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CHLORAMPHENICOL METABOLISM IN THE PHENOBARBITAL-INDUCED RAT. COMPARISON WITH THIAMPHENICOL

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Abstract—Blood levels, urinary excretion and *in vivo* chemotherapeutic activity of chloramphenicol in the rat have been studied under conditions of normal and stimulated drug metabolism (prior phenobarbital treatment over 3 days). Parallel studies have been conducted with the *p*-methylsulfonyl analogue, thiamphenicol, which is not extensively conjugated or metabolized in normal conditions. Blood levels and chemotherapeutic activity of chloramphenicol were distinctly reduced by pretreatment with phenobarbital, and urinary excretion was enhanced; the latter was entirely accounted for by an increase in the glucuronide fraction. The elimination and activity of thiamphenicol were not affected by phenobarbital pretreatment.

The relative importance of glucuronide formation and nitro-group reduction in the metabolism of chloramphenicol under stimulated conditions is discussed, and some implications of altered drug metabolism for chloramphenicol activity and toxicity in human therapy are considered.

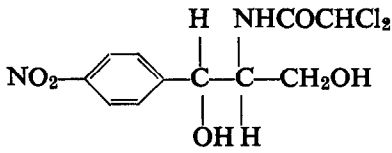
TWO PROPERTIES of chloramphenicol are particularly relevant to the induction of drug metabolizing enzymes in liver microsomes: (a) as an inhibitor of protein synthesis,[1] it might interfere with the induction process, which is known to be mediated by the synthesis of more enzyme protein and to be blocked by established protein synthesis inhibitors;[2, 3] (b) as a substrate, its biological disposition and activity might be altered under conditions of stimulated drug metabolism.

Results compatible with action (a) were obtained in the rabbit by Lange;[3] in these experiments, however, chloramphenicol (250 mg/kg i.p.) inhibited only some of the stimulating effects of a 40 mg/kg s.c. phenobarbital dose (microsomal *N*-hydroxylating and dealkylating activities), whereas a standard protein synthesis inhibitor, actinomycin D, under comparable conditions completely abolished all the enzyme responses studied, including the *p*-hydroxylating activities which were relatively unaffected by chloramphenicol. In some preliminary studies mentioned by Glazko,[4] three 50 mg/kg doses of chloramphenicol and phenobarbital were administered i.p. to rats over a 24-hr period, "with no indication of interference to the induction of microsomal enzyme activity".

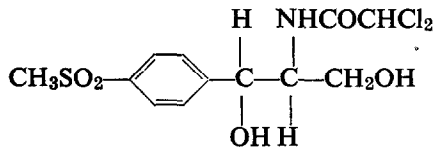
The available data thus do not provide a definite answer to the question (a), and the fact that chloramphenicol also exerts direct inhibitory effects on certain microsomal enzyme activities[5] may well contribute to these difficulties. Moreover, although protein synthesis in certain other mammalian systems is known to be inhibited by chloramphenicol,[1] protein synthesis in rat liver *in vivo*, according to recent data,[6] is not subject to chloramphenicol inhibition, a fact which would make the very

hypothesis of a chloramphenicol inhibition of microsomal enzyme synthesis less tenable.

It seemed to us that testing of hypothesis (b) could be put on firmer ground and could have more practical importance. Chloramphenicol metabolism in the rat is well defined,[4] as are the effects of standard phenobarbital stimulation on the two most relevant microsomal activities—glucuronic acid conjugation[7] and nitro group reduction.[8] Furthermore, the opportunity seemed very interesting of comparing under conditions of stimulated drug metabolism the physiological disposition of chloramphenicol with that of thiamphenicol, an active analogue in which the *p*-nitro group is replaced by the *p*-methylsulfonyl group (see formulas) and which, unlike the parent compound, is not subjected to extensive glucuroconjugation in the normal rat.[9]



Chloramphenicol



Thiamphenicol

Our experiments, carried out in parallel with the two compounds, indicate that the intensity and duration of chloramphenicol action are distinctly reduced by phenobarbital induction in the rat, whereas those of thiamphenicol are unaffected.

MATERIALS AND METHODS

These studies were carried out on male Wistar rats weighing 190–210 g. The animals were given 80 mg/kg of sodium phenobarbital intraperitoneally for 3 days to induce drug metabolizing enzymes. Chloramphenicol and thiamphenicol (products of Zambon S.p.A.) were administered by gastric tube or parenterally, as specified in each study. For each antibiotic the oral preparation consisted of a suspension in 2% gum arabic and the parenteral of a water solution of the glycine ester which is characterixed by high solubility, rapid hydrolysis and complete absorption.[10, 11]

1. *Study of the serum levels of chloramphenicol and thiamphenicol in induced and normal rats*

On the basis of preliminary studies, a single dose of 75 mg/kg of each antibiotic was administered i.m. to normal rats and to induced rats 48 hr after the last dose of phenobarbital.

At 1, 2 and 3 hr after the dose of thiamphenicol and at ½, 1 and 2 hr after that of chloramphenicol groups of 5 normal and 5 induced rats were exsanguinated. Quantitative determinations of the serum antibiotic levels were performed, based on standard microbiological assay techniques using serial broth dilutions and *Pasteurella bovi-septica*, Harvard strain, as the test organism.

The serum concentration values of each antibiotic were compared in normal and in induced rats and the statistical significance of the differences were determined by analysis of variance; the phenobarbital pretreatment by time interaction on the serum level curves was also calculated.

2. *Study on the urinary excretion of chloramphenicol and thiamphenicol and their metabolites in induced and normal rats*

Normal and induced rats, fasting for 18 hr, were given a single oral dose of 200

mg/kg of antibiotic. The animals were also given a water load of 6 ml orally at the time of antibiotic administration and again 2 hr later. The urine of each animal was collected for the first four hours after antibiotic administration in 2-hr fractions.

The following methods of chemical assay for the antibiotics and their metabolites in urine were employed.

Free antibiotic. Both methods are based on a preliminary extraction in ethyl acetate and only free antibiotic is determined. Chloramphenicol was determined by the method of Levine and Fischbach[12] and thiamphenicol by that of McChesney *et al.*[13]

Conjugated antibiotic. The conjugated fraction was hydrolyzed enzymatically using 2,000 Fishman units of β-glucuronidase (Ketodase, Warner–Chilcott) per 0·5 ml of urine after incubation for 18 hr at pH 5·2. The hydrolysis was followed by extraction in ethyl acetate and resulted in the total antibiotic. The conjugated fraction is equal to the difference between total and free antibiotic.

Chloramphenicol reduced. The free arylamines were determined by the method of diazotization and coupling described by Bratton and Marshall.[14] The results are reported as mg equivalents of chloramphenicol.

A preliminary acid hydrolysis at 100° for 1 hr was carried out for the determination of the acetylated arylamines.

3. *Evaluation of the chemotherapeutic activity of chloramphenicol and thiamphenicol in induced and normal rats*

A group of normal and induced rats, 48 hr after the last dose of phenobarbital, were each infected with *D. pneumoniae*, type I, by i.p. inoculation of 500×10^6 organisms. The organisms had been cultured in broth for 12 hr, centrifuged and resuspended in peptone water plus 3% mucin.

Two hours after the infecting inoculation, treatment was initiated with the water soluble esters of the antibiotics. Chloramphenicol was administered in two daily doses for 3 days and thiamphenicol for 5 days. Observations were noted until 24 hr after the last antibiotic dose. Mortality rates and the harmonic means of survival time, in hours, were recorded.

TABLE 1. CONCENTRATION OF CHLORAMPHENICOL IN THE BLOOD-SERUM OF PHENOBARBITAL-PRETREATED AND CONTROL RATS

Groups*	Chloramphenicol levels (mcg/ml)†		
	30 min	60 min	120 min
Control	21·6 ± 1·4	12·0 ± 1·6	6·3 ± 0·7
Phenobarbital	12·2 ± 0·7	4·5 ± 0·5	3·1 ± 0·4

* Phenobarbital was given i.p. (80 mg/kg) once daily for 3 days; chloramphenicol was given i.m. (75 mg/kg) 48 hr after the last dose of phenobarbital.

† Blood samples were obtained 30, 60 and 120 minutes after chloramphenicol administration. Chloramphenicol was determined by microbiological assay. Values shown are means of 5 rats ± S.E.

Analysis of variance was carried out on the logarithms of the concentration values and gave the results as set out overleaf.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Pretreatment with phenobarbital (P)	1	0·933	0·933	84·83	<0·001
Linear	1	1·428	1·428	129·82	<0·001
Quadratic	1	0·014	0·014	1·27	
Times (T)	2	1·442	0·712	65·55	<0·001
Interaction (P × T)	2	0·019	0·010	—	
Between groups	5	2·394	0·478	43·45	<0·001
Within groups	24	0·253	0·011		
Total	29	2·647			

TABLE 2. CONCENTRATION OF THIAMPHENICOL IN THE BLOOD-SERUM OF PHENOBARBITAL-PRETREATED AND CONTROL RATS

Groups*	Thiamphenicol levels (mcg/ml)†		
	60 min	120 min	180 min
Control	15·6 ± 1·4	10·2 ± 0·7	3·3 ± 0·3
Phenobarbital	15·6 ± 1·4	9·6 ± 0·6	3·0 ± 0·6

* Phenobarbital was given i.p. (80 mg/kg) once daily for 3 days; thiamphenicol was given i.m. (75 mg/kg) 48 hr after the last dose of phenobarbital.

† Blood samples were obtained 60, 120 and 180 min after thiamphenicol administration. Thiamphenicol was determined by microbiological assay. Values shown are means of 5 rats ± S.E.

Analysis of variance was carried out on the logarithms of the concentration values and gave the results as set out below.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Pretreatment with phenobarbital (P)	1	0·010	0·010	—	
Linear	1	2·557	2·557	170·41	<0·001
Quadratic	1	0·180	0·180	12·00	<0·01
Times (T)	2	2·737	1·368	91·20	<0·001
Interaction (P × T)	2	0·019	0·010	—	
Between groups	5	2·756	0·551	36·73	<0·001
Within groups	24	0·360	0·015		
Total	29	3·116			

RESULTS

1. *Serum levels of chloramphenicol and thiamphenicol*

The serum concentrations of chloramphenicol and thiamphenicol in normal and induced rats are reported in Tables 1 and 2, respectively. Comparison of the results shows clearly that after induction the serum levels of chloramphenicol are markedly reduced, while those of thiamphenicol show no change. In fact, the serum levels of chloramphenicol in the phenobarbital pretreated animals are 50 per cent lower than in the normal animals; analysis of variance confirms that this difference is highly significant. The pattern of the serum level curve of chloramphenicol is identical in both groups of animals, and the phenobarbital pretreatment by time interaction (P × R) on serum concentration levels is not significant. It can be concluded from this data that induction only alters the rate of chloramphenicol metabolism and has no significant effect on thiamphenicol metabolism.

TABLE 3. URINARY EXCRETION OF CHLORAMPHENICOL AND METABOLIC PRODUCTS IN PHENOBARBITAL-PRETREATED AND CONTROL RATS

Groups*	Urine collection (hr)	Urine volume (ml)	Arylamine† total (mg)	Arylamine† free (mg)	Chloramphenicol† total (mg)	Chloramphenicol† free (mg)	Chloramphenicol† conjugated (mg, % of total)	
Control	0–2	7·4 ± 0·4	0·18 ± 0·02	0·09 ± 0·01	1·91 ± 0·17	1·27 ± 0·12	0·63 ± 0·11	33
	2–4	7·5 ± 0·5	0·28 ± 0·03	0·24 ± 0·04	2·25 ± 0·46	1·73 ± 0·43	0·52 ± 0·12	23
	0–4	14·9 ± 0·9	0·46 ± 0·05	0·33 ± 0·05	4·16 ± 0·54	3·00 ± 0·46	1·15 ± 0·16	27
Phenobarbital	0–2	6·8 ± 0·5	0·21 ± 0·03	0·11 ± 0·01	4·02 ± 0·50‡	1·67 ± 0·35	2·35 ± 0·38‡	58
	2–4	7·8 ± 0·6	0·30 ± 0·03	0·26 ± 0·02	2·79 ± 0·61	0·99 ± 0·21	1·81 ± 0·53	64
	0–4	14·6 ± 0·8	0·51 ± 0·06	0·37 ± 0·03	6·81 ± 0·84§	2·66 ± 0·49	4·16 ± 0·63‡	61

* Phenobarbital was given i.p. (80 mg/kg) once daily for 3 days. Chloramphenicol (200 mg/kg) was given by gastric route 48 hr after the last dose of phenobarbital.

† Values are expressed in terms of chloramphenicol. Chloramphenicol was determined by chemical assay on untreated samples and on samples previously treated with β-glucuronidase. Values shown are means ± S.E. obtained from 10 rats.

‡ Significantly different from respective control ($P < 0·001$).

§ Significantly different from respective control ($P < 0·05$).

2. *Urinary excretion of chloramphenicol and thiamphenicol*

In the first 4 hr following a single oral dose of 200 mg/kg of chloramphenicol, there is a change in the urinary excretion of metabolites in induced rats compared to normal. In Table 3 the average values and standard errors of arylamines, free and conjugated chloramphenicol are reported as mg equivalents of chloramphenicol.

Particularly evident is the increase of the glucuronide-conjugated fraction in the induced animals, especially in the first 2 hr ($P < 0.001$). The excretion of arylamines in induced animals resulting from nitroreductase activity, is not modified. In contrast, there is no evidence of significant change in the urinary excretion of free and conjugated thiamphenicol after induction (Table 4).

TABLE 4. URINARY EXCRETION OF THIAMPHENICOL AND METABOLIC PRODUCTS IN PHENOBARBITAL-PRETREATED AND CONTROL RATS

Groups*	Urine collection (hr)	Urine volume (ml)	Thiamphenicol† total (mg)	free (mg)	conjugated (mg, % of total)	
Control	0–2	5·7 ± 0·4	5·00 ± 0·57	4·50 ± 0·53	0·50 ± 0·06	10
	2–4	4·2 ± 0·3	2·39 ± 0·37	2·24 ± 0·37	0·15 ± 0·03	6
	0–4	9·9 ± 0·5	7·39 ± 0·64	6·74 ± 0·64	0·65 ± 0·04	9
Phenobarbital	0–2	5·5 ± 0·6	4·40 ± 0·36	4·00 ± 0·31	0·40 ± 0·06	9
	2–4	3·1 ± 0·3	1·70 ± 0·35	1·58 ± 0·33	0·12 ± 0·03	7
	0–4	8·6 ± 0·5	6·10 ± 0·30	5·58 ± 0·24	0·52 ± 0·04	8·5

* Phenobarbital was given i.p. (80 mg/kg) once daily for 3 days. Thiamphenicol (200. mg/kg) was given by gastric route 48 hr after the last dose of phenobarbital.

† Thiamphenicol was determined by chemical assay on untreated samples and on samples previously treated with β-glucuronidase. Values shown are means ± S.E. obtained from 6 rats.

3. *Chemotherapeutic activity of chloramphenicol and thiamphenicol in induced and normal rats*

After phenobarbital pretreatment in the rat, the chemotherapeutic activity of chloramphenicol against the experimental infections with *D. pneumoniae* is notably reduced. The data in Table 5 show that with a dose of 200 mg/kg of chloramphenicol, the mortality rate in normals is 30 per cent, while in induced rats it rises to 70 per cent.

TABLE 5. EFFECTS OF CHLORAMPHENICOL ON THE EXPERIMENTAL INFECTION BY *D. Pneumoniae* IN PHENOBARBITAL-PRETREATED AND CONTROL RATS*

Chloramphenicol daily dose mg/kg	Mortality percentage Control	Phenobarbital	Survival time, hr (harmonic means) Control	Phenobarbital
0	100	100	24	24
100	85	100	39	27
150	50	85	93	51
200	30	70	185	69

* Phenobarbital was given i.p. (80 mg/kg) once daily for 3 days. The rats were infected i.p. with *D. pneumoniae* 48 hr after the last dose of phenobarbital. Chloramphenicol was given i.m. in two daily doses for 3 days beginning 2 hr after infection.

Groups of 20 phenobarbital-pretreated and 20 control rats were used for each dose-level of chloramphenicol.

The harmonic mean of the survival time in animals treated with chloramphenicol is only 69 hr in induced rats compared to 185 hr in normals.

The chemotherapeutic activity of thiamphenicol, unlike that of chloramphenicol, is equivalent in normal and induced animals (Table 6).

TABLE 6. EFFECTS OF THIAMPHENICOL ON THE EXPERIMENTAL INFECTION BY *D. Phneumoniae* IN PHENOBARBITAL-PRETREATED AND CONTROL RATS*

Thiamphenicol daily dose mg/kg	Mortality percentage		Survival time, hr (harmonic means)	
	Control	Phenobarbital	Control	Phenobarbital
0	100	100	24	24
40	90	90	62	62
80	30	20	230	530

* Phenobarbital was given i.p. (80 mg/kg) once daily for 3 days. The rats were infected i.p. with *D. pneumoniae* 48 hr after the last dose of phenobarbital. Thiamphenicol was given i.m. in two daily doses for 5 days beginning 2 hr after infection.
Groups of 10 phenobarbital-pretreated and 10 control rats were used for each dose-level of thiamphenicol.

DISCUSSION

The data presented indicate that following a rapidly absorbed chloramphenicol load, the antibiotic activity disappears from the blood at a significantly faster rate in phenobarbital-pretreated rats than in controls. Serum concentrations attained lower peak values in the induced animals (12·2 vs. 21·6 mcg/ml) and then followed parallel time courses with a similar apparent half-life for both groups (about 40 min).

The faster rates of disappearance of the antibiotic from the blood are reflected by the lowered *in vivo* chemotherapeutic activity shown against the experimental infection by *D. pneumoniae*. The protective efficacy of chloramphenicol injections repeated every 12 hr was distinctly reduced in the phenobarbital-pretreated rats, indicating that a smaller proportion of the administered dose was available to the body for combating the infection.

Both lines of evidence support the view that phenobarbital pretreatment enhances the rate of elimination of chloramphenicol from the rat. The urine studies provide additional evidence, showing a higher rate of urinary excretion of chloramphenicol in the induced than in the control animals. In the early 4-hr post-dose interval studied by us, the enhanced excretion took place clearly for the glucuroconjugated fraction only (which showed a more than 3-fold increase over the "non induced" values), while the fractions representing unconjugated chloramphenicol and arylamines were practically unaffected by phenobarbital pretreatment.

Our data are compatible with the hypothesis that treatment with phenobarbital induces the hepatic enzyme system catalysing the glucuronic acid conjugation of chloramphenicol, thereby facilitating the urinary excretion of the antibiotic. Indeed, increased formation of UDPGA is known to result from a 2-day phenobarbital treatment in the rat;[7] the drug, however, was shown to enhance glucuronide formation with some substrates but not with others, reflecting most probably the existence of several glucuronyl transferases with different substrate specificities and responses to phenobarbital.[15–17] Our findings are consistent with the hypothesis that chloramphenicol is a substrate of phenobarbital-enhanced glucuronidation in the rat, and that

enhancement of this metabolic mechanism is probably the only one responsible for accelerated elimination of the antibiotic in the induced rat.

The early rate of urinary excretion of arylamines after a dose of chloramphenicol was not affected in the induced rats. This is noteworthy in view of the fact that mammalian tissues contain a nitroreductase activity and that this activity toward chloramphenicol is known to be increased in the isolated liver microsomes of the phenobarbital-treated rat.[8] Our findings are consistent with the view[4] that tissue nitroreductases do not play a significant role in the metabolic transformation of chloramphenicol and that formation of arylamines results mainly from the activity of the intestinal flora on the biliary excretion products of the drug. However, since phenobarbital is known to increase bile flow in the rat,[18] one should also suppose that any such increase in our rats was compensated for by a lower concentration in bile, so that no greater proportion of chloramphenicol reached the intestinal flora as a result of the phenobarbital treatment. Preferential enhancement of chloramphenicol glucuronide excretion somehow may have successfully competed with the alternative route leading to arylamine formation and this may be a feature of the rat. Studies of species not responding to phenobarbital with increased urinary excretion of chloramphenicol glucuronide will show if enhanced arylamine excretion can take place in these conditions; some preliminary results of ours seem to indicate that this obtains in man (unpublished data).

The findings of our parallel studies with the *p*-methylsulfonyl analogue of chloramphenicol, thiamphenicol, are consistent with the several conclusions set out above. In contrast with chloramphenicol, this derivative is not significantly glucuroconjugated in the normal rat (nor in other species including man),[9] and our data indicate that this holds true in the phenobarbital-induced rat as well. In agreement with earlier findings,[9] in normal rats thiamphenicol compared to chloramphenicol exhibited a slower rate of disappearance from the blood as well as a higher rate of urinary excretion in chemically unmodified form. Both these parameters were unaffected by phenobarbital induction, as was the *in vivo* chemotherapeutic efficacy against the experimental infection by *D. pneumoniae*.

The significance of the present findings in rats for human therapy remains to be determined. However, some conjectures about possibly important clinical implications seem justified at this time, particularly since the mechanism of the rare but serious toxicity of chloramphenicol is completely obscure and it has been suggested[19] that the missing factor toward an elucidation could be the simultaneous administration of another drug. The pattern of chloramphenicol metabolism in man is characterized by prevailing glucuronidation, with very little arylamine formation.[4] In newborn and premature infants, deficient glucuronidation results in greater retention of chloramphenicol, and a characteristic toxicity, known as the "grey syndrome", has been associated with relative overdosage from this cause.[4] Knowing that chloramphenicol metabolism can be substantially altered by prior induction of drug metabolizing enzymes, and since the nature of the change may be expected to vary according to the prevailing metabolic pattern, a study to identify possible modifiers of chloramphenicol metabolism in man under different physiological conditions is obviously desirable. Aminopyrine, another widely used drug and known inducer, was shown by Patané *et al.*[20] to enhance the glucuronidation and the elimination of chloramphenicol in rabbits, and we were able to confirm this in rats (unpublished data). The risk of an

increased glucuronidation of chloramphenicol in man appears rather slight because this transformation normally prevails; moreover, any resultant loss of therapeutic efficacy would be probably overcome by adjusting the dose. Increased NO_2 reduction, should it occur (as is indicated by our preliminary results cited above), would probably be more relevant from a toxicological standpoint, as it is known that "reduced chloramphenicol" can act as haptene in eliciting chloramphenicol-specific antibodies;[21] moreover, the existence of nitrosamine metabolites is considered quite possible[6] and probably relevant to the occurrence of chloramphenicol-associated blood dyscrasias.[6]

It is known that a majority of patients with chloramphenicol-associated aplastic anemia received prior or concomitant treatments with other drugs. In epidemiological surveys,[22–25] these are usually classified according to their own hematopoietic toxicity as toxic or non-toxic. It is suggested that due consideration be given not only to the other drugs' independent toxicity, but also to their capacity of interacting with chloramphenicol metabolism. For example, phenobarbital is classified as innocuous to bone marrow, but its capacity of modifying chloramphenicol metabolism is high and some product of such altered metabolism might be responsible for the exceptionally high bone marrow toxicity experienced by these patients.

The greater stability of thiamphenicol to normal and stimulated drug metabolism is due to its physiological disposition not being mediated by transformation into glucuronides or into other known metabolic products. Thiamphenicol toxicity in newborn rats is considerably lower than that of chloramphenicol and much closer to the toxicity in adult animals;[26] moreover, no case of "grey syndrome" in infants to our knowledge has so far been reported from its clinical use. The erythropoietic depressing activity of thiamphenicol in men is greater than that of chloramphenicol[27] but the blood levels of the two compounds upon repeated administration were not studied to evaluate this difference in terms of therapeutic ratio; moreover, it has to be borne in mind that these studies did not cover the more important, irreversible aspects of bone marrow toxicity.[28] In this regard, no case of aplastic anemia associated with thiamphenicol has been reported after wide clinical use over several years. At least, this may be taken as evidence that increased erythropoietic depression is not paralleled by increased irreversible bone marrow toxicity, and it seems to be an additional argument in favour of the view[23, 29, 30] that two kinds of chloramphenicol toxicity should be separated: one is dose-related, reversible and predictable on the basis of the mode and duration of action of the active drug in the body ("pharmacological" toxicity); the other is delayed, relatively unrelated to dose, irreversible and unpredictable ("idiosyncratic" toxicity). Investigations in man along the lines suggested above will show how closely the number and nature of the transformation products, resulting from stimulated and not only from normal metabolism, may be related to the latter serious reactions.

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