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Statistical Evidence for Remnants of the Primordial Code in the Acceptor Stem of Prokaryotic Transfer RNA

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Summary. The specificity of interaction of amino acids with triplets in the acceptor helix stem of tRNA was investigated by means of a statistical analysis of 1400 tRNA sequences. The imprint of a prototypic genetic code at position 3–5 of the acceptor helix was detected, but only for those major amino acids, glycine, alanine, aspartic acid, and valine, that are formed by spark discharges of simple gases in the laboratory. Although remnants of the code at position 3–5 are typical for tRNAs of archaebacteria, eubacteria, and chloroplasts, eukaryotes do not seem to contain this code, and mitochondria take up an intermediary position. A duplication mechanism for the transposition of the original 3–5 code toward its present position in the anticodon stem of tRNA is proposed. From this viewpoint, the mode of evolution of mRNA and functional ribosomes becomes more understandable.

Key words: Transfer RNA — Acceptor helix stem — Primordial code — Statistics

Introduction

Life appears to be the result of a transition from a purely nonreplicative chemistry to a chemistry that propagates cells. In the course of replication, the language of nucleic acids is transformed into that of proteins. Transfer RNA is the agent of this transformation and therefore contains in its structure clues to the origin of the genetic code. Yet we can only surmise by inference how the genetic code was originally written into the structure of tRNA and how each amino acid attached itself to the appropriate tRNA.

Theories about the origin of the code are essentially of three kinds (Crick 1968; Eigen and Winkler-Oswatitsch 1981a): (1) The stereochemical theory postulates a steric fit between each amino acid and its corresponding codon present in a primitive tRNA. (2) In the frozen accident theory the code arose purely by a process of chance and, once established, remained unchanged because any alteration would be deleterious to the many proteins in the cell. (3) According to the third theory, the code evolved in two phases. First the primordial amino acids alanine, glycine, aspartic acid, and valine, and the primal codons, GCC, GGC, GAC, and GUC, selected each other through preferential binding to primitive tRNA adaptors, which carried the respective codon. Which of the three theories is closest to being correct?

The stereochemical model for primitive protein synthesis, as proposed by Crick (1968) and others (Jungck 1978), has been generally played down because the large separation between the aminoacylation site and the anticodon site in present-day tRNAs precludes a direct interaction between an amino acid and its codon. The purely random assignment of amino acids to protocodons is, as a theory, found to be unsatisfactory (Crick 1968), particularly when considering the wealth of information on the chemical specificity of protein–nucleic acid interactions in general and the occurrence of a specific amino acid binding site composed of RNA (Ya-

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rus 1988). To the detriment of a theory of a fixed code, it has been shown that the genetic code is not a frozen accident but instead is still evolving in mitochondrial and nuclear genomes (Jukes and Osawa 1991). Concerning the third theory, there may initially have been a few RNA adaptors carrying a few prototypic amino acids (Eigen and Winkler-Oswatitsch 1981a). As more amino acids appeared, they may have been encoded by first appropriating existing codons from their precursor amino acids before spreading out over the 64 triplets of the four bases (Wong 1988). Of these theories, the third seems most appealing also because the supposedly primordial codons, with guanine as the first base, code for the abundant amino acids formed during gas-sparking experiments (Miller 1986).

In a previous paper we proposed an ancient interaction of amino acids with triplets of the acceptor helix stem of tRNA based on a statistical analysis of 1400 tRNA sequences. A search through all triplets along the acceptor helix revealed that position 3–5 is unique in harboring a prototypic triplet code (Möller and Janssen 1990). The vestiges of this code in the acceptor helix raises new questions about universality and functionality. To try to answer these, we have now carried out an analysis for remnants of the prototypic code surviving in all types of organisms.

As a corollary, our detection of a prototypic code at position 3–5 of the acceptor helix immediately predicts a plausible mechanism by which the spatial separation between the aminoacylation site and the anticodon in present-day tRNAs may have arisen. In addition, this analysis of tRNAs sheds new light upon the possible evolution of the three kingdoms, archaebacteria, eubacteria, and eukaryotes, as proposed by Woese (1989).

Evidence for Protocodons at Position 3-5 in tRNAs

The statistical evidence for a possible prototypic code in the acceptor helix of tRNAs of different organisms is given in Table 1. This represents an extension of an earlier compilation (Möller and Janssen 1990), which did not take into account differences between organisms. A high level of correct code words for primordial amino acids at position 3–5 of prokaryotic tRNAs is evident, but doubts remain about its meaning and functionality. Why, for instance, is this code not universally preserved in tRNAs, instead of being apparently lost in eukaryotes, and why is it more marked in chloroplasts than in mitochondria? How far do eubacteria differ from archaebacteria in possessing a primordial code?

In Table 1, both eubacteria and archaebacteria are treated as a single group, the prokaryotes, thought to be the earliest and most simple identifiable life forms on earth (Vidal 1984). The data are pooled because the number of individual archaebacterial tRNA species sequenced is so small that a comparison of their sequences with those of eukaryotes does not afford the same statistical validity. The data in Table 2 illustrate this point explicitly, but it is obvious that archaebacteria also carry an old code.

The strength and consistency of this paper lies in the data of Table 3, which include the number of times that a protocodon occurs in present-day tRNAs for *old* as against *new* amino acids. This is tantamount to the frequency of a guanine base at position 3 for the two types of aminoacyl tRNA. The statistical frequencies (Table 3) are highly significant, with respect both to the type of tRNA and to the organism to which the protocodon frequency refers. Prokaryotes contain two major classes of tRNA structure, which are distinguished by the presence (class I) or absence (class II) of a guanine residue at position 3. Of the 86 sequenced prokaryotic species of $tRNA^{Ala}$, $tRNA^{Gly}$, $tRNA^{Asp}$, and $tRNA^{Val}$, 80% belong to class I; of the 287 sequenced species of tRNAs for the remaining amino acids 65% belong to class II. Transfer RNAs from chloroplasts behave like those of archae- and eubacteria, whereas mitochondrial tRNAs just because of their relatively higher mutation rates, can be understood to occupy an intermediate position. Eukaryotes do not carry a clear imprint of a prototypic code in their tRNAs. On the contrary, the observed frequency of protocodons in their tRNAs for new amino acids is on average even below statistical expectation.

In order to assess the distribution and levels of "wrong" code words, we also scored the code words at position 3–5 that did not accord with the amino acid bound by the tRNA. In the group of prokaryotic and chloroplast tRNAs for the four primordial amino acids, none of the code words for recent amino acids appear with greater than statistical expectation ($P < 0.001$). In mitochondria, a Glu code word is often found in $tRNA^{Val}$, whereas in eukaryotes, there is Ile in $tRNA^{Gly}$, Leu in $tRNA^{Asp}$, and Phe in $tRNA^{Val}$. As regards wrong code words for primordial amino acids, besides Gly in $tRNA^{Val}$ from chloroplasts, only Gly occurs in $tRNA^{Ala}$ with high frequency throughout all kingdoms of organisms, the cellular organelles, mitochondria and chloroplasts included. The converse—a high Ala codon use at position 3–5 of $tRNA^{Gly}$—does not occur. In all other cases wrong code words at position 3–5 were not observed. At least in statistical terms the existence of vestiges of the code for primordial amino acids at position 3–5 in the acceptor helix stem of tRNAs of present-day prokaryotes is demonstrated (Fig. 1).

Table 1. Transfer RNAs for primordial amino acids carry possible remnants of a primitive code at position 3–5, especially in prokaryotes

Type of aminoacyl tRNA	Frequency (%) of a match observed in[a]				Frequency expected (%)[b]
	Prokaryotes[c]	Chloroplasts	Mitochondria	Eukaryotes	
Ala	53 **[d]	0	0	30 *	6.2
Gly	72 **	73 **	9	5	6.2
Asp	20 *	100 **	30 **	0	3.1
Val	53 **	0	0	0	6.2
Glu	0	0	0	0	3.1
Pro	18	0	0	13	6.2
Lys	0	0	0	0	3.1
Arg	0	0	0	0	9.4
Thr	0	0	0	0	6.2
Phe	7	0	13 *	0	3.1
Leu	3	46 **	8	8	9.4
Ile	0	38 **	0	0	4.7
Met[e]	0	0	0	0	1.6
Ser	0	0	2	34 **	9.4
Asn	0	0	12	0	3.1
Trp	0	0	0	0	1.6
Gln	0	0	0	0	3.1
His	0	0	0	10	3.1
Tyr	0	0	0	0	3.1
Cys	0	0	0	0	3.1

[a] Percentages refer to the fractions of tRNAs with a triplet at position 3–5 corresponding to the amino acid on the tRNA

[b] Ratio of the number of codons for a given amino acid to the total number of codons (i.e., 64) expected on the basis of a random distribution of the four bases

[c] The group of prokaryotes consists of both eubacteria and archaebacteria, the latter representing 12–15% of each group figure

[d] Estimated significance of the difference between the observed and expected frequency. ** Denotes a tail probability, $P < 0.001$; *, $0.001 < P < 0.05$; no sign, $P > 0.05$. Frequencies less than expected on a random basis were always scored negatively (no sign). Statistics were performed by assuming a Poisson distribution of probabilities of finding x codons in a given set of n, the total number of specific tRNAs investigated (Goodman 1964)

[e] Transfer RNAs for initiator-methionine are included

Table 2. Transfer RNAs for primordial amino acids often bear a triplet at position 3–5 that reads as the codon for the amino acid on the tRNA. This remnant of a sequence signature is especially noticeable in the kingdoms of archaebacteria and eubacteria and in chloroplasts[a]

Type of aminoacyl tRNA	Frequency (%) of a match observed					Frequency expected (%)
	Archaebacteria	Eubacteria	Chloroplasts	Mitochondria	Eukaryotes	
Ala	100 (14)[b]	20 (20)	0 (9)	0 (18)	30 (10)	6.2
Gly	29 (7)	89 (18)	73 (11)	9 (22)	5 (20)	6.2
Asp	0 (3)	29 (7)	100 (6)	30 (23)	0 (15)	3.1
Val	71 (7)	40 (10)	0 (13)	0 (20)	0 (21)	6.2

[a] See notes with Table 1; in this table only tRNAs for the primordial amino acids are considered

[b] Values in parentheses are number of tRNAs examined in the given group. Total number of sequences was 1432

Evolutionary Model of tRNA Based on Protocodon Duplication

A model for the possible evolution of tRNA from a short RNA hairpin loop having a protocodon–anticodon pair is given in Fig. 2. In short, we propose that the code arose at position 3–5 of the stem of primordial tRNAs, and that codons gradually appeared in mRNA after duplication and cleavage of these primordial tRNAs. The model describes the evolution of tRNA in terms of having both a messenger adaptor and an amino acid loading function; this model is subject to experimental tests and does not invoke any new chemical principles.

The proposed duplication is consistent with recent information on the processing of RNA precursors. (1) RNA templates can direct the synthesis of the complementary strand with activated nucleotides and without the need for proteins (Inoue and Orgel 1983). (2) Transfer RNAs can be cleaved cat-

Table 3. Frequency of protocodons[a] in tRNAs from different kingdoms and cell organelles

Type of amino acid bound to aminoacyl tRNA	Observed frequency (%) of a protocodon[b]					Expected frequency (%)[c]
	Eubacteria	Archae-bacteria	Chloroplasts	Mitochondria	Eukaryotes	
Primordial amino acid	75 (55)[d]	84 (31)	90 (39)	41 (83)	29 (66)	22
Recent amino acid	35 (215)	35 (72)	42 (175)	14 (413)	11 (283)	22

[a] Protocodons are defined as codons for primordial amino acids at position 3–5 (Möller and Janssen 1990)
[b] Frequency of a match between the triplet at position 3–5 and a codon for Gly, Ala, Asp, or Val
[c] Sum of the codons for Gly, Ala, Asp, and Val (i.e., 14) divided by the total number of codons (i.e., 64)
[d] Values in parentheses are number of tRNAs investigated

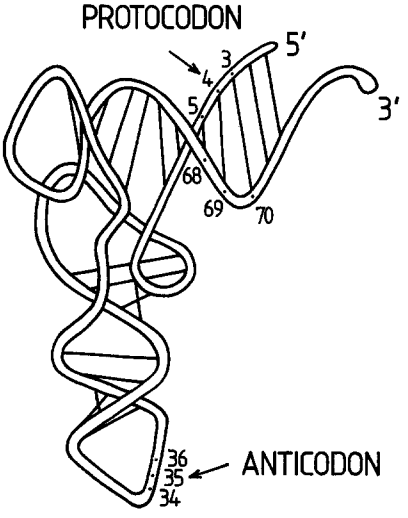

Fig. 1. Schematic drawing of the three-dimensional structure of a tRNA taken from Kim et al. (1974). The numbering of the bases is according to the system of Sprinzl et al. (1989) with the localization of the proposed primordial codon and anticodon indicated. The pair 3–70 corresponds to the critical G3–U70 base pair of Hou and Schimmel (1988) and includes the paracodon region proposed by de Duve (1988). Originally the amino acid may have recognized the tRNA through its primitive codon–anticodon conformation ("chargeon").

alytically by RNA (Guerrier-Takada et al. 1983). (3) A number of yeast tRNA genes contain a short intron located one or two bases from the 3′ end of the anticodon of the mature tRNA (Goodman et al. 1977; Valenzuela et al. 1978). (4) This intron possesses the codon for the amino acid carried by the tRNA, and this short piece can be folded around the anticodon loop in a manner suggesting that the codon–anticodon interaction exerted some function (Valenzuela et al. 1978). The site of cleavage—next to the 3′ end, rather than to the 5′ end of the anticodon loop of tRNA—is similar to that proposed here and supports the idea that protocodons originated near the 5′ rather than the 3′ end of the acceptor helix.

Thus, primitive protein synthesis may have started with short RNA hairpin loops that were recognized directly by the side chain of primordial amino acids according to stereochemical rules for the interaction between a specific amino acid and its cognate codon–anticodon pair. Template-guided synthesis is considered not to exist yet, but amino acid-specific hairpin loops could have reacted with each other, resulting in the synthesis of polypeptides with random sequence but nonrandom composition that depended on the relative abundance and strengths of binding of the different amino acids to their cognate hairpin and the frequency of interaction between the different aminoacyl hairpins. Only after duplication of these half-tRNA hairpins had occurred could the stage have been set for ordering amino acids in a nonrandom sequence on an RNA template. At this point, a synthetase having a defined amino acid sequence may have entered the picture. From then on, the genetic pressure for having a code at position 3–5 would be diminished as the synthetases became the primary determinants of the charging reaction. In itself it is therefore surprising that the primordial code at position 3–5 has left traces for at least 3.3×10^9 years. This period is based on the premise that the primordial code arose quite early, that is, before a progenote is believed to have appeared on this planet (Schopf and Packer 1987). This antiquity is hardly explainable, unless present-day synthetases would recognize it.

The proposed duplication scheme, however, disregards non-Watson and Crick pairings and does not therefore account for the fact that discrimination of alanine by a synthetase almost invariably depends on the presence of a G:U wobble base pair at position 3 of the cognate tRNA (Hou and Schimmel 1988). However, it is easily seen that the melting of an RNA hairpin structure in which the two paired alanyl triplets, including the G:U base pair, duplicate purely according to Watson and Crick base pair rules, gives rise to a new type of tRNA having an A:U instead of G:U base pair at position 3, whereas in the new structure at the position of the third letter of the anticodon, the correct base C appears. The proposed model possesses therefore an inherent tendency to duplicate hairpins with a G:U wobble base pair incorrectly unless there is some editing mech-

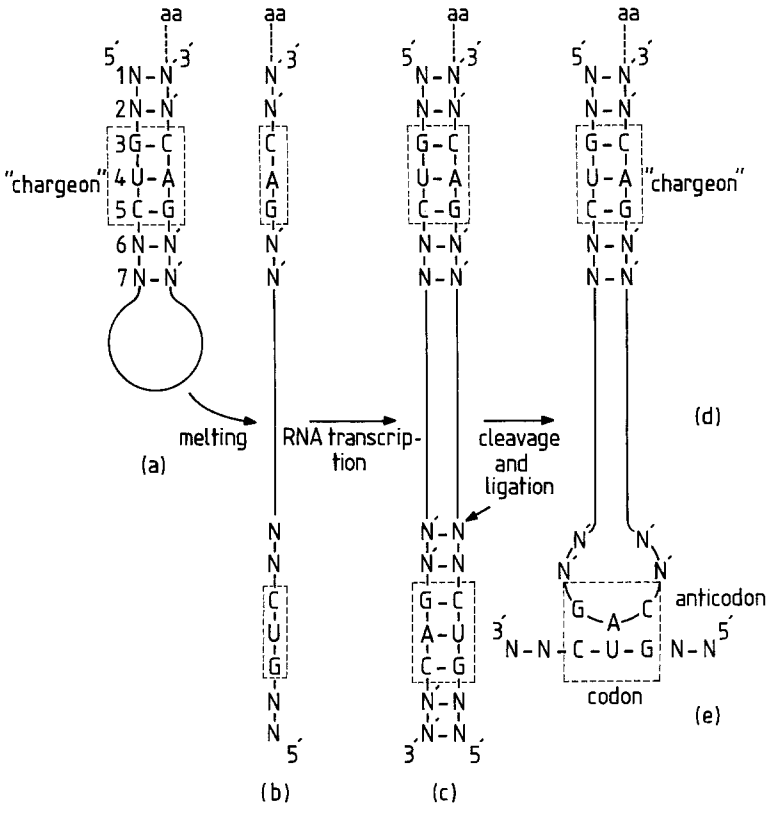

Fig. 2. Hypothetical scheme of the evolution of valine tRNA from a short amino acid-specific RNA hairpin loop (a). On melting of the double-stranded RNA, a single-stranded molecule (b) is formed, resulting in the separation of the two elements of the "chargeon," viz. the primitive codon (nucleotides 3, 4, and 5 in a) and the primitive anticodon (nucleotides opposing 5, 4, and 3, respectively). RNA-directed synthesis of the opposite strand yields a palindromic double-stranded RNA intermediate (c), in which the duplication of the chargeon has taken place. Transfer RNA may have evolved from this structure autocatalytically by cleavage (see arrow) of a codon-containing tract, followed by ligation of the 5′ end with the 3′ end of the opposing strand, so as to form a primitive tRNA (d) with an anticodon loop well separated from the amino acid attachment site. The short stretch of RNA containing the codon (e) would be available as a building block for mRNA. The same scheme applies to the formation of alanine, glycine, and aspartic acid tRNA.

anism that changes an A:U into a G:U base pair in the duplicate.

The Importance of Being Old

The statistical evidence that position 3–5 of tRNAs for the old amino acids glycine, alanine, aspartic acid, and valine are often filled up with triplets that read as code words for the cognate amino acid, is fairly persuasive. These amino acids are the major products generated in the laboratory by condensation of hydrogen, ammonia, water, and either methane or CO_2 (Miller 1986). These are also the four most abundant amino acids in carbonaceous meteorites (Kvenvolden et al. 1970), supporting the view that they played a dominant role in shaping early protein synthesis on this planet. Remarkably, the same four amino acids are also encoded by what Eigen and Schuster (1978) conjectured to be the earliest codons, assigned on the basis of the frequently recurring sequence motif, RNY, seen in many RNAs (R = purine, Y = pyrimidine, N = any of the four bases). As Eigen and Schuster observe: "the agreement between the abundance of natural amino acids and the order of the first codon assignments is striking." Indeed, it would be hard to imagine that such primordial codons with guanine as the first base were assigned purely on the basis of chance. It is far more likely that this universal correlate between glycine, alanine, aspartic acid and valine, on one hand, and the first codons, GGC, GCC, GAC, and GUC, on the other, derives from organic reactions involving a plethora of possible heterocyclic compounds, sugars, and α-amino acids, the precise identity of which cannot be explicitly determined (Joyce 1989). The selective character of the code—α-amino butyric acid, for example, is not coded—would be an argument in favor of stereochemical constraints between nucleotides and α-amino acids. Apparently, for the evolution of the code, both abundance and stereochemical fit were important. In this context it is remarkable that coded amino acids can be classified into three categories: (1) being small, (2) having a branched aliphatic side chain, and (3) carrying a polar or aromatic group in the side chain. Therefore it appears that size, branching, and polarity–aromaticity played a decisive role in the assignment, with straight aliphatic side chain amino acids like α-amino butyric acid, norvaline, and norleucine being presumably energetically less acceptable to nucleotide configurations.

Is the 3-5 Code in tRNA Presently Functional?

Does this 3–5 code still retain functional significance, and is our perception of its origin correct? The answer is not simple and depends on how the 3–5 code is defined. If one asks whether the acceptor

helix of tRNA contains critical bases or base pairs that determine the specificity of the charging reaction, the answer is definitely yes. In fact Hou and Schimmel (1988) were the first to demonstrate that a single base pair G3–U70 of tRNA determines the amino acid specificity for alanine. This led de Duve (1988) to define triplets in this region as the second genetic code and to propose these "paracodons" as a possible site for recognition by synthetases. We have shown that in prokaryotic organisms, tRNAs for primordial amino acids carry the remnants of the code at position 3–5, and that G3 is indeed the most critical base of this triplet (Fig. 1).

This leaves unanswered whether the 3–5 code is still functional in the sense of a direct noncovalent screening of the amino acid at this position. The G3–U70 base pair is a critical determinant for the alanine tRNA synthetase reaction even when it is part of a microhelix of only 7 bp, which suffices for correct alanine charging (Musier-Forsyth et al. 1991a). However, the possible extent to which the amino acid alanine itself senses the bases at position 3–5 during the charging reaction is less clear. Whether such a probing device still works, or has long since been subsumed by the synthetase, is worth testing. The unpaired, exocyclic amino group of the guanine in the G3–U70 base pair is a critical determinant for aminoacylation with alanine (Musier-Forsyth et al. 1991b). Insight into the origin of the code would be advanced by a critical reexamination of the total charging reaction of tRNAs and their fragments.

Two Possible Classes of tRNAs and Synthetases

It is tempting to think that the two classes of tRNAs as observed by us gave rise to the evolution of two distinct types of synthetases, whose members fall in the standard classification of present-day class I or II (Burbaum and Schimmel 1991). These two classes are defined on the basis of sequence comparisons and with exception of valine, synthetases for primordial amino acids belong to class II (Eriani et al. 1990). Moreover, the amino acids derived metabolically from primordial amino acids, like lysine, serine, and threonine, also react with synthetases of class II. Furthermore, amino acids, which do not form easily in gas-sparking experiments (Miller 1986), tend to show a preference for class I synthetases, which have the structural domain (the Rossman fold) that binds ATP (Rould et al. 1989). Judging from the lack of such a classical nucleotide-binding fold in class II enzymes (Cusack et al. 1990; Eriani et al. 1990), one can envision that protosynthetases for primordial amino acids were the precursors of present-day synthetases of class II and that class I enzymes evolved separately.

Conservation of the 3-5 Code in Different Kingdoms

Why are vestiges of the 3–5 protocode observed in prokaryotes but hardly at all in eukaryotes? The most simple explanation would be that this molecular fossil has only been preserved in prokaryotes because they constitute the most ancient forms of life on this planet. A clue may be provided by aerobic bacteria that have found refuge in anaerobic niches for eons with little adaptive pressure from outside: the same may apply to the methanogenic archaebacteria (Woese 1989). For some reason, in eukaryotes the specific contacts between tRNA and synthetase may have been less dependent on the 3–5 code, as a result of which they have lost this code under genetic pressure. The same type of division between the different kingdoms emerges when one analyzes two other properties, the conservation of the RNY code (Eigen and Winkler-Oswatitsch 1981a,b; Winkler-Oswatitsch et al. 1986) and the mode of ribosomal protein operon organization (Shimmin et al. 1989). Archaebacteria and eubacteria possess the RNY code and eukaryotes do not. The same trend is present for ribosomal protein gene organization. However, sequence homology between ribosomal proteins (Amons et al. 1977; Matheson et al. 1980; Ramirez et al. 1989) or translation factors (Auer et al. 1989) is greater between archaebacteria and eukaryotes than between either of these and eubacteria.

This apparent paradox, however, may be false because the evolution of tRNA, the RNY code, and RNA operon organization may have long preceded that of ribosomal protein and factors: the latter may have been refined to its present state later on when the ribosomal machinery increased its accuracy and rate. It could be that ribosomal proteins and factors of archaebacteria and eukaryotes have undergone and are still undergoing rapid evolution to arrive at the streamlined state reached much earlier by eubacteria (Darnell and Doolittle 1986).

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