarum*      | 368 ± 102 (3)               |
| PMNs + *L. plantarum* (unopsonized)     | 14 ± 4 (3)                  |
| PMNs + *L. plantarum* + serum           | 633 ± 124 (3)               |

The complete system contained 4 mM sodium phosphate buffer pH 7.4, 0.128 M NaCl, 12 mM KCl, 1 mM CaCl₂, 2 mM MgCl₂, 2 mM glucose, 12 μM [³⁵S]-estradiol (0.05 μCi; 6,000 pmol) and the supplements indicated below as follows: PMNs, 1.25 × 10⁶; preopsonized or unopsonized zymosan, 0.5 mg; pooled human serum, 0.05 ml; *L. plantarum, 2.4 × 10⁶ organisms. The PMNs were heated at 100°C for 15 min where indicated. Total vol, 0.5 ml. Incubation period, 30 min.
* Mean ± SE of (n) experiments.

loss of activity which was restored by the addition of serum. Serum was ineffective in the absence of zymosan. Heat-killed *L. plantarum* could replace zymosan as the ingestible particle. In Table I, [³⁵S]-estradiol in ethanol was evaporated to dryness in the test tube before the addition of the other components. Comparable results were obtained when estradiol, suspended in 0.154 M NaCl with a motor driven teflon-glass homogenizer, was employed without evaporation.

The binding of estradiol by phagocytosing PMNs was confirmed by autoradiographic studies. No binding of estradiol was observed when normal blood was incubated with tritiated estradiol in the absence of bacteria (Fig. 1 a). However when heat killed *L. acidophilus* was added, silver grains were seen overlying neutrophils (Fig. 1 b) and to a lesser degree monocytes (Fig. 1 c) and eosinophils which contained ingested organisms. No silver grains were seen over erythrocytes, lymphocytes (Fig. 1 b), or extracellular bacteria.

Conversion of estradiol to an alcohol-precipitable form by PMNs and either preopsonized zymosan or zymosan plus serum was increased by superoxide dismutase at a concentration of 5 μg/ml but not by the heated enzyme (120°C, 20 min) (Table II). Hypoxia induced by gassing with nitrogen markedly reduced the binding of estrogen by the PMN-preopsonized zymosan system [pmoles bound: air, 593 ± 8 (SE), n = 3; N₂, 98 ± 42 (SE), n = 3; P < 0.001].

Fig. 2 demonstrates the effect of azide, cyanide, aminotriazole, or catalase on the conversion of estradiol to an alcohol-precipitable form by the PMN-preopsonized zymosan system over a range of PMN concentrations. Estradiol binding at each PMN concentration is designated as 0 (dotted line), and the percent change caused by the addition of the supplements shown. Azide (1 mM) significantly inhibited binding at low PMN levels (1.25 × 10⁵–1.25 × 10⁶) but had no effect when 2.5 × 10⁶ PMNs were added and significantly increased binding when the PMN level was raised to 5 × 10⁶. Similar, although less striking, findings were obtained with 1 mM cyanide, i.e., cyanide was inhibitory at low and stimulatory
Table II

Effect of Superoxide Dismutase

| Supplements      | Estrogen conversion (pmol) | PMNs + preopsonized zymosan | PMNs + zymosan + serum |
|------------------|---------------------------|----------------------------|------------------------|
| None             | 886 ± 106 (7)*            | 702 ± 40 (5)                |                        |
| SOD              | 965 ± 106 (7)             | 926 ± 49 (5)                | <0.002                 |
| Heated SOD       | 858 ± 119 (6)             | NS                          | 712 ± 52 (5)           |

The reaction mixture was as described in Table I except that 2.5 μg of superoxide dismutase (SOD) (29 U) or SOD heated at 120°C for 20 min was added where indicated.

* Mean ± SE of (n) experiments.
‡ Significance level of difference from no SOD determined by paired analysis.

at high PMN concentrations. Aminotriazole increased the binding of estradiol throughout the range of PMN concentrations employed, with the percent increase being greatest at low cell levels, whereas catalase (67 μg/ml) inhibited binding at all but the highest PMN concentration employed, where no significant effect was observed.

Neutrophils defective in oxidative metabolism had a markedly reduced capacity for the phagocytosis-induced conversion of estradiol to an alcohol-precipitable form. Fig. 3 demonstrates this defect in five male patients with CGD, one female patient with familial lipochrome histiocytosis, and two male siblings with severe leukocytic G6PD deficiency. Binding in these patients was less than 5% of normal. Intermediate values were observed when the leukocytes of the mother of two male siblings with CGD (L. C.) and the mother of three male siblings with G6PD deficiency (E. J.) were employed as would be expected from the x-linked nature of the disease in these families.

The decreased binding of estradiol by leukocytes defective in oxidative metabolism was confirmed by autoradiographic techniques (Fig. 4). No silver grains were seen over neutrophils from patients with CGD which contained ingested organisms (Fig. 4a) and two populations of leukocytes, one with and one without overlying silver grains, were found in the blood of a CGD carrier (Fig. 4b). Silver grains were also absent in severe leukocytic G6PD deficiency (Fig. 4c).

Fig. 5 compares the conversion of estradiol to an alcohol-precipitable form by normal and MPO-deficient leukocytes over a range of PMN concentrations. With normal cells, estradiol conversion increased with an increase in PMN concentration to a maximum at about 2.5 × 10⁶ PMNs/0.5 ml reaction mixture. Conversion was significantly reduced when the cells of two siblings with MPO deficiency were employed, although conversion was observed at high PMN concentrations. The decreased binding of estradiol by MPO-deficient leukocytes was also demonstrated autoradiographically (Figure 4d).

Cell-Free Systems. Table III demonstrates the conversion of estradiol to an
alcohol-precipitable form by a system consisting of MPO, glucose, glucose oxidase, and zymosan and the requirement for each component of the system for optimum activity. Heat treatment (100°C, 15 min) of either MPO or glucose oxidase abolished conversion. MPO could be replaced by LPO; zymosan by either preopsonized zymosan, heat-killed *L. plantarum*, or to a lesser degree albumin; and glucose and glucose oxidase by either reagent *H₂O₂* or the xanthine oxidase system. Superoxide dismutase had no effect on the conversion of estradiol to an alcohol-precipitable form over a range of MPO concentrations when the glucose oxidase system was employed as the source of *H₂O₂* (Fig. 6). However, when the xanthine oxidase system was used, superoxide dismutase significantly increased estradiol conversion at the relatively high MPO levels.

Catalase at a concentration of 335 μg/ml inhibited estradiol conversion by the MPO-*H₂O₂*-zymosan system (Table IV). However, when *H₂O₂* was generated continuously and at low concentration by glucose and glucose oxidase, catalase significantly increased conversion to an alcohol-precipitable form (*P < 0.001*). A small amount of estradiol conversion was produced by the catalase-glucose-glucose oxidase system in the absence of MPO, presumably due to the peroxidatic activity of catalase under these conditions; however, the combined effect of catalase and MPO was considerably greater than additive.

Fig. 7 demonstrates the effect of azide, cyanide, and aminotriazole, on estradiol conversion by either the MPO- or catalase-glucose-glucose oxidase system at pH 7.0. When MPO was employed, conversion of estradiol to an alcohol-precipitable form was strongly inhibited by azide and cyanide, whereas aminotriazolc
at concentrations ranging from $10^{-2}$ to $10^{-4}$ M consistently increased conversion.
In contrast, azide had a marked stimulatory effect on estradiol conversion by the catalase-dependent system, whereas cyanide and aminotriazole had no effect under the conditions employed. In the absence of azide, conversion by the catalase system was low at neutral or alkaline pH and increased sharply as the pH was decreased to 4.5. Azide stimulated the conversion of estradiol to an alcohol-precipitable form at pH levels above 6.0–6.5, whereas at the more acid pH levels, azide was strongly inhibitory (Fig. 8).

Discussion
Estradiol was converted to an alcohol-precipitable form by leukocytes during phagocytosis and autoradiographic studies revealed the presence of the bound estrogen largely in neutrophils. Silver grains were also seen over the monocytes and eosinophils which contained ingested particles, but were not present over lymphocytes or erythrocytes.

Estradiol binding by intact PMNs was dependent on the phagocytosis-induced respiratory burst. It was inhibited by hypoxia and did not occur in leukocytes defective in oxidative metabolism, i.e., from patients with CGD and related conditions. The respiratory burst in PMNs is associated with the reduction of oxygen first to the superoxide anion and then to H$_2$O$_2$. The binding of estradiol by PMNs was stimulated by superoxide dismutase, an enzyme which catalyzes the conversion of the superoxide anion to oxygen and H$_2$O$_2$, and, except at the highest PMN concentration employed (Fig. 2), was inhibited by catalase, an
FIG. 5. Comparison of normal and MPO-deficient leukocytes. The reaction mixture was as described in Table I (PMNs plus preopsonized zymosan) except that the number of PMNs added was varied as indicated and either normal (O—O) or MPO deficient (I. F. Δ—Δ, n 4–7; B. F. ▲—▲, n 1–3) PMNs were employed. The number of experiments and the mean ± 1 SD are shown for normal PMNs. The significance of the difference between the MPO deficient and normal PMNs is shown for each patient except where n = 1.

enzyme which degrades H₂O₂. This suggests that H₂O₂ is the product of the respiratory burst required for the binding of estradiol. Estradiol binding by PMNs during phagocytosis can be added to the list of laboratory procedures which may be used for the diagnosis of CGD. The carrier state is revealed by intermediate levels of estradiol conversion to an alcohol-precipitable form and, as with nitroblue tetrazolium reduction (21, 22) and iodination (23), two populations of cells can be demonstrated cytochemically in the heterozygote, one which binds estradiol and one which does not (Fig. 4 b).

MPO catalyzes the oxidation of a variety of substances by H₂O₂ and therefore might be expected to participate with H₂O₂ in the conversion of estradiol to an alcohol-precipitable form. Evidence was obtained which suggested that MPO-dependent binding occurred in intact PMNs. Azide and cyanide are potent inhibitors of peroxidase-catalyzed reactions and these agents inhibited binding at relatively low PMN concentrations (Fig. 2). Binding by the leukocytes of two patients with MPO deficiency was below that of normal cells as measured either by the conversion of estradiol to an alcohol-precipitable form or by the autoradiographic technique. Finally, a highly purified preparation of MPO catalyzed the conversion of estradiol to an alcohol-precipitable form in the presence of reagent H₂O₂ or a H₂O₂ generating system (glucose plus glucose oxidase, xanthine plus
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Table III

| Supplements | Estradiol conversion (pmol) |
|-------------|----------------------------|
| MPO + glucose + GO + zymosan | 439 ± 25 (14)* |
| MPO omitted | 19 ± 5 (4) |
| Glucose omitted | 34 ± 17 (4) |
| GO omitted | 29 ± 13 (4) |
| Zymosan omitted | 62 ± 17 (4) |
| MPO heated | 19 ± 6 (4) |
| GO heated | 31 ± 17 (4) |
| MPO omitted, LPO added | 818 ± 47 (7) |
| Glucose + GO omitted, H₂O₂ added | 537 ± 56 (3) |
| Glucose + GO omitted, xanthine + XO added | 429 ± 52 (3) |
| Zymosan omitted, preopsonized zymosan added | 725 ± 103 (3) |
| Zymosan omitted, L. plantarum added | 398 ± 96 (3) |
| Zymosan omitted, albumin added | 122 ± 1 (3) |

The reaction mixture contained 10 mM sodium phosphate buffer pH 7.0, 12 μM ¹⁴C-estradiol (0.5 μCi, 6,000 pmol), water to a final vol of 0.5 ml and the supplements indicated below as follows: MPO, 40 mU; glucose, 10 mM; glucose oxidase, 0.12 U (0.6 μg); zymosan (unopsonized or preopsonized, 0.5 mg; LPO, 40 mU; H₂O₂, 0.1 mM; xanthine, 0.1 mM; xanthine oxidase, 2 mU (5 μg); L. plantarum, 2.5 × 10⁸ organisms; albumin, 0.5 mg.

* Mean ± SE of (n) experiments.

As in the intact cell, at least at low PMN concentrations, estradiol conversion by the MPO system was inhibited by azide and cyanide and was stimulated by aminotriazole and superoxide dismutase. Superoxide dismutase was effective with the xanthine oxidase but not the glucose oxidase system as the source of H₂O₂. The xanthine oxidase system generates both the superoxide anion and H₂O₂, whereas only H₂O₂ has been detected as a product of the glucose oxidase system.

Although these studies support a role for MPO in the binding of estradiol by PMNs, they also suggest the involvement of MPO-independent systems. At high PMN concentrations, azide and to a lesser degree cyanide, stimulated binding by intact PMNs despite their inhibitory effect on the MPO-catalyzed reaction. Further, binding of estradiol by MPO-deficient leukocytes increased with an increase in the cell concentration, although it remained below that of normal cells (Fig. 5). Eosinophils contain their normal complement of peroxidase in hereditary MPO deficiency (24), and thus binding by MPO-deficient (or normal) PMNs may be due in part to a peroxidase-dependent mechanism in these cells. Catalase also catalyzed the conversion of estradiol to an alcohol-precipitable form in the presence of a H₂O₂ generating system at acid pH. At neutral or alkaline pH, conversion by the catalase system was stimulated by azide, as was binding by intact cells at high cell concentrations. Azide combines reversibly mole for mole with catalase iron (25). When H₂O₂ is added to the catalase-azide complex, the heme iron is reduced from the trivalent to the bivalent state (25) and the azide is oxidized to nitrous oxide, nitric oxide, and nitrogen (26, 27). It is not clear how this sequence of events would lead to increased estradiol fixation.
The type of binding of estradiol described here appears to be similar to the covalent binding of estradiol to certain estrogen-sensitive tissues and can be distinguished from the high affinity noncovalent binding to specific receptors (28). Tchernitchin (29, 30) has distinguished two types of binding in the rat uterus, one to the cytoplasmic and nuclear receptors of a number of uterine cell types and the other to uterine eosinophils. Extraction with water removed the bound estrogen from the former but not the latter (30). Cowan et al. (31) have also distinguished between noncovalent and covalent binding to macromolecules in rabbit uterine preparations, with the latter having the properties of the
peroxidase-catalyzed reaction. H$_2$O$_2$ can increase the in vitro binding of estradiol to uterine tissue through covalent bonds at sites (eosinophil granules, cytoplasm of epithelial cells) which stain for peroxidase (32, 33). Finally, photo-induced covalent attachment of estrogen to uterine proteins or albumin unrelated to steroid binding to specific receptors has been described (34).

Estrogens can stimulate a number of peroxidase-catalyzed reactions by acting as an oxidation-reduction catalyst (35, 36). The estrogen is oxidized by peroxidase and H$_2$O$_2$ probably to the phenoxy radical and the latter can be reduced to the original estrogen by an electron donor whose oxidation is thus stimulated. In the absence of an appropriate electron donor or after its complete oxidation, irreversible inactivation of the estrogen occurs (37, 38). When albumin or other proteins are present in the reaction mixture under these conditions, a water-soluble, ether-insoluble conjugate is formed with the estrogen, involving a strong chemical bond (39-41). Conjugates may also be formed with tyrosine or tyrosine peptides (41, 42), thiols (39, 40, 43), polynucleotides (44), and possibly tryptophane (39, 45), although there are differences in the mechanism of conjugation and in the type of bond formed (40, 43, 44). The studies reported here suggest that estradiol is oxidized in phagocytosing PMNs, in part by MPO and H$_2$O$_2$, with attachment to macromolecules by covalent bonds. Thus PMNs may contribute to the inactivation of estrogens in vivo, particularly during bacterial infection where increased oxidation by phagocytosing PMNs would be expected.
Fig. 8. Effect of pH on estradiol conversion by the catalase-dependent system in the presence and absence of azide. The reaction mixture was as described in Table III except that MPO was replaced by catalase (8,000 U; 168 μg) and the pH was varied as indicated using either 10 mM sodium phosphate (○, ●) or 60 mM sodium lactate (△, ▲) buffer. Estradiol conversion was determined in the presence (open symbols) or absence (closed symbols) of 10 mM sodium azide. The pH was measured at the beginning of the incubation and the actual pH is shown.

MPO-catalyzed reactions contribute significantly to the antimicrobial activity of the PMN (46). Although effects of estrogens on some aspects of neutrophil function have been reported (see reference 47), it is not known whether MPO-mediated reactions are affected by estrogens in situ. The oxidation of estradiol by PMNs during phagocytosis raises this possibility.

Summary

Estradiol binds covalently to normal leukocytes during phagocytosis. The binding involves three cell types, neutrophils, eosinophils, and monocytes and at least two reaction mechanisms, one involving the peroxidase of neutrophils and monocytes (myeloperoxidase [MPO]) and possibly the eosinophil peroxidase, and the second involving catalase. Binding is markedly reduced when leukocytes from patients with chronic granulomatous disease (CGD), severe
leukocytic glucose 6-phosphate dehydrogenase deficiency, and familial lipo-
chrome histiocytosis are employed and two populations of neutrophils, one
which binds estradiol and one which does not, can be demonstrated in the blood
of a CGD carrier. Leukocytes from patients with hereditary MPO deficiency also
bind estradiol poorly although the defect is not as severe as in CGD. These
findings are discussed in relation to the inactivation of estrogens during infec-
tion and the possible role of estrogens in neutrophil function.

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