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In vivo production of steroids with central depressant actions by the ovary of the rat

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Summary

1. In addition to progesterone and 20-dihydroprogesterone, the following unconjugated steroids were isolated from ovarian tissue and ovarian venous blood of mature, non-pregnant rats: pregnenolone, 3βOH-5α-pregnan-20-one, 3αOH-5α-pregnan-20-one, 20αOH-5α-pregnan-3-one and 5α-pregnane-3α, 20α-diol.

2. Their concentration in the ovarian tissue and their secretion rates into the ovarian venous blood was of the same order of magnitude as that of progesterone.

3. In the evening of the day of pro-oestrus significant increases in the ovarian contents of the following steroids were observed: progesterone, +140%; 3αOH-5α-pregnan-20-one, +320%; 20αOH-5α-pregnan-3-one, +195%; sum of pregnenolone and 3βOH-5α-pregnan-20-one, +275%.

4. At the same time the ovarian progesterone secretion rate was increased by 365%, that of 3αOH-5α-pregnan-20-one by 190%.

5. A possible physiological role of the ovarian allopregnane derivatives as central depressant agents is discussed.

Introduction

In the course of experiments on rats in which the ovarian concentrations and secretion rates of progesterone had been studied, several unknown compounds of possible steroidal nature were consistently found in the tissue and blood extracts. As they occurred in quantities similar to those of progesterone they were studied in more detail. The structural analysis showed that they included pregnenolone and several allopregnane derivatives. The latter steroids are usually thought to be metabolites of progesterone which are formed in the liver. Wiest (1963), however, reported that ovarian tissue of rats can also metabolize [^{14}C]-progesterone *in vitro* to allopregnanes, an observation which was confirmed by Mason (1970) for 5α-pregnane-3α,20α-diol (allopregnanediol). Little was known as to whether these steroids were also produced *in vivo* in the ovary from intrinsic precursors, whether they were secreted into the ovarian venous blood, and whether their production rate changed during the oestrous cycle. The experiments described in this paper have provided some information on these questions.

Methods

Operative procedure

The experiments were carried out on virgin female Wistar rats (100–150 g body weight) which were kept under artificial lighting conditions with 12 h white light (200–1400 h) and 12 h red light. This time schedule was chosen in order to bring the beginning of the period of late pro-oestrus into a convenient hour of the working day. Only those rats were used for the experiments which had shown at least three regular cycles of 4 day duration. Ovarian venous blood was collected under pentobarbitone anaesthesia (30–50 mg/kg body weight) from a cannula introduced into the left renal vein after the renal peduncle, the adrenal vein, the lower part of the ovarian vein and the mouth of the renal vein into the vena cava had been ligated. Great care was taken not to interfere with the arterial blood supply to the ovary during these manipulations. Heparin (350 units/kg body weight) was injected into a femoral vein. Blood was collected for 15–30 min and the loss replaced by an intravenous infusion of either arterial blood from a male rat or a 0·9% solution of sodium chloride. The ovaries were dissected out immediately at the end of the blood collection period. In experiments in which only the ovarian tissue was extracted the rats were killed by rapid decapitation (unstressed rats).

Chemical procedures

The ovaries were homogenized in an all glass homogenizer with ethyl acetate. The addition of a small volume of water facilitated the breaking up of the tissue. The whole blood samples were extracted with ethyl acetate after dilution with water. The extracts were purified as described previously (Holzbauer & Newport, 1969; Fajer, Holzbauer & Newport, 1971). 4-[^{14}C]-Progesterone and 4-[^{14}C]-pregnenolone were added to the whole blood samples and to the ovarian homogenates to allow correction for losses. The amounts of pregnenolone recovered were in some samples somewhat lower than the amounts of progesterone recovered. Radioactively labelled preparations of the other steroids studied were not available at the time when the experiments were carried out. The values obtained for these steroids were therefore corrected by the figure obtained for the recovery of progesterone, which may have resulted in a slight underestimation.

The isolation of the individual steroids was carried out by descending paper chromatography in two systems (E_2B and E_1, Eberlein & Bongiovanni, 1955) and by gas-liquid chromatography. The latter technique was also used for quantitation. The samples were first freed from polar compounds in the E_2B system, in which pregnenolone, progesterone, 20-dihydroprogesterone and the allopregnane derivatives travelled with the solvent front. This region was eluted and the eluates rechromatographed in the E_1 system on 1 cm wide lanes of Whatman No. 50 paper which had been pretreated with NaOH (Holzbauer & Newport, 1969). On paper treated in this manner a distinct separation of steroids can be achieved and eluates of less than 3 cm^2 do not produce any ' blank peaks ' on the gas chromatography tracings. On separate control lanes 5 μg quantities of pure standards of the steroids studied were simultaneously developed. The chromatogram regions with the R_F values of the steroids in question were eluted. The eluates were analysed on 3·8% SE-30 columns (120 cm, 225°C) in an F + M model 402 gas chromatograph using

flame ionization detection. The quantitative estimations were based on the straight line relation which exists between the concentration ratio of two given steroids in a solution and the ratio of the heights of the peaks produced by the same steroids on the gas chromatography tracing. The dried residues of the paper eluates were dissolved in small quantities (10–20 μl) of ethanol containing 0·25–2 μg of the steroid used as internal standard. Details of the methods were described previously (Heap, Holzbauer & Newport, 1966; Holzbauer & Newport, 1969). The following steroids were measured in the extracts: pregnenolone, progesterone, 20αOH-pregn-4-en-3-one (20-dihydroprogesterone), 3αOH-5α-pregnan-20-one (3αOH-allopregnanolone), 3βOH-5α-pregnan-20-one (3βOH-allopregnanolone), 5α-pregnan-3α,20α-diol (allopregnanediol) and 20αOH-5α-pregnan-3-one. The structural analysis of the steroids was based on the R_F value on the paper chromatograms and on the retention times (relative to cholestane, RRT) of the free compounds, their acetates and their trimethylsilyl ethers on the gas chromatograph. Confimation of the structure is being obtained by combined gas chromatography with mass-spectroscopy with the help of Dr. Brooks (Department of Chemistry, University of Glasgow). The unknowns suspected to be pregnenolone, allopregnanediol and 3βOH-allopregnanolone produced mass spectra which were similar to those shown by the standards. Not enough material has been sufficiently purified to allow a reliable identification of 3αOH-allopregnanolone and 20αOH-5α-pregnan-3-one by mass-spectrography. Data on these experiments will be published elsewhere. In Table 1 several steroids are listed which were considered during the structural analysis of the unknowns in the extracts. Those steroids which are not underlined could not be detected in the ovarian blood samples or the ovarian tissue extracts.

TABLE 1. *Steroids tested for their possible identity with unknown compounds extracted from rat ovaries and ovarian venous blood*

Steroid	R_F	RRT	
		Free steroid	Acetate
Pregn-4-ene-3,20-dione (progesterone)	0·55	0·82	—
20αOH-pregn-4-en-3-one (20-dihydroprogesterone)	0·20	0·92	1·30
3βOH-pregn-5-en-20-one (pregnenolone)	0·40	0·61	0·93
Pregn-5-ene-3β,20α-diol (20-dihydropregnenolone)	0·15	0·67	1·47
3βOH-5α-pregnan-20-one (3βOH-allopregnanolone)	0·42	0·63	0·95
3αOH-5α-pregnan-20-one (3αOH-allopregnanolone)	0·60	0·60	0·84
3βOH-5β-pregnan-20-one	—	0·56	0·78
3αOH-5β-pregnan-20-one	—	0·56	0·82
5α-pregnane-3α,20α-diol (allopregnanediol)	0·20	0·68	1·33
5α-pregnane-3β,20α-diol	0·18	0·68	1·50
5β-pregnane-3α,20α-diol (pregnanediol)	—	0·61	1·33
5β-pregnane-3β,20α-diol	—	0·61	1·25
20αOH-5α-pregnan-3-one	0·40	0·73	0·95
20αOH-5β-pregnan-3-one	0·30	0·66	0·95
5β-pregnane-3,20-dione (pregnanedione)	0·70	0·62	—
5α-pregnane-3,20-dione	0·72	0·66	—

Evidence for the presence in biological extracts was obtained for those steroids which are underlined. The steroids for which no R_F values are given in the table have been ruled out by their retention times and those of their acetates on the gas chromatograph. R_F: mobility on paper relative to the solvent front in the E_1 system of Eberlein & Bongiovanni. RRT: retention times on the gas chromatograph (3·8% SE 30 column, 225° C) relative to that of cholestane. (The presence of 20-dihydropregnenolone could not be ruled out.)

Of the compounds studied 20-dihydroprogesterone had the lowest R_F value in the E_1 system, approximately 0·2. The other progesterone metabolite found in the same region of the paper chromatogram was allopregnanediol. The two steroids could be separated on the gas chromatograph: the RRT of 20-dihydroprogesterone was 0·92, that of allopregnanediol 0·68. The RRTs of the respective acetates were 1·30 and 1·33 and of their trimethylsilylethers (TMSiEs) 1·25 and 0·94. Androstenedione was used as internal standard for the quantitative estimation of these two steroids.

The R_F value of pregnenolone in the E_1 system was about 0·4. The ovarian extracts contained in addition to pregnenolone, 3βOH-allopregnanolone, which travelled slightly faster than pregnenolone (Holzbauer, 1969), but could not be quantitatively separated from it. Both steroids had the same RRT on the SE-30 column of the gas chromatograph (0·61) and the RRTs of their acetates (0·93 and 0·95) and TMSiEs (0·77, 0·79) were very similar. In the present experiments only the sum of these two steriods was therefore estimated. 3βOH-Allopregnanolone was only detected in the extracts of ovarian tissue, but not in ovarian venous blood.

A third steroid which had an R_F value of about 0·4 in the E_1 system and which was present in the extracts of ovarian tissue and blood was tentatively identified as 20αOH-5α-pregnan-3-one. Its RRT on the gas chromatograph was 0·73, and it could thus easily be separated from the other two steroids in this region. The RRT of its acetate was 0·95, that of the TMSiE, 1·00. Pregnenolone, 3βOH-allopregnanolone and 20αOH-pregnan-3-one were estimated in the same eluate using 11βOH-progesterone (RRT 1·44) as internal standard.

The R_F value of progesterone in the E_1 system was about 0·55 and its RRT 0·82. 3αOH-Allopregnanolone travels on paper only somewhat faster (R_F 0·58), but has a RRT of 0·61; the RRT of its acetate was 0·84 and that of the TMSiE 0·62. The two steroids were eluted together and 11βOH-progesterone was again used as the internal standard for the quantitative estimation.

Several experiments were carried out in which unlabelled and ^{14}C-labelled progesterone was added to rat arterial blood or to homogenates of rat hearts. The extracts were analysed in the same manner as the ovarian extracts. None of the progesterone metabolites could be detected in any of these extracts. These experiments ruled out the possibility that the metabolites had been artificially formed from progesterone during the processing of the biological samples.

With the methods used only unconjugated steroids were extracted and estimated. They were not sensitive enough to detect the steroids in 5 ml blood from the carotid arteries of female rats.

Results

The results summarized in Fig. 1 were obtained from a group of eleven rats from which ovarian venous blood and ovaries had been collected. The steroids were estimated in the blood and in the ovarian tissue of the same rats. The rats were chosen at random; six were in oestrus, three in metoestrus and two in early pro-oestrus. The columns in Fig. 1 represent mean values and standard errors of the mean. In this group of operated, stressed rats the ovarian concentrations of

allopregnanediol was nearly double that of progesterone, and so was its rate of secretion into the ovarian blood. The ovarian content of 3αOH-allopregnanolone was similar to that of progesterone, its secretion rate about half. In contrast, the ovarian concentration of 20αOH-5α-pregnan-3-one was less than a quarter of the progesterone concentration whereas its secretion rate was similar to that of progesterone. The ovarian concentration of the sum of pregnenolone and 3βOH-allopregnanolone was slightly lower than that of progesterone. 3βOH-Allopregnanolone was not detected in the ovarian blood of rats, pregnenolone was found in the blood of four out of eleven rats. In our strain of rats, 20-dihydroprogesterone was the steroid which was produced in largest quantities in the non-pregnant rat. The structural configuration of the substance measured as 20-dihydroprogesterone in the biological extracts was confirmed by combined gas chromatography and mass-

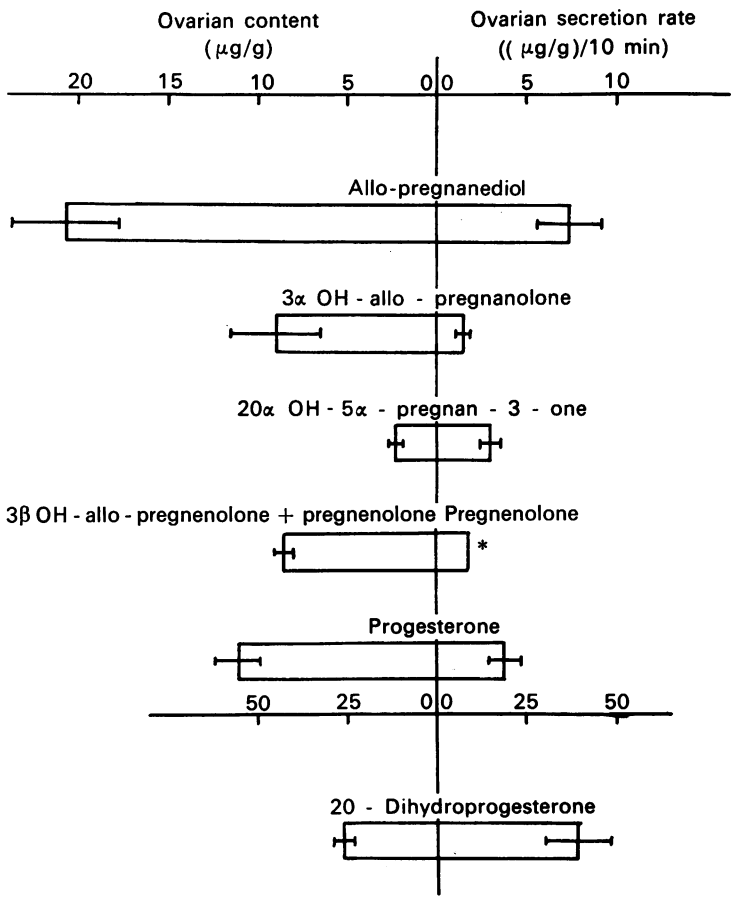

FIG. 1. Ovarian concentrations and ovarian secretion rates of six steroids. Ovarian blood collected under sodium pentobarbitone anaesthesia for 15–30 min ; ovaries dissected out at end of blood collection. The columns are mean values and standard errors of the means obtained from eleven rats (six oestrus, three metoestrus, two early pro-oestrus). * : Mean pregnenolone secretion in four rats. In the remaining seven, pregnenolone and 11βOH-5α-pregnan-20-one could not be detected.

spectroscopy. By comparing the left hand columns in Fig. 1 with the right hand columns it can be seen that the amounts of progesterone and allopregnanediol which were present in the ovaries at the end of the blood collection were equivalent to the amounts secreted during 20–30 min, those of 20-dihydroprogesterone and 20αOH-5α-pregnan-3-one equal to the amounts secreted in less than 10 minutes.

TABLE 2. *Concentrations of unconjugated steroids in the ovary of the rat during early and late pro-oestrus*

Steroid	Concentration in the ovary (μg/g ovary) Early pro-oestrus (after 8 h white light)	Late pro-oestrus (after 3 h red light)	Increase in late pro-oestrus (%)	Significance of difference (*P*-values)
Progesterone	1·40±0·43	3·00±0·47	+114	0·02–0·01
20-Dihydroprogesterone	16·60±1·13	22·20±2·13	+ 34	0·05–0·02
5α-Pregnane-3α,20α-diol (allopregnanediol)	3·68±0·42	4·10±0·42	+ 11	0·1 –0·2
3αOH-5α-Pregnan-20-one (3αOH-allopregnanolone)	0·70±0·20	2·50±0·39	+258	<0·001
20αOH-5α-Pregnan-3-one	0·52±0·10	1·20±0·19	+130	0·01–0·001
Pregnenolone+3βOH-5α-pregnan-20-one (3βOH-allopregnanolone)	0·60±0·10	1·79±0·37	+200	0·01–0·001
Ovarian weights: mg/pair	70·5 ±3·2	78·0 ±3·7		

The rats were killed by rapid decapitation (unstressed rats). Seventeen rats were studied in early pro-oestrus, seventeen rats in late pro-oestrus.

TABLE 3. *Secretion rates of unconjugated steroids from the left ovary of the rat during early and late pro-oestrus*

Steroid	Secretion rates from the left ovary ((μg/g ovary)/10 min) Early pro-oestrus (after 8–9 h white light)	Late pro-oestrus (after 3–4 h red light)	Increase in late pro-oestrus (%)	Significance of difference (*P*-values)
Progesterone	2·62±0·85	12·17± 3·3	+360	0·01–0·02
20-Dihydroprogesterone	22·40±3·66	24·74±12·3	+ 24	0·8 –0·9
5α-Pregnane-3α,20α-diol (allopregnanediol)	2·88±0·67	2·90± 1·06	+0·5	>0·9
3αOH-5α-Pregnan-20-one (3αOH-pregnanolone)	0·91±0·15	2·68± 0·47	+195	0·01–0·001
20αOH-5α-Pregnan-3-one	3·64±1·45	4·96± 1·02	+ 36	0·4 –0·5
Pregnenolone	0·80±0·27	1·35± 0·40	+ 69	>0·9
Ovarian weights: mg/left ovary	35·6 ±1·8	42·4 ± 1·5		

(Ovarian blood collected under sodium pentobarbitone anaesthesia; eight rats were studied in early pro-oestrus, seven rats in late pro-oestrus.)

Further experiments were carried out in order to investigate whether the production of the allopregnanes is different during phases of the oestrous cycle. From previous work (Hashimoto, Henricks, Anderson & Melampy, 1968; Fajer, *et al.*, 1971) it was known that there is a considerable increase in the secretion rate of progesterone during the evening of the day of pro-oestrus. This day was chosen to study, in addition to the production of progesterone, the production of its metabolites and of pregnenolone.

One set of experiments was carried out on thirty-four unstressed rats in which only the concentration of the steroids in the ovarian tissue was measured. Seventeen rats were killed by rapid decapitation 8 h after onset of white light (early pro-oestrus) and seventeen rats 3 h after onset of red light (late pro-oestrus). The results are listed in Table 2. Each sample analysed consisted of one pair of ovaries. In late pro-oestrus, as well as the rise in the ovarian progesterone concentration by 114%, there was also a significant rise of two allopregnane derivatives: 3αOH-allopregnanolone was increased by 258%, 20αOH-5α-pregnan-3-one by 130%. There was also a large increase in the ovarian concentration of the sum of pregnenolone and 3βOH-5α-pregnan-20-one. In contrast, the concentration of allopregnanediol was only slightly increased. Its ovarian concentration during early pro-oestrus was similar to the progesterone concentration during late pro-oestrus.

In another set of experiments the ovarian secretion rates of the same steroids on the day of pro-oestrus was measured in fifteen rats. Eight of them were operated 8–9 h after onset of white light (early pro-oestrus), seven of them 3–4 h after onset of red light (late pro-oestrus). The results are listed in Table 3. The rats in late pro-oestrus secreted 4·6 times as much progesterone as those in early pro-oestrus and the secretion rate of 3αOH-allopregnanolone was nearly trebled. The secretion of the other steroids was slightly but not significantly increased.

Discussion

The ovary of the rat produces *in vivo* several metabolites of progesterone which are also secreted into the ovarian venous blood in an unconjugated form (Holzbauer, 1969; Holzbauer & Mason, 1970; Holzbauer, 1971). Since the presence of enzymes required for their synthesis has been demonstrated in the rat ovary under *in vitro* conditions (Wiest, 1963; Zmigrod & Lindner, 1969; Mason, 1970) it could be expected to find traces of these steroids in the ovarian tissue. It was, however, surprising to see that they were present in quantities equal to, or even larger than, those of progesterone and that they were also secreted into the ovarian venous blood at rates comparable to the secretion rates of progesterone.

The ovarian production of some allopregnane derivatives showed in the evening of the day of pro-oestrus an increase similar to the rise in the progesterone production, which is assumed to be a consequence of an increased release of luteinizing hormone (LH). An exception was allopregnanediol. This is in agreement with results obtained *in vitro* by Mason (1970) on ovarian slices from immature rats pretreated with pregnant mare's serum. The addition of LH to the incubation medium caused an increase in the production of progesterone by about 450% whereas that of allopregnanediol was hardly affected.

By comparing Fig. 1 with Table 2 it can be seen that the ovaries of those rats from which ovarian venous blood had been collected (Fig. 1) contained larger quantities of the steroids measured, including allopregnanediol, than the ovaries of unstressed rats even in late pro-oestrus (Table 2). The question arises whether this increase is connected with the increase in the serum prolactin concentration which Neill (1970) found in rats after laparotomy under ether anaesthesia. The same manipulations were without effect on the serum concentrations of LH.

Why does the ovary metabolize progesterone, which is one of its major hormones, into compounds which do not any longer have its characteristic actions on the reproductive organs? Several explanations come to mind which might be valid either each on its own or in combination. First, the presence of the reducing enzymes in the ovary might be fortuitous and the formation of the metabolites a rather wasteful procedure. Second, the intra-ovarian metabolism of progesterone might help to regulate the secretion rates of progesterone by decreasing the quantities of progesterone available. Third, progesterone might fulfil a biological function on an intra-ovarian structure and be subsequently metabolized at its site of action. Fourth, the reduced steroids might be genuine secreted hormones and fulfil an extra-ovarian function like any other hormone. Whichever of these or any other consideration is correct, the fact remains that ring reduced progesterone metabolites are constantly released into the ovarian venous blood in an unconjugated form in which they can easily pass through any blood-tissue barrier.

The only biological action of ring-A reduced C-21 steroids which has so far been studied in any detail is their central depressant effect. In the past, large quantities of steroids had to be used in order to achieve an anaesthetic effect (Selye, 1942). This was mainly due to the difficulty to prepare steroid solutions from which they became readily available to the brain. Recently this problem was partly overcome by dissolving the steroids in propylene glycol. Using such solutions, Gyermek, Genther & Fleming (1967) were able to observe sleep spindles in the EEG of cats immediately after an intravenous injection of 0·25 mg/kg body weight of 3αOH-5β-pregnan-20-one, whereas 3·0 mg/kg body weight of sodium pentobarbitone were required to achieve the same effect. Sleep spindles occurred also 2 min after an intravenous injection of 1·0 mg/kg body weight of pregnanediol. Progesterone itself was found to be 10 times less effective than pregnanediol and to act only after a latency of 5–15 minutes.

It can be calculated that the rat ovary secretes daily on average 1·5–3·0 mg/kg body weight of allopregnanediol plus 3αOH-allopregnanolone. It is very probable that a constant intravenous infusion of centrally depressant compounds at this rate will exert a modulating effect on the activity of the central nervous system.

Our results also suggest variations of the secretions during the cycle. It is thus possible that cycle dependent changes in mood might be related to the amounts of ring-A reduced pregnane derivatives flooding the central nervous system. Progesterone has long been implicated in the state of sedation which sometimes occurs during pregnancy (P'An & Laubach, 1964). It was suggested that this might be due to metabolites formed from progesterone in the liver. However, in the liver progesterone is not only metabolized but the metabolites are also conjugated. These conjugates are hydrophilic and it seems improbable that they will readily penetrate

the blood-brain barrier. Therefore, the secretion of the unconjugated progesterone metabolites by the ovary might be of physiological significance, especially as their uptake by the brain is fairly rapid. Raisinghani, Dorfman, Forchielli, Gyermek & Genther (1968) found maximum brain concentrations 3 min after the intravenous injection of pregnanolone.

Experiments are in progress in which the sedative effect of allopregnane derivatives (having an α-hydrogen atom on carbon 5) is compared with that of pregnane derivatives (having a β-hydrogen atom on carbon 5). According to Selye (1942) allopregnanediol and pregnanediol are equally effective.

The isolation from rat ovarian plasma of 3αOH-5α-pregnan-20-one, 20αOH-5α-pregnan-3-one and 5-pregnane-3α,20α-diol and confirmation of their structure by mass-spectroscopy was recently also described by Ichikawa, Morioka & Sawada (1971).

The ovarian production of progesterone metabolites is not peculiar to the rat. We were able to extract 25 times more 3βOH-5α-pregnan-20-one than progesterone from the corpus luteum of a pregnant goat. It has also been isolated from the ovary of the pig and the whale (see Dorfman & Ungar, 1965).

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