on paper electrophoresis. Total insulin-binding capacities of antisera ranged from less than 10 to 5000 mμg/ml in guinea pigs and from 14 to 550 mμg/ml serum in rabbits.

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Hyperlipemia and Hemolysis III. Acceleration of Oleate Lysis of Human Erythrocytes by Homologous Plasma. (25075)

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In a previous study(1), human erythrocytes were found to compete *in vitro* with homologous plasma for binding exogenous sodium oleate. That portion of oleate bound by plasma was rendered hemolytically inert; even vigorous mechanical agitation failed to dissociate the binding appreciably. That portion of exogenous oleate bound by the erythrocyte constituted the active hemolytic moiety. The present report concerns the nature and mechanism of human plasma acceleration of hemolysis following erythrocyte-oleate binding; these findings extend earlier reports(2) indicating that once human erythrocytes bind oleate, homologous plasma reverses its hemolytic protective action and accelerates oleate lysis. Such studies are a continuation of investigations regarding possible intermediate pathways for *in vivo* hemolysis observed during conditions associated with rapid increases in plasma unesterified fatty acid(3-6). From our present findings, a scheme is inferred which may clarify certain events linking *in vivo* plasma unesterified fatty acid increases with subsequent hemolysis.

Materials and methods. Heparinized blood of all major groups was collected from normal human subjects, spun at 2500 rpm, 0°C, 15 minutes, and erythrocytes washed 3 times in sodium phosphate buffer, pH 7.2, ionic strength 0.15. Unless otherwise specified, standardized erythrocyte preparations were employed wherein addition of 0.5 ml of erythrocyte suspension to 2.5 ml of fatty acid or control solutions resulted in final concentration of 5 x 10^5 cells/cu mm. All fatty acid solutions were prepared in sodium phosphate buffer. The effect of temperature on pH of such buffered sodium oleate (Baker) solutions, measured with glass electrode, was as follows: 37°C, pH = 7.10; 25°C, pH = 7.20; 0°C, pH = 7.30. All fatty acid solutions were allowed to remain at room temperature at least 24 hours prior to use, since the hemolytic potency declined upon standing, rapidly at first, then more slowly. Human plasma albumin and globulin fractions were prepared from 3 individual citrated normal plasma specimens by overnight dialysis against ammonium sulfate solutions at 4°C employing magnetic stirring. Globulin-rich fractions were obtained at 50% ammonium sulfate saturation; albumin-rich fractions at 100% saturation. The fractions were washed with appropriate ammonium sulfate solutions, resuspended in buffered isotonic saline (pH 7.4) and cleared of ammonium ion (verified with Nessler's reagent) by dialysis at 4°C

TABLE I. Acceleration of Oleate Hemolysis by Plasma and Plasma Proteins.

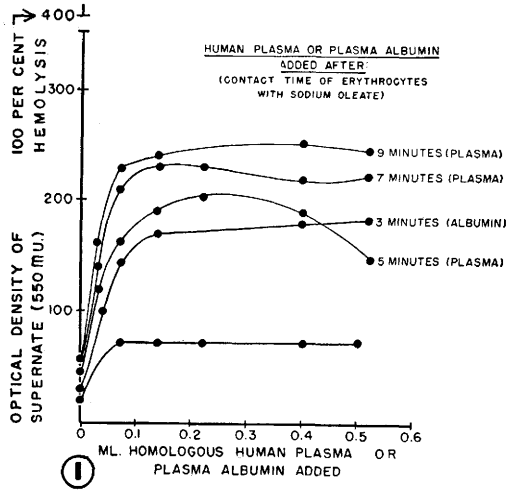
2.5 ml sodium oleate + 0.5 ml standardized human erythrocyte suspension +	Final oleate conc. ($\times 10^{-4}$ M) 4			2			1			0.5	
	°C										
	37	25	0	37	25	0	37	25	0	37	25
Isotonic buffered saline	1.1* (1.1)	1.5 (1.5)	18	1.5	3.0	50	2.0 (2.0)	5.0 (5.0)	350	9.0	22
Bovine serum albumin (40 mg/ml)	.16 (60)	.16 (60)	.50	.33	.42	.75	.75 (60)	1.5 (60)	90	6.0	12
Homologous human plasma	.25 (60)	.25 (60)	.75	.42	.50	1.5	1.0 (60)	1.5 (60)	90	7.0	12
Homologous human plasma albumin	.25 (4.0)	.25 (24)		.42	.50		1.0 (60)	1.5 (60)		7.0	12
Homologous human plasma globulin	.70 (1.7)	1.0 (3.2)		.83	1.0		1.0 (10)	3.5 (30)		8.5	16

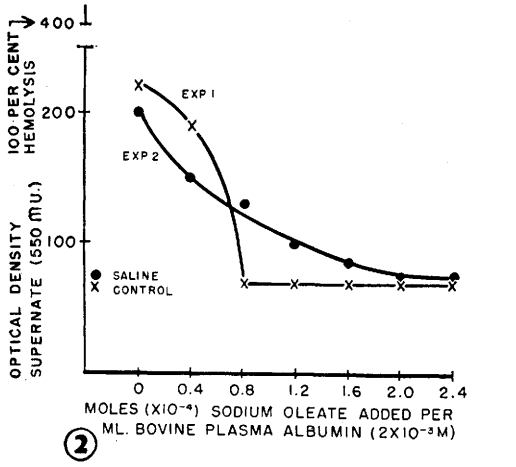
* Minimum oleate-erythrocyte contact time (min.) after which 0.10 ml of saline, plasma, or plasma proteins induce immediate 100% lysis. Figures in parentheses give 100% lysis time when sequence of combining is reversed, *i.e.*, when oleate and 0.10 ml saline, plasma, or plasma proteins are mixed first, followed by addition of erythrocytes. All values are means of 3 determinations.

against isotonic saline. Final volume of plasma fractions was adjusted to that of original plasma volume by addition of buffered isotonic saline. Cholesterol (Pfanstiehl) suspensions in the buffered saline were prepared by method of Lee and Tsai(8). Total cholesterol determinations in plasma and plasma fractions were performed according to method of Bloor(9).

Results. Acceleration of oleate hemolysis by plasma and plasma fractions. When a fixed number of washed human erythrocytes are suspended in sodium oleate, after a definite latent period, dependent at pH 7.2 ± 0.1 upon temperature and oleate concentration, addition of homologous plasma or plasma fractions induces immediate 100% hemolysis. Table I indicates *minimal latent period* required for immediate 100% lysis of standardized human erythrocyte suspensions by 0.10 ml homologous plasma and plasma fractions at varying oleate concentrations and temperatures. For example, 15 seconds *after* erythrocyte insertion in 2.5 ml sodium oleate (4 x 10^{-4} M, 37°C), addition of 0.10 ml homologous plasma induced immediate 100% lysis; such plasma addition to sodium oleate *prior* to erythrocyte insertion would have increased 100% lysis time from 1.10 to 60 minutes. From Table I it is apparent that minimal latent period required for immediate 100% lysis of standardized human erythrocyte suspensions by a fixed quantity of homologous plasma or plasma fraction is dependent upon initial oleate concentration and upon temperature; in general, the higher the oleate concentration or the lower the temperature, the relatively shorter the minimal latent period in relation to control 100% hemolysis time. Anticoagulants were not responsible for plasma hemolysis acceleration since homologous serum produced comparable results.

Intermediate phases of oleate lysis acceleration by human plasma. The preceding experiments indicate that a definite latent period is required following erythrocyte suspension in oleate solutions before addition of homologous plasma induces 100% hemolysis; the effect of plasma addition during the latent period was now determined. Small quantities (<0.10 ml) of homologous plasma or plasma albumin added rapidly to washed human erythrocytes suspended in 0.5 x 10^{-4} M sodium oleate at 25°C increased hemolysis in almost linear fashion; with larger plasma quantities, hemolysis attained a plateau or declined (Fig. 1). Time of oleate-erythrocyte contact prior to plasma addition determined the initial slope of the hemolysis curve as well as subsequent lysis plateau level. Under the same conditions, bovine serum albumin (2 x 10^{-3} M) and homologous human globulin produced similar families of hemolysis curves. Analysis of Fig. 1 suggests that as oleate-erythrocyte contact time increases, progressively more erythrocytes become "susceptible" to lysis by ho-





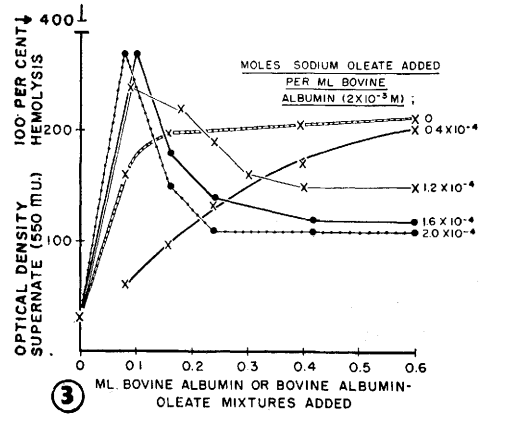


FIG. 1. Hemolysis accelerating activity of human plasma and human plasma albumin on homologous erythrocytes suspended in 3.0 ml sodium oleate (0.5×10^{-4} M, pH 7.2, 25°C). Lowest curve represents effect of plasma albumin addition 2 min. after erythrocyte-oleate contact. Each value is the mean of 4 determinations. (Optical density of supernates corrected for volume changes induced by varying quantities of human plasma or plasma albumin.)

FIG. 2. Effect of pretreatment of bovine serum albumin with sodium oleate on oleate hemolysis accelerating activity. Each test performed by adding 0.40 ml bovine serum albumin or bovine serum albumin containing added oleate to human erythrocytes suspended for 5 min. in 3 ml sodium oleate (1×10^{-4} M, pH 7.2, 25°C). Each value is mean of 4 determinations.

FIG. 3. Effect of concentration of bovine serum albumin-oleate mixtures on hemolysis accelerating activity for human erythrocytes suspended 5 min. in 3 ml sodium oleate (1×10^{-4} M, pH 7.2, 25°C). Each value is mean of 4 determinations. (Optical density of supernates corrected for volume changes induced by varying quantities of bovine serum albumin.)

mologous plasma and that this effect cannot be attributed simply to the increased control oleate lysis that occurs in the absence of plasma addition. Moreover, the initial linear relation between plasma quantity added and hemolysis (which can be demonstrated more precisely by studying the action of bovine albumin (2×10^{-3} M) on human erythrocytes in 1×10^{-4} M sodium oleate at 37°C) suggests a stoichiometric interaction between plasma factors and "susceptible" erythrocytes.

Nature of oleate hemolysis acceleration by homologous plasma. Two general mechanisms seem possible for oleate hemolysis acceleration by homologous plasma: 1) acceleration of *intrinsic* oleate lytic activity, or 2) acceleration of lysis by *superimposed* lytic mechanisms independent of intrinsic oleate hemolysis. Washed human erythrocytes were therefore exposed to increasingly dilute sodium oleate solutions at room temperature until thresholds were attained at which erythrocytes, after 5 saline washings, exhibited no increased tendency to lysis upon incubation at 37°C. Lysis of such erythrocytes by bovine or human albumin was not observed; erythrocyte binding of lytic quantities of oleate appears to be a prerequisite. Plasma and plasma fractions thus seem to act primarily by accelerating intrinsic oleate lytic activity, but this does not necessarily implicate the same intermediate pathways. Since oleate reduces aqueous surface tension, human plasma might accelerate erythrocyte lysis in oleate solutions by abruptly raising solution surface tension. This

possibility can be excluded since isotonic saline will raise oleate solution surface tension abruptly (measured with glass capillary tubes) without accelerating hemolysis; moreover, human erythrocytes pretreated with sodium oleate and washed repeatedly could be lysed immediately upon contact with homologous plasma as described previously(2). These observations do not exclude the possibility that abrupt surface tension alterations *within* the erythrocyte membrane may be concerned in the oleate hemolysis acceleration.

Inhibition of oleate hemolysis accelerating activity of human plasma. Pretreatment of human plasma or bovine serum albumin with increasing quantities of sodium oleate at 25°C progressively inhibited and finally abolished oleate hemolysis accelerating properties (Fig. 2). Such pretreatment simultaneously reduced the capacity to protect human erythrocytes from oleate hemolysis. By saturating the oleate binding sites, plasma and plasma fractions simultaneously lose ability to protect against or to accelerate oleate hemolysis. Incidentally, such findings strongly suggest that the oleate molecule on the surface of the erythrocyte membrane is directly responsible for lysis accelerating activity of homologous plasma. As anticipated from preceding findings, human plasma albumin, capable of binding more fatty acid than plasma globulin(10), exhibited greater oleate hemolysis protective and accelerating activity than the corresponding globulin fraction, (Table I). Nevertheless, oleate hemolysis protective and accelerating properties may not run parallel; thus whole human plasma afforded greater oleate hemolysis protection than did the corresponding plasma albumin fraction, yet was no more effective in accelerating oleate hemolysis, Table I. Although pretreatment of human plasma or bovine albumin with sodium oleate inhibits the oleate hemolysis accelerating mechanism, when such solutions are diluted with isotonic saline, the inhibitory effect wanes. Indeed, a sufficiently dilute solution of bovine albumin pretreated with oleate may exhibit more intense hemolysis accelerating activity than untreated albumin (Fig. 3). This observation suggests dissociation of the albumin and oleate upon dilution, so that both then act to accelerate lysis. Such findings would account for the frequent ability of small quantities (0.10 ml) of normal human plasma to induce more intense oleate hemolysis acceleration than larger quantities (0.2-1.0 ml) upon addition to 3.0 ml oleate-erythrocyte systems.

TABLE II. Acceleration of Oleate Hemolysis by Unesterified Cholesterol.

2.5 ml sodium oleate* + .5 ml standardized human erythrocyte suspension +	Exp. 1	Exp. 2
Isotonic buffered saline	1.0†(1.0)	1.5 (1.5)
Unesterified cholesterol in isotonic buffered saline (1 mg/ml)	.33 (30)	.33 (35)

* Final oleate concentration = 4×10^{-4} M.

† Minimum oleate-erythrocyte contact time (min.) at 25°C after which 0.10 ml of saline or cholesterol induce immediate 100% lysis. Figures in parentheses give 100% lysis time when sequence of combining is reversed, *i.e.*, when oleate and 0.10 ml saline or cholesterol are mixed first, followed by addition of erythrocytes. All values are mean of 3 determinations.

Effect of cholesterol on oleate hemolysis. Cholesterol has been reported to be important for the fatty acid hemolysis protective properties of plasma(11). The ability of cholesterol (in isotonic buffered saline colloidal suspension) to protect against fatty acid hemolysis was confirmed during this study. Cholesterol, however, behaves similarly to whole plasma in that when added *after* human erythrocyte-oleate contact, hemolysis is accelerated, (Table II). It is unlikely, though, that plasma cholesterol accounts for the entire oleate hemolysis accelerating activity of human plasma. The oleate lysis accelerating activity of human plasma albumin (total cholesterol = 116 mg %) was similar to that of the corresponding whole plasma (total cholesterol = 234 mg %) (Table I), yet the total cholesterol level was lower. Indeed, bovine serum albumin containing the lowest cholesterol concentration (50 mg %) was the most effective oleate hemolysis accelerator. It is appreciated that these measurements are limited to total cholesterol and that only the unesterified moiety affects fatty acid hemolysis(11); nevertheless the results suggest that plasma factors other than cholesterol are also impor-

tant in lysis acceleration of oleate pretreated human erythrocytes.

Specificity of oleate hemolysis acceleration by human plasma. Although marked species variation existed in erythrocyte susceptibility to oleate lysis, plasma from rats, rabbits, guinea pigs, and sheep were all found capable of accelerating hemolysis of homologous erythrocytes pretreated with oleate. Moreover, hemolysis accelerating activity of mammalian plasma is not restricted to oleate. Sodium palmitate and stearate though less effective than sodium oleate as hemolytic agents (at pH 7.2) yielded qualitatively similar relations with respect to hemolysis protective and accelerating influences of homologous plasma. Indeed, the capacity of the same substance to protect against and to accelerate fatty acid hemolysis (depending upon the sequence of fatty acid addition) was not restricted to plasma or plasma factors. Sodium cyanide retarded hemolysis when added to oleate solutions prior to human erythrocyte insertion (7); final concentrations of 1×10^{-3} M sodium cyanide consistently accelerated lysis of oleate pretreated human erythrocytes.

Discussion. Since 1940, numerous studies have indicated some relation between hyperlipemia and *in vivo* hemolysis. Hyperlipemia induced by high fat diets or by intravenous infusions of lipid emulsions or hyperlipemic plasma accelerates erythrocyte destruction; at times hemoglobinemia is evident(3-5). Frank anemia may be produced by repeated intravenous fat emulsion infusions(12). Such *in vivo* observations support the hypothesis that the hyperlipemic state constitutes an important physiologic mechanism in human erythrocyte destruction(13). The precise manner whereby *in vivo* hemolysis develops during hyperlipemia, however, has been incompletely defined.

The findings presented in this report indicate that once lytic quantities of fatty acid are bound *in vitro* by human erythrocytes, homologous plasma, the plasma albumin and globulin fractions, or unesterified cholesterol (as well as cyanide) reverse their protective action and accelerate hemolysis. This acceleration can be attributed to a stoichiometric plasma interaction with the altered erythrocytes. Ability to block simultaneously and progressively both hemolysis protection and acceleration by pretreatment of plasma fractions with increasing quantities of fatty acid suggests that plasma sites which bind fatty acid and protect against fatty acid hemolysis, accelerate lysis once the fatty acid has "coated" the erythrocyte membrane. Whether plasma acts by "tearing" the fatty acid from the cell wall or by combining at the cell surface to form a new complex is undetermined; the underlying mechanism, however, appears to depend in some way upon acceleration of intrinsic fatty acid lytic activity. Changes in surface tension of the suspension medium are not responsible for fatty acid hemolysis accelerating activity of homologous plasma. The mechanism of cyanide action is unknown.

Intensity of fatty acid hemolysis acceleration by human plasma or plasma fractions is not uniform. Variations can be attributed to differences in capacity of plasma and plasma fractions to bind fatty acid and to a plasma inhibitor which can be "inactivated" by dilution. Indeed, upon sufficient dilution, the inhibitor actually enhances lysis acceleration; this inhibitor possesses the activity of unesterified plasma fatty acid.

A hypothesis linking certain aspects of hyperlipemia, *i.e.* increases in unesterified plasma fatty acid, with *in vivo* hemolysis now may be suggested from the results of the present and related previous(1,2,7) studies: Introduction into the circulation of *free* fatty acid by infusion(14), from postprandial chyle(15), from hydrolysis of circulating chylomicra(16,17), and possibly from fat depots(18), would result at the introduction site, in competitive binding by plasma and erythrocytes. Those erythrocytes binding sufficient fatty acid to exceed the hemolytic threshold may potentiate lysis by releasing lytic factors upon hemolysis during transit. The surrounding homologous plasma not alone fails to be protective once erythrocyte fatty acid binding occurs, but accelerates hemolysis. The remainder of the exogenous fatty acid, that bound by plasma albumin, globulin, and unesterified cholesterol to form unesterified plasma fatty acid, is rendered virtually inert hemolytically. *It is not the increase in plasma unesterified fatty acid*

that abruptly accelerates hemolysis during hyperlipemia—it is the exogenous free fatty acid bound by erythrocytes before plasma binding occurs. Plasma unesterified fatty acid increase is merely an indicator that exogenous fatty acid has been added to blood; it is *during* addition that conditions for maximum hemolysis acceleration are attained. Once bound by plasma, exogenous fatty acid is rapidly transferred to tissues for oxidation or esterification(19,20), probably without constituting any further serious immediate erythrocyte threat. Increased plasma unesterified fatty acid would, however, constitute an insidious threat by a) decreasing plasma ability to protect against additional free fatty acid, and b) providing erythrocytes with a source of sparingly released fatty acid.*

Whether intact chylomicra can induce hemolysis is unknown. However, since chylomicra are chiefly triglycerides(21), they can be regarded as potential *free* fatty acid reservoirs. *In vivo* hemolysis acceleration during chylomicronemia would, in part, be a function of rate of intravascular chylomicra hydrolysis. Since heparin provides optimal conditions for such intravascular hydrolysis, maximum hemolysis acceleration during chylomicronemia should develop following heparin administration. This conforms with experimental observations(6). Normally, in absence of exogenous heparin, the bulk of chylomicra hydrolysis occurs in extravascular depots(19,21,22) where a large fraction of the triglyceride fatty acid is directly oxidized without reentering the circulation(19); this process thus apparently serves as a homeostatic mechanism for mitigating intravascular hemolysis. However, in certain human and animal subjects, exogenous heparin may not be necessary for intravascular chylomicra hydrolysis since the untreated plasma contains active enzymes capable of inducing significant fatty acid release (23-25). Competitive erythrocyte-plasma binding of such released fatty acid might explain, at least in part, ability of normal chylomicra-rich plasma to induce hemolysis *in vivo* and to increase erythrocyte mechanical fragility *in vitro*(5,26).

The above interpretations permit clearer analysis of the observations of previous investigators(5,6,15,26). The proposed fatty acid-erythrocyte-plasma interactions are not specific for one fatty acid, for one blood group, or for the human. Comparable results, at least *in vitro,* were obtained with the several fatty acids tested, with all major human blood groups, and with sheep, rat, rabbit, and guinea pig erythrocytes. The suggested hypothesis linking *in vivo* hemolysis with increases in unesterified plasma fatty acid appears to possess general applicability.

Summary. 1. Pretreatment of fatty acid with human plasma, plasma albumin or globulin, unesterified cholesterol, or cyanide retards or prevents hemolytic activity. Once lytic quantities of fatty acid are bound by human erythrocytes, the above substances reverse their protective activity and accelerate hemolysis. 2. Plasma and plasma factors appear to accelerate intrinsic oleate hemolytic activity rather than act *via* a secondary superimposed lytic mechanism; the mode of cyanide action is unknown. 3. The oleate hemolysis protective and accelerating properties of human plasma can be inhibited progressively and simultaneously by pretreatment with oleate. Both mechanisms appear to depend upon plasma ability to bind fatty acid. 4. The intensity of oleate hemolysis acceleration by human plasma or plasma fractions is not uniform. Variations can be attributed to differences in fatty acid binding capacities and to inhibitors which possess unesterified fatty acid activity. 5. A hypothesis is suggested for linking increases in mammalian plasma unesterified fatty acid with *in vivo* hemolysis.

* Erythrocytes removed from humans one hour after intravenous injection of albumin-bound C^{14} labelled unesterified palmitic or oleic acid contained 1% of the radioactivity of equivalent plasma volumes; by this time plasma fatty acid radioactivity had fallen to 1% of initial values(20), so that erythrocyte fatty acid content was now only 1/10,000 of that equivalent initial plasma volumes.

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Conversion of Corticosterone-4-C^{14} to Aldosterone by Human Adrenal Slices.* (25076)

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Investigations of the biosynthetic pathways of aldosterone have yielded conflicting results. Kahnt(1) demonstrated incorporation of desoxycorticosterone-21-C^{14} into aldosterone using beef adrenal homogenates. Addition of progesterone to the incubation medium did not increase aldosterone production. Rosemberg(2) has shown that perfusion of isolated calf adrenals with progesterone increased production of aldosterone while perfusion with corticosterone or desoxycorticosterone had little or no effect. Incubation of C^{14}-labelled progesterone, desoxycorticosterone or corticosterone with beef capsule strippings by Ayres(3) and later by Travis(4) demonstrated the incorporation of each into aldosterone. The data are conflicting in regard to the most efficient precursor. Little is known of the pathways of aldosterone biosynthesis in man. Seltzer(5) demonstrated that infusion of large quantities of corticosterone increased urinary excretion of aldosterone. However, it was not determined whether this increased aldosterone excretion resulted from a direct incorporation of corticosterone or by indirect effect of corticosterone upon adrenal cortical function. Moreover, despite continuous infusion of corticosterone, aldosterone excretion diminished. The present report describes incorporation of corticosterone-4-C^{14} into aldosterone by human adrenal slices.

Methods. A patient with metastatic cancer of the breast underwent a bilateral adrenalectomy. The right adrenal was immediately immersed in cold isotonic saline, then freed of fat, weighed, and sliced with a Stadie-Riggs microtome. Approximately 2.22 x 10^5 DPM (1.4 x 10^5 CPM)/24 μg of corticosterone-4-C^{14}§

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