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THE INFLUENCE OF DIETHYLENETRIAMINEPENTA-ACETATE ON THE SYNTHESIS OF DNA, RNA AND PROTEINS IN THE REGENERATING RAT LIVER

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Abstract—Administration of toxic doses of Ca-DTPA (diethylenetriaminepentaacetate) after partial hepatectomy inhibits the synthesis of DNA, RNA and proteins in the regenerating rat liver. Zn-DTPA proved to be ineffective. Impairment by Ca-DTPA of DNA synthesis can be completely restored by subsequent joint administration of Zn^{2+} and Mn^{2+}. The dependence of the inhibitory action of Ca-DTPA on dosage and on the time of its administration is consistent with the assumption that the inhibition of DNA synthesis is not the primary reaction but the consequence of an impaired synthesis of proteins which is tentatively ascribed to a disturbed conformation of RNA due to removal of Zn^{2+} and Mn^{2+}. Parallel studies on cell-free systems revealed a direct inhibitory action of Ca- and Zn-DTPA on the syntheses. The different activity pattern *in vitro* and *in vivo* is explained by the assumption that in the former case the inhibitory action is partly due to the formation of ternary complexes; a mechanism which is not operative *in vivo*, because the distribution of the chelates is confined to the extracellular space.

THE Na-salts and Ca-chelates of EDTA and diethylenetriaminepentaacetate (DTPA) impair the synthesis of DNA in tissue cultures of kidney cells[1,2] and of chicken embryos,[3] in phytohaemagglutinin-stimulated lymphocytes,[4,5] in the intestinal mucosa,[6–8] kidneys[8] and regenerating liver of the rat.[9] There is ample direct and indirect evidence[2,7,9] (for further ref. see 10) that this effect is due to an interference with the Zn^{2+}-requirement of DNA synthesis, although the chelation of other metal ions, such as Mn^{2+} and Fe^{2+}, may also be significant.[7] The fact that in all aforementioned studies a maximum effect was attained 12–15 hr after administration of the chelator suggests that the impairment of DNA synthesis is not the primary reaction, but the consequence of an influence on the reaction(s) preceding DNA synthesis.[7]

The elucidation of this mechanism may contribute to a better understanding of the toxic side-effects of DTPA, which is now widely used as metal antidote in medicine, and, thus, to offer a starting point for the optimization of chelate therapy.

The present study concerns the effect of DTPA on the synthesis of DNA, RNA and proteins in the regenerating liver of rats following partial hepatectomy. Because of the eventual goal of the investigation, DTPA was administered in toxic doses. In order to obtain supplementary information, experiments were performed with cell-free systems.

MATERIALS AND METHODS

Animal experiments. Female albino rats of the Heiligenberg strain with an average body weight of 145 g were used. They had free access to food (altromin-R-pellets) and tap water. Partial hepatectomy was performed according to Higgins and Anderson.[11]

A single dose of the chelates Na_3Ca-DTPA and Na_3 Zn-DTPA (4 and 8 m-mole $\times$ kg^{-1} in 1·5–2 ml H_2O, pH 7·3–7·6) was injected subcutaneously at different times after the operation. The control animals received physiological saline.

The radioactive precursors were injected intraperitoneally. To assay DNA synthesis, thymidine-(methyl-^3H) (0·15 μCi per g body weight, sp. act. 25 Ci $\times$ m-mole^{-1}) was used, in the case of RNA synthesis orotic acid-6-^{14}C hydrate (0·01 μCi per g body weight, sp. act. 50 mCi $\times$ m-mole^{-1}) and, for protein synthesis, leucine-1-^{14}C (0·01 μCi per g body weight, sp. act. 62 mCi $\times$ m-mole^{-1}).

The time at which chelates and precursors were injected, is shown in Table 1. The animals were killed (by exsanguination under ether anaesthesia) when the

TABLE 1. DESIGN OF ANIMAL EXPERIMENTS

Synthesis	Hours after partial hepatectomy: Injection of chelate	Injection of labelled precursor	Sacrifice
DNA	0*; 4; 8; 12; 15; 18; 21	25	26
RNA	0*; ½; 4; 8	11⅔	12
Proteins	0*; 4; 8; 12; 15	17⅚	18

* Immediately after the operation.

synthesis rates attain a maximum.[12–14] The liver was quickly removed, chilled, homogenized in H_2O and DNA, RNA and proteins were extracted by a modified Schmidt–Thannhauser procedure.[15] After a 20-min-pulse of orotate the specific activity of nuclear RNA is higher than that of cytoplasmatic RNA.[16] Extraction of whole cellular RNA, as in our experiments, leads consequently to a lower specific activity but does not invalidate the conclusions derived from the results. DNA was determined as described by Burton,[17] RNA by the orcinol reaction[18] and proteins by means of the Folin-reagent.[19] Radioactivity was assayed using Instagel$^{(R)}$ and liquid scintillation counting with an automatic correction for quenching. The results (dis/min per mg DNA, protein or RNA per g liver) were expressed as percentage of the corresponding control.

In vitro *experiments.* The RNA synthesis was tested using a suspension of nuclei of the regenerating liver (12 hr) as described by Moulé.[20] However, to avoid binding of metals, the Tris-buffer was replaced by HEPES-buffer.[21] The final system contained in 0·5 ml: 25 μmole HEPES [pH 7·9 (37°)], 1·6 μmole $MgCl_2$, 0·25 μmole each of ATP, GTP, CTP, UTP, 0·2 ml of the nuclei suspension (1·5–3 mg proteins) and, with the exception of the control, the chelate. UTP was labelled by 0·25 μCi uridine-4-^{14}C-triphosphate (sp. act. 43 mCi $\times$ m-mole^{-1}). The assay system was incubated at 37°. The reaction was stopped after 20 min by adding 1 ml of ice-cold 10% trichloracetic acid and 2 mg bovine serum albumin as carrier. One incubation was stopped

immediately after adding labelled UTP to determine the background. After standing for 15 min at 0°, the precipitate was washed according to Canellakis,[22] dissolved in 0·5 N NaOH and adjusted with HCl to pH 7·0. The radioactivity was counted after adding 10 ml Instagel.®

DNA synthesis was assayed using the 105,000 *g* supernatant of an extract of a 24 hr-regenerating rat liver.[23] Tris-buffer was replaced by HEPES-buffer. The assay system contained in 0·5 ml: 25 μmole HEPES [pH 7·5 (37°)], 10 μmole KCl, 15 μmole $MgCl_2$, 0·05 μmole each of dATP, dGTP, dCTP and TTP (labelled by 0·5 μCi thymidine-(methyl-^{3}H)-$^{5'}$triphosphate, sp. act. 3·4 Ci × m-mole^{-1}), 50 μg of rat liver DNA (prepared according to Kay *et al.*[24]), 0·15 ml of the 105,000 *g* supernatant (3–5 mg of protein) and, with the exception of the control, the chelate. The incubation (37°) was stopped after 1 hr by addition of 1 ml ice-cold trichloracetic acid and 100 μg carrier DNA. The background was determined by stopping one incubation immediately after addition of the radioactivity. The pellet obtained after centrifugation was washed with 5% trichloracetic acid and dissolved in 0·5 N NaOH, hydrolysed for 10 min at 80° and reprecipitated with ice-cold trichloracetic acid. The DNA was dissolved after washing in 0·5 N NaOH and the radioactivity measured as for the RNA synthesis.

Protein synthesis was assayed, as described by Richardson *et al.*,[25] using an 18 hr regenerating liver extract. In order to remove Zn from Sephadex used in this experiment, it was washed with 0·3% Na_4EDTA.[26]

To avoid metal contamination of the assays, in all experiments the glassware was washed with Na_4EDTA and subsequently thrice with deionized quartz-bidistilled water.

RESULTS

Animal experiments. As can be seen from Table 2, the inhibition of DNA synthesis by the lower dose of Ca-DTPA had two distinct peaks. A maximum effect was attained if the chelate was administered either immediately or 12–15 hr after the partial hepatectomy. All animals injected with 8 m-mole × kg^{-1} died after 12–15 hr; therefore, the effect upon DNA synthesis could be tested only up to 12 hr after the operation. In intact animals, lethality was lower and did not occur until 48 hr.[7] The

TABLE 2. EFFECT OF DTPA ON DNA SYNTHESIS IN THE REGENERATING RAT LIVER (PER CENT OF CONTROL). ARITHMETIC MEANS ± S.E. THE NUMBER OF ANIMALS IS INDICATED IN BRACKETS. THE CONTROL GROUP COMPRISED 17 RATS, AND THE SPECIFIC ACTIVITY OF THE CONTROL WAS 184,769 ± 9653 dis/min per mg DNA/g liver

Time of injection (hr after part. hep.)	Ca-DTPA		Zn-DTPA
	4 m-mole × kg^{-1}	8 m-mole × kg^{-1}	8 m-mole × kg^{-1}
0	29·6 ± 7·6 (11)	Exitus	114·7 ± 15·2 (4)
4	57·1 ± 5·6 (6)	Exitus	
8	34·7 ± 7·2 (6)	Exitus	
12	25·4 ± 5·1 (6)	9·1 ± 3·8 (3)	89·1 ± 19·1 (7)
15	25·2 ± 3·8 (4)	12·2 ± 2·9 (7)	
18	33·4 ± 5·9 (6)	16·5 ± 1·5 (6)	
21	57·6 ± 4·8 (5)	29·8 ± 3·0 (5)	

inhibition of the synthesis was markedly higher than with the lower dose and again showed a maximum when the chelate was given after 12 hr.

Zn-DTPA proved to be ineffective. In order to test whether the impaired DNA synthesis could be restored by subsequent administration of Mn^{2+} and/or Zn^{2+}, the following experiment was performed: Ca-DTPA (4 m-mole $\times$ kg^{-1}) was injected immediately after the partial hepatectomy, the metal acetates 4 hr later. The bulk of DTPA is excreted within 4 hr.[27] The dose (19 μmole Zn $\times$ kg^{-1}, and 2 μmole Mn $\times$ kg^{-1}) corresponds to the amounts which are removed by 4 m-mole $\times$ kg^{-1} Ca-DTPA from the body.[28,29] As can be seen from Table 3, neither Zn^{2+} nor Mn^{2+} completely reversed the inhibitory effect of Ca-DTPA. Joint administration of both metal ions, however, was followed by a normal synthesis rate.

TABLE 3. EFFECT OF Ca-DTPA (4 m-mole $\times$ kg^{-1}) ON DNA SYNTHESIS IN THE REGENERATING RAT LIVER AS INFLUENCED BY THE SUBSEQUENT ADMINISTRATION OF Mn-ACETATE (2 μmole $\times$ kg^{-1}) AND/OR Zn-ACETATE (19 μmole $\times$ kg^{-1}). THE CHELATE WAS INJECTED IMMEDIATELY AFTER THE PARTIAL HEPATECTOMY, THE METAL SALTS AFTER 4 hr. ARITHMETIC MEANS $\pm$ S.E. THE NUMBER OF ANIMALS IS INDICATED IN BRACKETS. THE CONTROL GROUP COMPRISED 17 RATS

Zn^{2+}	Mn^{2+}	Per cent of control
−	−	29·6 ± 7·6 (11)
+	−	68·1 ± 7·1 (10)
−	+	74·7 ± 11·7 (6)
+	+	116·6 ± 27·4 (4)

The influence of Ca-DTPA on protein synthesis (Table 4) was less pronounced than on DNA. In other respects, however, the same efficacy pattern was obtained, i.e. a maximum inhibition, when the chelate was administered immediately or 12–15 hr after the partial hepatectomy, as well as a clear-cut dose dependence. Zn-DTPA is ineffective.

TABLE 4. EFFECT OF DTPA ON PROTEIN SYNTHESIS IN THE REGENERATING RAT LIVER (PER CENT OF CONTROL). ARITHMETIC MEANS $\pm$ S.E. THE NUMBER OF ANIMALS IS INDICATED IN BRACKETS. THE CONTROL GROUP COMPRISED 17 RATS, AND THE SPECIFIC ACTIVITY OF THE CONTROL WAS 302 $\pm$ 21 dis/min per mg PROTEIN/g LIVER

Time of injection (hr after part. hep.)	Ca-DTPA		Zn-DTPA
	4 m-mole $\times$ kg^{-1}	8 m-mole $\times$ kg^{-1}	8 m-mole $\times$ kg^{-1}
0	86·2 ± 9·0 (9)	49·7 ± 7·0 (3)	83·1 ± 7·7 (5)
4	93·1 ± 21·7 (5)	53·4 ± 6·9 (3)	
8	106·6 ± 20·6 (5)	57·7 ± 16·9 (6)	
12	112·4 ± 13·5 (5)	31·4 ± 2·4 (7)	
15	66·7 ± 8·8 (7)	39·6 ± 6·2 (6)	81·6 ± 11·4 (8)

The inhibition of RNA synthesis by Ca-DTPA, administered either immediately or after 8 hr, was relatively small and there was no statistically significant difference in the action of either dose used (Table 5). Once more, no effect was observed with Zn-DTPA.

Histological investigations did not reveal any pathological alterations of the liver after administration of Ca-DTPA.[30]

TABLE 5. EFFECT OF DTPA ON RNA SYNTHESIS IN THE REGENERATING RAT LIVER (PER CENT OF CONTROL). ARITHMETIC MEANS ± S.E. THE NUMBER OF ANIMALS IS INDICATED IN BRACKETS. THE CONTROL GROUP COMPRISED 8 RATS, AND THE SPECIFIC ACTIVITY OF THE CONTROL WAS 13,405 ± 528 dis/min per mg RNA/g LIVER

Time of injection (hr after part. hep.)	Ca-DTPA		Zn-DTPA
	4 m-mole × kg^{-1}	8 m-mole × kg^{-1}	8 m-mole × kg^{-1}
0	74·7 ± 8·3 (5)	71·3 ± 8·3 (5)	90·2 ± 9·8 (6)
½	107·7 ± 6·5 (6)		
4	88·4 ± 12·6 (3)	102·4 ± 5·2 (3)	
8	81·0 ± 6·0 (4)	68·7 ± 10·6 (5)	

In vitro *experiments*. The results are presented in Figs. 1–3; polynomial regression analysis was used to obtain the concentration-effect curves. The most striking difference between the *in vivo*- and *in vitro*-studies is that in the latter case Zn-DTPA leads to an impaired synthesis of DNA, RNA and proteins. The dependence of the RNA-synthesis rate on the concentration of Ca-DTPA is noteworthy; a maximum is obtained with a concentration of 20 mM.

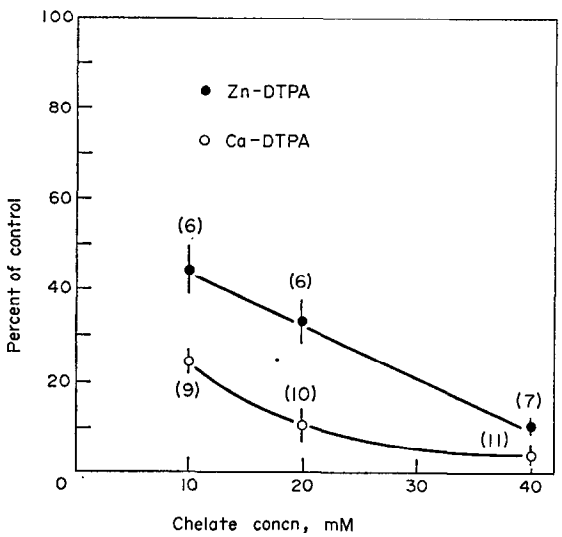

FIG. 1. Effect of DTPA chelates on the DNA synthesis *in vitro*. Arithmetic means ±S.E. The number of experiments is indicated in brackets. The control comprises 23 experiments.

DISCUSSION

Zn-DTPA is not able to chelate any relevant metal ions, such as Mn^{2+} or Fe^{2+}, because of its higher stability constant.[10] As the stability of the bimetallic species Zn_2-DTPA is rather low,[31] mobilization of Zn^{2+} can be neglected. Consequently, the inhibitory action of Zn-DTPA, as revealed in cell-free systems, cannot be ascribed to the mobilization of essential metals. Zn^{2+} has a coordination number of 4 whereas

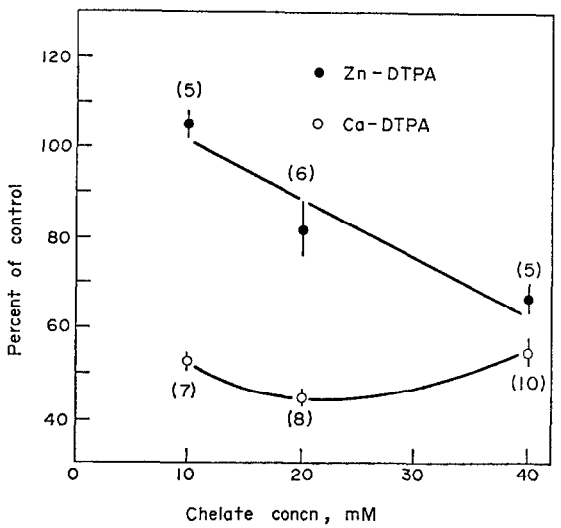

FIG. 2 Effect of DTPA chelates on the RNA synthesis *in vitro*. Arithmetic means ±S.E. The number of experiments is indicated in brackets. The control comprises 15 experiments.

DTPA is an octadentate ligand. Taking into account also the pronounced affinity of most constituents of the assay systems toward metal ions[32,33] we may assume in accordance with other experimental evidence[31] that Zn-DTPA is subject to the formation of simple or bimetallic ternary complexes which interfere with the syntheses. Formation of ternary complexes may concern the template DNA and RNA-polymerase in the case of the RNA synthesis, and DNA and DNA-polymerase in the

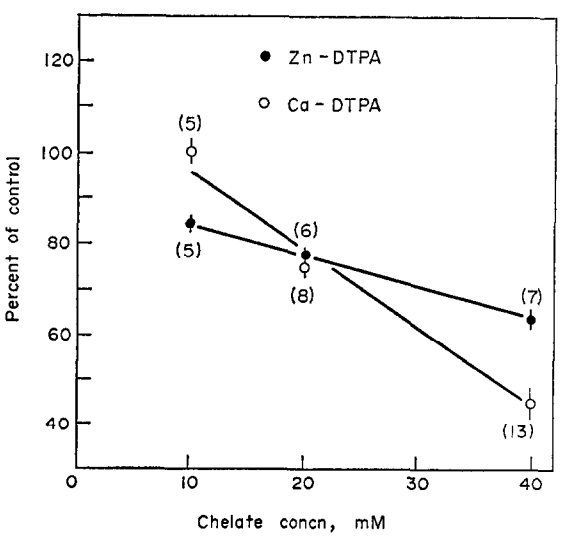

FIG. 3. Effect of DTPA chelates on the proteins synthesis *in vitro*. Arithmetic means ±S.E. The number of experiments is indicated in brackets. The control comprises 16 experiments.

case of the DNA synthesis.[30] The inhibition of protein synthesis could be due to a binding of Zn-DTPA by mRNA and/or tRNA.[30]

As can be seen from Figs. 1–3, the slope or shape of the concentration-effect curves for Zn-DTPA distinctly differ from those for Ca-DTPA. This may be taken as evidence that, in the latter case, a basically different mechanism is operative, i.e. that inhibition of synthesis has mainly to be ascribed to a genuine removal of metals from the system.

The inhibition by Ca-DTPA of the DNA synthesis is easily explained by the removal of Zn^{2+} from DNA and/or DNA-polymerase.[30]

The activity of the RNA-polymerase depends on Mg^{2+} and Mn^{2+}. As Mg-DTPA is less stable than Ca-DTPA, its inhibition can be ascribed to the removal of Mn^{2+}. Somewhat unexpected is the shape of the concentration-effect curve, i.e. a maximum effect with a chelate concentration of 20 mM (Fig. 2). The assay system contains ribonuclease and it has been demonstrated that its activity displays a maximum in a limited range of metal concentration (Mn^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}).[34] Thus, the actual inhibition of RNA-synthesis could be due to the interaction of Ca-DTPA with RNA-polymerase and ribonuclease.[30]

No metalloenzymes or metal activated enzymes (with the exception of Mg^{2+}) participate in protein synthesis. On the other hand, the conformation of RNA strongly depends on various metals, in particular on Zn^{2+}, Mn^{2+} and Fe^{2+}.[31] Whereas Fe^{2+} is tightly bound, Zn^{2+} and Mn^{2+} are bound rather loosely and should be easily removed by Ca-DTPA.[32,33] Due to the disturbed conformation, the impaired function of RNA as template or amino acid carrier should bring about an inhibition of protein synthesis. Bearing in mind that the extracellular water is the dilution space of the DTPA chelates,[35] the failure of Zn-DTPA to affect the synthesis *in vivo* (Tables 2, 4, 5) is by no means unexpected, as the main prerequisite for its inhibitory action *in vitro*, i.e. the direct interaction with intracellular constituents, is not fulfilled.

DNA synthesis in the regenerating liver and the appearance of relevant enzymes, e.g. DNA-polymerase and thymidine-kinase, do not start until 15–18 hr after the partial hepatectomy.[12,13] In view of the fact that Ca-DTPA shows maximum effectiveness if administered immediately or 12–15 hr after the operation (Table 2), a direct interference of the chelate with the DNA assembly is rather unlikely otherwise, one should expect that DTPA, administered after 12–15 hr, would bring about a delay of approximately 4 hr in the onset of the synthesis.[27] According to the time course, as determined by Rabes and Brändle,[14] this would reduce the synthesis rate by about 20 per cent, which is at variance with the experimental value (75 per cent). Other findings, quoted in the introduction, are in agreement with the conjecture that inhibition of DNA synthesis is a secondary reaction.

It does not appear that the inhibition of RNA synthesis is responsible for the impaired synthesis of DNA as the dose dependence of the RNA inhibition is only marginal while the dose dependence of DNA synthesis is very pronounced. Another argument is given by experiments with actinomycin D. Also actinomycin D, a specific blocker of RNA synthesis, affects DNA synthesis only if it is administered within a few hours after the partial hepatectomy or, in other systems, after the appropriate stimulus.[2,36,37] Our experiments, however, show that DTPA still inhibits DNA-synthesis at a time where RNA synthesis is decreased.[14]

Protein synthesis, however, shows a distinct dependence on DTPA dosage and

the inhibition by DTPA of DNA synthesis is correlated rather closely with the time course of protein synthesis, maximum impairment being attained when the administration of the chelate coincides with an increased rate of synthesis.[14,36] Although the effect on DNA synthesis is more pronounced than on protein synthesis, this fact is not at variance with the assumption that the inhibition of the latter is the key-reaction. The level of the protein turnover in the liver is very high and inhibition of the additional protein synthesis, induced by partial hepatectomy, will, have a profound effect on the synthesis of DNA. We have shown that the effect of Ca-DTPA can be completely reversed only by joint administration of Zn^{2+} and Mn^{2+}. The explanation given for the influence of Ca-DTPA on the protein synthesis *in vitro*, i.e. the removal of both metals from RNA, holds, consequently, also for the effect observed *in vivo*.

The conclusion that inhibition of protein synthesis is the primary reaction leading to impaired DNA synthesis, needs to be substantiated by measurement of the activity of specific enzymes, such as DNA polymerase, ribonucleotide reductase or kinases involved in thymidylate synthesis, as influenced by Ca-DTPA *in vivo*.

However, studies[38–42] with various inhibitors of protein synthesis (and, as a secondary reaction, of DNA synthesis) failed to reveal a correlation between the activity of specific enzymes and the total protein synthesis rate.

The *in vivo*-inhibition of RNA synthesis may be due to impaired protein synthesis as well as to direct interference due to the diminution of the intracellular concentration of Zn^{2+} and Mn^{2+}.

Different explanations can be proposed for the differences in DNA synthesis *in vivo* and *in vitro* (Fig. 1). Firstly, the concentrations of DTPA in the cell-free systems are markedly higher than in the animal. Secondly, the removal of metals, which was assumed to be the responsible reaction, may not necessarily proceed through a dissociation mechanism, i.e. via the *free* metal ion; the formation of intermediary ternary complexes followed by displacement of the ligands may be an alternative mechanism and this mechanism cannot become operative *in vivo*.

The conclusions derived from our results have some implications which are significant from the theoretical as well as practical point of view: Firstly, they stress the importance of the protein synthesis as a key-reaction for the initiation and maintenance of the DNA synthesis. Secondly, we were able to susbtantiate that Zn-DTPA has no toxic effects *in vivo* and, thus, has advantages for therapeutic purposes as has been already suggested.[10]

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