Xanthine Oxidase

VI. INFLUENCE OF pH ON SUBSTRATE SPECIFICITY*

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Milk xanthine oxidase exhibits a broad substrate specificity that includes purines, aldehydes, and pteridines (1). The mechanism of substrate oxidation, particularly that of purines, has been the subject of several studies (2-4). The present report concerns the observation of a new class of substrates, quaternary heterocyclic compounds, which are oxidized at pH values above 9.6. A partial list of substrates that are oxidized at pH 7.8 and 10.8 is given in Table I. The data to be presented indicate that all substrates are oxidized at the same active center and binding site and support the view that quaternary compounds become substrates owing to the titration of a group on the enzyme that normally prevents their approach to the substrate-binding site.

These findings should provide additional information on which to base a reaction mechanism, and they will be useful in attempts to establish the nature of the substrate-binding site.

EXPERIMENTAL PROCEDURE

Enzyme—Milk xanthine oxidase, obtained from Worthington Biochemical Corporation, was further purified either by the method of Fridovich (6) or by that of Rajagopalan and Handler.1 As prepared by the latter procedure, the enzyme showed two minor impurities in the ultracentrifuge, one heavier and one lighter than the main component. The specific activity (8) varied from 12.5 to 15.0.

Substrates—Xanthine and hypoxanthine were obtained from California Corporation for Biochemical Research, purine from Mann Research Laboratories, Inc., acetaldehyde from Mallinckrodt Chemical Works, salicylaldehyde from Fisher Scientific Company, and benzaldehyde from Eastman Organic Chemicals Department (Eastman Kodak Company). N1-Methylnicotinamide iodide was obtained from Merek and Company, Inc., and from Sigma Chemical Company and was converted to the chloride by passage through Dowex 1-chloride. N-Methylquinolinium chloride, from Eastman Organic Chemicals Department, was recrystallized according to Lund and Wise (7); its extinction in absolute methanol agreed with that reported by Sarett, Perlzweig, and Levy (9), was kindly provided by Dr. J. G. Joshi. Attempts to purify 1-(4-pyridyl)pyridinium chloride hydrochloride from Eastman Organic Chemicals Department by vacuum sublimation (10) were unsuccessful, and it was used without further purification. The absorption spectrum agreed with the data reported by Bak and Christenson (11).

Assay Methods—All assays at pH 7.8 were conducted in 0.05 m potassium phosphate containing 0.005% Versene Fe-3 (Bershworth Chemical Company); assays in the pH range 9.4 to 11.0 were conducted in 0.1 m sodium carbonate containing 10-4 m EDTA. A Gilford model 2000 absorbance recorder equipped with a thermostatted cell holder set at 25° and a Beckman DU monochromator was used for most spectrophotometric studies. A Cary model 14 spectrophotometer was employed to obtain absorption spectra and for assays when the absolute optical density of the sample exceeded 2.0. Purine oxidation was most conveniently followed by measuring the rate of reduction of cytochrome c (Sigma Chemical Company, Type III) at 550 mλ. At pH 9.6 or 10.8, the change in absorbance at 246 mλ could also be used, since xanthine and hypoxanthine are isosbestic at that wave length and uric acid absorption differs only slightly. The difference in extinction coefficients was 6.1 x 104 liters per mole per cm. Determinations of Km by both methods were in agreement. Hypoxanthine oxidation was assayed at 281 mλ, where xanthine and uric acid are isosbestic. Xanthine oxidation was followed at 290 mλ for pH 7.8, or at 300 mλ for pH 9.4 to 11.0. Acetaldehyde oxidation rates were measured by cytochrome c reduction. Salicylaldehyde oxidation was followed at 290 mλ for pH 7.8, and at 295 mλ for pH 9.6 and 10.8. Benzaldehyde oxidation was followed by measuring cytochrome c reduction. However, at pH 10.8, benzaldehyde reacted with both cytochrome c and xanthine oxidase and thereby inhibited the observed catalysis. At pH 8 to 9.6, the inhibitory reaction was sufficiently slow to permit determinations of the Km value if cytochrome c and enzyme were added last, in rapid succession. At pH 10.8, Km determinations were not feasible; instead, the Ki value for benzaldehyde as a competitive inhibitor was measured. This inhibition is discussed in detail in the following report (12).

N1-Methylnicotinamide oxidation was followed at 300 mλ (13). Oxidation of N-methylquinolinium and N-ethylquinolinium were followed by cytochrome c reduction, or by the change in absorbance at 270 mλ. Fig. 1 shows the absorption spectra of N-ethylquinolinium chloride and of its oxidation product. For convenience, the oxidation product was obtained in quantity by using rabbit liver aldehyde oxidase, for which we are grateful.

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to Dr. K. V. Rajagopalan. The product so obtained was identical with that formed by xanthine oxidase activity, and the spectra of Fig. 1 were essentially identical with those observed with the N-methyl homologue. Trigonelline oxidation was measured at 300 m\(\mu\). The absorption spectrum of the product is shown in Fig. 2; the extinction change at 300 m\(\mu\) was \(5.8 \times 10^{-3}\) m per cm. The oxidation of 1-(4-pyridyl)pyridinium chloride hydrochloride was conveniently followed at 290 m\(\mu\). The absorption spectra of this compound and the product of its oxidation by xanthine oxidase are shown in Fig. 3. For \(K_m\) determinations at pH 9.4 to 11.0, the Na\(_2\)CO\(_3\) buffer was titrated to the desired pH value with HCl; a Beckman model 76 pH meter with a general purpose glass electrode standardized to pH 10.0 was used, and allowance was made for the sodium ion correction. In some cases, NaCl was added to maintain the desired ionic strength; however, the ionic strength was never high enough to affect the pK of the buffer. The carbonate buffers were adjusted to the desired pH at the beginning of the working day; these pH values were stable for about 24 hours.

**Table I**

<table>
<thead>
<tr>
<th>Substrates oxidized by xanthine oxidase</th>
<th>pH 7.8</th>
<th>pH 10.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purine</td>
<td>Purine</td>
<td></td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>Hypoxanthine</td>
<td></td>
</tr>
<tr>
<td>Xanthine</td>
<td>Xanthine</td>
<td></td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>Acetaldehyde</td>
<td></td>
</tr>
<tr>
<td>Salicylaldehyde</td>
<td>Salicylaldehyde</td>
<td></td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>Benzaldehyde</td>
<td></td>
</tr>
<tr>
<td>N*-Methylnicotinamide</td>
<td>N*-Methylnicotinamide</td>
<td></td>
</tr>
<tr>
<td>N-Methylnicotininilium</td>
<td>N-Methylnicotininilium</td>
<td></td>
</tr>
<tr>
<td>N-Ethylquinolinilium</td>
<td>N-Ethylquinolinilium</td>
<td></td>
</tr>
<tr>
<td>N-Methylnicotinic acid</td>
<td>N-Methylnicotinic acid</td>
<td></td>
</tr>
<tr>
<td>1-(4-Pyridyl)pyridinium</td>
<td>1-(4-Pyridyl)pyridinium</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Absorption spectra of N-ethylquinolinium (A) \(1.1 \times 10^{-4}\) m in 0.05 m potassium phosphate, pH 7.8, and of the product of its oxidation (B) by aldehyde oxidase.

**Fig. 2.** Absorption spectrum of trigonelline 6-pyridone, \(5 \times 10^{-5}\) m in 0.1 n Na\(_2\)CO\(_3\), pH 10.8.

**Fig. 3.** Absorption spectrum of 1-(4-pyridyl)pyridinium (lower peaks) and of the product of its oxidation by xanthine oxidase (upper peak) each at \(10^{-4}\) m in 0.1 n Na\(_2\)CO\(_3\), pH 10.8.
The reactions catalyzed by the two enzymes when acting on liver aldehyde oxidase are quite similar to those of xanthine oxidase. Both oxidize a variety of aldehydes, but aldehyde oxidase exhibits no activity with the usual purine substrates. Xanthine oxidase exhibits no activity with the usual purine substrates. Oxidase-The flavin, iron, and molybdenum contents of rabbit liver aldehyde oxidase were determined by Dr. J. G. Joshi. The absorption spectrum of the product of 1-(4-pyridyl)pyridinium chloride hydrochloride was distinctively different from that of xanthine oxidase, allowing for the difference in solvents.

Comparison of Milk Xanthine Oxidase with Liver Aldehyde Oxidase—The flavin, iron, and molybdenum contents of rabbit liver aldehyde oxidase (13) are quite similar to those of xanthine oxidase (19). Both oxidize a variety of aldehydes, but aldehyde oxidase exhibits no activity with the usual purine substrates with xanthine oxidase. Judging from the reaction products, the reactions catalyzed by the two enzymes when acting on quaternary substrates are quite similar. However, aldehyde oxidase can accomplish the latter reactions at pH 7.8, whereas the related xanthine oxidase activity is only evident at high pH. Since similar enzymes catalyze the same reactions with both aldehydes and quaternary compounds, it seems possible that they do so by similar mechanisms. Hence, the apparent differences in substrate specificity might well relate to differences in their substrate-binding sites. In addition to the differences cited above, it is noteworthy that trigonelline is not oxidized by aldehyde oxidase but, at high pH, can be oxidized by xanthine oxidase. Thus, the negatively charged carboxyl group of trigonelline may be repelled by the binding site of aldehyde oxidase, but not from that of xanthine oxidase. Differences in size and shape of the substrate-binding sites of the two enzymes are also evident. Phenazine methosulfate is an excellent substrate for aldehyde oxidase, but it is not oxidized by xanthine oxidase. Similarly, the comparison of $K_m$ values for N-methyl- and N-ethylquinolinium shown in Table II indicates that the addition of a methylene group in a critical position markedly lowers the binding affinity. In contrast, aldehyde oxidase oxidizes N-ethylquinolinium chloride readily (21). It is, therefore, surprising that 1-(4-pyridyl)pyridinium chloride is bound and oxidized by xanthine oxidase, since the pyridyl group attached to the quaternary nitrogen is much larger than an ethyl group. Nevertheless, the $K_m$ value for this compound is considerably lower than that for N-ethylquinolinium chloride. Since 1-(4-pyridyl)pyridinium chloride probably cannot bind to the enzyme with its 2-position available for reaction, it may bind with a different orientation from the other quaternary compounds, presenting its 4-position for oxidative attack. Since aldehyde oxidase also attacks the 4-position of this substrate, it is likely that this mode of binding is common for both enzymes.

Influence of pH on $K_m$ of Various Substrates—Table II lists the substrates tested together with their $K_m$ values determined at three different pH values: 7.8, at which the enzyme is normally studied; 9.6, the lower limit of observable activity with quaternary substrates; and 10.8. For both purines and aldehydes, $K_m$ values increased over the entire range with increasing pH.
whereas the $K_m$ values of the quaternary compounds decreased as the pH was raised. Since the quaternary compounds and aldehydes undergo no change in ionization in this pH range, the observed changes in $K_m$ must reflect changes in the enzyme itself. In fact, the magnitude of change in $K_m$ of the purines and quaternary compounds, except trigonelline and 1-(4-pyridyl)pyridinium chloride, was approximately equivalent from pH 9.6 to 10.8, but opposite in direction. The fact that the purines ionize to negatively charged compounds within this pH range is probably responsible for some of the apparent discrepancies. Purine and hypoxanthine dissociate with a $pK_a$ value of 8.9, while xanthine exhibits a $pK_a$ at 7.4, and another at 11.1 (22). The effects of these ionizations on the $K_m$ value have not been carefully studied, but coupled with the changes in the enzyme itself, they make the variation of $K_m$ with pH more complex than is the case with quaternary compounds or simple aldehyde substrates. The $K_m$ for salicylaldehyde, which is largely un-ionized at pH 7.8, partly ionized at pH 9.6, and completely ionized at pH 10.8, undergoes a large change from pH 9.6 to 10.8. Benzaldehyde, an un-ionized substrate, exhibits only a small change in binding affinity. Acetaldehyde shows little or no change in $K_m$ from pH 7.8 to 10.8, its stability in this pH range may be due partly to its being un-ionized and partly to its being a much smaller molecule than the other substrates. The anomalous behavior of trigonelline and 1-(4-pyridyl)pyridinium chloride remain unexplained.

**Variation of $V_{max}$ with pH**—A careful study of the variation of $K_m$ with pH was made for xanthine and $N^1$-methylnicotinamide from pH 9.4 to pH 11.0, at constant temperature and ionic strength. Fig. 4 shows a series of double reciprocal plots for xanthine, and Fig. 5 presents a similar series for $N^1$-methylnicotinamide. $K_m$ values were calculated from the data of Figs. 4 and 5 and were plotted against pH, as shown in Fig. 6. The $V_{max}$ value was constant over this pH range for both substrates, as was the case with all other substrates studied. It is apparent, therefore, that the changes occasioned in the enzyme by high pH are related only to changes in the substrate-binding affinity.

**Fig. 4.** Influence of pH on the rate of oxidation of xanthine by xanthine oxidase. Data are shown as a double reciprocal plot for the pH range 9.4 to 11.0, each line corresponding to the pH stated. Reaction rates were determined by following the change in absorbance at 300 m$\mu$, and were corrected for the pH variation in total extinction change from xanthine to uric acid. Ionic strength was held constant at 0.079 by addition of appropriate amounts of NaCl. Temperature was 25°. Sodium carbonate buffer, 0.03 M, containing 6 X $10^{-4}$ M EDTA was used at each pH. All pH values were corrected for sodium ion effect.

**Fig. 5.** Effect of pH on the rate of oxidation of $N^1$-methylnicotinamide by xanthine oxidase. Data are shown as a double reciprocal plot for the pH range 9.4 to 11.0, each line corresponding to the pH stated. Reaction rates were measured by following the change in absorbance at 300 m$\mu$. Ionic strength was held constant at 0.079 by addition of NaCl. Temperature was 25°. Sodium carbonate buffer, 0.03 M, containing 6 X $10^{-4}$ M EDTA was present in all incubations. All pH values were corrected for sodium ion effect.

**Fig. 6.** Competitive inhibition by $N$-methylquinolinium of the xanthine oxidase-catalyzed oxidation of xanthine. The reaction was followed at 290 m$\mu$, where $N$-methylquinolinium and $N$-methyl-2-quinolone are isosbestic. The reaction mixture contained 0.1 N Na$_2$CO$_3$, pH 10.8, EDTA, and 10$^{-4}$ M xanthine (O—O); and the inhibited system contained, in addition, 6 X $10^{-4}$ M $N$-methylquinolinium chloride (●—●).

With increasing pH, a change occurs at the substrate-binding site that permits the enzyme to bind positively charged, quaternary compounds.

**Excess Substrate Inhibition**—Inhibition of xanthine oxidase activity at elevated concentrations of its conventional substrates has frequently been reported. All quaternary substrates here studied also displayed excess substrate inhibition. As indicated by the experiment in Table III, changes in the $K_m$ caused by changing the pH resulted in parallel changes in the substrate inhibition range. Thus, for $N$-methylquinolinium and $N$-methyl nicotinamide, the substrate concentration required for an inhibition was lower at pH 10.8, where the $K_m$ was also lower.
Influence of pH on excess substrate inhibition of xanthine oxidase

All rates were measured as change in absorbance at 550 nm due to reduction of 2 × 10⁻⁴ M cytochrome c. Reactions were run at 25°, in 0.1 N Na₂CO₃, pH 9.6 or 10.8, containing 10⁻⁴ M EDTA. The rates shown are actual changes in absorbance in 3 minutes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>Rates</th>
<th>pH 9.6</th>
<th>pH 10.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Methylquinolinium chloride</td>
<td>3 × 10⁻² M</td>
<td>0.065</td>
<td>0.170</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁻³ M</td>
<td>0.115</td>
<td>0.130</td>
<td></td>
</tr>
<tr>
<td>N-Methylnicotinamide</td>
<td>3 × 10⁻² M</td>
<td>0.230</td>
<td>0.540</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁻¹ M</td>
<td>0.340</td>
<td>0.350</td>
<td></td>
</tr>
<tr>
<td>Xanthine</td>
<td>3 × 10⁻³ M</td>
<td>0.645</td>
<td>0.345</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10⁻¹ M</td>
<td>0.645</td>
<td>0.485</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 × 10⁻² M</td>
<td>0.480</td>
<td>0.480</td>
<td></td>
</tr>
</tbody>
</table>

Evidence for Common Substrate binding Site—The possibility was considered that the changes in substrate specificity and associated phenomena could be due to the gradual exposure or unmasking of a second site of activity as pH is increased. The data of Figs. 4 and 5 were employed to determine whether the turnover number for both substrates was the same. Because the same amount of enzyme was used with both substrates, Vₘₐₓ could be calculated from the intercepts. Values of 2.57 × 10⁻⁵ M per 3 minutes for xanthine, and 3.25 × 10⁻⁴ M per 3 minutes for N-methylnicotinamide were obtained. Thus, within experimental error, the turnover number for the enzyme was the same with either substrate. The effect of high pH on the substrate specificity of xanthine oxidase appears to be due solely to a change at the substrate-binding site.

That purines and quaternary compounds are bound at the same site was demonstrated by observing competitive inhibition of the oxidation of xanthine by N-methylnicotinolinium as shown in Fig. 7. The Kᵢ value calculated for N-methylnicotinolinium chloride was 1 × 10⁻³ M, which is in reasonable agreement with the Kᵢ value of 0.64 × 10⁻² M at pH 10.8.

Further evidence for a single site of action towards both conventional and quaternary substrates was afforded by the failure to detect differential sensitivity to inhibition by cyanide or dinitrofluobenzene. Dinitrofluobenzene inhibition is discussed in detail in the following paper (12). Table IV indicates that activity toward xanthine and N-methylnicotinolinium was inhibited to the same extent by these inhibitors.

Effect of inhibitors on xanthine oxidase activity toward different substrates

For cyanide inhibition, 10⁻³ M sodium cyanide was preincubated with xanthine oxidase in the assay cuvette for 5 minutes at pH 10.8; enzyme activity was assayed at the same pH by adding either xanthine, 2.5 × 10⁻⁴ M, or N-methylnicotinolinium chloride, 10⁻³ M. For dinitrofluobenzene inhibition (12), xanthine oxidase, dinitrofluobenzene (3.3 × 10⁻⁴ M final concentration) and substrate (3 × 10⁻⁴ M xanthine or 3 × 10⁻³ M N-methylnicotinolinium, final concentrations) were added in rapid succession. The rates were measured at 5 minutes after reaction began, for both control and inhibited reactions. Oxidation rates are expressed as the change in absorbance per 3 minutes at 300 nm for xanthine oxidation, or at 290 nm for N-methylnicotinolinium oxidation.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Oxidation rates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xanthine</td>
</tr>
<tr>
<td>None</td>
<td>0.052</td>
</tr>
<tr>
<td>Cyanide</td>
<td>0.028</td>
</tr>
<tr>
<td>Inhibition</td>
<td>12.5%</td>
</tr>
<tr>
<td>None</td>
<td>0.120</td>
</tr>
<tr>
<td>Dinitrofluobenzene</td>
<td>0.038</td>
</tr>
<tr>
<td>Inhibition</td>
<td>68%</td>
</tr>
</tbody>
</table>

Fig. 7. Influence of pH on the Kᵢ values for xanthine and N¹-methylnicotinamide as substrates of xanthine oxidase. The values for Kᵢ were calculated from the data in Figs. 4 and 5.

Fig. 8. Dixon plot (25) of the effect of pH on the oxidation of N¹-methylnicotinamide (O—O) and xanthine (□—□) by xanthine oxidase. The data are taken from those shown in Fig. 7. Straight line sections of integral slope were drawn through the points. The intersections of these lines indicate the approximate pKᵢ of a titratable group on the enzyme.
Comparison of the enzyme in the ultracentrifuge at pH 7.8 and pH 10.8 failed to reveal any evidence of aggregation, disaggregation, or gross conformational change.

It has been known for some time that only about 50% of the FAD in preparations of xanthine oxidase is reducible by xanthine (23, 24). If quaternary compounds react at a different site than conventional substrates, the activity of which becomes manifest only at high pH, then simultaneous anaerobic incubation of the enzyme with both a purine and a quaternary substrate at high pH might effect reduction of 100% of the FAD. This experiment was performed, with the use of xanthine, 2.5 × 10⁻⁴ M, and N⁴-methylnicotinamide, 2.1 × 10⁻² M, 2.5 × Kₐ for each substrate. The initial, rapid reduction, measured 1 minute after the enzyme and combined substrates were mixed, was only 60% of the total FAD, a value identical with that observed with either substrate alone. This observation renders unlikely the possibility of an independent site for oxidation of quaternary substrates.

Nature of Effect of pH on Xanthine Oxidase—The data cited above support the conclusion that, at high pH, xanthine oxidase undergoes a change at its substrate-binding site that permits the binding of quaternary heterocyclic compounds. Clearly, such a change could reflect the loss of a proton from a group at or near the substrate-binding site. An approximate pKₐ value for the group responsible was determined from the data of Fig. 7. When these data were plotted according to Dixon (25), as shown in Fig. 8, an approximate pKₐ of 10.7 was obtained for the ionizing group; this value is compatible with the dissociation of an amino group, a sulfhydryl group, or a phenolic hydroxyl group. As yet, it has not been possible to distinguish among these possibilities.

Perhaps the most likely possibility is an amino group at or near the substrate-binding site. In the protonated form, this group would prevent the binding of quaternary compounds by electrostatic repulsion, but it would contribute to the binding of the negatively charged purines. Dissociation of this group with increasing pH would allow quaternary compounds to approach the active center and be oxidized and, at the same time, would diminish the binding affinity for purines. Although this hypothesis seems most attractive, the data are also compatible with the creation, by titration, of a negatively charged sulfide or phenoxide group, which would attract quaternary compounds and tend to repel purines. However, this model does not adequately account for the complete lack of binding of quaternary compounds at neutral pH.

Another possibility is that the titration of a group or groups, with a pKₐ of about 10.7 but remote from the substrate binding site, results in a conformational change that removes a positively charged group from the vicinity of the substrate-binding site. This would similarly allow quaternary compounds to be bound and diminish the binding affinity for purines.

Future experimental work will be directed toward elucidating the mechanism of the substrate specificity changes and toward determination of the nature of the responsible titratable group.

SUMMARY

1. At pH values above 9.0, milk xanthine oxidase catalyzes the oxidation of quaternary, heterocyclic compounds such as N⁴-methylnicotinamide, N-methylquinolinium, and trigonelline, which are hydroxylated ortho to the quaternary nitrogen, whereas 1-(4-pyridyl)pyridinium is hydroxylated para to the ring nitrogen.

2. In the pH range 9.4 to 11.0, V_max remained constant for all substrates tested. The Kₐ value for purines and aldehydes increased with increasing pH, whereas that for quaternary compounds decreased. The magnitude of the increase in Kₐ for purines approximately equaled the decrease in Kₐ for quaternary compounds throughout this pH range.

3. From these and other data, it was concluded that the changes in specificity reflect an altered ability of the enzyme to bind substrates while the reaction mechanism and turnover rate are unchanged.

4. From data concerning the susceptibility of the enzyme to inhibitors when measured with quaternary and conventional substrates, ultracentrifugation studies, and anaerobic reduction of the enzyme by substrates, it is concluded that the same active center is involved in the oxidation of all types of substrates.

5. From the variation with pH of Kₐ for xanthine and N⁴-methylnicotinamide, it was determined that these changes are due to the titration of a group, or groups, on the enzyme with a pKₐ of about 10.7.

Acknowledgment—We wish to thank Miss Mary Mason for performing the ultracentrifugation.

REFERENCES


Xanthine Oxidase: VI. INFLUENCE OF pH ON SUBSTRATE SPECIFICITY
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