Influence of Interstitial Cell–stimulating Hormone on the Conversion of Cholesterol to Progesterone by Bovine Corpus Luteum*

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ABSTRACT: Slices of bovine corpora lutea were incubated in Krebs-Ringer bicarbonate buffer containing glucose and [7α-^{3}H]cholesterol of high specific activity (4.8 c/mmole) in Tween (Polysorbate 80). Addition of interstitial cell–stimulating hormone (ICSH or LH) to the medium increased the conversion of [7α-^{3}H]cholesterol to [^{3}H]progesterone. Addition of adenosine 3′,5′-cyclic monophosphate (3′,5′-cyclic AMP) produced a similar response.

These findings indicate that ICSH stimulates steroidogenesis in the corpus luteum at some step(s) beyond cholesterol. The action of 3′,5′-cyclic AMP reported here suggests that this nucleotide plays some part in the response of corpus luteum to ICSH.

The biosynthetic pathway between cholesterol and progesterone in adrenal cortex and corpus luteum appears to be similar with respect to the intermediates involved (Halkerston *et al.*, 1961; Constantopoulos and Tchen, 1961; Tamaoki and Pincus, 1961; Hall and Koritz, 1964a). Moreover, in response to their respective tropic hormones, ACTH and ICSH,[1] both tissues show, in addition to increased biosynthesis of steroids, concomitant depletion of ascorbic acid (Sayers *et al.*, 1948; Parlow, 1961) and cholesterol (Long, 1947; Parlow, 1958; Franchimont, 1962). These similarities suggest the attractive hypothesis that, in stimulating steroidogenesis in their respective target organs, ACTH and ICSH may share a common mechanism.

In the case of the response of the adrenal cortex to ACTH, evidence has been presented to show that the resulting increase in steroid biosynthesis involves stimulation of step(s) in the biosynthetic pathway beyond cholesterol. Such evidence comes from studies with the isolated perfused adrenal (Stone and Hechter, 1954), and experiments in which the adrenal cortex has been exposed to the action of ACTH *in vivo* (Krum *et al.*, 1964) and *in vitro* (Yudaev and Razina, 1964; Karaboyas and Koritz, 1965).

It has been shown that ICSH *in vitro* increases the biosynthesis of progesterone by slices of bovine corpus luteum (Mason *et al.*, 1962) and by slices of rat ovary (Armstrong *et al.*, 1964b). It was therefore decided to study the response of bovine corpus luteum to ICSH in order to determine whether ICSH acts by stimulating some step(s) in the pathway to progesterone beyond cholesterol. Moreover, it has been proposed that 3′,5′-cyclic AMP is involved in the response of the adrenal to ACTH (Haynes *et al.*, 1958; Koritz, 1962) and that this nucleotide stimulates the conversion of cholesterol to corticosteroids in the adrenal (Karaboyas and Koritz, 1965). Since 3′,5′-cyclic AMP has been shown to stimulate steroidogenesis in bovine corpus luteum (Marsh and Savard, 1964), the site of action of this nucleotide on steroidogenesis was also examined in bovine luteal tissue.

Experimental Procedure

Conditions of Incubation. Bovine ovaries were obtained from a slaughterhouse and corpora lutea were removed and stripped of their fibrous capsules. Corpora lutea selected for the present experiments were orange-yellow in color and highly vascular, with capsules which peeled freely from the corpus. According to criteria reported by several workers (McNutt, 1924; Asdell *et al.*, 1949; Hunt, 1961; Foley *et al.*, 1964) the corpora selected were young, probably within 2 weeks of the first day of estrus.[2] Corpora lutea from pregnant cows were not used in these studies. Slices of corpora were prepared by means of a Stadie-Riggs microtome and weighed on a torsion balance. The slices (500 mg/beaker) were incubated at 37° in Krebs-Ringer bicarbonate buffer containing glucose (2 mg/ml) unless otherwise stated, with constant agitation, and gassed with a mixture of oxygen (95%) and carbon dioxide (5%). The procedure referred to under Results as

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[1] The following abbreviations are used: ACTH, adrenocorticotropic hormone; 3′,5′-cyclic AMP, adenosine 3′,5′-cyclic monophosphate; ICSH, interstitial cell–stimulating hormone (or luteinizing hormone, LH); FSH, follicle-stimulating hormone.

[2] Dr. Keith Inskeep, Department of Animal Industry and Veterinary Science, West Virginia University, Morgantown, kindly advised the authors concerning the appearance of corpora at different stages of the cycle.

TABLE I: Recrystallization of [^{3}H]Progesterone Isolated Following Incubation of Slices of Corpus Luteum with [7α-^{3}H]-Cholesterol.

Sample A[a]			Sample B[b]		
	Specific Activity (dpm/mg)			Specific Activity (dpm/mg)	
Recrystallization	Crystals	Mother Liquor	Recrystallization	Crystals	Mother Liquor
After addition of carrier	24,400		After addition of carrier	3400	
(1) Hexane/chloroform	26,100	24,300	(1) Hexane/chloroform	3200	2800
(2) Ligroin/acetone	25,800	24,800	(2) Ligroin/acetone	2900	3100
(3) Heptane/benzene	24,900	24,600	(3) Heptane/benzene	3200	2900
(4) Aqueous ethanol	25,200	25,000	(4) Aqueous ethanol	3000	3200

[a] Sample A represents pooled eluates from paper chromatograms. To the pooled eluates 10 mg of progesterone was added and crystals were allowed to form from the solvents shown. After each recrystallization aliquots of crystals and mother liquors were taken for measurement of mass by absorbance at 240 mμ and of radioactivity by liquid scintillation. [b] Sample B consists of pooled fractions from the peak region of countercurrent chromatography (see Figure 1).

standard conditions involved 1 hour of incubation in buffer (called preincubation) and 2 hours of incubation in buffer with [7α-^{3}H]cholesterol and other additions when indicated. Between preincubation and incubation slices were blotted, washed in buffer, blotted, and transferred to breakers containing fresh buffer. Final *p*H of incubation was 7.4, and other additions to the medium are shown in the accompanying tables. [7α-^{3}H]Cholesterol (4.8 c/mmole) was suspended in Tween 80[3] and to each beaker 25 μc/2 μg was added. Each experiment was performed with slices from one corpus only.

Extraction and Measurement of Progesterone. At the end of incubation, beakers were placed on ice and the tissue was homogenized in the incubation medium. The homogenized tissue and medium were extracted four times with 30 ml of diethyl ether, and the combined extracts were taken to dryness under nitrogen and submitted to a 3-transfer countercurrent distribution with stripping, in the system petroleum ether/methanol–water (90:10, v/v) (Savard and Casey, 1964). Aqueous methanol fractions were dried and applied in benzene to a column of silicic acid (800 mg) which was washed with increasing concentrations of ethyl acetate in benzene (Savard and Casey, 1964). Under the conditions used more than 97% of progesterone was removed by ethyl acetate 50% in benzene (v/v). This fraction was taken to dryness under nitrogen and applied to paper in the system ligroin–propylene glycol for 6.5 hours (Brady, 1951; Savard, 1954). Dry chromatograms were examined by means of a Haines ultraviolet scanner (Haines and Drake, 1950) and the area of the chromatogram occupied by progesterone was eluted. Aliquots of the eluate were taken for determination of mass by absorbance at 240 mμ and of radioactivity by liquid scintillation counting.

In order to measure the recovery of progesterone from the tissue by these methods, [4-^{14}C]progesterone was added to corpus luteum homogenized in buffer as in the experiments to be described. Recovery was measured after elution of [4-^{14}C]progesterone from paper chromatograms.

Identification of [^{3}H]*Progesterone.* Samples of [^{3}H]-progesterone isolated as described were pooled after aliquots had been taken for determination of mass and radioactivity, in order to provide sufficient radioactivity for further purification. To the pooled samples progesterone (500 μg) was added, and the mixture was submitted to 99-transfer countercurrent distribution in the system cyclohexane–ethyl acetate (80:20, v/v)/ethanol-water (60:40, v/v). Aliquots of selected fractions were examined for absorbance at 240 mμ and for radioactivity. The data from fractions in the region of [^{3}H]progesterone were analyzed according to the method reported by Baggett and Engel (1957). Fractions containing progesterone were pooled and dried. Following the addition of 10 mg of progesterone the sample was recrystallized from a number of solvents, and mass and radioactivity of crystals and mother liquors were measured after each crystallization by absorbance at 240 mμ and liquid scintillation spectrometry, respectively. Other samples of [^{3}H]progesterone were recrystallized without previous purification by countercurrent distribution.

Measurement of Radioactivity. Elution of samples from paper chromatograms was performed by cutting the paper into small fragments and extracting by means of 10 ml of methylene chloride–chloroform (1:1, v/v) for 4 hours with constant agitation, followed by two further extractions of 2 hours each in the same solvent, and the pooled extracts were filtered and evaporated to

[3] Dr. T. T. Tchen, Department of Chemistry, Wayne State University, Detroit, Mich.; personal communication.

dryness. An aliquot was dried in a counting vial and examined by liquid scintillation spectrometry as reported previously (Hall and Koritz, 1964a). Counting efficiency for tritium was 41–45% and the expression "dpm" used here indicates that values have been corrected to 100% efficiency.

Chemicals. [7α-^{3}H]Cholesterol was obtained from New England Nuclear Corp. (lot 66-129-36) and was purified before use in the present studies by a method previously described (Hall and Koritz, 1964b). It was found that this procedure removed a polar contaminant but that the findings to be reported could be obtained without preliminary purification of this batch of [7α-^{3}H]-cholesterol. ICSH (NIH-LH-S-8) was a gift of the Endocrine Study Section of the National Institutes of Health. 3′,5′-Cyclic AMP was purchased from Sigma Chemical Corp. and silicic acid (Unisil, 200–325 mesh) was obtained from Clarkson Chemical Co., Williamsport, Pa. Bovine ICSH deactivated at room temperature according to the method of Reichert (1961) (referred to here as deactivated ICSH) was generously provided by Dr. L. E. Reichert, Emory University, Atlanta, Ga. The deactivated hormone was found to produce no demonstrable response in slices of rabbit testis *in vitro* at levels of 100 μg/beaker in a system which has been shown elsewhere (Hall and Eik-Nes, 1962) to respond to less than 0.01 μg of untreated ICSH per beaker.

Results

Radiochemical Purity of [^{3}H]*Progesterone.* The substance reported here as [^{3}H]progesterone was detected on paper chromatograms following extraction, partition, and column chromatography as described under Experimental Procedure. Proof that this compound was radiochemically pure was obtained by recrystallization to constant specific activity in the presence of authentic progesterone (Table I, sample A). It will be seen that the specific activity of crystals and mother liquors remained constant through four recrystallizations from different solvent systems. Since the first crystallization was not accompanied by significant change in specific activity it appears that the [^{3}H]progesterone measured in these experiments was radiochemically pure after paper chromatography.

A second line of evidence for the purity of [^{3}H]progesterone is shown in Figure 1 and Table I. Figure 1 shows the result of countercurrent distribution on pooled samples of [^{3}H]progesterone. It will be seen that in the peak region radioactivity and mass coincide. At the 5% level of significance no regression was found between the curves for radioactivity and mass (F 0.95 [1, 9] = 0.211) (Baggett and Engel, 1957), and specific activity was constant within the limits of experimental error over the span ±2 standard deviations. Table I, sample B, shows the result of recrystallizing the sample shown in Figure 1 after pooling fractions from the countercurrent distribution and adding authentic progesterone. Again it will be seen that the compound was radiochemically pure.

Recovery of [4-^{14}C]progesterone added to corpus luteum up to the stage of elution from paper chromatograms was 58–64% in ten estimations. Data presented here are not corrected for losses incurred during the isolation of progesterone.

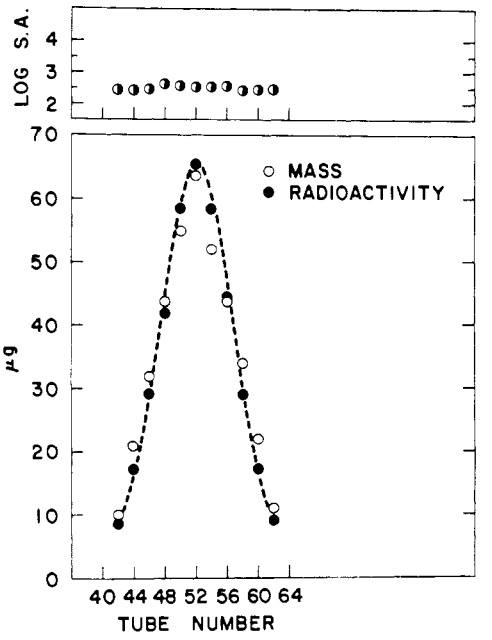

FIGURE 1: The 99-transfer countercurrent distribution of progesterone in cyclohexane–ethyl acetate (80:20, v/v)/ethanol-water (60:40, v/v). Values on the ordinate are mass (μg) and radioactivity divided by mean specific activity (also expressed in μg). Mean specific activity was calculated from the specific activities of alternate tubes in the peak region. The theoretical normal distribution curve was constructed with the following parameters: K = 1.11 and standard deviation ±4.97. Log specific activity (S.A.) of [^{3}H]progesterone in alternate tubes (dpm/μg) is plotted above the distribution curve.

Action of ICSH and 3′,5′-Cyclic AMP on Bovine Corpora Lutea. Table II shows the results of six consecutive experiments (one corpus per experiment) in which the response of slices of corpora lutea to ICSH and 3′,5′-cyclic AMP *in vitro* is shown. It can be seen that ICSH caused marked stimulation in both the production of progesterone from endogenous precursors (mass) and the incorporation of [7α-^{3}H]cholesterol into [^{3}H]progesterone (radioactivity). A similar but less pronounced effect upon both parameters was seen with 3′,5′-cyclic AMP. The unstimulated level of both production of progesterone from endogenous precursors and incorporation of tritium from [7α-^{3}H]cholesterol was variable but the responses to ICSH and 3′,5′-cyclic AMP were unequivocal in every case. Table III shows that this response to ICSH is specific in that FSH, ACTH, and deactivated ICSH produced no detectable stimulation.

Conditions of Incubation. Experiments in which the

TABLE II: Effect of ICSH and 3′,5′-Cyclic AMP upon the Biosynthesis of [^{3}H]Progesterone from [7α-^{3}H]Cholesterol by Slices of Bovine Corpus Luteum.[a]

Experiment	Addition	[^{3}H]Progesterone		
		Ratioactivity (dpm)	Mass (μg)	Specific Activity (dpm/μg)
		17,000	8.1	2,100
		14,000	9.2	1,500
		140,000	32.0	4,400
1	ICSH	120,000	24.4	4,900
		70,000	16.0	4,400
	3′,5′-Cyclic AMP	50,000	16.4	3,100
		29,000	13.0	2,200
		33,000		
		200,000	63.1	3,200
2	ICSH	188,000	53.4	3,500
		76,000	23.0	3,300
	3′,5′-Cyclic AMP	71,000	20.2	3,500
		24,000	8.8	2,700
		21,000	7.6	2,800
		116,000	31.2	3,700
3	ICSH	118,000	32.4	3,700
		82,000	18.4	4,400
	3′,5′-Cyclic AMP	81,000	16.8	4,800
		19,000	7.5	2,500
		18,000	7.1	2,500
		132,000	15.9	8,300
4	ICSH	168,000	19.2	8,800
		142,000	14.0	10,100
	3′,5′-Cyclic AMP	128,000	14.2	9,000
		10,000	9.6	1,100
		9,000	8.4	1,100
		90,000	34.8	2,600
5	ICSH	80,000	37.2	2,200
		46,000	19.2	2,400
	3′,5′-Cyclic AMP	44,000	18.4	2,400
		40,000	19.2	2,100
		36,000	14.8	2,400
		240,000	43.2	5,600
6	ICSH	215,000	40.8	5,300
		102,000	26.8	3,800
	3′,5′-Cyclic AMP	90,000	28.0	3,300

[a] Slices of corpus luteum were incubated in Krebs-Ringer bicarbonate buffer with [7α-^{3}H]cholesterol as described under Experimental Procedure. Where indicated, ICSH (200 μg/beaker) or 3′,5′-cyclic AMP (80 μmoles/beaker), dissolved in buffer, was added to the incubation medium.

influence of various conditions used to demonstrate the response to ICSH and 3′,5′-cyclic AMP in experiments 1–6 (Table II) was examined are reported in Table IV. It is clear that the response to both substances was not entirely dependent upon preincubation and removal of the medium at the end of preincubation (expt 7), upon the presence of glucose in the medium (expt 8, lines 3 and 4), or upon addition of [7α-^{3}H]cholesterol in Tween

TABLE III: Effect of Various Hormones upon the Biosynthesis of [^{3}H]Progesterone from [7α-^{3}H]Cholesterol by Slices of Bovine Corpus Luteum (Expt 9).[a]

Addition	[^{3}H]Progesterone Mass (μg)	Radioactivity (dpm)	Specific Activity (dpm/μg)
	9.2	13,000	1400
ICSH	21.4	44,000	2100
ICSH	25.9	51,000	2000
Deactivated ICSH	10.8	14,000	1300
Deactivated ICSH	8.1	10,000	1200
FSH	10.8	15,000	1400
FSH	8.8	10,000	1100
ACTH	7.8	8,000	1000

[a] This experiment was performed as described beneath Table II except for the hormones added. ICSH, deactivated ICSH, and FSH were each dissolved in buffer and 200 μg/beaker was added where shown. ACTH (1 unit) was added to one beaker.

as opposed to propylene glycol (expt 8, lines 5–8), since in each case a response to ICSH was observed. However addition of [7α-^{3}H]cholesterol in propylene glycol was followed by much less incorporation of tritium into progesterone than was observed when Tween was used. The response to ICSH in the absence of glucose (Table IV) appears to be less than that observed in the presence of glucose, although further studies would be necessary to establish a possible role of glucose in the response to ICSH reported here. In two experiments, [^{3}H]progesterone was measured in luteal slices from which incubation medium had been removed following incubation with [7α-^{3}H]cholesterol. In both experiments stimulation by ICSH was observed in both mass and radioactivity.

Discussion

Heretofore it has been uncertain whether ICSH increases steroidogenesis by stimulating a single enzymatic step of the pathways involved or whether several steps are stimulated, and in either case whether the stimulated step(s) is (are) before cholesterol or between cholesterol and steroid hormones. The number of steps stimulated by ICSH remains uncertain, but the findings reported here clearly demonstrate that the hormone can stimulate the conversion of cholesterol to progesterone by slices of bovine corpus luteum. Moreover, this action of ICSH is specific in that other tropic hormones failed to produce stimulation, and a preparation of ICSH found to be without effect on slices of testis *in vitro* (deactivated ICSH) did not increase the conversion of cholesterol to progesterone by slices of bovine corpus luteum (Table III). These observations are in keeping with the findings of Parlow (1958) that ICSH *in vivo* causes depletion of ovarian cholesterol, a response which forms the basis of one method of assay for the hormone (Bell *et al.*, 1964).

Studies of cholesterol biosynthesis in the testis are compatible with a site of action of ICSH on that organ, beyond cholesterol (Hall, 1963). Furthermore, evidence reviewed in the introduction of this paper indicates that the action of ACTH upon the adrenal cortex involves stimulation of some step(s) beyond cholesterol. Moreover, 3′,5′-cyclic AMP increases the production of corticosteroids by the adrenal *in vitro* (Haynes *et al.*, 1958) and the conversion of [7α-^{3}H]cholesterol to [^{3}H]corticosteroids by slices of adrenal cortex *in vitro* (Karaboyas and Koritz, 1965). In keeping with the similarities between the responses of corpus luteum and adrenal cortex to their respective tropic hormones, it has been shown here that 3′,5′-cyclic AMP stimulates the conversion of cholesterol to progesterone by slices of bovine corpus luteum (Table II). The fact that both the nucleotide and the tropic hormones act beyond cholesterol is a point in favor of the idea that 3′,5′-cyclic AMP plays some part in the action of both ICSH and ACTH upon corpus luteum and adrenal cortex, respectively. It would be interesting to examine these similarities further by determining whether ICSH increases the concentration of 3′,5′-cyclic AMP in corpus luteum since ACTH has been shown to produce this change in the adrenal (Haynes, 1958).

The present findings are in conflict with those of Mason and Savard (1964), who observed that ICSH failed to produce consistent stimulation of the conversion of [7α-^{3}H]cholesterol to [^{3}H]progesterone by slices of bovine corpus luteum, in spite of consistent stimulation of the biosynthesis of progesterone from endogenous precursors. It can be seen from Table IV of the present paper that this difference in the findings of the two laboratories cannot be entirely attributed to preincubation of tissue, to addition of glucose to the medium, or to the addition of [7α-^{3}H]cholesterol in Tween (in contrast to propylene glycol used by Mason and Savard, 1964); however, these differences in experimental procedure may play some part in the results (Table IV). Since our findings with respect to the biosynthesis of progesterone from endogenous precursors are in agreement with the observations of Mason and Savard, it is unlikely that the source of corpora lutea (pregnant as opposed to nonpregnant cows) accounts for the difference mentioned. In our experiments, considerably more radioactivity was found in [^{3}H]progesterone than in those of Mason and Savard. Moreover, in two of six experiments reported by these authors addition of ICSH was accompanied by unequivocal increase in conversion of cholesterol to progesterone. Determinations were not made in duplicate, and in view of the variations among the six experiments reported (see Mason and Savard, 1964, Table II) it is not possible for these authors to prove that ICSH was without effect in their experiments. It would appear that the effect of ICSH *in vitro* upon the conversion of

TABLE IV: Influence of Conditions of Incubation upon the Response of Slices of Bovine Corpus Luteum to ICSH *in Vitro*.[a]

Experiment	Conditions	ICSH	[^{3}H]Progesterone Radioactivity (dpm)	Mass (μg)	Specific Activity (dpm/μg)
	Standard	−	42,000	38.4	1100
		+	196,000	81.8	2400
	Standard	−	42,000	41.4	1000
		+	320,000	110.0	2900
7	Medium unchanged after preincubation	−	50,000	40.5	1200
		+	194,000	84.0	2300
	No preincubation	−	72,000	40.2	1800
		+	232,000	75.0	3100
	Standard	−	30,000	18.6	1600
		+	69,000	36.1	1900
	No glucose added	−	48,000	20.1	2400
		+	66,000	32.3	2000
8	[7α-^{3}H]Cholesterol added in propylene glycol	−	14,000	19.8	700
		+	34,000	48.5	700
	[7α-^{3}H]Cholesterol added in propylene glycol	−	18,000	28.1	640
		+	44,000	67.5	650

[a] Slices of corpora lutea were incubated with [7α-^{3}H]cholesterol. Standard conditions (i.e., those used in expts 1–6, Table I) involved preincubation in buffer containing glucose and addition of fresh medium and of [7α-^{3}H]cholesterol in Tween at the beginning of incubation. In expt 7, the phrase "medium unchanged" indicates that at the end of preincubation [7α-^{3}H]cholesterol was added without change of medium and "no preincubation" indicates that [7α-^{3}H]-cholesterol was added at the beginning of the experiment. In every case [7α-^{3}H]cholesterol was present in the medium for 2 hours. The expression "standard conditions" is explained under Experimental Procedure.

cholesterol to progesterone is more readily demonstrated when large amounts of [7α-^{3}H]cholesterol of high specific activity are added in Tween than under the conditions reported by the group at Florida; the [7α-^{3}H]-cholesterol used in our studies was of more than 20-fold greater specific activity than that used by Mason and Savard (1964).

It was pointed out under Experimental Procedure that corpora lutea were selected for the studies reported here upon the basis of morphological criteria which suggested that the tissue was relatively young, i.e., within 2 weeks of the first day of estrus. Age of corpora lutea appears to be an important factor in their capacity to respond to ICSH (Armstrong *et al.*, 1964a). This factor may be important in demonstrating the changes reported here, although the point can only be proved by using corpora from cows of known reproductive history.

The present experiments show that ICSH can act beyond cholesterol but do not in themselves exclude a second action before cholesterol. However, if the hormone does stimulate before cholesterol, such an action would tend to lower the specific activity of the [^{3}H]progesterone isolated after incubation with [7α-^{3}H]cholesterol, since unlabeled precursors would enter the biosynthetic pathway in greater amounts. In these experiments (with the possible exception of expt 8) the specific activity of [^{3}H]progesterone in the presence of ICSH was never less than that formed in the absence of the tropic hormone. It is therefore clear that any action of ICSH upon the corpus luteum before cholesterol is quantitatively insignificant compared to the action of the hormone upon the conversion of cholesterol to progesterone.

Acknowledgments

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