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EFFECT OF COFACTORS AND LUTEINIZING HORMONE ON TESTOSTERONE PRODUCTION BY RAT TESTIS PREPARATIONS

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SUMMARY

The aim of the present study was to investigate the possible cause of reported differences in testosterone production by rat testis preparations *in vitro*. These differences appear to reflect differences in tissue preparations and in incubation conditions, rather than differences in the steroidogenic potential of the tissue.

In vitro production of testosterone by rat testis preparations was shown to be stimulated by the addition to the incubation medium of calcium and magnesium ions, succinate and a NADPH regenerating system. This effect was seen with intact and homogenized preparations of both total testis tissue and isolated interstitial tissue and with isolated testicular cell suspensions enriched in Leydig cells.

Testosterone production in homogenates of seminiferous tubules was very low when compared with interstitial tissue and was not stimulated by the addition of cofactors.

Luteinizing hormone (LH) stimulated testosterone production in testicular cell suspensions. The effects of cofactors and of LH on testosterone production in these suspensions were not additive.

On the assumption that LH would act on intact cells only and that NADPH would not enter intact cells, a comparison was made of the effect of cofactors on cell suspensions before and after disruption of the cells. It was not possible, however, to calculate the amount of damaged cells in testicular cell suspensions from the response to incubation with cofactors.

INTRODUCTION

Testosterone production by the rat testis has been the subject of many studies and there is general agreement that the interstitial tissue is the main testosterone producing testicular compartment[1–8]. It has also been shown, that testosterone production by testis tissue *in vivo* as well as *in vitro* can be stimulated by Luteinizing Hormone (LH)[6–11, 20].

Homogenates of whole testis or homogenized isolated interstitial tissue in the presence of appropriate cofactors[6] can produce steroids at a level comparable to the rate of testosterone production *in vivo*[11]. In contrast there are several reports[3, 7, 10, 20] that steroid production by presumed intact testis tissue *in vitro* is much lower than steroid production *in vivo*. These observations may be interpreted in the light of the requirement of NADPH as an essential cofactor for steroid production. NADPH will not easily pass the intact cell membrane, hence exogenous NADPH will not enter intact cells[15]. After disruption of cells endogenous cofactors may be diluted and exogenous additions are essential to maintain steroid production at normal levels. In this respect the first aim of the present study was to compare the effect of various combinations of cofactors on testosterone production in homogenized and intact testis and on intact isolated Leydig cells.

It is generally accepted that only intact cells can respond to trophic hormones because many coupled intracellular events are involved (activation of adenyl cyclase, activation of protein kinase, phosphorylation of specific proteins, protein synthesis, activation of mitochondrial side-chain cleaving activity). As a second aim it was tried to discriminate between intact and broken testicular interstitial cells from a comparison between the effects of LH on testosterone production by intact and damaged cells. Therefore we have studied the effects of LH and different cofactors on testosterone production by either homogenized testis cells, deliberately damaged testis cells and presumed intact testis cells.

EXPERIMENTAL

Materials. All chemicals used were of analytical grade. Calcium and magnesium chloride were obtained from Merck, Darmstadt, Germany. Disodium succinate, $NADPNa_2$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49, 140 IU/mg) were purchased from Boehringer, Mannheim, Germany. Luteinizing Hormone (Ovine LH, NIH-LH-S18, 1.03 units/mg) was a gift from the Endocrinology Study Section, National Institute of Health, Bethesda, Maryland, U.S.A.

Animals. Male adult Wistar rats weighing 200–300 g were used in the experiments with testis tissue. For

the preparation of testis cell suspensions male adult rats of the R-Amsterdam strain (a Wistar substrain) were used. All animals were kept in a light (14 h light 10 h darkness) and temperature (20–22°C) controlled room and received standard dry pellets (Hope Farms, Woerden, The Netherlands) and tap water *ad lib.*

Tissue preparation. The animals were killed by decapitation, the testes were removed, decapsulated and weighed. Interstitial tissue and seminiferous tubules were separated by wet dissection[1, 12]. Seminiferous tubules were purified by suspending them in Krebs–Ringer Buffer, pH = 7.4, and allowing them to settle. The supernatant was decanted and discarded. This procedure was repeated five times.

Homogenization of tissue was performed by sonication at 20,000 kHz with an amplitude of 6.5 μm for 30 s.

Testis cell suspensions were prepared according to the method of Moyle and Ramachandran[13] as modified by Janszen *et al.*[14]. Unless indicated otherwise, they were preincubated for 60 min in Krebs–Ringer Buffer, pH = 7.4, containing 0.2% (w/v) glucose at 32°C under agitation. Purification of cell suspensions was performed by mixing the suspension carefully with an equal vol. of a solution of 26 g Ficoll (Pharmacia) and 400 mg BSA in 100 ml Krebs–Ringer Buffer, pH = 6.5, containing 0.2% (w/v) glucose and centrifuging for 10 min at 3000 rev./min at 4°C.

The term "intact tissue" will be used for testis tissue which has been decapsulated and slightly teased with jeweller's forceps in order to divide the tissue in pieces suitable for incubation (one-sixth of a testis).

Incubations. Tissue preparations originating from one-sixth of a testis were incubated in 2 ml of incubation medium in round bottomed glass tubes at 32°C in an atmosphere consisting of 95% oxygen and 5% carbon dioxyde. These tubes were constantly shaken. Cell suspensions were incubated in 0.2 ml of incubation medium in plastic tubes. Cell density during incubation was 2–5 × 10^6 cells/ml. Incubations were carried out for 2 h. The concentrations of added cofactors, when used, were: Ca^{2+}: 0.7 mM, Mg^{2+}: 5 mM, succinate: 20 mM, glucose-6-phosphate: 5 mM, $NADP^+$: 1 mM and glucose-6-phosphate dehydrogenase: 1 IU/incubation tube.

At the end of the incubation, intact tissues and cell suspensions were sonicated in the incubation medium and 2 ml acetone was added. For the estimation of extraction recoveries 20,000 d.p.m. of tritiated testosterone were added in 10 μl ethanol. After thorough mixing the precipitate was separated from the acetone fraction and washed with another 2 ml of acetone. The precipitate was dried at ambient temperature and dissolved in 1 ml of 1 M potassium hydroxide for protein estimation. The combined acetone fractions were concentrated at 45°C under nitrogen. Whenever necessary, the volume of the remaining aqueous phase was adjusted to 2 ml with distilled water. Steroids were then extracted with 3 times 2 ml diethyl ether. The combined ether fractions were evaporated to dryness under nitrogen at 45°C. The residue was dissolved in 5 ml ethanol and stored in the refrigerator for testosterone assay.

Estimations. Testosterone was measured by radioimmunoassay as described by Verjans *et al.*[16]. This method is essentially a modification of the method of Furuyama *et al.*[17].

Lactate dehydrogenase (LDH, E.C. 1.1.1.27) was measured according to the method of Kornberg[18].

Protein was measured according to Lowry *et al.*[19].

RESULTS

Experiments with homogenized testis tissue. The effect of the different compositions of the incubation buffer was first examined with homogenates of total testis tissue. There was no significant difference between testosterone production in Krebs–Ringer Buffer containing 0.2% glucose (KRBG)[3, 7] and in Tris–HCl buffer[6]. Testosterone levels (means ± S.E.M., n = 4) before incubation were 6.6 ± 2.4 pmol/mg protein and 4.8 ± 1.7 pmol/mg protein for KRBG and Tris–HCl respectively. After incubation these values were 40.8 ± 12.1 pmol/mg protein and 30.8 ± 9.0 pmol/mg protein for the respective buffers. There was an indication of slightly higher testosterone productions in KRBG than in Tris–HCl. In further experiments KRBG was used as an incubation medium.

The results in Table 1 show the effect of the addition to the incubation medium of extra calcium ions, magnesium ions, succinate and a NADPH generating system on testosterone production by homogenates of total testis tissue, interstitial tissue and seminiferous tubules. The addition of cofactors was essential for any significant testosterone production by homogenates of both total testis tissue and interstitial tissue. The small increase observed in testosterone production by homogenates of seminiferous tubules (Table 1) in the presence of cofactors was not statistically significant. The possible effect of individual cofactors and of any combination of the cofactors, was subsequently investigated on the basis of the testosterone production by homogenates of interstitial tissue. The results in Table 2 indicate that the presence of a NADPH generating system was essential, although the observed effect of the NADPH generating system alone does not explain the large testosterone production observed in the presence of all cofactors. Especially calcium and magnesium ions cooperate with the NADPH generating system.

Experiments with intact testis tissue. The results in Table 3 show, that addition of cofactors also stimulated the *in vitro* production of testosterone by intact total testis tissue and by intact isolated interstitial tissue obtained by wet dissection[1, 12].

Experiments with testis cell suspensions. The results in Tables 1 and 2 show that testosterone production

Table 1. Testosterone (T) levels in homogenates of total testis tissue, interstitial tissue and seminiferous tubules before and after incubation in Krebs–Ringer Buffer, pH = 7.4, either in the presence or in the absence of additional cofactors*

Tissue	Incubation time, min	Cofactors added	$\frac{\text{pmol T}}{\text{mg protein}} \pm$ S.E.M. (n = 8)
Total Testis	0	–	9.7 ± 2.1
	120	–	9.7 ± 1.7
	120	+	50.3 ± 6.6†
Interstitial	0	–	22.2 ± 2.4
Tissue	120	–	18.4 ± 2.8
	120	+	302 ± 67†
Seminiferous	0	–	1.7 ± 0.3
Tubules	120	–	2.1 ± 0.3
	120	+	2.4 ± 0.3

* Cofactors: $CaCl_2$: 0.7 mM, $MgCl_2$: 5 mM, succinate: 20 mM, glucose-6-phosphate: 5 mM, $NADP^+$: 1 mM, glucose-6-phosphate-dehydrogenase: 1 IU/2 ml.
† Significantly different from corresponding control ($P < 0.001$).

by homogenized testis tissue preparations could be increased by the addition of cofactors to the incubation medium. The different results in Table 2 were obtained in independent experiments, so that only data occurring on one line (T_0 vs T_{120} vs T_{cof}) may be compared with each other. Since it is thought[15] that pyridine nucleotides do not enter intact cells, it should be possible, in theory at least, to calculate the amount of damaged cells in testis cell suspensions from a comparison of the effect the response of cofactors on cell suspensions before and after disruption of the cells. In this way it was attempted to correct for the amount of damaged cells when studying the quantitative effect of LH on steroid production by isolated testis cells. Testis cells were obtained by enzymic digestion of testis tissue[13, 14]. The effect of the addition of cofactors and/or LH to the incubation medium on the testosterone production by a suspension of intact (i.e. not deliberately damaged) testis cells is shown in Table 4. Disruption of cells was considered successful, when the testosterone production upon incubation of the damaged suspension did not respond to LH, but only to cofactors. It was not possible to homogenize testis cell suspensions by either sonication, or with a Potter–Elvehjem homogenizer, both with a Teflon and a glass pestle. Either the tes-

Table 2. Testosterone levels in homogenates of rat testis interstitial tissue before incubation (T_0), after 120 min incubation in buffer* only (T_{120}) and after 120 min incubation in the presence of various cofactors ($T_{cof.}$). All results are given as means ± S.E.M. (n = 4) and are expressed as pmol testosterone/mg protein

Cofactors used	T_0	T_{120}	$T_{cof.}$
Ca^{2+}	6.6 ± 0.3	7.6 ± 0.3	6.9 ± 2.8
Mg^{2+}	8.7 ± 0.3†	8.0 ± 0.3	7.3 ± 0.3§
Succinate	14.9 ± 0.7	14.9 ± 0.7	15.9 ± 1.4
NADPH	5.2 ± 0.3	8.3 ± 0.7§	8.7 ± 0.3¶
Ca^{2+} + Mg^{2+}	6.9 ± 0.3	7.3 ± 0.3	8.0 ± 0.3
Ca^{2+} + Succinate	16.6 ± 4.9	16.3 ± 4.2	11.4 ± 1.7†
Ca^{2+} + NADPH	14.6 ± 3.1	16.3 ± 4.2	62.7 ± 11.4¶
Mg^{2+} + Succinate	8.0 ± 0.7	11.1 ± 2.4	9.4 ± 2.1
Mg^{2+} + NADPH	9.4 ± 2.8	8.3 ± 2.1	147.4 ± 12.5¶
Succinate + NADPH	7.6 ± 1.4	6.2 ± 0.7	161.2 ± 35.7§
Ca^{2+} + Mg^{2+} + Succinate	16.3 ± 7.3	14.2 ± 6.6	13.7 ± 8.3
Ca^{2+} + Mg^{2+} + NADPH	16.6 ± 4.2	15.3 ± 4.9	288.8 ± 52.7¶
Ca^{2+} + Succinate + NADPH	8.0 ± 0.7	7.3 ± 1.4	124.1 ± 10.1¶
Mg^{2+} + Succinate + NADPH	40.2 ± 6.6	39.9 ± 7.6	75.9 ± 18.7
Ca^{2+} + Mg^{2+} + Succinate + NADPH‡	22.2 ± 2.4	18.4 ± 2.8	302 ± 67¶

* Krebs–Ringer Bicarbonate buffer pH = 7.4, containing 0.2% glucose (w/v).
† n = 3.
‡ n = 8.
§ significantly different from corresponding control ($P < 0.01$).
¶ significantly different from corresponding control ($P < 0.001$).
In this table NADPH is used to describe the entire NADPH generating system, as described in the experimental section.

Table 3. Testosterone levels in intact and homogenized rat testis tissue before and after incubation in the absence or presence of additional cofactors*. All results are given as means ± S.E.M. (n = 4) and are expressed as pmol testosterone/mg protein

I. Total testis tissue Incubation time min	Co-factors added	pmol testosterone/mg protein Intact tissue	Homogenates
0	–	9.4 ± 4.5	4.9 ± 1.7
120	–	10.1 ± 4.2	6.2 ± 1.7
120	+	110.3 ± 36.1†	123.1 ± 43.3†
II. Interstitial tissue			
Incubation time min	Co-factors added	pmol testosterone/mg protein Intact tissue	Homogenates
0	–	30.9 ± 11.1	46.1 ± 21.1
120	–	75.6 ± 34.7	67.3 ± 21.8
120	+	1626 ± 220‡	1050 ± 297‡

* Cofactors: $CaCl_2$: 0.7 mM, $MgCl_2$: 5 mM, succinate: 20 mM, glucose-6-phosphate: 5 mM, $NADPNa_2$: 1 mM, glucose-6-phosphate dehydrogenase: 1 IU/2 ml.
† significantly different from control value ($P < 0.025$).
‡ significantly different from control value ($P < 0.01$).

tosterone synthetizing capacity was lost completely (sonication) or the cells were mainly undamaged (Potter), as judged by microscopic observation.

However, disruption of cells was achieved when the cell suspension was frozen quickly in dry ice and allowed to thaw at room temperature. The residual steroidogenic capacity of the homogenized cell suspension appeared to reside in the pellet which was obtained after centrifuging the frozen suspension for 10 min at 100 *g*, since the supernatant showed hardly any testosterone production upon incubation in the presence of cofactors (Fig. 1). Testosterone production in the presence of cofactors was always higher in the original suspensions than in the frozen and thawed suspensions. For this reason it was not possible to calculate the amount of damaged cells in the original suspension from the response of the frozen and thawed suspension to incubation in the presence of cofactors.

Table 4. Testosterone levels in a testis cell suspension before and after incubation in Krebs–Ringer Buffer, pH = 7.4, either in the absence or presence of cofactors*, LH or both. All values are given as means ± S.E.M. (n = 4)

Incubation time min	Co-factors added	LH† added	pmol Testosterone/ 10^6 cells
0	–	–	25.0 ± 0.3
120	–	–	40.6 ± 1.4‡
120	+*	–	173.0 ± 6.2‡
120	–	+	93.6 ± 2.1‡
120	+	+	184.8 ± 2.8‡

* Cofactors: $CaCl_2$: 0.7 mM, $MgCl_2$: 5 mM, succinate: 20 mM, $NADPNa_2$: 1 mM, glucose-6-phosphate: 5 mM, glucose-6-phosphate dehydrogenase: 1 IU/2 ml.
† LH: 100 ng/ml.
‡ significantly different from control ($P < 0.001$).

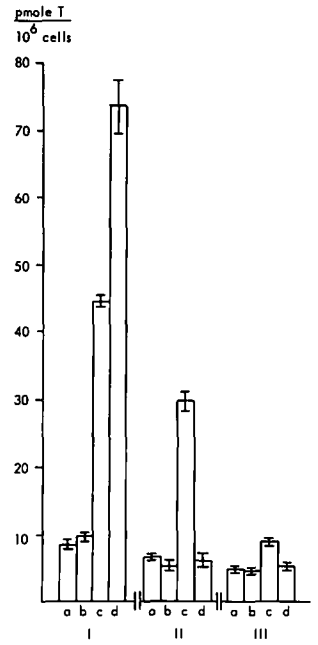

Fig. 1. Testosterone levels in suspensions of intact (I) and frozen and thawed (II) testis cells and in the 100 *g* supernatant of a frozen and thawed suspension (III). (a) not incubated, (b) incubated for 120 min in Krebs–Ringer Buffer, pH = 7.4 (c) incubated for 120 min in the presence of cofactors, (d) incubated for 120 min in the presence of LH (100 ng/ml) Vertical lines extend to the individual results of duplicate incubations.

A greater response of intact cells, both to cofactors and LH was observed in the Ficoll purified cell suspensions, as shown in Fig. 2. The Ficoll treated cell suspensions showed a relatively better response to LH than to cofactors, which is just the opposite of the situation with the untreated suspensions, and which may be an indication that the Ficoll treated suspensions contain a larger percentage of viable cells than the untreated suspensions. With Ficoll treated cell suspensions it was also not possible to calculate the amount of damaged cells from a comparison of the responses of the original suspension with a frozen and thawed one to incubation with cofactors, for the same reason as was mentioned for the untreated cells. A time study was done without preincubation in order to exclude the possibility that degradation of the cell suspension during incubation might account for the high response of the so called "intact" cell suspension to incubation with cofactors. Incubations were carried out in the presence of either buffer alone, buffer and cofactors or buffer and LH. The results depicted in Fig. 3 indicate that the cell suspension still produced testosterone after 3 h incubation. Testosterone production in the presence of cofactors increased rapidly, when compared with the incubations in buffer only, whereas, there was a time lag of about 45 min for the response of the suspension to LH.

Preincubation with cofactors also had no effect on the response to LH (results not shown). Therefore,

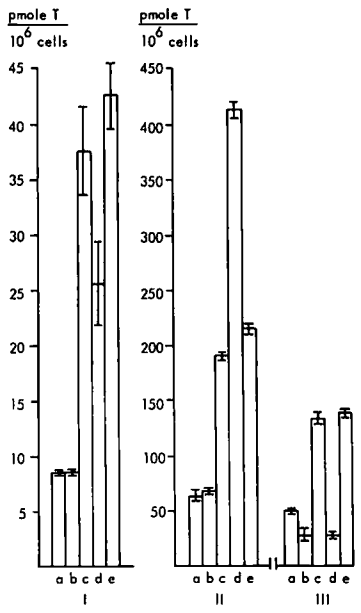

Fig. 2. Testosterone levels in crude (I), Ficoll purified (II) and Ficoll purified, frozen and thawed (III) testis cell suspensions. (a) through (d) see Figure 1, (e) incubated for 120 min in the presence of LH and cofactors. Vertical lines extend to the individual results of duplicate incubations.

a possible degradation of the cells through the addition of cofactors can be excluded. Furthermore, the LDH content of the cells was found not to change during incubation, with either buffer (osmolarity 280 mOsmol), cofactors (350 mOsmol), LH, or LH + cofactors (results not shown).

DISCUSSION

Our observations confirm previous results that the addition of cofactors or LH can stimulate steroid production by testis tissue preparations *in vitro*[6, 10]. Our studies were started in the belief that (the stimulation of) steroid production by intact testis tissue or intact cells is the result of a compartmentalized sequence of reactions. This would involve the possible effect of LH on the intact cell membrane resulting in increased c-AMP levels which would ultimately affect the mitochondrial conversion of cholesterol to pregnenolone requiring NADPH (and O_2) as obligatory cofactors. Addition of LH *in vitro* would only stimulate the steroid production of cells with intact cell membranes[22]. It has been observed in other experiments (Van der Vusse, unpublished results) that in the presence of sufficient Ca^{2+}-ions NADPH is the limiting factor for steroid production by testis tissue homogenates or isolated mitochondrial fractions. NAPDH *in vitro* was expected to have little effect on intact cells because it will not be transferred across the intact cell membrane. In cells with disrupted membranes endogenous cofactors may have leaked out, hence an exogenous supply of cofactors may be necessary for proper steroid production.

It can be concluded from the results in Table 2 that additional calcium and magnesium ions appear to cooperate with the NADPH generating system, but that the NADPH generating system has the largest effect on steroid production. Both homogenized and supposedly "intact" testis tissue showed an increase in testosterone production when calcium and magnesium ions, succinate and a NADPH generating system were added to the incubation medium (Table 3). This casts some doubts on the intactness of cells in the tissue preparations, because if NADPH really cannot enter intact cells[15] this could indicate that many cells of the tissue preparation were damaged. Tissue damage might have occurred during the isolation procedure of the tissue or during incubation. It seems very unlikely, however, that testicular tissue, which has only been decapsulated, would be damaged to the same extent as tissue which was intentionally disrupted by sonication (cf. Table 3).

As an alternative to the entering of NADPH into damaged cells leakage of enzymes involved in steroidogenesis from the tissue into the medium, either during the tissue preparation or during the incubation, might explain the increase in testosterone production in the presence of NADPH. The data given in Fig. 1 (III) and the finding that the LDH-content of the cells does not change during incubation do not support this assumption. For adrenals[15] and ovaries[23] observations have been reported which seem to parallel our findings with testes. Halkerston[15] tried to remove damaged cells from rat adrenal slices by enzymic digestion with collagenase,

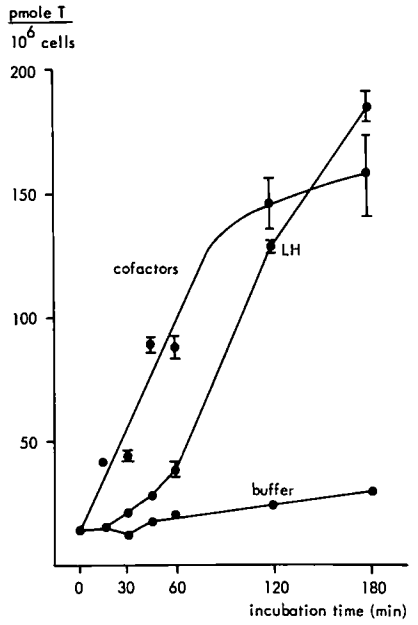

Fig. 3. Time course of testosterone levels in suspensions of Ficoll purified testis cells, incubated with either Krebs Ringer Buffer alone, or in the presence of cofactors or LH. Each point represents the mean of duplicate incubations. The range of these duplicate incubations is represented by vertical lines.

trypsin, DNase and RNase. Nevertheless, he still found a stimulatory effect of the NADPH generating system on corticosteroid synthesis by his preparations. Savard *et al.*[13] incubated bovine corpus luteum slices and observed a stimulation of progesterone production when $NADP^+$ and glucose-6-phosphate were added. From our observations it appears that the assumption made by Halkerston[15] that NAPDH would not enter intact cells does not hold for testis cells.

The present results (Table 4) also suggest the requirement of cofactors for testosterone production by suspensions of isolated testis cells. From the effect of LH on testosterone production by such cell suspensions it appears that addition of cofactors may inhibit the LH effect to a greater or lesser extent. In almost all experiments the effects of LH and cofactors were not additive (Table 4). This is in contrast with the results for adrenal cells reported by Neher and Milani[21], who observed that NADPH and (monobutyryl) cyclic AMP had a marked synergistic effect on the stimulation of corticosteroid production. This lack of additivity of the effects of LH and cofactors on our testis cell suspensions may indicate, that there is no clear distinction between intact and disrupted cells, i.e. that cells whose testosterone production can be stimulated by cofactors also can respond to LH. It was therefore not possible to calculate the amounts of intact and damaged cells from the effect of NADPH on testosterone production.

The testosterone production by intact total testis tissue in the presence of cofactors can be calculated from the data in Table 3 to be about 100 pmol/mg protein per two hours. This result is in good agreement with the *in vivo* testicular testosterone production rate estimated by De Jong *et al.*[11].

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