Effect of Heparin Injection on Plasma Protein Binding of Bilirubin and Salicylate in Rats

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Abstract □ Intravenous heparin injection significantly increased the free bilirubin and salicylate fractions in the plasma of rats. This effect occurred within 2 min after injection of 500 U of heparin/kg and lasted for 15–45 min (bilirubin) or for >45 min (salicylate). *In vitro* addition of heparin to plasma had no quantitatively significant effect on the protein binding of bilirubin and salicylate. The *in vivo* effect of heparin on protein binding was reversible by treating the plasma with activated charcoal, a procedure known to remove fatty acids from albumin. Since protein binding affects the pharmacokinetic characteristics and the pharmacological activity of drugs, the heparin–drug interaction may have significant clinical implications. Use of heparinized plasma for exchange transfusion in the treatment of neonatal jaundice may be hazardous.

Keyphrases □ Heparin—effect on plasma protein binding of bilirubin and salicylate, rats □ Anticoagulants—heparin, effect on plasma protein binding of bilirubin and salicylate, rats □ Bilirubin—plasma protein binding, effect of heparin, rats □ Salicylate—plasma protein binding, effect of heparin, rats

Heparin is a widely used injectable anticoagulant. It is not only administered for direct therapeutic purposes but also as a concomitant of blood exchange transfusions and to facilitate hemodialysis. There have been reports that heparin injection or infusion decreases the plasma or serum protein binding of endogenous substances such as triiodothyronine (1), of the neutral drugs digoxin and digitoxin (2), and of the basic drug propranolol (3). On the other hand, the plasma protein binding of warfarin, a weak acid, has been reported to increase greatly after heparin injection (4).

Plasma protein binding can have a pronounced effect on the pharmacokinetic characteristics and pharmacological activity of drugs that are normally extensively bound (5). Sudden displacement of such drugs or of a potentially toxic endogenous substance from plasma protein binding sites could have serious consequences (6, 7). Heparin effects on plasma protein binding, if not recognized and accounted for, may also influence pharmacokinetic and toxicological findings in animals. It is desirable, therefore, to assess the magnitude, time course, dose dependence, and mechanism of the heparin effect on the plasma protein binding of important drugs and endogenous substances.

In this investigation, the effect of heparin injection on the plasma protein binding of bilirubin and salicylate was determined as a function of time in rats. Bilirubin was selected because its total (sum of free and bound) concentration in plasma is often used as a diagnostic index of liver function; a decrease in the protein binding of this heme pigment causes a decrease of total concentration (6, 7) and may lead to erroneous clinical interpretations.

Unconjugated hyperbilirubinemia in neonates is often treated with exchange transfusions of heparinized blood (8, 9); a displacement of protein-bound bilirubin during this procedure may be hazardous. Salicylic acid was employed in this study as a representative and widely used weakly acidic drug. Its binding to plasma proteins is affected by age and disease (10, 11), and there are indications that its pharmacological effect is a function of the free drug concentration (12).

Previous investigations of the effect of heparin injection on plasma protein binding of drugs revealed a strong correlation between the change in protein binding and the concentration of free fatty acids (2, 3). It has been suggested, therefore, that the heparin effect is the result of drug displacement from plasma protein binding sites by free fatty acids whose concentration in plasma has been elevated due to the increased lipoprotein lipase activity caused by heparin administration (2, 3). Fatty acids adsorbed on serum albumin can be removed by treatment with activated charcoal (13). This treatment was employed to determine if heparin-induced changes in the plasma protein binding of bilirubin and salicylate are reversible upon removal of fatty acids (and possibly other bound substances) from plasma proteins.

EXPERIMENTAL

The effect of heparin injection on the plasma protein binding of bilirubin as a function of time was determined by administering 200 or 500 U of heparin/kg[1] or an equal volume of saline solution by rapid intravenous injection to male Sprague–Dawley rats[2] weighing 300–325 g and maintained on a standard diet[3]. The solutions were injected into the right jugular vein through a cannula implanted 1 day earlier (14). Crossover experiments were carried out 2 weeks later.

Mainstream blood samples (0.7–0.8 ml) were withdrawn into a plastic syringe[4] through one outlet of a three-way stopcock as described previously (14), except that the cannulas were filled with saline solution rather than with heparin between blood withdrawals. The blood samples were immediately transferred to heparinized glass capillary tubes[5], which were centrifuged for plasma separation. These blood withdrawals were made before injection and 5, 10, 15, and 45 min after injection.

In a subsequent study on other rats, blood was collected before and 2, 3, and 4 min after injection. The jugular cannulas of some rats did not remain patent for 2 weeks. These animals were recannulated on the left side 1 day before the crossover study.

An aqueous bilirubin[6] solution was prepared by dissolving the pigment in a small volume of distilled water with the aid of a few drops of 2.5 *N* NaOH and adding sufficient distilled water and 0.5 *N* HCl to yield a pH 7.8–8.0 solution containing 6 mg of bilirubin/ml. The solution was always freshly prepared and protected from light. Five microliters of this solution was added to 300 μl of plasma to produce a plasma bilirubin concentration of 10 mg/100 ml. The free bilirubin fraction in plasma was determined in triplicate by the peroxidase-catalyzed reaction rate method (6, 15). The reaction time was always 2 min.

The effect of heparin injection on the plasma protein binding of salicylate was studied in another group of rats, using the same *in vivo* tech-

[1] Heparin sodium injection USP, 1000 U/ml, from beef lung, The Upjohn Co., Kalamazoo, Mich.
[2] Blue Spruce Farms, Altamont, N.Y.
[3] Charles River formula 4RF.
[4] Plastipak, 1 cc TB, Becton Dickinson Co., Rutherford, N.J.
[5] Red Tip Caraway, HR1 8889-306000, Sherwood Medical Industries, St. Louis, Mo.
[6] Crystalline bilirubin from bovine gallstones, Sigma Chemical Co., St. Louis, Mo.

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Table I—Effect of Heparin Injection, 500 U/kg, on Plasma Protein Binding of Bilirubin in Rats

Minutes	Free Fraction × 10^4, mean ± *SD*, *n* = 7		Statistical Significance of Difference[a]
	Heparin	Saline	
0	5.8 ± 1.8	5.6 ± 1.6	NS
5	23.1 ± 12.1	6.9 ± 1.5	$p < 0.0025$
10	11.2 ± 3.5	6.2 ± 1.2	$p < 0.025$
15	9.5 ± 4.0	6.4 ± 2.1	$p < 0.05$
45	6.6 ± 1.9	5.6 ± 1.5	NS

[a] Paired *t* test.

Table II—Effect of Heparin Injection, 200 U/kg, on Plasma Protein Binding of Bilirubin in Rats

Minutes	Number of Animals	Free Fraction × 10^4, mean ± *SD*		Statistical Significance of Difference[a]
		Heparin	Saline	
0	5	5.1 ± 1.7	5.3 ± 1.2	NS
5	3	7.9 ± 0.9	5.4 ± 1.2	
10	5	7.5 ± 2.4	4.7 ± 0.6	$p < 0.025$
15	3	6.4 ± 0.5	5.3 ± 0.8	
45	5	5.0 ± 2.0	4.8 ± 1.1	NS

[a] Paired *t* test. Done only for groups with $n = 5$.

niques as those employed in the bilirubin experiment. The free salicylate fraction was determined by equilibrium dialysis of 400 μl of plasma against an equal volume of 0.134 *M* phosphate buffer, pH 7.4, containing 30 mg of sodium salicylate/100 ml. Dialysis was carried out for 4 hr at 37° with Plexiglas cells rotated in a water bath. The cell chambers were separated by a membrane made from natural cellulose with a molecular weight cutoff between 12,000 and 14,000[7]. Salicylate was assayed by the method of Brodie *et al.* (16), using carbon tetrachloride for extraction.

Plasma for experiments to determine the effect of treatment with activated charcoal on the protein binding of bilirubin and salicylate was obtained by withdrawing ~10 ml of blood from the abdominal aorta of ether-anesthetized rats into a plastic syringe[8] containing ~250 U of heparin. Plasma was separated by centrifugation in polypropylene tubes. Such blood withdrawals were made from rats that had not received heparin or saline injections (representative of conditions before injection) and from other rats 5 min after intravenous injection of heparin, 500 U/kg, or of an equal volume of saline solution.

Treatment of plasma with activated charcoal was carried out by a literature procedure (13), which consisted of acidifying the plasma to pH 3 after charcoal addition, separation of charcoal by centrifugation (20,000×*g* for 20 min at 2°) after 1 hr of stirring in an ice bath, and readjustment of the plasma pH to 7.4. To minimize plasma dilution, 1 *N* rather than 0.2 *N* HCl and NaOH (as specified in Ref. 13) were used for pH adjustment. The charcoal[9] was prewashed by preparing a slurry in 2 *N* HCl. The acid was removed by decanting, and the procedure was repeated several times with distilled water until the decanted water pH was nearly neutral. This process also removed very fine charcoal particles, which would have been difficult to separate from plasma. The charcoal was dried at room temperature. Another group of plasma samples was acidified, stirred, centrifuged, and readjusted to pH 7.4 without addition of charcoal to determine the effect of pH manipulation on binding characteristics. Total plasma protein concentrations were determined by the biuret method (17) with crystalline rat serum albumin as the standard. The albumin fraction was determined by electrophoresis[10].

Protein binding of bilirubin and salicylate in the charcoal-treated plasma samples and in the appropriate control plasma was determined as described in a preceding paragraph.

To determine the possible effect of ether anesthesia on protein binding, rats were anesthetized with ether, and a cannula was implanted in the right jugular vein (14). Blood was withdrawn immediately after implantation (after ~25 min of anesthesia) and 45 min and 24 hr after recovery. Plasma was separated and used for bilirubin binding determinations as described previously.

[7] Spectrapor 2, Spectrum Medical Industries, Los Angeles, Calif.
[8] Plastipak, 10 ml, Becton Dickinson Co., Rutherford, N.J.
[9] DARCO activated carbon, grade G 60, Atlas Chemical Ind., Wilmington, Del.
[10] Sepratek system, Gelman Instrument Co., Ann Arbor, Mich.

Table III—Effect of Heparin Injection, 500 U/kg, on Plasma Protein Binding of Salicylate in Rats

Minutes	Number of Animals	Free Fraction × 100, mean ± *SD*		Statistical Significance of Difference[a]
		Heparin	Saline	
0	6	53.6 ± 8.9	58.4 ± 7.2	NS
5	5	92.2 ± 7.4	60.1 ± 6.3	$p < 0.0005$
10	6	85.5 ± 6.7	59.8 ± 7.2	$p < 0.0005$
15	6	80.3 ± 6.7	59.2 ± 1.6	$p < 0.0005$
45	5	78.0 ± 7.2	58.4 ± 2.4	$p < 0.0005$

[a] Paired *t* test.

Table IV—*In Vitro* Effect of Heparin, 25,000 U/liter, on the Protein Binding of Bilirubin and Salicylate in Rat Plasma

	Bilirubin Free Fraction × 10^4		Salicylate Free Fraction × 100	
	Heparin	Control	Heparin	Control
	6.22	5.07	66.6	68.2
	5.93	5.32	65.2	65.8
	6.30	5.53	67.3	63.9
	6.13	5.67	65.4	65.6
Mean	6.15	5.40	66.1	65.9
	$p < 0.01$		NS	

RESULTS

Heparin, 500 U/kg iv, caused a pronounced increase in the free bilirubin fraction for at least 15 min after injection. At 45 min after injection, the free bilirubin fraction value had returned to near normal (Table I). A considerably less pronounced effect was obtained when only 200 U of heparin/kg was injected (Table II). Since the largest effect of heparin was observed at the first postinjection blood withdrawal (*i.e.*, at 5 min), additional studies were carried out on another group of rats at 2, 3, and 4 min after heparin injection. The most pronounced effect on bilirubin binding was observed at 2 min after injection of 500 U of heparin/kg. The average free bilirubin fraction at that time was 32×10^{-4} in three rats.

Heparin, 500 U/kg iv, also significantly increased the free salicylate fraction in plasma. This effect was still quite pronounced at the end of the experiment, *i.e.*, at 45 min after injection (Table III). Additional studies on three other rats revealed that the maximum heparin effect was already evident 2 min after injection. The average salicylate free fraction at that time was 0.922.

To determine if the heparin effect on the plasma protein binding of bilirubin was direct or indirect, a large amount of heparin was added to plasma *in vitro*. An equal volume of saline solution (25 ml/liter) was added to control plasma from the same animals. Heparin had no apparent effect on the plasma protein binding of salicylate (Table IV). *In vitro* addition of heparin had a statistically significant effect on bilirubin binding, but the increase in the free bilirubin fraction was only 14% on the average and, therefore, quantitatively insignificant compared to the effects observed *in vivo* (Table IV).

Activated charcoal treatment of plasma obtained 5 min after heparin injection completely reversed the heparin-induced bilirubin binding decrease (Table V). Even the process of plasma acidification and subsequent neutralization caused a partial reversal of the heparin effect but not as much as that produced by activated charcoal. Similar effects were obtained with respect to the protein binding of salicylate (Table VI).

Table V—Effect of Activated Charcoal Treatment on Heparin-Induced Changes in Plasma Protein Binding of Bilirubin in Rats

Type of Plasma[a]	Bilirubin Free Fraction × 10^4	
	Heparin	Saline
No injection, untreated	5.7 ± 1.3 (9)[b]	4.6 ± 0.6 (5)
After injection, untreated	16.4 ± 9.3 (9)[c]	5.8 ± 1.1 (7)
After injection, treated with activated charcoal	5.4 ± 1.2 (9)	6.2 ± 0.6 (6)
After injection, acidified and neutralized	9.5 ± 2.6 (9)	7.6 ± 1.3 (6)

[a] Blood to yield plasma was drawn from uninjected animals and from injected animals after intravenous injection of heparin, 500 U/kg, or of an equal volume of saline solution. [b] Mean ± *SD*; number of animals in parentheses. [c] Significantly different from plasma obtained from uninjected animals and from activated charcoal-treated plasma obtained after injection ($p < 0.01$).

Table VI—Effect of Activated Charcoal Treatment on Heparin-Induced Changes in Plasma Protein Binding of Salicylate in Rats

Type of Plasma[a]	Salicylate Free Fraction × 100	
	Heparin	Saline
No injection, untreated	46.9 ± 4.6 (8)[b]	43.0 ± 3.1 (5)
After injection, untreated	87.8 ± 6.1 (8)[c]	50.9 ± 3.8 (5)
After injection, treated with activated charcoal	54.7 ± 6.1 (8)	46.6 ± 3.7 (5)
After injection, acidified and neutralized	64.8 ± 10.4 (8)	48.2 ± 1.7 (5)

[a,b] See Table V. [c] Significantly different from plasma obtained from uninjected animals and from activated charcoal-treated plasma obtained after injection ($p < 0.001$).

The dilution of plasma caused by acidification and neutralization and the additional effect of some albumin adsorption on activated charcoal reduced the albumin concentration of the treated plasma samples (Table VII). This obviously will have affected the results presented in Tables V and VI and makes the observed charcoal treatment effects even more significant. To permit an assessment of the charcoal treatment effect on the protein binding of salicylate from a different perspective, the results are also presented as the ratio of the amount of bound salicylic acid to the amount of albumin in plasma (Table VIII). Treatment with activated charcoal actually increased this ratio to a value greater than that of plasma from rats that did not receive heparin. A similar tabulation for bilirubin is not informative, because the latter is >99% bound under all of the experimental conditions.

Since blood for the charcoal treatment experiments was obtained from ether-anesthetized rats, it was desirable to determine the effect of ether anesthesia on the plasma protein binding of bilirubin. Plasma samples obtained through a jugular catheter after 25 min of ether anesthesia yielded essentially the same free bilirubin fraction values as did plasma samples collected from the same animals 45 min and 24 hr after anesthesia (Table IX).

DISCUSSION

The results of this investigation show that an intravenous heparin injection can significantly decrease the plasma protein binding of bilirubin and salicylate. This effect occurred within 2 min after injection. It was relatively brief in the case of bilirubin, a compound with very high affinity to albumin, and of appreciably longer duration in the case of salicylate, a drug with much less affinity to albumin.

Heparin, even at a very high concentration, had no quantitatively significant direct *in vitro* effect on the plasma protein binding of bilirubin and salicylate. Thus, an *in vivo* competition by heparin or by the preservative contained in the heparin solution (9 mg of benzyl alcohol/ml) with bilirubin or salicylate for plasma protein binding sites can be ruled out. Benzyl alcohol in relatively high concentration can displace bilirubin from albumin binding sites (18, 19) and is possibly responsible for the small displacing effect observed *in vitro* (Table IV). However, the plasma concentrations of benzyl alcohol produced by heparin injections are so low that apparently no significant bilirubin displacement by benzyl alcohol will occur *in vivo*.

The heparin-induced reduction of bilirubin and salicylate binding to plasma proteins can be reversed by treating the plasma with activated charcoal. Such treatment is known to remove fatty acids from albumin binding sites but may also remove other endogenous inhibitors. Some reversal of the heparin effect can be produced simply by acidification and subsequent neutralization of the plasma. Acidification to pH 3 causes a conformational change (*i.e.*, N-F transformation and acid expansion) of albumin (20), which can facilitate the dissociation of a bound substance from the protein (21). Thus, the results of this investigation are consistent with a mechanism involving the formation or release into the blood of competitive inhibitor(s) of plasma protein binding as a consequence of heparin administration. These competitors may be free fatty acids in view of the temporal relationship between plasma protein binding changes and free fatty acid concentrations in plasma demonstrated by previous investigators (2–4).

It appears that a rapidly injected intravenous dose of 200 U/kg is approximately the minimum dose of heparin required to decrease the binding of bilirubin in rat plasma since this dose barely affected the protein binding of the pigment (Table II). On that basis, and assuming the heparin effect to be rapidly reversible, the duration of the effect of a 500-U/kg dose should be equal to one to two times the biological half-life of heparin. A recent study (22) showed that the biological half-life of 500 U of heparin/kg in rats is 69 ± 13 min (mean ± *SD*, $n = 12$). The duration of the effect of 500 U of heparin/kg on the plasma protein binding of bilirubin, as observed in this investigation (Table I), appears to be somewhat less than the 69-min average biological half-life of the anticoagulant. This finding suggests that there may be a limited amount of precursor of the endogenous displacing agent(s) or a limited depot of releasable endogenous displacing agent accessible or responsive to injected heparin. Once this amount of displacer has been discharged into the circulation, further discharge may be controlled by another rate-limiting step and may proceed at a much lower rate than that occurring immediately after heparin injection. It is also quite evident that the displacing agent itself must have a very short half-life in plasma. These characteristics are not inconsistent with previous suggestions that heparin affects plasma protein binding by causing a transient elevation of free fatty acid concentration in plasma (2–4).

The drug displacement from plasma protein binding sites caused by heparin administration can have significant pharmacokinetic and toxicological implications, as pointed out previously (3). In the case of bilirubin, such a displacing effect is of particular concern because heparinized blood in large quantities is administered to severely jaundiced infants in exchange transfusions. The heparin effect on salicylate binding is also striking since a 500-U/kg dose almost abolishes the plasma protein binding of salicylate during the first 5 min after rapid injection. The effect of the heparin injection on the plasma protein binding of salicylate lasts much longer than on the binding of bilirubin, probably because of the greater affinity of the latter to binding sites on albumin. It will be important to determine if the salicylate binding in tissues is reduced also by heparin administration.

Ongoing clinical studies indicate that significant alterations in the plasma protein binding of bilirubin are produced by considerably lower heparin doses than those required to decrease the plasma protein binding of this compound in rats.

Table VII—Effect of Activated Charcoal Treatment on Albumin Concentration in Rat Plasma

Treatment of Plasma[a]	Albumin Concentration, g/100 ml	
	Heparin ($n = 8$)	Saline ($n = 5$)
None	3.1 ± 0.2	3.1 ± 0.3
Acidification and neutralization	2.3 ± 0.2	2.4 ± 0.1
Activated charcoal	1.9 ± 0.2	2.1 ± 0.1

[a] Blood to yield plasma was drawn 5 min after intravenous injection of heparin, 500 U/kg, or of an equal volume of saline solution.

Table VIII—Effect of Activated Charcoal Treatment on the Amount of Salicylate Bound per Gram of Albumin in Plasma of Rats after Heparin Injection

Type of Plasma[a]	Bound Salicylic Acid/Albumin			
	Heparin ($n = 8$)		Saline ($n = 5$)	
	Milligrams per Gram	Molar Ratio	Milligrams per Gram	Molar Ratio
No injection, untreated	3.1 ± 0.8[b]	1.5	3.2 ± 0.2	1.5
After injection, untreated	0.8 ± 0.3	0.4	3.2 ± 0.4	1.5
After injection, treated with activated charcoal	4.8 ± 1.3	2.3	5.2 ± 0.9	2.5
After injection, acidified and neutralized	3.1 ± 1.2	.1.5	4.2 ± 0.6	2.0

[a] Blood to yield plasma was drawn from uninjected animals and from injected animals 5 min after intravenous injection of heparin, 500 U/kg, or of an equal volume of saline solution. [b] Mean ± *SD*.

Table IX—Plasma Protein Binding of Bilirubin in Rats during and after Ether Anesthesia

Time	Bilirubin Free Fraction × 10^4
During anesthesia[a]	5.1 ± 0.7[b]
45 min after anesthesia	4.5 ± 1.3
24 hr after anesthesia	4.6 ± 0.7

[a] Ether anesthesia for ~25 min. Blood was collected just before withdrawal of ether. [b] Mean ± *SD*, $n = 3$.

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Correlation of Urinary Excretion with *In Vitro* Dissolution Using Several Dissolution Methods for Hydrochlorothiazide Formulations

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Abstract □ Four different hydrochlorothiazide formulations were prepared, and cumulative urinary hydrochlorothiazide excretion was determined in a crossover study using six volunteers. A comparison of *in vivo* results showed that one formulation (Formulation D) was significantly different from the others at 2, 3, 4, 5, 8, and 14 hr. A dissolution study was conducted on each formulation using the flask, USP basket, and magnetic basket methods at agitation speeds of 50, 100, and 150 rpm. Formulation D was significantly different from other formulations when determined using the USP basket method at 150 rpm and a sampling time of 10 min; the USP basket method at 100 rpm and a sampling time of 100 min; the flask method at 100 rpm and sampling times of 30, 40, 60, and 120 min; and the flask method at 150 rpm and sampling times of 30 and 40 min. Significant *in vitro* and *in vivo* correlations were found using a regression analysis and *F* test. With a correlation coefficient and 95% confidence intervals, it was established that the USP basket method at 150 rpm was the best predictor of urinary hydrochlorothiazide excretion among the dissolution methods tested.

Keyphrases □ Hydrochlorothiazide—urinary excretion correlated with *in vitro* dissolution, various methods, bioavailability □ Diuretic agents—hydrochlorothiazide, urinary excretion correlated with *in vitro* dissolution, various methods, bioavailability □ Dissolution testing—hydrochlorothiazide, urinary excretion correlated with *in vitro* dissolution

Hydrochlorothiazide is a widely used diuretic and antihypertensive agent. Due to its limited aqueous solubility, this drug has a potential for poor absorption from the GI tract. In January 1977, the Food and Drug Administration (FDA) issued final regulations on bioequivalency and bioavailability (1). Hydrochlorothiazide was included in a list of drug entities described as having "known or potential bioequivalency or bioavailability problems."

In this publication, FDA also reported that: "A bioequivalence requirement for the majority of products should be an *in vitro* test in which the drug product is compared to a reference material. Preferably, the *in vitro* test should be an *in vitro* bioequivalence standard, *i.e.*, a test that has been correlated with human *in vivo* data. In most instances, the *in vitro* test should be a dissolution test" (1). FDA further stated that since *in vivo* testing requires an enormous number of human subjects and clinical investigators, the *in vitro* test as a valid predictor of bioequivalency can greatly reduce human subject risk and cost involved with *in vivo* testing. With these regulations, correlation between *in vitro* dissolution and bioavailability for drugs having bioavailability problems becomes more important.

McGilveray *et al.* (2) studied hydrochlorothiazide tablets manufactured by 39 Canadian companies. *In vitro* dissolution times using the USP method and bioavailability using urinary excretion were measured. Very poor correlation was found between these two parameters.

Meyer *et al.* (3) studied 14 different commercial hydrochlorothiazide formulations marketed in the United States. This work also did not reveal any apparent rela-

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