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# *In Vitro* Evaluation of Circadian Patterns of Bone Collagen Formation (42192)

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*Abstract.* The circadian patterns of bone collagen formation were studied *in vitro* to ascertain whether the biorhythmic profiles previously measured *in vivo* reflect true differences in collagen synthesis. Alteration of amino acid pool sizes did not negate the circadian-stage differences in bone collagen production. Evaluations of proline uptake and transport, as well as collagenous protein turnover, demonstrated that the intracellular assembly and secretion of bone collagenous protein during the dark span is truely decreased relative to that during the light period. It was further affirmed that PTH is essential for maintenance of the normal circadian collagen synthesis rhythms. 

In the *ad libitum* fed rat, there is evidence that the activities of the cells involved in hard tissue formation and mineralization operate within a circadian framework (1–4). For bone formation, the data indicate that rat bone cells form significantly more collagen during the midday than at night. The phasing of the bone matrix formation rhythm (= net collagen synthesis) appears to be constrained by the light/dark cycle and an endocrine stimulus (1–3). The integrity of the collagen synthesis rhythm (sCOLL) is dependent upon and antiphasal to the rhythm of serum parathyroid hormone, for it is ablated in chronically parathyroidectomized animals (5). Meal timing studies failed to demonstrate an association between the biorhythms of bone formation, serum calcium, or phosphorus, and serum corticosterone titers.

The mechanisms which may govern the circadian changes in the rates of net collagen synthesis *in vivo* have not been established. Because the times in the body rhythm for peak and minimal net collagen synthesis are the reciprocal of the activity and *ad libitum* feeding rhythms, it is possible that the 24 hr sCOLL periodicity only reflects differences in synthesis or differences in amino acid pool sizes, transport, and/or protein degradation. This report addresses these questions in intact and chronically parathyroidectomized rats. The results indicated that the circadian changes in sCOLL in intact rats were due to true changes in bone cell activity, that they were not the consequence of altered rates of amino acid transport or degradation of newly synthesized collagen, and that parathyroid hormone (PTH) is essential to the maintenance of those rhythms.

**Materials and Methods.** *Animal maintenance.* Two series of 100 young male rats (Sprague–Dawley, 4 weeks of age, 100–120 g body wt) were conditioned to a 12-hr light:12-hr darkness for 4 weeks (light on from 08.00 to 20.00 hr). Purina laboratory rat chow was available *ad libitum.* One group was parathyroidectomized surgically, at 4 weeks of age, under light ether anesthesia as previously described (6). The adequacy of the parathyroidectomy was determined by measuring serum calcium concentrations (6) from tail vein samples of rats fasted overnight. Only animals with a serum calcium less than 1.75 mmole/liter were considered to be parathyroidectomized. The animals were supplemented with 2% Ca lactate in water to raise the serum Ca toward normal values (5, 7). At the end of the 4-week conditioning period, animals were killed at 12.00 and 24.00 hr to assess bone proline transport and collagen turnover/degradative parameters. These time points represent, respectively, the peak and nadir of net bone collagen synthesis *in vivo* (2, 5). Body weights at killing were intact controls, 302.1 ± 3.6 g; parathyroidectomized, 284.4 ± 4.6 g.

*Sample preparation.* Tibial cortical bone was excised from 8-week-old intact or chronically parathyroidectomized rats and freed of

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all adhering muscle and marrow constituents. The bones from individual rats were preincubated for 180 min in 3.0 ml of a modified Kreb's Ringer bicarbonate buffer (6) containing 1.75 mmole/liter of $Ca^{2+}$ and 5 m*M* glucose, at 37°C. A pH of 7.5 was maintained by gassing with 95% $O_2$/5% $CO_2$. This preincubation assured steady-state conditions (8) in each of the individual protocols, as detailed below. All radioisotopes were obtained from New England Nuclear, Boston, Massachusetts: [U-$^{14}$C]proline, 269.0 mCi/mmole; [*carboxyl*-$^{14}$C]inulin, 13.68 mCi/mmole; [G-$^{3}$H]proline, 4.6 Ci/mmole.

*Proline pool-size effects on collagen synthesis.* Subsequent to the 180 min preincubation in buffer containing either 0.03 or 1.0 m*M* "cold" proline, the bones were transferred to identical buffer containing 1.0 $\mu$Ci of [$^{14}$C]proline/3 ml. The samples were incubated for an additional 15- to 120-min period to allow quantitation of the rate of collagen protein synthesis. At termination of the incubation, the bone tissue was rinsed with medium containing 1.0 m*M* "cold" proline, immediately frozen in liquid $N_2$, lyophilized, and stored at −80°C. For extraction and analysis of tissue DNA according to the indole method of Keck (9), bone was powdered in liquid $N_2$ (Spex Mixer/Mill, Metuchen, N.J.) and homogenized at 4°C in 1 *M* NaOH. Subsequently, the protein was precipitated and washed with 25% trichloroacetic acid (TCA) to exclude the acid-soluble amino acid pools.

The newly synthesized collagenous protein of the remaining bone matrix was solubilized with acidic pepsin digestion and purified via successive NaCl precipitations at acid and neutral pH as described by Webster and Harvey (10). Nonspecific "trapping" or adherence of isotope was determined by incubation of "dead," freeze-dried bone. The residual bone matrix, that not solubilized with acidic pepsin digestion, was hydrolyzed in 6 N HCl, 108°C for 18 hr, and designated the noncollagenous protein fraction (NCP). The [$^{14}$C]collagen and -NCP were counted in a Packard Tri-Carb liquid scintillation counter with external standardization (11), and expressed in disintegrations per minute (dpm) per milligram cellular DNA. Six individual bones were incubated at each of the time points. The determination of the percentage collagen synthesized by the bones was corrected for the relative abundance of proline residues in collagen compared to NCP (12).

*Proline uptake and transport.* At the end of the preincubation period, each sample was transferred to medium containing generally (G) labeled [$^{3}$H]proline (1.0 $\mu$Ci/3 ml) and [$^{14}$C]inulin (0.5 $\mu$Ci/3 ml), the latter to define the extracellular tissue space for each bone sample. Following the specified periods and conditions of incubation, the bone tissue was briefly rinsed in media containing 10 m*M* nonradioactive proline and boiled for 2 min in 2 ml of distilled water to extract free amino acids (8). Aliquots of the media and bone-water extracts were quantitated by liquid scintillation spectrometry as described above.

The intracellular to extracellular distribution ratio was calculated according to the method of Akedo and Christiansen (13). For each individual bone sample, the following calculations and corrections for extracellular tissue space were made. The percentage of extracellular space (ECS) was defined as the ratio of ([$^{14}$C]inulin dpm)/([$^{3}$H]proline dpm) × $R$ in the bone-water extracts, where $R$ = the molar ratio of [$^{14}$C]inulin/[$^{3}$H]proline in the incubation media. The proportion of the bone-water extract which represented intracellular proline uptake was determined following subtraction of that percentage accounting for the ECS: ([$^{3}$H]proline dpm) − ([$^{3}$H]proline dpm)(ECS) = [$^{3}$H]proline uptake intracellularly. The distribution ratio represents the ratio of intracellular proline uptake (dpm)/milligram DNA to media proline dpm/milliliter of media, designated as $P_c/P_f$, respectively. The ECS of the bone samples averaged 11.16 ± 0.87 and 12.16 ± 0.95% at 12.00 and 24.00 hr, respectively.

The transport kinetics of proline uptake were evaluated following 5 min of incubation in media containing 0.30, 0.50, 1.0, 3.0, and 5.0 m*M* proline. Following determination of the distribution ratio ($P_c/P_f$) as described above, $P_c/P_f$ was plotted against 1/[Pro], and the slope of the line described by the data was determined by linear regression analysis (14). Using this slope and intercept, $V$ ($\mu$mole/mg DNA/hr) was calculated for each of the respective proline concentrations and measured distribution ratios. The apparent $K_m$ and $V_{max}$ were derived from Lineweaver–Burke recip-

rocal plots of $1/V$ (μmole/mg DNA/hr) vs 1/[Pro] ($mM^{-1}$).

*Collagen protein turnover/degradation.* Following a preincubation (180 min) and a 60-min "pulse" period, the "chase" periods of 10–60 min allowed an assessment of collagen degradation. Unlabeled proline was 0.3 m*M* during the "pulse" period, for this is the average free serum proline concentration in our rat population (data not shown) during a 24-hr period. 1.0 m*M* "cold" proline was used during the "chase" period. Both [$^{14}$C]proline and -hydroxyproline in the acidic pepsin-extractable bone collagen (10) and low molecular weight peptides of the media (15) were measured. Determination of the percentage collagen synthesized by the bones or detected in the media was calculated after allowing for the relative abundance of proline residues in collagen compared to NCP (12).

Statistical analyses were performed according to the Student *t* test, as required by the data series.

**Results.**[$^{14}$C]*Proline incorporation vs proline pool size.* Intact rats: Table I shows that at the two biopsy times, the *in vitro* rate of $^{14}$C-collagenous protein synthesis exhibits the expected inverse relationship to medium proline concentrations, but that independent of the proline concentrations, nearly twice as much collagen is produced by the incubated bone tissue at 12.00 hr than at 24.00 hr. The *in vitro* rates of collagen production were not linear; the 12.00 hr vs 24.00 hr (circadian) differences in collagen synthesis appeared after an incubation time of 1 hr. The 12.00-hr biopsies showed a four- to six-fold increase in collagen production at the end of the first hour of incubation (95% of total) with little change thereafter. Bones incubated at 24.00 hr showed a similar overall performance, but the major pulse of collagen formation was delayed until the second hour of incubation. The percentage day/night difference in sCOLL synthesis after 120 min of incubation in 0.03 m*M* medium proline was much less than that observed with 1.0 m*M* medium proline (16% vs 34%), presumably because 0.03 m*M* proline provided insufficient substrate for collagen synthesis and was thereby rate limiting (16). Since no differences were noted in the percentage collagen synthesized, with either high or low concentrations of proline in the media, the percentage

TABLE I. *In Vitro* Circadian Patterns of [$^{14}$C]Proline Incorporation into Bone Collagenous Protein from Intact and PTX Rats[a]

| Animal group | Clock hour | [$^{14}$C]Collagen (dpm/mg DNA) | | | | | | % Collagen synthesized |
|---|---|---|---|---|---|---|---|---|
| | | 0.03 m*M* proline | | | 1.0 m*M* proline | | | |
| | | 30 min | 60 min | 120 min | 30 min | 60 min | 120 min | |
| Intact | 12.00 | 10,627 ± 2481 | 53,674 ± 16,725* | 55,990 ± 11,728 | 1940 ± 273 | 5014 ± 1184* | 6048 ± 1020 | 11.17 ± 0.90** |
| | 24.00 | 11,794 ± 1342 | 13,844 ± 163 | 46,831 ± 9721 | 1323 ± 283 | 1430 ± 296 | 4073 ± 725 | 19.51 ± 2.44 |
| PTX | 12.00 | 2138 ± 537 | 3432 ± 527 | 9476 ± 990 | 293 ± 44 | 1124 ± 158 | 2136 ± 340 | 5.40 ± 2.08 |
| | 24.00 | 3200 ± 1194*** | 7256 ± 2015*** | 13,415 ± 2625*** | 331 ± 74*** | 679 ± 166*** | 1705 ± 827*** | 6.28 ± 2.85 |

*Note.* Significantly different from corresponding 24.00 hr value, according to unpaired Student's *t* test. *$P < 0.05$; **$P < 0.025$. Significantly different from corresponding "intact" value, according to unpaired Student's *t* test. ***$P < 0.025$.

[a] The mean [$^{14}$C]proline incorporation into collagen (±SEM), six bones from intact and parathyroidectomized (PTX) rats incubated at each of the time points from 30 to 120 min.

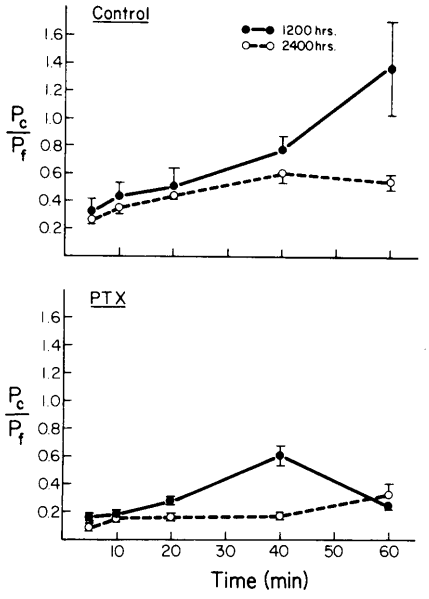


FIG. 1. [$^3$H]Proline uptake by the cortical tibial diaphysis *in vitro*, from intact (control) and Ca-lactate-supplemented parathyroidectomized (PTX) rats. Values are expressed as the ratio of the intracellular ($P_c$) to extracellular ($P_f$) proline concentration over 60 min of incubation. Each calculated value represents the mean ±SEM of six bones. $P_c/P_f$ was determined according to the method of Akedo and Christiansen (1962) (13).

collagen synthesis (Table I) is a compilation of the two experiments. The increase in percentage collagen synthesized at 24.00 hr vs 12.00 hr was due to a proportionally greater decrease in the noncollagen protein synthesized at 24.00 hr.

Parathyroidectomized rats: In the bones from parathyroidectomized rats at both concentrations of proline, the level of collagen formation sustained was only 50% of that of the "intact" rat bone. No circadian changes in bone collagen synthesis were detected (Table I) at the different incubation times.

*Proline uptake studies. In vitro* analysis of proline uptake (intra- vs extra-cellular distribution ratios) demonstrated a significant decrease ($P < 0.05$) in the presence of physiologic proline concentrations (0.30 m*M*; Fig. 1). Bone from parathyroidectomized rats exhibited a diminished uptake of proline at both 12.00 and 24.00 hr. Analysis of initial (5 min) uptake rates over a 0.3–5.0 m*M* proline concentration range (Fig. 2) revealed that the apparent $V_{max}$ was unchanged while a diminution in the apparent $K_m$ was evident at 2400 hr compared to 1200 hr (Table II). No significant differences between intact and parathyroidectomized rat bones were noted.

*Collagen turnover studies.* The *in vitro* circadian patterns of $^{14}$C-collagenous protein turnover in cortical bone (Table III) did not demonstrate an enhanced degradation of newly synthesized collagen at 2400 hr compared to 1200 hr. No evidence of accelerated turnover was discernable in bone from parathyroidectomized rats. The percentage of [$^{14}$C]collagen measured in the media [% = (media [$^{14}$C]collagen incorporation/bone

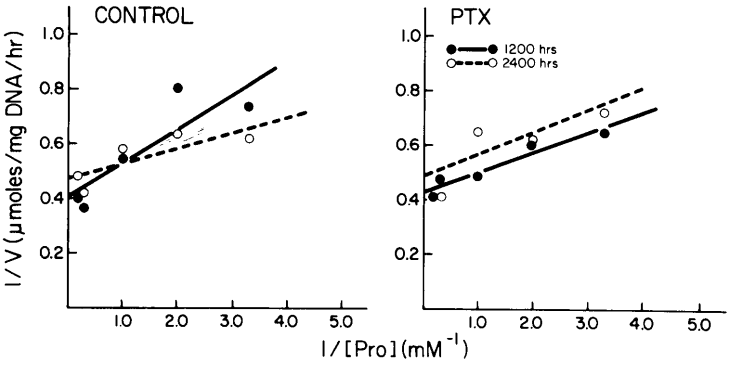


FIG. 2. Lineweaver–Burke plot of the *in vitro* proline uptake (5 min) by bone from intact or parathyroidectomized (PTX) rats, at 12.00 and 24.00 hr. Each calculated value represents the mean of six bones at each concentration of unlabeled proline (0.3, 0.5, 1.0, 3.0, and 5.0 m*M*), in the presence of 1.0 μCi [$^3$H]proline and 0.5 μCi [$^{14}$C]inulin. Cortical bone was preincubated for 180 min in Kreb's Ringer bicarbonate buffer, with the appropriate unlabeled proline concentration, to attain steady-state conditions.

TABLE II. APPARENT $K_m$ AND $V_{max}$ VALUES FOR PROLINE TRANSPORT BY CORTICAL BONE *IN VITRO*[a]

| Animal group | Clock hour | $K_m$ (m$M$) (mean ± SEM) | $V_{max}$ ($\mu$mole/mg DNA/hr) (mean ± SEM) |
|---|---|---|---|
| Intact | 12.00 | 0.312 ± 0.046* | 2.480 ± 0.367 |
| | 24.00 | 0.127 ± 0.015 | 2.137 ± 0.247 |
| PTX | 12.00 | 0.229 ± 0.029* | 2.311 ± 0.291 |
| | 24.00 | 0.109 ± 0.011 | 2.053 ± 0.203 |

[a] Each calculated value represents the mean ± SEM of six bones from intact or parathyroidectomized (PTX) rats, each group of which was incubated in concentrations of unlabeled proline from 0.3 to 5.0 m$M$ for 5 min, in the presence of 1.0 $\mu$Ci [$^3$H]proline and 0.5 $\mu$Ci [$^{14}$C]inulin. Cortical bone was preincubated for 180 min in Kreb's ringer bicarbonate buffer, with the appropriate unlabeled proline concentration, to attain steady-state conditions. A Lineweaver–Burke plot of $1/V$ vs $1/S$ (Fig. 2) was then constructed from the data and the intercepts determined.

* $P < 0.005$, according to the Student $t$ test, from the corresponding 24.00-hr value.

+ media incorporation) × 100] was 71.99 ± 2.01 at 12.00 hr and 73.75 ± 1.78 at 24.00 hr in intact rat bone. Comparable values of 66.60 ± 4.00 at 12.00 hr and 73.26 ± 1.75 at 24.00 hr were noted for those bones from parathyroidectomized rats.

**Discussion.** In this study, we have explored the cellular mechanisms which confer the *in vivo* circadian rhythm of bone collagen synthesis in rats. Importantly, the magnitude and patterns of the day/night changes in sCOLL detected *in vitro* were qualitatively and quantitatively identical to those which could be demonstrated *in vivo*. It was evident in both types of studies that the rate of sCOLL in intact rats proceded most actively during the daytime and least actively at night (2, 5). Our new data also affirmed that the parathyroid glands were required to maintain the integrity of the sCOLL rhythm and normal osteoblastic activity (2, 5). Parathyroidectomized rats lacked a sCOLL rhythm, and their level of osteoblastic bone formation was only 50% of that of intact rats. The device of feeding parathyroidectomized rats Ca-lactate supplements to obviate the stress and influences of severe hypocalcemia on bone metabolism does not in itself raise the rate of sCOLL or restore the sCOLL rhythm (6).

The present *in vitro* studies examined the proposition that the presence and phasing of the bone collagen synthesis rhythm, its daytime peak and nocturnal nadir, reflected indirectly an efffect of dietary amino acid pool sizes, membrane transport, and/or intracellular protein degradation. In terms of (changes in the) pool size, we (2) and others (17) have detected a circadian rhythm for serum proline. In the strain of rats in our vivarium, proline titers peak at midday (0.44 $\mu M$) and reach their nadir (0.28 m$M$) in the middle of the night. While the nocturnal serum proline level was always greater than the value of 0.15 m$M$ which has been shown to be rate limiting for collagen synthesis in *in vitro* isolated bone cultures (16), this factor remained a possible explanation for the nocturnal nadir of sCOLL in "intact" rats. The work comparing the effects of preincubating bones with 0.03 and 1.0 m$M$ medium proline (Table I) addressed this possibility, and the data indicated that alterations in proline intracellular pool sizes did not ablate the day/night circadian differences in collagenous matrix production. The significant (50%) impairment in sCOLL in chronically parathyroidectomized rats also could not be explained by a specific effect of pool size, i.e., proline availability. Nor, as shown in Fig. 1, could the normal circadian rhythm seen in intact rats, and the absence of the rhythm in parathyroidectomized rats, be ascribed to a change in the rate of intracellular substrate transport.

The present studies also addressed the prospect that the circadian patterns of sCOLL, in bone, might derive from changes in intracellular protein degradation. Since, as shown in Table III, the media [$^{14}$C]collagen (% of bone + media; 73.75 ± 1.78) at 24.00 hr, the time at which the nadir in collagen formation is reached, did not exceed that ratio measured during the light span (71.99 ± 2.01 at 12.00 hr), it can be concluded that the decrease in net bone collagen formation at 24.00 hr is not

TABLE III. *IN VITRO* CIRCADIAN PATTERNS OF $^{14}$C-COLLAGENOUS PROTEIN TURNOVER IN CORTICAL BONE FROM INTACT AND PTX RATS[a]

| Animal group | Clock hour | Media [$^{14}$C]collagen (dpm/mg DNA) Minutes of chase period 0 | 10 | 20 | 40 | 60 | % Collagen in media protein Minutes of chase period 0 | 10 | 20 | 40 | 60 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Intact | 1200 | 78,790 ± 13,121 | 131,434 ± 27,254 | 152,563 ± 37,788 | 305,739 ± 58,075 | 380,858 ± 55,349 | 14.6 ± 0.9 | 14.3 ± 0.6* | 14.2 ± 0.6** | 15.2 ± 0.5* | 15.3 ± 0.3 |
| | 2400 | 68,283 ± 14,136 | 151,069 ± 14,291 | 234,321 ± 44,343 | 577,463 ± 168,568 | 469,062 ± 70,996 | 16.9 ± 1.4 | 18.2 ± 0.7 | 18.2 ± 0.5 | 17.9 ± 0.7 | 17.0 ± 0.9 |
| PTX | 1200 | 76,869 ± 9,804 | 97,770 ± 14,295 | 118,311 ± 10,411*** | 145,013 ± 5,794*** | 184,031 ± 22,296† | 11.6 ± 2.1 | 11.7 ± 1.8* | 15.8 ± 0.9 | 14.4 ± 0.3** | 16.8 ± 0.8 |
| | 2400 | 82,637 ± 8,576 | 142,865 ± 17,281 | 260,802 ± 22,812 | 321,497 ± 99,465 | 226,556 ± 23,795† | 15.0 ± 2.8 | 16.6 ± 0.2 | 15.6 ± 0.3† | 17.8 ± 0.3 | 14.7 ± 0.8 |

*Note.* Significantly different from corresponding 2400-hr value, according to the Student *t* test: $*p < 0.025$; $**P < 0.001$. Significantly different from corresponding "intact" value, according to the Student *t* test: $***P < 0.025$; $\dagger P < 0.01$.

[a] Each calculated value represents the mean ± SEM of six bones from intact or parathyroidectomized (PTX) rats. Each group was preincubated for 180 min in Kreb's ringer bicarbonate buffer with 0.3 m*M* unlabeled proline to attain steady-state conditions. Thereafter the media was replaced with identical buffer containing 1.0 $\mu$Ci of [G-$^{14}$C]proline/3 ml and the bones labeled for an additional 60-min "pulse" period. The "chase" period, for times as indicated above, was carried out in buffer containing 1.0 m*M* unlabeled proline. Full details of the incubation and analytical procedures are as delineated under Materials and Methods.

attributable to enhanced degradation of newly synthesized collagen. Likewise, no evidence of increased collagen turnover was measurable in bones from parathyroidectomized rats (Table III). Although this method of assessing collagen turnover (15) may tend to overestimate the degradative rate, we do know from the investigations of Kream *et al.* (18) and Bienkowski *et al.* (19) that as much as 40–60% of the newly synthesized collagen in fetal tissue may be extensively degraded shortly after its synthesis. Collagenous protein degradation, *in vitro,* in these 2-month-old growing rats exceeds that in fetal tissue; our results nevertheless support the premise that the circadian pattern of net bone collagen formation is not conditioned by changes in collagen degradation. Thus, the intracellular assembly and secretion of bone collagenous protein during the dark span (24.00 hr) is truly decreased relative to that during the light period (12.00 hr), and it is affirmed that PTH per se is essential for maintenance of the normal circadian collagen synthesis rhythms. These observations do not exclude, and do in fact reinforce, the level of regulation demonstrated by Kream *et al.* (18) for fetal rat calvaria. In their studies, PTH decreased the functional procollagen mRNA levels; within a circadian framework, this would be operative during the dark period when PTH serum titers are elevated (20, 21) and collagen synthesis has reached its nadir (2, 5).

In summary, these *in vitro* studies affirm the character and phase relationships of the bone collagen synthesis rhythm to the light/dark cycle, and reinforce the concept that the integrity of the rhythm is parathyroid dependent.

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