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N-OXYGENATION OF ARYLAMINES IN MICROSOMES PREPARED FROM CORPORA LUTEA OF THE CYCLE AND OTHER TISSUES OF THE PIG*

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Abstract—Microsomes prepared from the corpora lutea of pigs in cycle were found to *N*-oxygenate aniline, *N*-ethylaniline, and *N*,*N*-dimethylaniline as rapidly as liver microsomes. Lower oxygenating activities were observed with microsomes from other tissues. Pig liver microsomes *p*-hydroxylate aniline and acetanilide at rates nearly as high as they *N*-hydroxylate aniline. Corpus luteum microsomes, however, do not *p*-hydroxylate aniline and acetanilide. The *N*-oxygenations in corpus luteum microsomes correspond with those in liver microsomes in their sensitivity to some inhibitors and in the insensitivity to carbon monoxide. Except in the *N*-hydroxylation of aniline by corpus luteum microsomes, all *N*-oxygenations need high oxygen pressures for maximal activity.

N-OXYGENATION of arylamines has been observed with microsomes prepared from livers, lungs, and kidneys of rabbits[1] and from livers of various species.[2–5] Among the other tissues, bladder mucosa has been tested for its capacity of *N*-hydroxylating arylamines.[6] Recently Machinist, Dehner and Ziegler[5] published data on the *N*-oxygenation of *N*,*N*-dimethylaniline by microsomes prepared from liver, kidney, lung and adrenal of the pig. We prepared microsomes from several tissues and measured the *N*-oxygenation of aniline, *N*-ethylaniline and *N*,*N*-dimethylaniline. Using the tissues of pigs we obtained sufficient amounts of microsomes also from small organs.

METHODS

The tissues were collected at the slaughterhouse immediately after the animals had been killed. For transportation they were covered with ice. Within less than an hour the tissues were homogenized in the cold room. Tissues of the consistency of liver were squeezed through a sieve according to Von Jagow, Kampffmeyer and Kiese.[7] The pulp was mixed with two volumes of cold 1·15% potassium chloride solution and stirred for 20 min. Other tissues were cut to small pieces, covered with 2 volumes of 1·15% potassium chloride solution, and homogenized in an ice bath with an ultraturrax for 10 sec. The homogenation was repeated three times with 1 min intermission.

The homogenates were centrifuged for 20 min at 9000 *g*. From the supernatant the microsomes were separated by 60 min centrifugation at 78,000 *g*. After being washed twice with 1·15% potassium chloride solution the microsomes were suspended in a small volume of 0·1 M sodium phosphate or Tris buffer solution pH 7·4 and kept at 3°. They were used one day after the preparation had begun.

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In most experiments the reaction mixtures contained

Magnesium chloride	6	mM
Nicotinamide	12	mM
NADP	0·12	mM
Glucose-6-phosphate	4·8	mM
Glucose-6-phosphate-dehydrogenase	700 I. U. per litre	
Substrate	1	mM

Sodium phosphate buffer solution pH 7·4 or Tris buffer solution pH 7·4 was added to a final concentration of 0·05 M.

As glucose-6-phosphate is hydrolyzed by microsomal glucose-6-phosphatase, part of the experiments was carried out with 10 mM glucose-6-phosphate. In order to avoid the degradation of the ester before the hydroxylation reaction started, glucose-6-phosphate was added to the microsome suspension together with the substrate.

The protein concentration was 3 mg/ml in the experiments which measured the *N*-oxygenation and 7 mg/ml in the experiments for determining the *p*-hydroxylation of acetanilide; exceptions are mentioned in the text.

Experiments at low oxygen pressures or with carbon monoxide were carried out as described by Hlavica and Kiese.[8] Glucose-6-phosphate and substrate solutions were equilibrated with the gas mixtures separately from the microsome suspensions.

The microsome suspensions were incubated with the substrates at 37° for 10–30 min in the experiments at low oxygen pressures or carbon monoxide and for 40 min in most of the other experiments.

The following analytical methods were used for the determination of
N-Hydroxylation products: Herr and Kiese,[9]
N,*N*-Dimethylaniline-*N*-oxide: Ziegler and Pettit,[10]
Aniline and *p*-aminophenol: Brodie and Axelrod,[11]
p-Acetylaminophenol: Kerekjarto and Staudinger,[12]
Protein: Gornall, Bardawill and David,[13]
Cytochrome P-450: Omura and Sato.[14]

RESULTS

1. N-*Oxygenation in microsomes from various tissues*

In the experiments with microsomes prepared from several tissues of the pig all microsome suspensions were incubated for 40 min in order to obtain measurable amounts of oxygenation products also from microsomes of low activity. As may be seen in Table 1, in liver microsomes the concentration of *N*-oxygenation products increases for 40 min, though not strictly proportionally with time. The *N*-oxygenation activities observed with microsomes prepared from various tissues are shown in Table 2. Pig liver microsomes were found to surpass dog, cat and rat liver microsomes in *N*-oxygenation activity. Microsomes from corpora lutea of the cycle are as active as liver microsomes in *N*-hydroxylating aniline and *N*-ethylaniline. They produce more *N*-oxide from *N*,*N*-demethylaniline, but are only half as active in dealkylating *N*-ethylaniline. Microsomes from ovaries whose corpora lutea were removed produce very small concentrations of *N*-hydroxy derivatives. Microsomes from ovaries of young pigs, which had not yet ovulated, were found to be inactive.

The production of *N*-hydroxy aniline from aniline and *N*-ethylaniline by liver and corpus luteum microsomes was also demonstrated by the spectrum of nitrosobenzene

TABLE 1. RELATIONSHIP BETWEEN INCUBATION TIME AND CONCENTRATION OF *N*-OXYGENATION PRODUCTS FOUND IN SUSPENSIONS OF PIG LIVER MICROSOMES INCUBATED WITH 1 mM *N*-ETHYLANILINE OR *N*,*N*-DIMETHYLANILINE

Incubation time (min)	*N*,*N*-Dimethylaniline-*N*-oxide from *N*,*N*-dimethylaniline (μg/ml)		Phenylhydroxylamine from *N*-ethylaniline (μg/ml)	
	1 mg microsomal protein per ml	3 mg microsomal protein per ml	1 mg microsomal protein per ml	3 mg microsomal protein per ml
10	3·8	10·2	0·51	1·32
20	7·2	18·0	0·92	2·46
40	13·8	28·5	1·61	4·47

The figures are the means of experiments with four microsome preparations.

TABLE 2. *N*-OXYGENATION OF ANILINE, *N*-ETHYLANILINE, *N*,*N*-DIMETHYLANILINE, *N*-DEALKYLATION OF *N*-ETHYLANILINE AND CONTENT OF CYTOCHROME P-450 IN MICROSOMES PREPARED FROM VARIOUS TISSUES OF THE PIG

	Homogenation	From aniline 10^{-3}M	From *N*-ethylaniline 10^{-3}M		From *N*, *N*-dimethylaniline 10^{-3}M	Cytochrome P-450 (nmole/mg protein)
		Phenylhydroxylamine (μg/ml)		Aniline (μg/ml)	*N*,*N*-Dimethylaniline-*N*-oxide (μg/ml)	
Liver	S	2·0	5·0	1·3	20·0	0·61
Corpus luteum	U	3·2	4·8	0·63	66·7	0·1
Ovary (Stroma)	U	0·08	0·14	—	—	0
Ovary (Young pig)	U	0	0	—	—	0
Testicle	U	0·28	0·38	0·14	3·1	0·19
Retained testicle	S	0	0	0·18	—	0·12
Hermaphrodite testicle	U	0·63	1·1	0·37	6·3	0·22
Lung	U	0·43	0·56	0·38	5·5	0
Kidney	S	0·07	0·1	0·18	3·3	0·3
Pancreas	S	0·03	0·02	—	2·1	0
Spleen	S, U	0	0	—	0	0
Bladder mucosa	U	0·15	0·22	—	4·1	0
Thymus	U	0·04	0·03	—	0·17	0
Thyroid	U	0·03	0·02	0·12	0·3	0
Adrenal	U	0·04	0·02	0·08	0·8	0·3
Lymph node	U	0·03	$<0·01$	0·4	—	0
Brain	U	0	0	—	0	0

The concentrations of the oxygenation products were determined after 40 min incubation.
All data are means of 3 or more experiments, excepted the data for testicle which refer to a single experiment, and for hermaphrodite testicle which are the means of 2 experiments.
S: Homogenation of the tissue by squeezing through a sieve.
U: Homogenation with the ultraturrax.

found in carbon tetrachloride extracts after the phenylhydroxylamine had been oxidized with hexacyanoferrate (III). The *N*-oxygenating activity in microsomes prepared from several other tissues is much lower than that observed with liver or corpus luteum microsomes. No activity was found in microsomes prepared from brain and spleen.

The cytochrome P-450 content of corpus luteum microsomes amounts to only one sixth of the content found in liver microsomes. Cytochrome P-450 was also found in the microsomes prepared from kidney, testicle, and adrenal. In view of the low oxygenating activity of the adrenal microsome preparations it was not investigated which

portion of the cytochrome P-450 was contributed by fragments of mitochondria. In the microsomes from other tissues the cytochrome P-450 content could not be determined, as other carbon monoxide binding pigments (hemoglobin) with a peak at 420 nm in the difference spectrum were present.

2. *Some properties of the microsomal oxygenating systems in corpora lutea of the cycle and in liver*

(a) *Disposal of* N,N-*dimethylaniline*-N-*oxide and phenylhydroxylamine.* In this study the concentration of *N,N*-dimethylaniline-*N*-oxide or phenylhydroxylamine found after the incubation with the substrate is taken as a measure of the rate of *N*-oxygenation. Since the *N*-oxide may be dealkylated in liver microsomes,[15] the effect of this reaction on the estimations of the rate of *N*-oxide formation had to be determined. *N,N*-dimethylaniline-*N*-oxide was found to react very slowly when added to the microsomes suspensions in concentrations as appear during the incubation of microsomes with *N,N*-dimethylaniline. In three experiments, corpus luteum and liver microsomes fortified as in oxygenation experiments were incubated for 40 min with 100 μg *N,N*-dimethylaniline-*N*-oxide per ml suspension. The decrease in *N*-oxide concentration was less than 5 per cent and could not be determined very accurately. Suspensions of rat liver microsomes slowly reduce phenylhydroxylamine to aniline[16] and dispose of it through other reactions. The data presented in Table 3 show that microsomes from the liver and corpus luteum of the pig eliminate phenylhydroxylamine more rapidly than microsomes from livers of rats and rabbits. The rate of disposal is not so high as to prevent the use of the phenylhydroxylamine concentration as a measure of the rate of *N*-hydroxylation. As may be seen in Table 3, phenylhydroxylamine was found to be more stable in the microsomes suspensions if air was replaced by nitrogen or carbon monoxide as the gas phase.

TABLE 3. DISAPPEARANCE OF PHENYLHYDROXYLAMINE AND PRODUCTION OF ANILINE IN SUSPENSIONS OF MICROSOMES PREPARED FROM VARIOUS TISSUES DURING 40 min INCUBATION WITH 5 μg PHENYLHYDROXYLAMINE PER ml OF SUSPENSION UNDER AIR, NITROGEN, OR CARBON MONOXIDE AT 37°

		Air		Nitrogen		Carbon monoxide	
		Phenylhydroxylamine (μg/ml)	Aniline (μg/ml)	Phenylhydroxylamine (μg/ml)	Aniline (μg/ml)	Phenylhydroxylamine (μg/ml)	Aniline (μg/ml)
Liver	Pig	− 2·8	+ 0·2	− 0·7	+ 0·3	− 0·1	+ 0·1
Liver	Rabbit	− 1·7	+ 0·4	− 0·2	+ 0·2	− 0·1	+ 0·1
Liver	Rat	− 0·5	+ 0·1	− < 0·1	+ < 0·1	− < 0·1	+ < 0·1
Corpus luteum	Pig	− 3·7	+ 0·3	− 0·2	+ 0·1	− < 0·1	+ < 0·1

The microsomes were suspended in Tris solution and fortified as in the oxygenation experiments. Protein content 3 mg per ml of suspension. The figures are the means of three or four experiments.

(b) N-*Oxygenation by microsomes prepared from corpora lutea in various phases.* The data presented in Table 4 show that the *N*-oxygenating activity of microsomes from corpora lutea is highest at the 10th to 12th day. Microsomes from freshly ruptured follicles were found to be nearly half as active. During the regression of the corpora lutea the microsomal activity readily disappears.

(c) p-*Hydroxylation of aniline and acetanilide.* In view of the high rates of *N*-oxygenation of arylamines by corpus luteum microsomes and the low cytochrome P-450 content it appeared of interest to study the *p*-hydroxylation of aniline and acetanilide by corpus luteum and liver microsomes. Corpus luteum microsomes did not *p*-hydroxylate aniline or acetanilide to a measurable degree.

Liver microsomes, in three experiments, were found to produce an average of 1·1 μg *p*-aminophenol per ml from aniline and, with 7 mg microsomal protein per ml, 2·1 μg *p*-acetylaminophenol from acetanilide.

TABLE 4. *N*-OXYGENATION OF ANILINE, *N*-ETHYLANILINE, *N,N*-DIMETHYLANILINE AND DEALKYLATION OF *N*-ETHYLANILINE BY MICROSOMES PREPARED FROM CORPORA LUTEA IN VARIOUS PHASES

	From aniline	From *N*-ethylaniline		From *N,N*-dimethylaniline	
Age of corpora lutea (days)	Phenylhydroxylamine (μg/ml)		Aniline (μg/ml)	*N,N*-Dimethylaniline-*N*-oxide (μg/ml)	Cytochrome P-450 (nmole/mg)
1–4	2·2	2·3	0·2	17·1	—
10–12	4·4	6·3	0·6	62·0	0·09
18–20	0·6	0·6	0·04	2·6	—

The figures are the means of three experiments.

Because of their content of cytochrome P-450 adrenal microsome preparations were also tested. In three experiments, no *p*-hydroxylation of aniline and acetanilide was observed. Microsomes prepared from lungs and kidneys, in three experiments, were not found to produce detectable concentrations of *p*-hydroxylation products from aniline and acetanilide.

(d) *Effect of various inhibitors.* Some substances whose effect on the hydroxylations of aniline and *N*-alkylaniline by rabbit liver microsomes has been studied[3] were tried with corpus luteum and liver microsomes. The inhibition pattern of these substances was found to be different from that observed with rabbit liver microsomes. *p*-Chloromercuribenzoate and *N*-ethylmaleimide inhibit the *N*-hydroxylation of aniline and *N*-ethylaniline as well as the dealkylation of *N*-ethylaniline; see Table 5.

(e) *Oxygenation at low oxygen pressures, and the effect of carbon monoxide.* The *N*-oxygenation of aniline, *N*-ethylaniline and *N,N*-dimethylaniline was studied at oxygen pressures of 4, 14 and 75 Torr. The results are summarized in Table 6. Except for the *N*-hydroxylation of aniline by corpus luteum microsomes, all oxygenating reactions at 14 Torr oxygen proceeded with rates below 50% of the rate observed under air.

The effect of carbon monoxide was examined with oxygen pressures of 4, 14 and 75 Torr. With the lowest oxygen pressure the carbon monoxide pressure was nearly 200 times higher than the oxygen pressure. At the highest oxygen pressure, i.e. 75 Torr, the reaction velocity was nearly maximal and the carbon monoxide pressure was nearly ten times higher than the oxygen pressure. As may be seen in Table 6, under all conditions tried, carbon monoxide did not inhibit the *N*-oxygenation reactions

TABLE 5. THE EFFECT OF 8-HYDROXYQUINOLINE, SEMICARBAZIDE, *p*-CHLOROMERCURIBENZOATE, AND *N*-ETHYLMALEIMIDE ON THE *N*-HYDROXYLATION OF ANILINE AND *N*-ETHYLANILINE AND ON THE DEALKYLATION OF *N*-ETHYLANILINE

		Tissue	From aniline: Phenylhydroxylamine (μg/ml)	From *N*-ethylaniline: Phenylhydroxylamine (μg/ml)	From *N*-ethylaniline: Aniline (μg/ml)
A		Liver	2·3 ± 0·8	5·0 ± 1·2	2·5 ± 0·34
		Corpus leutum	3·3 ± 0·3	5·1 ± 0·7	0·66 ± 0·07
			% of A	% of A	% of A
B	8-Hydroxyquinoline 10^{-3}M	Liver	59 ± 14	58 ± 3	25 ± 4
		Corpus luteum	44 ± 3	77 ± 17	59 ± 17
C	Semicarbazide 10^{-2}M	Liver	111 ± 12	78 ± 12	50 ± 10
		Corpus luteum	95 ± 6	71 ± 18	48 ± 2
D	*p*-Chloromercuribenzoate 4×10^{-4}M	Liver	11 ± 2	6 ± 2	14 ± 3
		Corpus luteum	16 ± 6	24 ± 8	13 ± 1
E	*N*-Ethylmaleimide 5×10^{-3}M	Liver	16 ± 3	15 ± 1	9 ± 4
		Corpus luteum	16 ± 1	23 ± 4	32 ± 10

The substances were added to the microsome suspensions 5 min before the substrates. The figures indicate the means of three and more experiments and the standard error.

TABLE 6. *N*-OXYGENATION OF ANILINE AND *N*-ALKYLANILINES BY MICROSOMES FROM PIG LIVER AND CORPUS LUTEUM UNDER REDUCED OXYGEN PRESSURES AND IN THE PRESENCE OF CARBON MONOXIDE

	Phenylhydroxylamine				*N,N*-Dimethylaniline-*N*-oxide	
	from aniline		from *N*-ethylaniline		from *N,N*-dimethylaniline	
Torr O_2	O_2–N_2	O_2–CO	O_2–N_2	O_2–CO	O_2–N_2	O_2–CO
			Liver			
4	8	12	7	12		
14	27	36	13	21	13	16
75	80	87	93	90	70	67
			Corpus luteum			
4	17	19	10	15		
14	74	97	24	28	22	25
75					67	70

The microsome suspensions were incubated with the substrates for 10 min at 37°. The figures, which are the means of three or more experiments, indicate the activity in per cent of the activity observed under air.

tested. Further experiments were carried out with microsome suspensions containing only 1 mg microsomal protein per ml. In these experiments the suspensions were incubated with the substrate for 20 min. The results confirm the data presented in Table 6. Neither with 75 Torr oxygen and a nearly 10-fold excess of carbon monoxide (five experiments) nor with 14 Torr oxygen and a 45 times higher pressure of carbon monoxide (four experiments) an inhibiting effect of carbon monoxide was observed.

The investigation of the effect of carbon monoxide on the *N*-oxygenation of arylamines in microsome suspensions with as little as 1 mg protein per ml appeared

necessary, because the *N*-hydroxylation of aniline in rabbit liver microsomes was found to be sensitive to carbon monoxide, if microsome suspensions with less than 5 mg protein per ml are used. Figure 1 shows the results of experiments with suspensions of rabbit liver microsomes containing from 1 to 20 mg protein per ml. In the absence of carbon monoxide, an increase in the content of microsomal protein beyond 5 mg/ml did not increase the yield of phenylhydroxylamine but diminished it. Carbon monoxide, in a pressure 50 times higher than that of oxygen, inhibited the *N*-hydroxylation of aniline by nearly 50 per cent in microsome suspensions with less than 5 mg protein per ml. In suspensions with high protein contents carbon monoxide was found to increase the yield of phenylhydroxylamine. Using microsomes prepared from the livers of young rabbits, Kröber *et al.*[18] observed the same relationship between the protein content of microsome suspensions and the effect of carbon monoxide on the yield of phenylhydroxylamine.

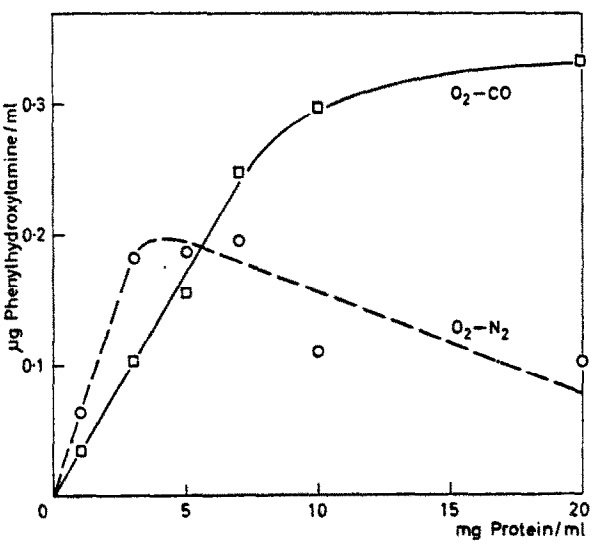

FIG. 1. *N*-Hydroxylation of aniline by rabbit liver microsomes in reaction mixtures with various microsome contents and the effect of carbon monoxide.

The microsomes were incubated at 37° for 10 min with 10^{-3}M aniline under 13 Torr oxygen and 650 Torr nitrogen or 13 Torr oxygen and 650 Torr carbon monoxide. The ordinate shows the concentration of phenylhydroxylamine determined after 10 min incubation. The symbols indicate the means of 5–7 experiments.

○, dotted line: yields of phenylhydroxylamine under 13 Torr oxygen and 650 Torr nitrogen.
□, full line: yields of phenylhydroxylamine under 13 Torr oxygen and 650 Torr carbon monoxide.

In order to find out, whether other oxygenations in pig liver microsomes are also insensitive to carbon monoxide, the dealkylation of *N*-ethylaniline was studied with microsome suspensions containing 1 and 3 mg protein per ml. The suspensions were incubated for 20 min under 75 Torr oxygen and 590 Torr nitrogen or carbon monoxide. In three series of experiments an average of 35 per cent inhibition by carbon monoxide was observed.

DISCUSSION

The high *N*-oxygenating activity of microsomes prepared from corpora lutea of the cycle is of particular interest, because these microsomes do not *p*-hydroxylate aniline and acetanilide. Lange and Thun[17] in our laboratory found that corpus luteum microsomes do not hydroxylate progesterone either. Microsomes from adrenals, however, which also failed to hydroxylate aniline and acetanilide, readily hydroxylate progesterone. Similar to the corresponding system in liver microsomes, corpus luteum microsomes produce *N,N*-dimethylaniline-*N*-oxide from *N,N*-dimethylaniline at much higher rates than they produce phenylhydroxylamine from aniline or *N*-ethylaniline.

The occurence of *N*-oxygenation of *N*-alkylaniline and *N,N*-dialkylaniline in microsomes which do not C-hydroxylate aniline gives further support to the hypothesis advanced by Hlavica and Kiese[8] that the *N*-hydroxylation of *N*-ethylaniline and the *N*-oxide formation from *N,N*-dimethylaniline are closely related in their enzymic mechanism. Both oxygenating systems are also similar in the need of high oxygen pressures for maximal activity and they are not inhibited by carbon monoxide. The *N*-hydroxylation of aniline is not inhibited by carbon monoxide either, whereas the *N*-hydroxylation of aniline by rabbit liver microsomes was found to be inhibited by carbon monoxide. The difference in sensitivity for carbon monoxide cannot yet be explained. Conney *et al.*[19] observed differences in the sensitivity for carbon monoxide also with the 6β-, 7α- and 16α-hydroxylation of testosterone by rat liver microsomes.

The results of this study are in line with others showing that an unspecific oxygenase cannot explain the specificities in oxygenations observed. Presently, the existence of a large number of microsomal oxygenases with narrow specificities appears unlikely. If there are only one or a few oxygenases, certain specificities of the oxygenating systems must be caused by additional factors. These are affected by various substances and changed by induction. They may act as substrate guides or substrate blocks and thereby determine which compounds or which sites of compounds that may be oxygenated in several positions get access to the unspecific oxygenase.

In a recently published paper Machinist *et al.*[5] report on *N,N*-dimethylaniline oxidase activity in microsomes from pig liver, lung, kidney, and adrenal. Since "dimethylaniline oxidase activity" is expressed as the sum of *N,N*-dimethylaniline-*N*-oxide and formaldehyde produced in 1 min by 1 mg microsomal protein with saturating concentrations of substrate, the results cannot be compared with the data presented in Table 1. *N,N*-Dimethylaniline may be demethylated and formaldehyde produced by mechanisms not involving the formation of *N,N*-dimethylaniline-*N*-oxide. Formaldehyde also originates from substrates in the microsomes which are not removed during the preparation.[20,8] This probably explains why the ratios between the activities found in the four tissues differ from the respective ratios which may be calculated from our data.

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