Some Properties of Oxaloacetate-Synthesizing Enzyme

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Utter & Kurahashi (1954; see also Kurahashi, Pennington & Utter, 1957) discovered an enzyme in pigeon liver which catalyses the reaction:

where IDP and ITP stand for inosine di- and triphosphate and GDP and GTP stand for guanosine di- and triphosphate. They refer to it as 'oxaloacetate-synthesizing enzyme' (Utter & Kurahashi, 1955). Relatively little is known of the kinetics of the enzyme and of the effects of inhibitors. The enzyme is assumed to play a role in the synthesis of glycogen from pyruvate (see Krebs, 1954; Hiatt, Goldstein, Lareau & Horecker, 1958), and possibly in the supply of oxaloacetate in liver. For the reasons stated by Stickland (1959*a*) it was of interest to investigate the inhibition of the enzyme by those carboxylic acids which are known to cause an accumulation of acetoacetate in respiring liver.

EXPERIMENTAL

Preparation of enzyme. Except for the first stages, the method used was that of Utter & Kurahashi (1955). From the observations of Bartley & Avi-Dor (1955), it was thought that an initial purification might be obtained by preparing an extract of mitochondria instead of an acetone-dried powder of liver as was used by Utter & Kurahashi. Kurahashi *et al.* (1957) have prepared the enzyme from an acetone-dried powder of chicken-liver mitochondria. All procedures were carried out at 0° unless otherwise stated.

The livers of 20 pigeons were removed and immediately placed in ice-water mixture. The livers (180 g.) were homogenized (in a stainless-steel homogenizer of the Potter-Elvehjem type) in 180 ml. of 0.9% KCl and spun at 700 *g* for 2 min., this removing unbroken cells, nuclei and cell debris. The mitochondria were then separated from the supernatant fraction by centrifuging for 15 min. at 3000 *g*. The mitochondria were suspended in 5 vol. (550 ml.) of water in a Waring Blendor and blended for eight periods of 2 min. each during 1 hr. To the disrupted mitochondria was added sufficient potassium phosphate buffer (0.25 M, pH 7.8) to bring the phosphate concentration to mM. This mixture was then shaken at 38° for 10 min. and the residue spun off (20 min., 10 000 *g*). The supernatant was used for step 2 of the procedure of Utter & Kurahashi (1955).

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The purification was continued to the end of the calcium phosphate gel stage. This enzyme fraction was diluted 10- to 20-fold when required and 0.1 ml. of the diluted solution was used for each experiment.

The protein content of the concentrated enzyme solution was 7.2 mg./ml., as estimated by the biuret method, bovine serum albumin (The Armour Laboratories) being used as standard.

Special chemicals. The disodium salt of adenosine triphosphate (ATP) and the Ba salt of adenosine diphosphate (ADP) were obtained from Schwarz Laboratories Inc., New York 17, N.Y., U.S.A. As assayed by the method of Krebs & Hems (1953), the ATP was 86% pure and no phosphate-containing impurities could be detected in the ADP. Inosine diphosphate (IDP) was prepared from ADP by the method of Kleinzeller (1942) for the conversion of ATP into ITP. Phosphopyruvate (Ba-Ag salt) was prepared by the method of Ohlmeyer (1951). The Ba or Ba-Ag salts of these compounds were converted into the Na or K salts by shaking them at 0° with an excess of Dowex-50 resin (25-40 mesh) in the appropriate form.

ATP, ADP, inosine triphosphate (ITP) and IDP were assayed by the method of Krebs & Hems (1953).

Disodium fluoromalonate (2-fluoro-3-hydroxysuccinate) and fluorocitrate (triethyl ester) were kindly supplied by Dr P. W. Kent. The triethylfluorocitrate was hydrolysed by heating with the theoretical amount of NaOH at 100° for 1 hr.

Measurement of enzyme activity. The activity of the enzyme was measured by determining colorimetrically the amount of oxaloacetate formed from sodium phosphoenolpyruvate in unit time.

The reaction mixture, which had a total volume of 1.5 ml., contained 2-amino-2-hydroxymethylpropane-1:3-diol chloride (tris) buffer, pH 7.4 at 30°, sodium phosphoenolpyruvate, sodium salt of IDP, NaHCO₃, enzyme, activators and inhibitors as given in the details of the individual experiments. The usual concentrations were as follows: tris (0.1 M), phosphoenolpyruvate (mM), IDP (0.4 mM), NaHCO₃ (16 mM) and MnCl₂ (mM). The stock enzyme solution (0.05-0.07 ml.) was diluted to 1 ml. as required and 0.1 ml. of this solution was placed in the hollow stopper of a Thunberg tube. All other constituents of the reaction medium were placed in the Thunberg tubes.

The hollow stopper containing the enzyme was cooled in ice for 5 min., and then placed in the tube, which was evacuated and filled with CO₂ + N₂ (5:95) three times. The tube was placed in a bath at 30° and allowed to equilibrate for 5-7 min., when the reaction was started by mixing the tube and stopper contents. After a suitable time interval (usually 5 min.) the reaction was stopped by the addition of 0.5 ml. of 25% (w/v) trichloroacetic acid.

In some experiments the NaHCO_3 concentration was varied. In order to maintain the pH at 7.4 the percentage of CO_2 in the gas phase was changed according to the data given by Umbreit (1949).

The amount of oxaloacetate present in the trichloroacetic acid extract was measured as given below. It was not found necessary to remove the precipitated protein as the small amount present (less than 0.05 mg.) did not interfere with the estimation.

Experiments on the removal of added oxaloacetate by the enzyme were conducted in a similar manner. Bicarbonate was omitted and the gas phase was N_2 . Other details are given with the individual experiments.

Estimation of oxaloacetate. Oxaloacetate was measured by a modification of the method of Kalnitsky & Tapley (1958). Since neither ascorbic acid nor acetoacetate was present here, the Kalnitsky & Tapley method was simplified by omitting the addition of NaNO_2 and the controls used to determine the acetoacetate. The procedure was as follows.

Immediately after the reaction was stopped by the addition of trichloroacetic acid, the tubes and contents (total volume 2 ml.) were placed in ice. When the tubes had been in ice for 5 min., 1.2 ml. of cold (5°) 1.66M-sodium acetate was added, followed by 3 ml. of the diazo reagent of Walker (1954). The solution was incubated in a water bath at 25° for 20 min., placed in ice for 5 min. and 4 ml. of ethyl acetate, followed by 2 ml. of 5N-HCl, were added. The tube was shaken vigorously within $7\frac{1}{2}$ sec. of adding the HCl and the shaking was continued for 15 sec. The extinction of the ethyl acetate layer was read at 450 m μ . At this wavelength, 100 μm -moles of oxaloacetate gave an extinction of 0.690 in 1 cm. cells. Malonate, fluoromalate, fluorocitrate and citrate gave colours with this procedure, 1 μmole of the compounds giving respectively as much colour as 140, 2.5, 21 and 35 μm -moles of oxaloacetate. The phosphopyruvate preparation used gave about 0.2% of the colour given by an equal concentration of oxaloacetate. Under the test conditions none of the other compounds interfered with the estimation at the highest concentrations at which they were used.

Analysis of kinetics. Values of the apparent Michaelis constant (K_m) for one set of conditions only were obtained by the method of Lineweaver & Burk (1934) and values of K_i (the dissociation constant of the enzyme-inhibitor complex) by the method of Dixon (1953). The terms v , $[S]$ and $[I]$ refer respectively to the initial velocity and substrate and inhibitor concentrations.

RESULTS

Time course of formation of oxaloacetate from phosphopyruvate. Preliminary experiments showed that the rate was linear for at least 6 min. under the test conditions (Fig. 1). In subsequent experiments an incubation period of 5 min. was taken as a convenient standard time.

Effect of substrate concentration. The rate of oxaloacetate formation increased with the concentration of phosphopyruvate but no maximum rate was reached by mM-phosphopyruvate (Fig. 2). When the IDP concentration was varied in the presence of mM-phosphopyruvate the rate reached a maximum at mM-IDP concentration. The values

of the apparent K_m for these two substrates were $2 \times 10^{-4}\text{M}$ for phosphopyruvate and $4 \times 10^{-5}\text{M}$ for IDP (Table 1).

When IDP was replaced by ADP the rate fell to about one-tenth and this remaining activity was

Fig. 1. Formation of oxaloacetate with time. Each tube contained, in a total volume of 1.5 ml., tris buffer, pH 7.4 (0.1M), NaHCO_3 (16.5 mM), MnCl_2 (mM), phosphoenolpyruvate (mM), IDP (0.4 mM) and enzyme (0.05 ml.). Gas phase, $\text{CO}_2 + \text{N}_2$ (5:95).

Fig. 2. Effect of phosphoenolpyruvate and IDP concentrations. Tube contents were as given in Fig. 1, except for the component which was varied. ●, Effect of IDP concentration in the presence of mM-phosphoenolpyruvate; ○, effect of phosphoenolpyruvate concentration in the presence of 0.4 mM-IDP. In the absence of either phosphoenolpyruvate or IDP 0.7 μmole of oxaloacetate was formed in 5 min.

probably due to traces of IDP or guanosine diphosphate and to nucleoside diphosphokinase, the presence of which was shown by the experiment recorded in Table 2: when ATP or IDP was incubated alone, no other nucleotides were formed, but when added together ITP and ADP appeared.

In the absence of added CO₂-bicarbonate very little oxaloacetate was formed (Fig. 3). Increase in the CO₂-bicarbonate concentration resulted in an increase in the rate of oxaloacetate formation. A maximum rate was reached at about 30 % of CO₂ in the gas phase (0.1M-HCO₃⁻ ion). Higher concentrations of CO₂-bicarbonate produced inhibition (about 50 % with a gas phase of 100 % CO₂). This

Table 1. Values of *K_m* and *K_i*

Values of the apparent *K_m*, for one set of conditions only, were obtained by the method of Lineweaver & Burk (1934) and values of *K_i* by the method of Dixon (1953).

	<i>K_m</i> (M)
Phosphoenolpyruvate	2×10^{-4}
IDP	4×10^{-5}
CO ₂	2×10^{-3}
Mn ²⁺	1×10^{-4}
	<i>K_i</i> (M)
Tartronate	2×10^{-4}

Fig. 3. Effect of CO₂ on oxaloacetate formation. Tube contents were as given in Fig. 1, except for the NaHCO₃, which was varied with the CO₂ to keep the pH at 7.4.

may be due to the high ionic strength, since NaCl also inhibited (see below). The apparent *K_m* for CO₂ was 2 mM-CO₂ in solution or 25 mM-bicarbonate under the conditions used.

Effect of cations. Mn²⁺ ions were essential for the reaction to proceed at maximum rate (Table 3, Expt. 1). The rate of oxaloacetate formation with Mg²⁺ and Co²⁺ ions was about 10 % of that with an equal concentration (mM) of Mn²⁺ ions. A tenfold increase in the Mg²⁺ ion concentration had no effect. Little or no oxaloacetate was formed in the presence of Ni²⁺, Cu²⁺, Zn²⁺ or Ca²⁺ ions.

The rate of oxaloacetate formation increased with the MnCl₂ concentration up to a maximum of mM-MnCl₂ (Fig. 4). Higher concentrations were inhibitory; at 33 mM-MnCl₂ the rate of oxaloacetate formation was only 20 % of the maximum rate. The apparent *K_m* for Mn²⁺ ions was 0.1 mM with reference to the overall Mn²⁺ ion concentration.

Mn²⁺ ions are very tightly chelated by phosphopyruvate (binding constant 560 l./mole; Wold & Ballou, 1957). With mM-MnCl₂ and mM-phosphopyruvate the concentration of free Mn²⁺ ions will be low, but when the amount of added MnCl₂ exceeded that of phosphopyruvate free Mn²⁺ ions are present in appreciable quantities and these ions catalyse the decarboxylation of oxaloacetate (Krebs, 1942). The apparent inhibition of oxaloacetate formation might be due to the decarboxylation of oxaloacetate by Mn²⁺ ions and this was tested by incubating oxaloacetate with enzyme and varying amounts of MnCl₂. In the absence of MnCl₂ there was a small decomposition (3 %) of oxaloacetate in 5 min. (Table 4), but this was increased to 7 % by mM-MnCl₂ and to 37 % by 10 mM-MnCl₂. The addition of mM-phosphopyruvate in the presence of mM-MnCl₂ decreased the decomposition of oxaloacetate to 3 %.

When added in the presence of MnCl₂, Co²⁺, Cu²⁺ and Zn²⁺ ions inhibited the synthesis of oxaloacetate but Mg²⁺ and Ca²⁺ ions did not (Table 3, Expt. 2). The total concentration of bivalent cations in these experiments was 2 mM, a concentration at which Mn²⁺ ions inhibited about 10 % (Fig. 4).

Potassium chloride inhibited oxaloacetate formation at concentrations of 10 mM and above

Table 2. Evidence for the presence of nucleoside diphosphokinase

Tubes contained tris buffer, pH 7.4 (0.1 M), MnCl₂ (3.3 mM), enzyme (0.05 ml.) and additions as shown, in a total volume of 0.9 ml., and were incubated for 15 min. at 30°. Reaction was stopped with 0.1 ml. of 30 % (w/v) trichloroacetic acid.

Additions	Nucleotides present (μmoles)			
	ATP	ADP	ITP	IDP
ATP + IDP, unincubated	1.63	0	0	1.67
ATP, incubated	1.60	0	0	0
IDP, incubated	0	0	0	1.60
ATP + IDP, incubated	0.98	0.72	0.93	0.69

(Fig. 4). There was 90% inhibition by 0.1M-KCl. The curve shown in Fig. 4 was obtained in the presence of approximately 0.02M-Na⁺ ion (added as the cation equivalent of bicarbonate, phosphopyruvate and IDP). Sodium chloride inhibited at concentrations of 0.1M (14%) and 0.22M (33%).

Table 3. *Effect of cations on oxaloacetate formation*

Each tube contained 0.1 ml. of enzyme and metal salts (mm) as stated. Other additions (except MnCl₂) were as given in Fig. 1. In Expt. 2 mm-MnCl₂ was present in all tubes in addition to the salt stated in the Table.

Expt. no.	Metal salt added	Oxaloacetate formed (μm-moles/5 min.)
1	None	0.7
	MnCl ₂	27.8
	MgCl ₂	2.5
	CoCl ₂	3.9
	NiSO ₄	0
	CuSO ₄	1.6
	ZnSO ₄	1.0
	CaCl ₂	0
Decrease in the rate compared with that for MnCl ₂ alone (%)		
2	MgCl ₂	0
	CoCl ₂	30
	NiSO ₄	13
	CuSO ₄	37
	ZnSO ₄	90
	CaCl ₂	4

Fig. 4. Effect of MnCl₂ and KCl on oxaloacetate formation. Tube contents were as given in Fig. 1, except for the component varied as above. In the absence of MnCl₂, 0.3 μm-mole of oxaloacetate was formed in 5 min. The KCl curve was obtained in the presence of approx. 0.02M-Na⁺ and mm-Mn²⁺ ions. The rate in the absence of added KCl was 29.1 μm-moles of oxaloacetate formed in 5 min.

When the bicarbonate, phosphopyruvate and IDP were added as potassium instead of sodium salts, the inhibition by added KCl was exactly the same if the total K⁺ ion concentration was taken into account.

Effect of carboxylic acids. L-Malate and meso-tartrate (10 mm) both had a slight inhibitory effect (10 and 14% respectively) with mm-phosphopyruvate as substrate. A threefold increase in the MnCl₂ concentration brought about a considerable reduction (to 4%) in this inhibition. The action of these inhibitors might thus have been due to the chelation of cations.

At a concentration of 10 mm, tartronate was a much stronger inhibitor than malate, giving about 70% inhibition. However, at tartronate concentrations above mm, plots of 1/v against [I] were non-linear. At lower concentrations, plots of 1/v against [I] were linear (see Fig. 5) and gave a value for K_i of 2 × 10⁻⁴M. The plots also showed that tartronate was a competitive inhibitor: a tartronate concentration of 0.8 mm inhibited 30% at mm-phosphopyruvate and 52% at 0.4 mm-phosphopyruvate (Table 5).

Oxalate also inhibited the formation of oxaloacetate. The inhibition was intermediate between purely competitive and purely non-competitive inhibition. Thus 0.2 mm-oxalate inhibited 16% at mm-phosphopyruvate and 32% at 0.1 mm-phosphopyruvate. However, 0.8 mm-oxalate inhibited 54% whether the phosphopyruvate concentration was 0.4 mm or 0.1 mm (Table 5). No value for K_i could be obtained because plots of 1/v against [I] and of 1/v against 1/[S] were non-linear (Fig. 5).

The effect of mesoxalate had to be studied with MgCl₂ to activate the enzyme instead of MnCl₂, owing to the formation of an insoluble compound between MnCl₂ and mesoxalate. Since the activity of the enzyme was very low in the presence of MgCl₂ (Table 3), the effect of mesoxalate could not be

Table 4. *Effect of enzyme on oxaloacetate*

Each tube contained tris buffer, pH 7.4 (0.1M), enzyme (0.1 ml.) and additions as stated below, in a total volume of 1.5 ml. Incubations were for 5 min. at 30°. Oxaloacetate concentration was 33 μM (50 μm-moles/tube) and phosphopyruvate concentration was mm.

Additions	Concn. of MnCl ₂ (mm)	Oxaloacetate present (μm-moles)
Oxaloacetate	0	49.0*
Oxaloacetate	0	47.6
Oxaloacetate	1	44.6
Oxaloacetate	10	30.2
Oxaloacetate + phosphopyruvate	1	47.6
Phosphopyruvate	1	0.4

* Not incubated.

studied in detail. A concentration of 10 mm-mesoxalate (with mm-phosphopyruvate) inhibited 52 %.

Malonate gave a colour in the oxaloacetate estimation. As a result, 0.2 mm was the highest concentration at which it could be used without giving a blank reading which was too high. At this concentration, and in the presence of an equal concentration of phosphopyruvate, malonate inhibited 15 %. If the MnCl_2 concentration was increased from mm to 3 mm, the inhibition was reduced to 8 %.

Fluoromalate, fluorocitrate and citrate also interfered with the oxaloacetate estimation. Under the

same conditions as for Fig. 1, but with 0.1 mm-phosphopyruvate, 10 mm-fluoromalate gave complete inhibition but 0.1 mm-fluoromalate gave almost none. With mm-fluoromalate, a threefold increase in phosphopyruvate concentration had almost no effect on the inhibition, which was reduced from 22 to 20 %. The figure of 22 % was not altered if the MnCl_2 concentration was increased from mm to 3 mm.

Fluorocitrate and citrate both gave 21 % inhibition with both inhibitor and substrate at a concentration of mm.

Of these weaker inhibitors, malonate, fluorocitrate and mesotartarate had no effect on the formation of phosphopyruvate from oxaloacetate (Stickland, unpublished work). The effect of citrate and L-malate on this latter reaction was not tried.

The figures given for the small inhibitions by malonate, fluorocitrate and citrate are not very reliable owing to the large blank which these compounds gave in the oxaloacetate estimation.

DISCUSSION

Co^{2+} , Ni^{2+} , Cu^{2+} and Zn^{2+} ions catalyse the non-enzymic decarboxylation of oxaloacetate to a greater extent than do Mn^{2+} ions (Speck, 1948), and it is possible that they decrease oxaloacetate formation not by inhibiting the enzyme but by decomposing the oxaloacetate once it is formed. Support for this assumption is that Mg^{2+} and Ca^{2+} ions, which are poorer catalysts than are Mn^{2+} ions for the decarboxylation of oxaloacetate, caused little decrease in the formation of oxaloacetate (Table 3, Expt. 2).

Boyer, Lardy & Phillips (1943) found that pyruvic kinase was activated by K^+ ion in concentrations up to 0.1 M, but that higher concentrations were inhibitory. In contrast, the oxaloacetate-synthesizing enzyme is not activated by K^+ ions, but concentrations greater than 10 mm inhibited (Fig. 4). Both enzymes are inhibited by 0.1 M- Na^+ ion.

The K^+ ion concentration in the water of the cells of dog liver is about 0.15 M and the Na^+ ion concentration about 3 mm (see Harris, 1956). This Na^+ ion concentration is not sufficient to cause inhibition of pyruvic kinase or oxaloacetate-synthesizing enzyme, but marked inhibition would be expected by 0.15 M- K^+ ion. However, it is possible that some of the K^+ ion is bound and the concentration of K^+ ion free to influence the activity of these two enzymes may be less than 0.15 M. Relatively small variations in K^+ ion concentration would affect the activity of both these enzymes.

Six ketogenic substances have been found to inhibit malic enzyme (Stickland, 1959*a, b*). These are malonate, tartarate, mesoxalate, oxalate,

Fig. 5. Effect of tartronate and oxalate on oxaloacetate formation. Top graphs are for tartronate; bottom graphs are for oxalate. Left-hand graphs are plots of $1/v$ against $1/[S]$; the figures on the graph indicate the inhibitor concentration. Right-hand graphs are plots of $1/v$ against inhibitor concentration; the figures on the graph indicate the substrate concentration. v , Initial velocity as μ moles of oxaloacetate formed in 5 min.; $[S]$, substrate concentration.

Table 5. Effect of tartartrate and oxalate on oxaloacetate formation

Tube contents were as given in Fig. 1, except where stated below.

Concn. of phosphopyruvate (mm)	Inhibition (%)			
	Tartartrate		Oxalate	
	0.8 mm	0.2 mm	0.8 mm	0.2 mm
1	30	15	46	16
0.4	52	20	54	28
0.1	—	42	54	32

mesotartrate and fluorocitrate. In addition Gal (1958) has found fluoromalate to inhibit malic enzyme. Of these substances only tartronate, mesoxalate, oxalate and fluoromalate give appreciable inhibition of oxaloacetate-synthesizing enzyme.

The ketogenic effect of tartronate, mesoxalate, oxalate and fluoromalate could be accounted for by the assumption that it is due to an inhibition of the formation of oxaloacetate via either malic enzyme or oxaloacetate-synthesizing enzyme. However, the ketogenic effect of fluorocitrate and malonate could be explained by inhibition of malic enzyme but not of oxaloacetate-synthesizing enzyme. In addition, malonate and oxalate inhibit succinic and malic dehydrogenases (Quastel & Wooldridge, 1928; Green, 1936; Lardy, 1949). Fluorocitrate inhibits aconitase (Morrison & Peters, 1954). Davies & Kun (1957) found that D- and mesotartrate, tartronate and mesoxalate inhibit the oxidation of malate by malic dehydrogenase. It is therefore possible that the ketogenic action of these compounds is due to inhibition of some stages of the citric acid cycle and thus to a decrease in the formation of oxaloacetate.

SUMMARY

1. The effect of inhibitors on the formation of oxaloacetate from phosphopyruvate by the oxaloacetate-synthesizing enzyme of pigeon liver has been studied, and data have been obtained on the kinetics of the reaction.

2. Under the conditions studied, the values of the apparent K_m were as follows: phosphopyruvate ($2 \times 10^{-4} M$), inosine diphosphate ($4 \times 10^{-5} M$), carbon dioxide ($2 \times 10^{-3} M$) and Mn^{2+} ion ($10^{-4} M$).

3. It is confirmed (Utter & Kurahashi, 1954) that for the maximum rate of formation of oxaloacetate added Mn^{2+} ions were required; Mg^{2+} and Co^{2+} ions were almost inactive. High concentrations (10 mM) of Mn^{2+} ions decreased the oxaloacetate yield, probably owing to the non-enzymic decarboxylation of the oxaloacetate. Co^{2+} , Ni^{2+} and Cu^{2+} ions also decreased the yield.

4. Potassium chloride (0.1 M) inhibited about 90%. Sodium chloride at the same concentration inhibited 14%.

5. Tartronate and oxalate inhibited the reaction about 50% at a concentration twice that of phosphopyruvate. The inhibition was partially competitive and partially non-competitive. A value for K_i of $2 \times 10^{-4} M$ was obtained graphically for tartronate. Mesoxalate (10 mM) inhibited 52% with

mm-phosphopyruvate and fluoromalate (mm) 22% with 0.1 mm-phosphopyruvate. There was less inhibition (approx. 15%) by L-malate, mesotartrate, malonate, fluorocitrate and citrate.

6. The ketogenic effect of tartronate, mesoxalate, oxalate and fluoromalate could be accounted for by the assumption that it is due to an inhibition of the formation of oxaloacetate via either malic enzyme or oxaloacetate-synthesizing enzyme. However, the ketogenic effect of fluorocitrate and malonate could possibly be explained by inhibition of malic enzyme but not of oxaloacetate-synthesizing enzyme.

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