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CHANGES IN COLLAGEN METABOLISM ASSOCIATED WITH THE ADMINISTRATION OF PENICILLAMINE AND VARIOUS AMINO AND THIOL COMPOUNDS*

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Abstract—D-Penicillamine and BAPN (β-amino propionitrile) were found to be the most active agents able to cause an accumulation of neutral salt-soluble collagen in the skin of rats. Most analogues of penicillamine had very little or no effects. Blocking the α-amino group (*N*-acetyl-penicillamine) or removal of the sulfhydryl group (valine) render inactive compounds. A higher homologue (mercaptoisoleucine) was ineffective. It would seem that the structure of D-penicillamine meets all the criteria for effectiveness, such as extracellular distribution, lack of metabolic deamination, sufficient amount of steric hindrance of the sulfhydryl group to retard its oxidation to disulfide, but yet not enough to prevent completely its reactivity. D-Penicillamine totally blocks the cross-linking of newly formed tropocollagen and is able to degrade a certain fraction of the more recently sythesized process insoluble collagen which has not yet completed its maturation D-Penicillamine affects equally both sexes, is more effective in young rats than in older ones and is not antagonized by copper if this metal is administered by a different route. If given simultaneously, mixed in the diet, the effect on collagen is inhibited and the toxicity of copper reduced, probably due to the marked affinity of both compounds and possible inactivation in the gastrointestinal tract. The urinary excretion of hydroxyproline in the D-penicillamine-treated rats falls within normal limits. Only the BAPN-treated animals showed an increase in urinary hydroxyproline. Since BAPN causes severe osseous abnormalities, whereas penicillamine does not, these findings would tend to support the view that an increase in urinary hydroxyproline is primarily a manifestation of abnormal metabolism of bone collagen.

COLLAGEN is a fibrous protein which can be regarded as the primary structural material of all vertebrates and of many invertebrates.[1] The recently assembled macromolecular aggregates of tropocollagen are readily dispersed by dilute solutions of neutral salts but, with time, fibers are formed which become very insoluble. These marked changes which are accompanied by increased mechanical[2] and thermal stability,[3] are attributed to the formation of new covalent bonds which stabilize the closely packed structure.

The lathyrogens comprise a group of natural and synthetic compounds capable of producing physiological changes comparable to those induced by the seeds of the flowering sweet pea (*Lathyrus odoratus*). These involve marked skeletal changes, consisting of thickening and deformation of the long bones, spinal curvature, disruption

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of the epiphyseal plate and many other connective tissue abnormalities described by Dasler.[4] Because of the prominence of skeletal abnormalities, these compounds have been called osteolathyrogens. The involvement of collagen in the pathogenesis of lathyrism was demonstrated by Levene and Gross,[5] who found a marked increase of soluble collagen in tissues of lathyritic chick embryos receiving β-amino-propionitrile (BAPN), a powerful osteolathyrogen. The underlying mechanism seems to be related to a blockage in the processes of intermolecular and intramolecular cross-linking.[6]

In the process of investigating the role of chelating agents and trace elements in different aspects of collagen metabolism, it was observed that penicillamine (β,β-dimethylcysteine), when administered to rats, was able to cause an accumulation of soluble collagen in their skin, a change which was accompanied by a marked reduction of its tensile strength and a delay in wound healing.[7] These changes, which have subsequently been confirmed in humans receiving penicillamine,[8] have been further investigated and these results are reported in the present communication.

MATERIALS AND METHODS

Holtzman rats of various ages were used in these experiments. D-Penicillamine (Aldrich) and the various other compounds tested were administered, either mixed in their diets or by injection, as indicated in each particular case. When the drugs were fed with the diets, the food consumption of each animal was recorded daily and the dose received calculated. The dose reported represents an average for the experimental periods. Because penicillamine, particularly the L-form, will induce a pyridoxine deficiency, particularly if this vitamin is present in marginal amounts in the diet,[9] all animals were given a supplement of 2 mg vitamin B_6 per day.

Collagen fractionation. Dorsal skin was cleaned of adhering tissue and aliquots were weighed and frozen until the time of analysis. After mincing with scissors, they were homogenized for 20 min with a VirTis model 25 homogenizer at half the full setting with 20 vol. of 0·15 M NaCl. They were shaken overnight and centrifuged at 40,000 *g* for 1 hr. The residue was re-extracted successively for 24 hr with 0·5 M NaCl and 0·5 M citrate, pH 3·6. The supernatant after each centrifugation was dialyzed overnight against water. All these operations were performed at 4°. The final residue, containing the insoluble collagen, was solubilized with 0·3 M trichloracetic acid at 90°[10] and dialyzed to remove the latter.

Aliquots after acid hydrolysis (5 N HCl; 2 hr at 25 psi) were analyzed for hydroxyproline by an automated version of the Stegemann procedure.[11] For expression as collagen, the hydroxyproline values were multiplied by 7·46.

To determine the total amounts of collagen present in the skin mass of animals, the total skin mass of the animal was removed at the time of sacrifice, cleaned from adhering tissue and weighed. A representative aliquot was fractionated according to solubility, as previously described.

Urinary hydroxyproline. Control and D-penicillamine-treated rats were placed in metabolic cages. Urine was collected daily, filtered and frozen. Hydroxyproline was measured by the method of Prockop and Udenfriend.[12]

RESULTS

The capacity of various compounds to affect the solubility of dermal collagen was evaluated in young growing rats (Table 1). Of all the compounds tested, D-penicillamine and BAPN fumarate were the most effective. Cysteamine and oxalyl dihydrazide

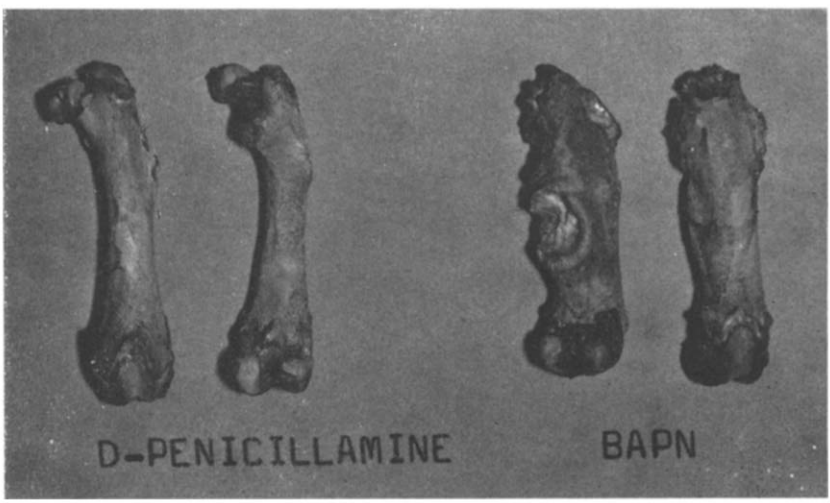

FIG. 1. Flexor and abductor aspects of femurs from D-penicillamine-and BAPN-treated rats.

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showed some effects, in agreement with the observations of Dasler[4] and those of Levene,[13] who reported that many hydrazides exhibited lathyrogenic activity. The other compounds did not significantly affect collagen metabolism. In addition to the compounds listed in Table 1, some of which were quite toxic at the dose indicated,

TABLE 1. COLLAGEN FRACTIONS EXTRACTED FROM THE DORSAL SKIN OF YOUNG RATS TREATED WITH VARIOUS LATHYROGENS, D-PENICILLAMINE AND RELATED ANALOGUES*

Group	Dose (mg/100 g body wt.)	Body wt. gain (g/d†)	Collagen fractions (mg/100 mg wet skin) 0·15 M NaCl	0·5 M NaCl	0·5 M Citrate (pH 3·6)	Insoluble
Control		6·3	0·62 ± 0·07	2·15 ± 0·15	1·00 ± 0·08	10·90 ± 1·2
Dithioglycolic acid	65	2·8	1·09 ± 0·11	4·77 ± 0·32	1·36 ± 0·12	9·70 ± 0·8
Cysteamine	100	3·0	0·92 ± 0·07	3·84 ± 0·30	0·95 ± 0·09	8·90 ± 0·9
N-acetyl-L-cysteine	150	5·8	0·92 ± 0·08	2·54 ± 0·18	1·05 ± 0·11	10·14 ± 0·9
Dimercaptopropanol (BAL)	5‡	2·0	1·10 ± 0·09	3·26 ± 0·22	1·00 ± 0·11	13·00 ± 2·1
D-Penicillamine	40	6·1	1·50 ± 0·10	8·60 ± 0·35	1·50 ± 0·20	1·90 ± 0·28
DL-Mercaptoisoleucine	50	6·3	0·70 ± 0·06	2·20 ± 0·15	1·10 ± 0·08	11·00 ± 0·70
N-acetyl-DL-penicillamine	50	6·4	0·65 ± 0·04	2·10 ± 0·14	1·20 ± 0·07	11·20 ± 1·4
Procainamide	65	5·6	0·66 ± 0·08	2·19 ± 0·14	1·00 ± 0·10	10·20 ± 1·3
Oxyalyl dihydrazide	55	4·0	0·98 ± 0·07	4·02 ± 0·35	1·30 ± 0·13	7·20 ± 2·2
ε-Amino-caproic acid	200	5·0	0·72 ± 0·06	2·49 ± 0·25	0·80 ± 0·06	9·20 ± 1·3
BAPN fumarate	40	3·0	1·40 ± 0·03	6·30 ± 0·31	1·30 ± 0·15	4·20 ± 0·9
DL-Homocysteine thiolactone	70	6·4	0·51 ± 0·05	1·74 ± 0·15	1·03 ± 0·12	10·80 ± 1·03
AET (2-amino-ethyl isothiouromium bromide hydrobromide)	70	3·5	0·49 ± 0·11	1·95 ± 0·28	0·98 ± 0·07	10·90 ± 1·25

* The results are expressed as mean ± S.E.
† Average change in body weight over the experimental period (14 days); initial weight 70–80 g.
‡ Injected subcutaneously; all other compounds given orally.

other compounds tested and found to be ineffective were: apresoline HCl, sulfanilamide, *trans*-1,2-diaminocyclohexane sulfate, *p*-aminosalicylate and 1,1,3-tricarbonitrile. The animals receiving BAPN began to show the typical signs of osteolathyrism, reflected by scolioses, kyphoses, sternal curvatures and thoracic cage deformities. Figure 1 shows the opposite aspects of femurs of D-penicillamine- and BAPN-treated rats. The D-penicillamine-treated animals did not differ from normal controls, whereas the BAPN rats showed the characteristic thickening, exostoses and changes in the femoral head. The ends of the femurs are very readily detached from the shaft at the epiphyseal lines.

The changes in collagen solubility seen in 3-month-old rats who were treated with D-penicillamine for periods of 1–4 weeks are shown in Fig. 2. Insoluble collagen drops during the first 2 weeks of treatment and levels off thereafter. The neutral salt-soluble collagen (combined material extracted by neutral 0·15 and 0·5 M NaCl) accumulates in increasing amounts throughout the experimental period.

Our previous experience using D-penicillamine seemed to indicate that dose and age of the animals were important parameters in evaluating the response to this compound. Figure 3 summarizes the responses obtained at different dose levels when young rats (70–80 g at the beginning of the experiment) of either sex were given D-penicillamine orally. The response can be examined adequately by establishing

a ratio between soluble and insoluble collagen. The ratio calculated is plotted on a logarithmic scale on the ordinate and the dose on the abscissa. The maximum response was recorded at a level of 400 mg/kg of body weight. When similar studies were performed on older animals, which are growing at a slower rate (210–230 g rats), the overall pattern observed is similar but the absolute changes are less pronounced

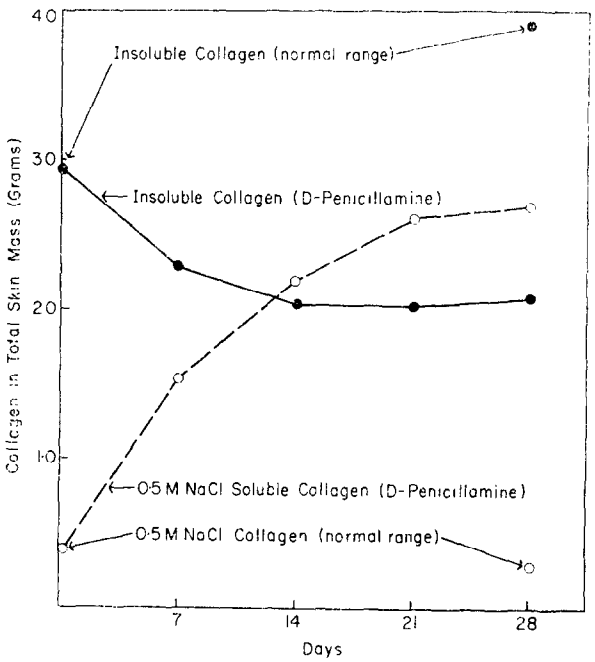


FIG. 2. Changes in the pattern of collagen solubility during treatment with D-penicillamine. A total of 24 rats, weighing between 220 and 245 g at the beginning of the experiment, were given D-penicillamine mixed with their diet (average dose 41 mg/day/100 g of body wt.). Groups of 6 animals were sacrificed at weekly intervals, together with a similar number of controls. The values represented by the 0·5 M NaCl-soluble collagen represent the combined material extracted by 0·15 M and 0·5 M NaCl. The insoluble collagen represents the residue left after extracting with 0·5 M citrate buffer, pH 3·6. The value corresponding to the collagen solubillized by the citrate buffer was not included, since it only accounted for a small fraction and was not significantly affected by treatment. The values on the ordinate represent the grams of collagen present in the total skin mass of the animals.

(Fig. 4). Because penicillamine is a very effective chelating agent and copper and other trace elements have been attributed various roles in connection with the metabolism of various connective tissue components, in particular collagen, the possible involvement of this cation was investigated. The results are summarized in Table 2. D-Penicillamine is as effective when administered orally as when given parenterally. When copper supplement was mixed with the same diet which contained penicillamine, it counteracted the latter's capacity to affect collagen metabolism, but if these two compounds were administered by different routes, e.g. copper in the diet and penicillamine by injection, they did not antagonize each other. On the other hand, dietary copper alone does not affect collagen metabolism. The parenteral route for copper could not be adequately investigated due to its high toxicity.

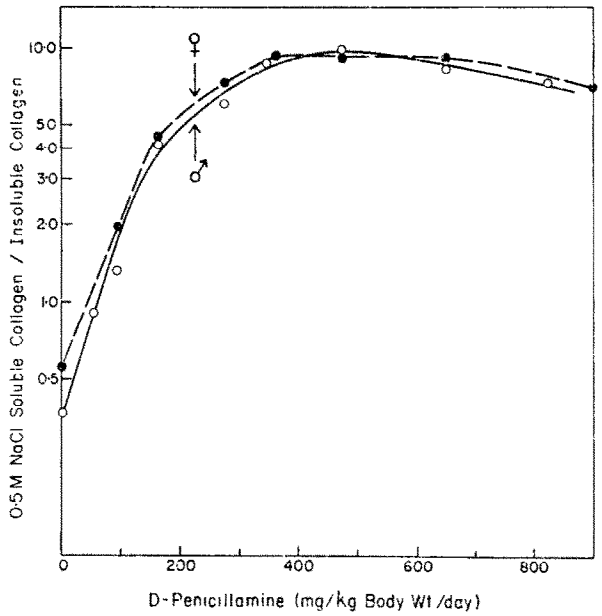


FIG. 3. Effect of dose on the changes in skin collagen solubility caused by D-penicillamine. The values are plotted as a ratio between the collagen extractable by neutral salt solutions to that which remains insoluble in citrate buffer. Young rats, males and females, initially weighing between 70 and 80 g, were used and the D-penicillamine was given orally over a 2-week period (four rats per experimental point).

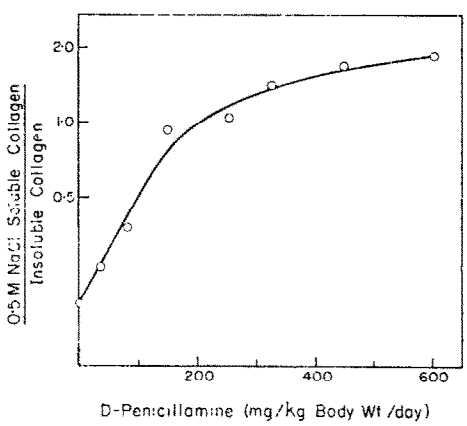


FIG. 4. Effect of dose on the changes in skin collagen solubility caused by D-penicillamine. The values obtained are plotted as a ratio between the collagen extractable by neutral salt solutions and that which remains insoluble in citrate buffer. Older rats than those used in Fig. 2 were used in this experiment (initial weight, 210–230 g).

The urinary excretion of hydroxyproline, determined for control rats and animals treated with BAPN or D-penicillamine, is summarized in Table 3. The average excretion for the D-penicillamine-treated and control animals did not differ significantly, whereas the BAPN animals showed a marked increase in hydroxyproline output.

TABLE 2. ROLE OF COPPER ON MODIFYING THE CHANGES INDUCED BY D-PENICILLAMINE FOLLOWING VARIOUS ROUTES OF ADMINISTRATION

Group*	Dietary supplement of $CuSO_4$ (mg/day)	D-Pencillamine (mg/day)		Collagen fractions (mg/100 mg skin)			
		Injected†	Oral	0·15 M NaCl	0·5 M NaCl	0·5 M Citrate	Insoluble
Control	—	—	—	0·51±0·06	1·80±0·17	0·62±0·03	10·11±0·92
D-Penicillamine	—	50	—	0·76±0·07	8·66±0·42	0·68±0·10	2·29±0·40
D-Penicillamine	—	—	50	1·10±0·07	8·80±0·44	1·00±0·06	2·00±0·32
Penicillamine + Cu	83	50	—	0·79±0·05	8·32±0·49	0·71±0·03	2·86±0·42
Penicillamine + Cu	160	—	95	0·60±0·03	2·02±0·15	0·75±0·04	10·90±1·04
Copper	77	—	—	0·49±0·05	1·82±0·13	0·64±0·08	11·48±1·09

* Seven rats per group (initial weight, 70–80 g); experimental period, 2 weeks.
† Subcutaneously in 2 ml of 0·15 M NaCl.

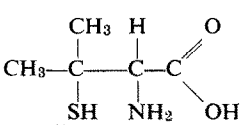
TABLE 3. HYDROXYPROLINE LEVELS IN RAT URINE

Group*	Urinary hydroxyproline‡ (mg/rat/24 hr)
Controls	0·77 ± 0·05‡
D-Penicillamine	0·82 ± 0·04
BAPN	1·47 ± 0·12§

* 10 rats per group. The dose, changes in body weight and skin collagen values are described in Table 1.
† These values represent the mean of the average hydroxyproline excretion for each animal monitored between days 7 and 14 of treatment.
‡ Standard error.
§ $P<0·001$.

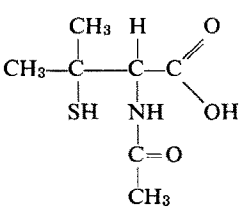
DISCUSSION

Of all the compounds tested in our laboratory, D-penicillamine is the most active in causing an accumulation of neutral salt-soluble collagen in the soft tissues of the rat. Most analogues of penicillamine had no effect. Blocking the α-amino group

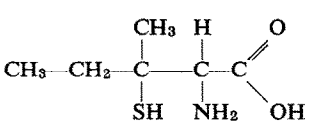


Penicillamine

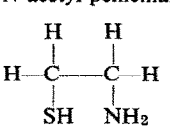
(β,β-dimethylcysteine)



N-acetyl-penicillamine



Mercaptoisoleucine
β-methyl, β-ethyl-cysteine)



Cysteamine
(mercaptoethylamine)

FIG. 5. Chemical structure of D-penicillamine and related compounds.

(*N*-acetyl-DL-penicillamine) or elimination of the sulfhydryl group, which gives rise to the amino acid valine, render inactive compounds. The methyl, ethyl derivative of cysteine (β-mercaptoisoleucine) was also completely inactive (Fig. 5). It seems that the mechanism by which penicillamine exhibits its effect requires a great deal of stereospecificity. In addition, it should meet the requirements of predominant extracellular distribution,[14] should not be desulfhydrated by cysteine desulfhydrase,[15] and should not be readily deaminated by amino acid oxidases[15] or susceptible to oxidation to the disulfide. When D- or L-penicillamine were incubated at 37° in a Krebs–Ringer bicarbonate medium in the presence of ascites tumor cells, 95 per cent of the penicillamine was still present in the sulfhydryl form after 1 hr.[14]

It has been previously demonstrated that penicillamine blocks almost quantitatively the conversion of soluble tropocollagen into insoluble collagen.[16] There seems to be no change in the rate of collagen synthesis and therefore all the newly synthesized material accumulates in a soluble form. In addition, a fraction of the pre-existing insoluble collagen may be degraded by penicillamine. The size of this degradable pool is inversely proportional to the age of the animal. When penicillamine is given to young animals, it exhibits its maximum effect because the rate of collagen synthesis and turnover is at its maximum and because the pool size of the thiol-degradable material is extremely large.

By using the total skin mass of animals as an assay for the absolute changes caused by penicillamine, it can be seen that rats weighing about 230 g at the beginning of the experiment (their growth rate now slowing down) show a decline in the amount of total insoluble collagen during the first 2 weeks of treatment. After that, continuing administration of D-penicillamine failed to affect the remaining insoluble collagen, but because the synthetic process continues at a normal rate, the soluble form of collagen keeps accumulating.

We have previously demonstrated that penicillamine, as well as analogues such as mercaptoethylamine, is able to solubilize *in vitro*, at neutral pH and at 4°, significant amounts of collagen which remained insoluble in citrate buffer, pH 3·6.[17, 18] This thiol-soluble collagen shows an age-dependent relationship, since the insoluble collagen originating from older rats is much more resistant to solubilization by these agents, whereas that obtained from young animals can be almost completely solubilized under these conditions.

This form of readily dispersed collagen may be either part of a rapidly turning-over pool or may correspond to the more recently synthesized material which has not been allowed to mature by means of the process of "normal" cross-linking. It is quite likely that both of these factors may be contributing to the existence of a form of collagen, insoluble in 0·5 M citrate, pH 3·6, but yet not completely cross-linked and readily dispersable to its monomeric components by various specific reagents.

Because of the many variables involved in a experiment *in vivo*, such as the rates of turnover and synthesis during different stages of the rats' development, it is difficult to estimate the time required for complete insolubilization of collagen.

Based on the extent to which the pre-existing insoluble collagen was degraded (about 33 per cent decline in 2 weeks), it would seem that this fraction of collagen, probably synthesized several weeks prior to the administration of penicillamine, had not been allowed enough time to crosslink adequately. In any case, it looks as if the formation of stable crosslinks may require several weeks to transpire.

Further studies involving the comparison of such systems *in vivo* with ageing *in vitro* of purified collagen gels, as well as the evaluation of compounds which accelerate or retard this process, may provide a clue to the mechanism underlying these events.

Chelation of copper does not seem to be of primary importance in connection with the mechanism of action of penicillamine. When administered by different routes (copper in the diet and D-penicillamine parenterally), copper was not able to prevent the penicillamine effect. It was only when both compounds were administered simultaneously mixed in the diet that copper blocked the penicillamine action, probably by direct chemical interaction in the gastrointestinal tract, since blocking the sulfhydryl group by alkylation or by steric hindrance (as in the case of *N*-acetyl-D-penicillamine) will render penicillamine inactive.

The urinary excretion of hydroxyproline in D-penicillamine-treated rats falls within normal limits, while those receiving BAPN doubled their total urinary output of hydroxyproline in the second week, an observation which is in agreement with the findings of Jasin and Ziff[19] and of Orbison *et al.*[20] Because BAPN produces marked skeletal changes, whereas D-penicillamine at the dose used does not, it is reasonable to attribute the excessive hydroxypyroline excretion to a specific disturbance of the metabolism of collagen in bone. Another possibility is that the nature of the molecular abnormality caused by both agents may be different. We know that the collagen from the penicillamine-treated animals behaves essentially like normal collagen once the penicillamine is removed from the environment[21] (*in vivo* by discontinuing the drug, or *in vitro* during the process of collagen purification), whereas the collagen from the BAPN rats is defective. It will not form intermolecular crosslinks *in vitro*[22] and has a low aldehyde content,[23, 24] and may therefore be preferentially degraded in the tissues.

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