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Changes in linoleic acid during follicular development and inhibition of spontaneous breakdown of germinal vesicles in cumulus-free bovine oocytes

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Summary. The fatty-acid composition of follicular fluid from small and large developing follicles was analysed and the effects of saturated and unsaturated fatty acids on spontaneous breakdown of germinal vesicles were investigated. Fatty acids were bound to bovine serum albumin and cultured with oocytes at 100 μmol/l. Linoleic acid (18:2) was the only fatty acid tested that significantly inhibited breakdown of germinal vesicles ($P < 0.01$). The effect was dose-dependent and was greatest at 50 μmol fatty acid/l (% breakdown of control, 81·1 ± 6·8 vs. 50 μmol linoleic acid/l, 35·4 ± 7·3; $P < 0.02$). Linoleic acid was the major fatty acid, constituting about a third of the total fatty acid in the follicular fluid; followed by 18·9 ± 1·0% and 16·9 ± 1·3% oleic acid (18:1) in small and large follicles, respectively. Saturated fatty acids accounted for <30% of the total fatty acid composition. There was a marked absence of tetraenoic acids in small and large follicles. Proportions of linoleic acid were significantly lower in follicular fluid from large follicles (31·1 ± 1·2% of total fatty acid) than from small follicles (34·8 ± 0·7% of total fatty acid) ($P < 0.05$) and there was a significant inverse correlation between follicle diameter and percentage of linoleic acid in the follicular fluid ($r = -0.6966$; $P < 0.05$). There was no significant alteration in any other fatty acid during follicular development. The results suggest that (i) a critical relative concentration of linoleic acid may be required for maintenance of meiotic arrest in bovine oocytes and (ii) the decline in the proportion of linoleic acid during follicular development may contribute to the relief of the inhibition of breakdown of germinal vesicles.

Keywords: cow; fatty acid; meiosis; germinal vesicle; oocyte; follicle

Introduction

Oocytes develop in the primordial follicle of the embryo where they begin to undergo meiosis. In mammals, this process is arrested at the diplotene stage, or germinal vesicle stage, immediately before or shortly after birth (reviewed by McGaughey, 1983). Meiotic arrest persists until sexual maturity, when continued follicular growth and development is stimulated by follicle-stimulating hormone during each reproductive cycle. The preovulatory surge of luteinizing hormone results in the completion of follicular development accompanied by the resumption of meiosis (Ayalon *et al.*, 1972). At this stage, the germinal vesicle breaks down, the chromatin condenses and the oocyte progresses to the metaphase of the second meiotic division.

Mammalian oocytes can spontaneously resume meiosis when removed from the follicular environment (reviewed by McGaughey, 1983); it was therefore suggested that components of follicular fluid regulate the maintenance of meiotic arrest (Chang, 1955). Several such factors have been reported to contribute to the maintenance of meiotic arrest, such as a peptide of low M_r in the

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follicular fluid (Gwatkin & Anderson, 1976; Tsafriri *et al.*, 1976), proportions of intrafollicular steroids (Rice & McGaughey, 1981), and hypoxanthine and adenosine (Downs *et al.*, 1985).

A previous study revealed that during the development of porcine follicles, actual concentrations of free fatty acid dramatically declined, accompanied by changes in the percentage distribution of fatty acids in the follicular fluid (Yao *et al.*, 1980). The fatty acid composition of meiotically immature porcine oocytes reflects that of the follicular fluid, being strikingly rich in polyunsaturated fatty acids (Homa *et al.*, 1986). Data from a preliminary investigation suggested that linoleic acid (18:2), a polyunsaturated fatty acid, inhibits meiotic maturation in porcine oocytes (Homa, 1987). Since mammalian oocytes are bathed in follicular fluid and fatty acids play such a crucial role in cellular function (reviewed by Stubbs & Smith, 1984), it was important to investigate further the relationship between fatty acids in follicular fluid and their influence in arresting meiosis in oocytes.

This study determined the fatty-acid composition of follicular fluid in small and large developing bovine follicles and investigated the effects of a range of saturated and unsaturated fatty acids on spontaneous breakdown of germinal vesicles in oocytes.

Materials and Methods

Experiment 1: determination of fatty-acid composition of follicular fluid

Collection of follicular fluid for fatty-acid analysis

Cow ovaries were purchased from a local abattoir. Follicle diameters were measured and follicular fluid was aspirated using an 18-gauge needle attached to a syringe. Care was taken to aspirate fluid only from follicles that appeared normal. Most antral follicles in mammalian ovaries are destined to undergo atresia (Himelstein-Braw *et al.*, 1976; McGaughey *et al.*, 1979; McGaughey, 1983); therefore, we cannot exclude the possibility that this process may have been initiated in a small percentage of our selected follicles, which may be reflected in the fatty-acid composition.

Fluid was pooled from several small follicles (1–3 mm diameter) of any one ovary to obtain a sufficient volume for analysis of fatty acids in any one sample. Large follicles (>7 mm diameter) contained sufficient fluid for us to carry out analysis without pooling. Five samples from five separate ovaries were prepared for each of the small- and large-follicle categories. The follicular fluid was centrifuged for 15 min at 13 000 *g*. Total lipids were extracted from 200-μl aliquants of the supernatant.

Fatty-acid analysis

Total lipid extraction was achieved with the addition of 3·75 ml chloroform/methanol (1:2 v/v), followed by 1·25 ml chloroform and 1·25 ml water (Bligh & Dyer, 1959). The organic phase was evaporated to dryness under a stream of O_2-free N_2, and saponified for 1 h at 80°C with 2 ml of 33% KOH in ethanol (6:94 v/v) containing 40 μg hydroquinone/ml. After addition of 2 ml water, the sample was washed twice with 3 ml hexane. The fatty acids were extracted with two washes of 3 ml hexane, after acidification with 1 mol HCl/l, and evaporated to dryness under a stream of O_2-free N_2. Fatty-acid methyl esters were prepared by treatment with 3 ml boron trifluoride in 14% methanol for 7 min at 80°C and extracted with three washes of 2 ml hexane. The fatty-acid methyl esters were separated isothermally by gas–liquid chromatography (Hewlett-Packard model no. HP 5840A; Avondale, PA, USA) using a 30-m Supelcowax column at 210°C, with N_2 as the carrier gas. The fatty-acid methyl esters were detected by flame ionization. The proportions of fatty acids were integrated automatically.

Experiment 2: effects of fatty acids on breakdown of germinal vesicles

Collection of oocytes

Oocytes were collected in chemically defined medium (2A-BMOC; McGaughey, 1977) using a procedure described by Homa (1988). Ovaries were macerated with double-edged razor blades, taking care to exclude corpora lutea and follicles >5 mm in diameter. Large tissue debris was removed by passing the suspension through a 1 mm screen. Cumulus-enclosed oocytes were collected on a stainless steel screen with 106-μm pores. The oocytes were denuded of adherent cumulus cells by vortexing with 1% (w/v) sodium citrate in 1 mol EDTA/l (pH 7·4) for 30 s and then resuspended in 2A-BMOC medium. The suspension was passed through a 106-μm screen and the oocytes were washed on the screen surface with 2A-BMOC medium. The oocytes were then transferred to Petri dishes and selected for culture with the aid of a light microscope. Only oocytes exhibiting a uniform distribution of organelles within the cytoplasm were cultured.

Preparation of fatty acids bound to albumin

Fatty acids (Sigma Chemical Co., St Louis, MO, USA, and NuCheck-Prep, Inc. Elysian, MN, USA) were bound to 10% (w/v) essentially fatty-acid-free bovine serum albumin (BSA, Sigma) in 2A-BMOC medium using a modification of the method of Spector & Hoak (1969). Fatty acids were dissolved in hexane (2 mg/ml) and added to celite (10 mg fatty acid/0·5 g celite). The hexane was evaporated under O_2-free N_2, and 10% BSA was added (1 ml 10% BSA/2 mg fatty acid). The albumin solution was left for 30 min at room temperature with occasional agitation and then decanted and centrifuged for 15 min at 13 000 ***g***. The solutions were sterilized by passing them through a 22-μm millipore filter (Millipore Corp., Bedford, MA, USA). The amount of fatty acid bound to albumin was determined by gas–liquid chromatography. Concentrations were adjusted with 10% BSA in 2A-BMOC medium.

Oocyte culture

(a) ***Effect of C_{16} and C_{18} fatty acids on breakdown of germinal vesicles.*** Groups of oocytes (6–10/group representing one replicate) were incubated under sterile conditions in 190 μl 2A-BMOC medium with 10 μl palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2) or α-linolenic (18:3) acid bound to albumin to give 100 μmol fatty acid/l. This concentration was predicted to be well within the physiological range compared with values calculated for fatty-acid concentrations in porcine follicular fluid (Yao *et al.*, 1980). The BSA concentration was 0·5% (w/v); in control experiments, 0·5% BSA was used alone. Generally, two or three replicates were carried out for each treatment, and the experiments were carried out three times. Incubations were carried out for 24 h in Lab-Tek chamber slides (Nunc, Inc., Naperville, IL, USA) at 38°C under 5% CO_2 + 5% O_2 + 90% N_2 in a humidified atmosphere.

(b) ***Effect of C_{20} and C_{22} fatty acids on breakdown of germinal vesicles.*** Groups of oocytes were cultured with 100 μmol arachidonic (20:4), eicosapentaenoic (20:5) or adrenic (22:4) acid/l under the conditions described for Expt 2a.

(c) ***Effect of concentration of linoleic acid on breakdown of germinal vesicles.*** The dose response was investigated by culturing groups of oocytes with 0–200 μmol linoleic acid/l. The BSA concentration was maintained at 0·5%. Culture conditions were as described for Expt 2a and 2b.

Determination of stage of meiosis

Oocytes were placed in 1% sodium citrate and were subsequently fixed in ethanol:acetic acid (3:1 v/v), allowed to air-dry and stained in Wright's stain in 2·9% (w/v) glycerol in methanol (Rice & McGaughey, 1981). Oocyte maturation was scored cytogenetically: at the germinal vesicle stage, chromatin was classified as either normal (fibrous or diffuse) or degenerate, as described by McGaughey *et al.* (1979) and Homa (1988). Oocytes which had progressed to or beyond diakinesis were considered to have undergone breakdown of germinal vesicles and to be maturing (McGaughey *et al.*, 1979).

Statistical analysis

Statistical analyses were carried out using Student's *t* test. Values of $P < 0·05$ were considered significant.

Results

Experiment 1: fatty-acid composition of follicular fluid

Saturated fatty acids accounted for <30% of the total fatty acids in both small and large follicles (Table 1); polyunsaturated fatty acids constituted the major components (48·0% in small follicles, 46·7% in large follicles). The primary constituent in bovine follicular fluid was the dienoic fatty acid linoleic acid (18:2), contributing about a third of the total fatty acid composition, almost twice as much as the secondary fatty acid component, the monoenoic oleic acid (18:1) (Table 1). The saturated fatty acids palmitic (16:0) and stearic (18:0) acids were present in similar proportions, representing 13·3 and 11·0%, respectively, in fluid from small follicles, and 14·8 and 12·3%, respectively, in fluid from large follicles. There was a notable absence of arachidonic acid (20:4) in fluid from small and large follicles, and adrenic acid (22:4) was negligible. The fatty-acid profile of fluid from large follicles appeared to contain greater proportions of palmitic, palmitoleic (16:1), stearic and linolenic (18:3) acids than fluid from small follicles, but these differences were not statistically significant; the lower proportion of oleic acid was not significant; but the percentage of linoleic acid was significantly lower in fluid from large follicles (31·1%) than in that of small follicles (34·8%; $P < 0·05$; Table 1). When the proportion of linoleic acid, expressed as a percentage of the total fatty acid in follicular fluid, was plotted against the average follicle diameter (Fig. 1), a significant inverse correlation was revealed ($P < 0·05$).

Table 1. Fatty acid composition of bovine follicular fluid

Fatty acid	Small follicles (1–3 mm diameter)	Large follicles (7–13 mm diameter)
Palmitic (16:0)	13·3 ± 0·7	14·8 ± 1·4
Palmitoleic (16:1)	5·0 ± 0·5	6·4 ± 1·3
Stearic (18:0)	11·0 ± 1·1	12·3 ± 0·9
Oleic (18:1)	18·9 ± 1·0	16·9 ± 1·3
Linoleic (18:2)	34·8 ± 0·7*	31·1 ± 1·2*
γ-Linolenic (18:3 n-6)	1·1 ± 0·3	0·6 ± 0·4
α-Linolenic (18:3 n-3)	6·1 ± 0·5	8·1 ± 1·4
Arachidic (20:0)	1·3 ± 0·4	0·6 ± 0·4
Eicosenoic (20:1)	1·3 ± 0·4	0·8 ± 0·4
Eicosadienoic (20:2)	1·0 ± 0·4	0·6 ± 0·3
Eicosatrienoic (20:3)	3·2 ± 0·5	3·0 ± 0·4
Eicosapentaenoic (20:5)	1·0 ± 0·5	1·7 ± 0·3
Heneicosanoic (21:0)	1·2 ± 0·4	1·6 ± 0·2
Docosapentaenoic (22:5)	0·8 ± 0·3	1·6 ± 0·3

Values are the means ± s.e.m. of five separate sample determinations, and are expressed as a percentage of the total fatty acid composition. Only fatty acids constituting > 1% of the total in either follicle category were included.

*Value of linoleic acid in small follicles significantly different from value of linoleic acid in large follicles ($P < 0·05$).

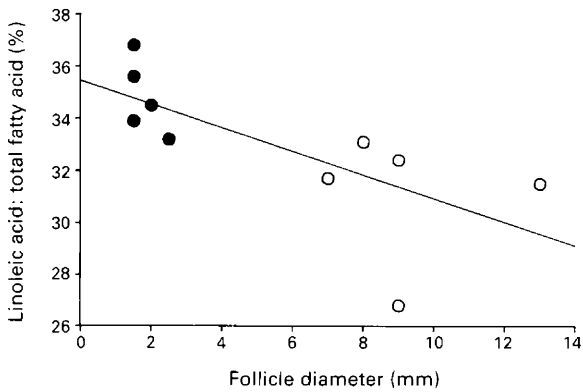

Fig. 1. Correlation between the proportion of linoleic acid in the follicular fluid and diameter of bovine follicle; (●) averaged for pooled samples, 1–3 mm diameter; (○) individual diameters for unpooled samples; $r = -0·6966$, $P < 0·05$.

Experiment 2: effects of fatty acids on breakdown of germinal vesicles

In the absence of fatty acid, 85·6–94·6% of oocytes resumed meiosis (controls for Expt 2a and 2b, respectively; Table 2). Linoleic acid (18:2) significantly inhibited resumption of meiosis; only 48·6% of oocytes demonstrated breakdown of germinal vesicles in the presence of the fatty acid ($P < 0·01$) (Table 2). However, this fatty acid did not affect the ability of oocytes that did undergo breakdown in its presence to progress to anaphase I or beyond (Table 2; 47·8% of total maturing oocytes in the absence of fatty acid vs. 51·4% of total maturing oocytes in the presence of linoleic acid). None of the other fatty acids tested had any significant effect on the resumption or progression of meiosis. The proportion of oocytes which degenerated during culture in the presence of any fatty acid was not significantly different from that in the absence of fatty acid (Table 3).

Table 2. Effects of fatty acids on breakdown of germinal vesicles and meiotic progression in bovine oocytes

Fatty acid	Number of replicates	Breakdown of germinal vesicles (%)*	Anaphase I to metaphase II (%)†
Experiment 2a			
None (control)	8	85·6 ± 5·3[a]	47·8 ± 10·4
Palmitic (16:0)	2	70·1 (75·0, 66·7)	50·0 (100, 0)
Palmitoleic (16:1)	8	63·6 ± 12·8	65·3 ± 11·1
Stearic (18:0)	9	61·7 ± 9·4	41·5 ± 12·7
Oleic (18:1)	8	70·2 ± 5·5	66·4 ± 7·9
Linoleic (18:2)	8	48·6 ± 10·3[a]	51·4 ± 15·3
α-Linolenic (18:3)	8	74·6 ± 7·4	59·1 ± 14·2
Experiment 2b			
None (control)	8	94·6 ± 4·5	83·3 ± 6·4
Arachidonic (20:4)	6	84·9 ± 6·0	74·2 ± 9·8
Eicosapentaenoic (20:5)	4	92·2 ± 5·3	85·9 ± 5·8
Adrenic (22:4)	5	82·7 ± 7·8	61·4 ± 12·5

Oocytes were cultured with, or without, 100 μmol fatty acid/l bound to bovine serum albumin.

*Percentage of total non-degenerate oocytes ± s.e.m.

†Percentage of total oocytes undergoing breakdown ± s.e.m. for % GVBD in the presence of linoleic acid vs. control, $P < 0·01$.

[a]Percentage breakdown of germinal vesicles in the presence of linoleic acid vs. control, $P < 0·01$.

Table 3. Incidence of degenerate bovine oocytes in the presence of fatty acids

Fatty acids	Degenerate oocytes (%)*
Experiment 2a	
None (control)	20·7 ± 4·3
Palmitic (16:0)	20·0 (0, 40·0)
Palmitoleic (16:1)	28·6 ± 7·2
Stearic (18:0)	26·1 ± 4·4
Oleic (18:1)	27·4 ± 2·2
Linoleic (18:2)	25·3 ± 6·4
α-Linolenic (18:3)	25·6 ± 3·9
Experiment 2b	
None (control)	12·9 ± 4·9
Arachidonic (20:4)	16·3 ± 8·5
Eicosapentaenoic (20:5)	18·5 ± 5·0
Adrenic (22:4)	18·7 ± 6·0

*Values are means ± s.e.m. of all oocytes cultured in Expts 2a and 2b.

The effect of linoleic acid was dose-dependent (Fig. 2), reaching maximum inhibition of breakdown of germinal vesicles at 50 μmol/l (% breakdown of control, 81·1 ± 1·1 vs. 50 μmol linoleic acid/l, 35·4 ± 7·3; $P < 0·02$). The proportion of oocytes prevented from undergoing breakdown of germinal vesicles did not increase significantly at higher doses. Incubation of bovine oocytes with linoleic acid bound to BSA revealed that the concentrations of fatty acid used in these experiments were not toxic, as the proportion of oocytes that were degenerate was not significantly different in the control and the groups treated with fatty acid (Table 2).

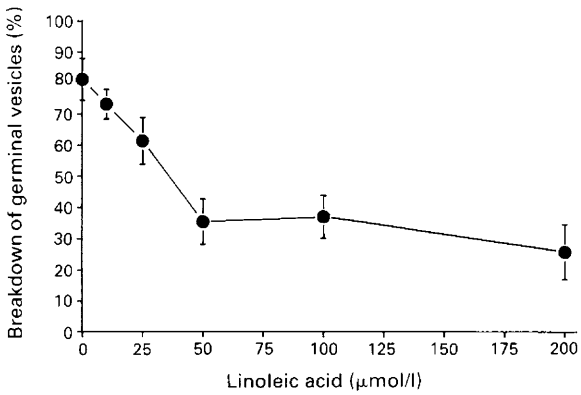

Fig. 2. Response of breakdown of germinal vesicles in bovine oocytes to concentration of linoleic acid. Values are means ± s.e.m. of percentage breakdown in the presence of 50 μmol linoleic acid/l vs. control, $P < 0{\cdot}02$.

Discussion

The observation that linoleic acid significantly inhibited breakdown of germinal vesicles is intriguing. *In vivo*, oocytes in small follicles are developmentally immature and meiosis is arrested. It is only after the preovulatory follicle has grown to a large stage that the inhibitory influence upon resumption of meiosis in oocytes is released, under the influence of luteinizing hormone (see McGaughey, 1983). The effect of linoleic acid may be physiologically relevant: first, this fatty acid is a natural constituent of follicular fluid in which the oocyte is bathed and, secondly, the actual concentration of free fatty acid in porcine follicular fluid is ~1 mmol/l (Yao *et al.*, 1980) and we have demonstrated inhibition of breakdown of germinal vesicles in bovine oocytes by only 50 μmol linoleic acid/l.

The results from the present study indicate that the fatty-acid environment of immature bovine oocytes in small follicles is more conducive to maintaining meiotic arrest than that of developing follicles, since the proportion of linoleic acid is smaller in the latter. This suggests that a critical relative concentration of linoleic acid may be necessary for the maintenance of meiotic arrest in bovine oocytes. The actual concentrations of fatty acids were not measured in this study, but it is interesting that the concentration of total fatty acid declines significantly in porcine follicular fluid as the follicle increases in size (Yao *et al.*, 1980).

Cyclic adenosine monophosphate (cAMP) plays a significant role in the maintenance of oocyte meiotic arrest in several mammalian species (mouse, Cho *et al.*, 1974; rat, Magnusson & Hillensjö, 1977; pig, Rice & McGaughey, 1981, Racowsky, 1985a; cow, Ball *et al.*, 1983; hamster, Racowsky, 1985b; cow, Homa, 1988). Incorporation of unsaturated fatty acids into membrane phospholipids increases basal and stimulated activity of adenylate cyclase (Engelhard *et al.*, 1978; Colard *et al.*, 1980; Poon *et al.*, 1981; Chambaz *et al.*, 1983). The stimulatory effect is specific for incorporation of linoleic acid (Colard *et al.*, 1980; Chambaz *et al.*, 1983). Evidence supports the existence of adenylate cyclase in the mammalian oocyte plasma membrane (Racowsky, 1985a, b), and it is therefore tempting to speculate that the inhibitory effect of linoleic acid on spontaneous breakdown of germinal vesicles in bovine oocytes may be due, in part, to its specific stimulatory effect on activity of adenylate cyclase.

Linoleic acid stimulates protein kinase C (Murakami *et al.*, 1986; Dell & Severson, 1989), which plays a significant role in cell growth and differentiation (Nishizuka, 1988) and may be important in regulating meiotic arrest. Tumour-promoting phorbol esters, which permanently activate protein kinase C, inhibit the resumption of meiosis in murine oocytes (Urner & Schorderet-Slatkine, 1984;

Bornslaeger *et al.*, 1986) and promote the inhibitory effects of a cAMP phosphodiesterase inhibitor on breakdown of germinal vesicles in bovine oocytes (Homa, 1991). Thus, the effects of linoleic acid may result from a direct activation of protein kinase C, thereby mimicking the effect of tumour-promoting phorbol esters on oocyte maturation.

Alternatively, linoleic acid may exert its effect through prostaglandins and/or leukotrienes, which have potent effects as local hormones (Smith, 1989) and have been implicated in ovarian function (reviewed by Armstrong, 1981). Lipoxygenase activity has been detected in oocytes of several invertebrates and lower vertebrates (sea-urchin, Perry & Epel, 1985; starfish, Meijer *et al.*, 1986; frog, Hawkins & Brash, 1989). Although arachidonic acid (20:4), the primary substrate for both lipoxygenase and cyclooxygenase (Smith, 1989), does not occur in bovine follicular fluid, linoleic acid can serve as an alternative precursor (Claeys *et al.*, 1985; Hawkins & Brash, 1989).

Irrespective of the mechanisms involved in inhibition by linoleic acid of the resumption of meiosis, dietary alterations may modify the fatty-acid composition of follicular fluid, since it is partly derived from serum (Yao *et al.*, 1980). As oocytes are bathed in follicular fluid, a high intake of polyunsaturated fatty acids in the diet may potentially influence the ability of oocytes to mature *in vivo*. The fatty-acid patterns of individual lipids of meiotically immature porcine oocytes (Homa *et al.*, 1986) are similar to that of porcine follicular fluid, suggesting a direct relationship between concentrations of fatty acids in oocytes and follicular fluid.

The fatty-acid profiles of bovine follicular fluid differ from those reported for total lipid extracts of porcine follicular fluid (Yao *et al.*, 1980). The most prominent fatty acid in porcine follicular fluid was oleic acid (18:1), followed by palmitic acid (16:0), compared with linoleic acid (18:2), followed by oleic acid in bovine follicular fluid. Porcine follicles contained ~8% arachidonic acid (20:4) and measurable amounts of adrenic acid (22:4), but there was a marked absence of these tetraenoic acids in bovine follicular fluid. During development of bovine follicles, only the proportion of linoleic acid changed significantly; whereas, during development of porcine follicles, proportions of polyunsaturated fatty acids and stearic acid (18:0) increased, and palmitic and oleic acids decreased. These results suggest that the fatty-acid distribution in mammalian follicular fluid is species-specific.

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