

**GENE STRUCTURE AND UPSTREAM REGULATORY REGIONS OF HUMAN
CYP2C9 AND *CYP2C18*¹**

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There is a genetic polymorphism in humans in the metabolism of *S*-mephenytoin which has been suggested to be mediated by either *CYP2C18* or *CYP2C9*. We have isolated genomic clones for *CYP2C9* and *CYP2C18* from the liver of an individual phenotyped *in vitro* as an extensive metabolizer of *S*-mephenytoin. Analysis of the genes reveals nine coding exons spanning approximately 55 kb. The intron-exon organization was similar to that of other members of the *CYP2C* subfamily. Analysis of 2200 bp of 5' upstream sequence for *CYP2C9* and 1300 bp 5' upstream sequence for *CYP2C18* reveals canonical TATA boxes situated 57 bp upstream from the first codon, multiple consensus sequences for glucocorticoid regulatory elements, and identification of a 15 base sequence with high homology to a 5'-flanking sequence responsible for barbiturate-inducible expression of P450_{BM-3} in *Bacillus megaterium*. The upstream region for *CYP2C9* was highly homologous (75%) to that of human *CYP2C8* through most of the 2200 bp sequenced, but the upstream region of *CYP2C18* was similar to *CYP2C8* and *CYP2C9* for only the first 200 bases. The availability of the sequences of the upstream regions and intron-exon junctions of *CYP2C9* and *CYP2C18* will allow future analysis of these genes in humans which differ in their ability to metabolize *S*-mephenytoin and other drugs.

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The human cytochrome P4502C family metabolizes drugs such as mephenytoin, tolbutamide and barbiturates (1,2). There is a genetic polymorphism in the 4'-hydroxylation of the *S*-enantiomer of mephenytoin in which approximately 3% of Caucasians and 20% of Orientals are deficient in the ability to metabolize this drug (3,4). This reaction is thought to

¹The sequences reported in this paper have been submitted to GenEMBL/Genbank, under accession numbers: L16869 through L16876 for *CYP2C18*, and L16877 through L16883 for *CYP2C9*.

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be metabolized by a member of the CYP2C subfamily. Both recombinant CYP2C9 (5) and CYP2C18 (6) have been reported to 4'-hydroxylate *S*-mephenytoin. However, other conflicting reports suggesting little or no metabolism of *S*-mephenytoin by recombinant CYP2C9 when expressed in a variety of cDNA expression systems (6-8) and the intermediate turnover numbers of recombinant CYP2C18 for 4'-hydroxylation of *S*-mephenytoin compared to human liver microsomes make the identity of the CYP2C gene responsible for this polymorphism still uncertain.

To date, the only information available on the gene structure of the human CYP2C subfamily is the sequence of the 5' regulatory region for CYP2C8 (9) and a partial gene structure for CYP2C18 covering 932 bases of 5'-flanking region and the intron/exon junctions of exons 1-4 (10). As a first step in the analysis of the molecular basis for the polymorphism in *S*-mephenytoin metabolism, we have isolated and sequenced genomic clones for the CYP2C9 and 2C18 genes from an individual with high hepatic *S*-mephenytoin 4'-hydroxylase activity.

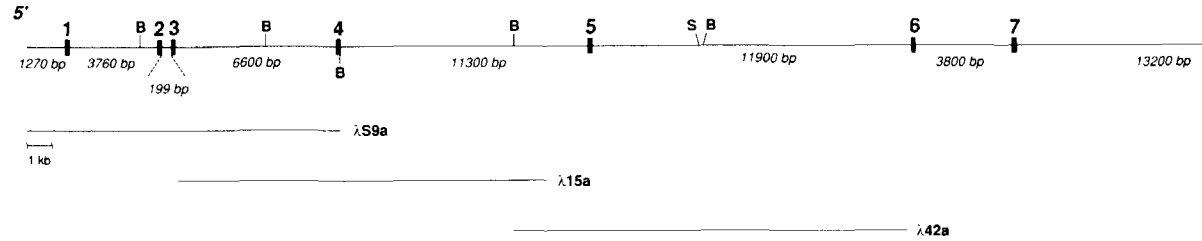
MATERIALS AND METHODS

Preparation and Screening of Genomic Library: A genomic library was constructed in bacteriophage EMBL3 (Stratagene) from liver DNA of an individual phenotyped *in vitro* as an extensive metabolizer of *S*-mephenytoin (0.8 nmol/min/nmol P450). The library was screened by *in situ* plaque hybridization (11) using CYP2C18 or CYP2C9 cDNA probes (12,13). Additional probes used included a 5'-fragment of CYP2C18 (from -199 to +127 bp of exon 1) and a PCR fragment containing exons 5 and 6 of CYP2C18. Fragments of positive clones derived from restriction analysis were subcloned into pBluescript SK vectors (Stratagene) and double-stranded DNA templates were isolated and sequenced using exon-specific synthetic oligonucleotides as primers, and Sequenase kits (Stratagene) or an automated sequencer (Applied Biosystems, Inc.). Additional primers were synthesized to completely sequence the 5'-flanking regions in both directions.

RESULTS AND DISCUSSION

A map of the genomic clones and the gene organization for CYP2C9 and CYP2C18 is shown in Figure 1. We obtained a complete set of overlapping clones for CYP2C18. Exon 6 of CYP2C9 was missing, but the nucleotide sequences of the remaining exons were identical to those of the Leu³⁵⁹ variant of CYP2C9 cDNA (12). Both genes are much larger than their rabbit and rat counterparts (14-17), with 2C18 spanning approximately 55 kb. The intron-exon junctions and polyadenylation sites for CYP2C9 and 2C18 are shown in Table 1. The intron-exon structure appears to be conserved in the CYP2 family, comparing the human genes to the rabbit (14) and rat CYP2 genes (15-17). All CYP2 genes have nine exons, and there is a very high homology in the amino acids at the exon-intron junctions. A variant cDNA clone for CYP2C9 has been reported which has a six-base deletion at positions 820-826 (18). The missing

CYP2C18



CYP2C9

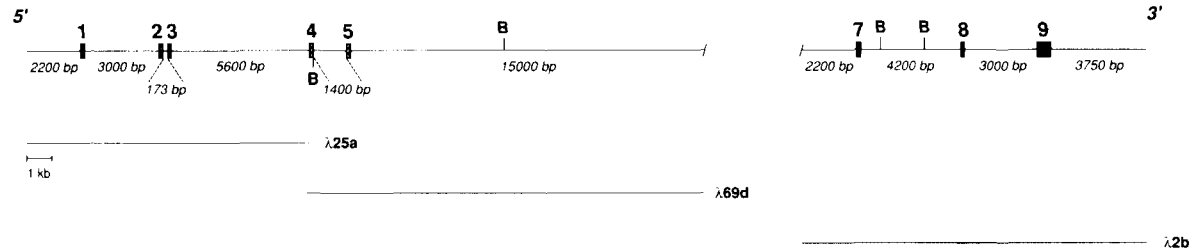


Fig. 1. Representation of the structure of *CYP2C9* and *CYP2C18* genes, and alignment of λ -EMBL 3 clones. Clones were isolated and characterized by restriction analysis, partial sequencing, and by hybridization studies to determine overlapping regions. DNA fragments were subcloned into pBluescript vectors for sequence analysis. Exons were located by restriction analysis and by PCR analysis using exon specific primers and primers located in the vector cloning sites. Overlapping areas are indicated by the positions of the clones on the diagram.

TABLE 1. LOCATION OF INTRON-EXON BOUNDARIES IN *CYP2C9* AND *CYP2C18*

Intron	Gene	Donor	Intron size	Acceptor
1	2C9	<u>N56</u> CTTAACCAATgtaagtatgc	3000	L57 actttccttagCTCTCAAAGG
	2C18	CTTAACCAATgtaagtatgc	3750	ctatgttagTTCTCAAAG F57
2	2C9	<u>G111</u> AGAGGATTGgtaggtgtgc	173	G111 ttcctgttagGAATTGTTTT
	2C18	AAAGGACTTgtaaatgtgc	191	gttctgttagGAATCCTTT
3	2C9	<u>A161</u> AAAACCAAGGgtgggtgacc	5600	A161 aattatttagCCTCACCTG
	2C18	AAAACCAATggtgggtgact	6600	aatccttagCCTCACCTG
4	2C9	<u>Q214</u> CTGGATCCAGgtaaggccaa	1400	I215 tttctcttagATCTGCAATA
	2C18	ATGGATCCAGgtgagatcaa	11300	aatctttaagGTCTGCAATA V215
5	2C9	<u>K273</u> AATGGAGAAgtaaaatgta	>17200	E274 GAAAAGCACACA
	2C18	AATGGAAACgtaaaatggtt Q273	11900	aattttttagGAAAAGCACACA
6	2C9	<u>A321</u> GAGGTCACAG	>2200	A321 gtcttatcagCTAAAGTCCA
	2C18	GAGGTCACAGgtatgatgat	3800	gtcttatcagCTAAAGTCCA
7	2C9	<u>K383</u> CATCCCAAGgtaagtttgt	4200	G384 ttgtttctagGGCACACCA
	2C18	CATCCCAAGgtaagcttgt	13200	ctattttcagGGCATGACCA
8	2C9	<u>G431</u> TTCTCAGCAGtaataataaa	3000	G431 ctattttcagGAAAACGGAT
	2C18	TTCTCAGCAGgtaatagata	1750	ttattttcagGAAAACGGAT
Poly(A) Site	2C9	TTCTAAATAAAAAGCATTATTATTGCTGAvgtcagtttat		
	2C18(1976 ^a)	TGATTAATAAATGACAATTCAGAGCCvATTTATTCTCT		
	2C18(2197 ^a)	GTCTAAATATATGCTTTCATATGGC•TAATCATgtgttaatga		

Exons are shown in upper case and introns in lower case. Codons at junctions and polyadenylation sites are underlined and the amino acids indicated above the sequences. vLocation of polyA tail or *longest cDNA clone. ^aRefers to positions in the *CYP2C18* (6b allele) cDNA.

bases are located at the intron 5-exon 6 junction and contain a possible alternative splice acceptor site (GAAAAG), explaining the probable mechanism by which this aberrant 2C9 cDNA was generated. The *CYP2C18* gene isolated in the present study was identical to the 6b allelic variant (Met³⁸⁵) (12,13). An additional clone was isolated which contained exons 8 and 9 of the 29c allelic variant of *CYP2C18* (not shown). Both clones contained the additional 420 bases found in the 3'-noncoding region of the cDNA for the 6b variant but not found in the cDNA for the shorter 29c variant. Exon 9 of both *CYP2C18* clones contained two alternative polyadenylation signals, making this exon longer (≥ 925 bases) in *CYP2C18* than in *CYP2C9* (545 bases).

The nucleotide sequences for the 5'-flanking regions of *CYP2C9* and *CYP2C18* are shown in Figure 2. *CYP2C9* contains several consensus sequences for glucocorticoid response elements

CYP2C9

GATCTCAGATATCCCTTCTATCTACACATTATCTATAATCTCTTT

-2100 TTTCGTAAATGAAAGGTCCTAGAAGGAGCCGACGCTCAGCAGGAGAGAGGAGGAGCTGAGCTGGGACCCTACCTCCTGAGGAATGAAATGATTATTA

-2000 TAAAGACAGCAACCGAGCTTATTTTACCCAAAATAAGGTAGTATATTTCTGTTAGAGTTTAGAGTTTCATGAGTCAGGGACCAAGTTATTGCTTTCTTT
AP-1 HNF-1

-1900 GCCTGTATAAAGGCTTCTCCAAAGCCTTTGACTTACCTAAGTACTAAATGTTATAAAACCAAACCTCTTCTGACCTCTCAACTAGTCAACTGGGGCTGT
C/EBP/Rev TATA

-1800 AATTATTAATGAAATTAATGTTTATTTGAAAATAATTACTAGACTGAATACCGAACTCTGAATCATTTGACACTATCAGTAAATATTGGTGGACCCA
C/EBP/Rev AP-1

-1700 ACTGAATCAATGTTTGGCTTGAATGAAACCTTTGAGATGCAGGGCTTATGGGTTCTAGTCCCACTGACTAGCAGCAGCATGTTCTTGGCTAA
GRE

-1600 GATACTGAATCTCAAGGCTCAGCTTCTCATTCGGAAATGGGTCAATTTTATGTAAGCAGAGGTAATTGAGAGATTCAAAGGGACATGAGGTGTAA

-1500 CAATCTCTGTAATTTGTTAGAAATCCCTGTTAAAAATGACCAGTAAAGCTTTGTGCAACTGTGTCTTGACATAAATTTATTTCTTAAATAAAGAAATG
Barbie Box

-1400 GAAATAACCTCACTAGGAAATTTAGAAACAAATATGATGATATCTTTAAAGAAATGGCTTTGCACAAGTATTGACATTAATGATCTAGTAAAGTGTATCT
GRE/Rev

-1300 TTCTAGTGTATTTAGATCCCTCAACTCAGTATGTCAGCTCCTGTTAAGGCTATACATTTGGTGGTCTGTGCTGTGGGTCCATTTAGTGATTTCCCTA

-1200 CCTCCCATCTCTTATGCAATCCACAACCTGTGGTCTGTCATTAATTTCTCTTTGCTTTCTGTGCATTATTACATCATATCTGAAAATGAGAAACCAAAAAAC
C/EBP/Rev

-1100 AATAGAAAGCAGCCATGCTGGAGGTGACTGGGGGTCGAGAAGCCCTAGTTTCTCAAACCTTAGCACCAAAATTTTCCCTCAGTTACACTGAGCGTTT
Barbie Box

-1000 CACCTCTGCAGTGTAGGAAAGGGAGATCCCTTATTTCTCTCATGAGCATCTCTGGTGTCTTTCCCTTAGAGACAAAATAAGGGGTCTTATTTAATGTG

-900 AAGCCTGTTTATGAACAGAATAAATGTGGTGTATTTAGAAATAAATGTTGGAAAGTGGTTTATTTTTCGTAATAAATTTGTTCTCAAGCGAGCTCT
GRE

-800 GCTGTAAAGAGATAATACACCAGATGGGCAATCAGAAGCCTCAGCTCAATCCCACTTCTCCAGCTATGAGCTGTGTGGCCACCAAGGTTGCTCTGTTT
C/EBP GRE

-700 TCCACAGGCTCTCCCTTTTCCCAATTTGAAAAATAAAAAATAACAATTCCTGCCTTCAGGAATTTTTTTTAGGGGGTTTAAATGGTAAAGGTGTTATATCTG

-600 CTAAGGTAATTTACTTTGATATATGTTGGTTATTTAAGATATATGAGTATATGTTAGCTATTTTCATGTTTAGGCTGCTGATTTTTAGTAGCTATATTA

-500 ATATTTGAAAGGATTTTCATTTATAAAGAACAAAGTCTCCTAATCTTTGATATAGCATTGACATACTTTTTAAATATACAAGGCATAGAAATGGCCATTTC
GRE/Rev TATA AP-1

-400 TGTTAAATCATATATTTCCCACTGGTATTAAATCTAAGAATTCAGAATTTTGAGTAATTTGCTTTTGCATCAGATATTACTTTCAGTGTCTCAATATAG
C/EBP

-300 ATGGTGCATTAGAACCATCTGGGTTAACATTTGTTTTTATTACCAATACCTAGGCTCCAACCAAGTACAGTGAACCTGGAATGTACAGAGTGGACAAATG

-200 GAACGAAGGAGAAACAAGACCAAAGGACATTTTATTTTATCTGTATCAGTGGGTCAAAGTCTTTTTCAGAAGGACATATAGTGGACCTAGGTGATTTGGTC
GRE/Rev HNF-1 CAAT/Rev

-100 AAATTTATCCATCAAGAGGCCACACCCGAATTAGCATGGAGTGTATAAAGGCTTTGGAGTGCACGCTCATGGTTGCTTTACAAGAAAGAGAGGCTTCA
TATA

1 ATG

CYP2C18

GATCTCATTTTCAAGGAAAATGATCATAGTGTGGAGAAAGCATTATAGTGTGAGTCTTTCGACATCCT

-1200 TAGCTCAGCTAGGTCGAGTGTCTTATGCAAGGAAATAGGCACACAGACACTGGAGAGTGAAGTGGAGTAGAATTTATTAAGTAAAGGAAAACCTCTC
HNF-1

-1100 AGCAAAGAGAGAGGCCCTGAAGCAGGTTGCAGATTTGCCACTTCACAGTTGAATACCAGGACTTTTATAGAAAAGCTGATGAGGCTGGGATACATTATTTG
Barbie Box Sp1

-1000 CATAAGGCGTGAATTCCTGGTGTTCACACCCTTCCCTCCATTGGCCGCTGTGGGCTCTTAGTCTAAGCCACTCCATATGATTTATTTCCCTTCCTGCCG
CAAT

-900 ATGTGTTAAAGTAGGAATTTTCACTGTGGCATATTTAGGCAAGCTCCCTGTGCAAGTCCCTTATCTGCACAAAACATCTAGTSTAAGTACTTGGGG
C/EBP

-800 TTTTTGTGGATTGGCCAAATGACCTGGAAGGGTTGGAGGTTCTCTGGGGACCCTTCCCTTACTGCCTGCCTAATGCAAGCTGGCTAACTCCTCAATAGG
C/EBP AP-1

-700 ATAAACATTTATTTATGTACAAGGAATATAATACACAGATTTGCCCTCAAAGTCATATTTCCCACTGCTCATCAATCTAAAAATCCAAAATTTTGAATAAT
TATA HNF-1

-600 TTTTTGATGAAATAATTTATTTTCAATTTTGGCTGCACAGTGGAAACCCCTGGGCTGTTTGA AAAAACCTAGCCCTAGCCAGGCACGGTGG

-500 CTCATGCCTGTAATCCAGCACCTTAGGGAGACAGCCTGGCCAAACATGGTGAACCCCTGTCTCTGCTAAAAATACAAAATCACCTGGGCTAGTTGTGCA
AP-2 DBP/Rev Sp1

-400 TTCTGTAAATCCAGCCACGCCAGAAGCTGAGGCAGAATCGTTCGAATCCAGGAAGTGGAGGCTGCAGTGAATTTGAGATCTTGCCATTGCATCCAGCCT

-300 GGCAACAAGAGCGAAATCCATCTCAAGGAAAAACAACAACAACAACAATAAATCCTGGGCTCTGCTTCCAGAGTAGTTAAACAGAAATCTCCAGGGTG

-200 GGCACCGGAAAGAACAGAAAAAAGAACCTTATTTTATCTTCTCAGTGCAGCAATGTTCAATCAAAGAGAGATTAAGTGTCTTTTGTCTGACTA
GRE GRE CAAT

-100 GTCACACTCAGAGTCAGAATCACAGGTGGATTAGTAGGAGTGTATAAAGCCTTGAAGTGAAGCCCCAGTGTCTTACTAAGAAAGAGAACCTTCA
TATA

1 ATG

Fig. 2. Nucleotide sequence of the 5'-flanking region of the *CYP2C9* and *CYP2C18* genes. Nucleotides are arbitrarily numbered in negative numbers from the ATG coding for the initiation codon. The sequences of putative binding sites for transcription factors are underlined, as are presumed TATA and CAAT boxes. Arrows indicate palindromic sequences.

(GREs) within the 2200 bp of 5'-upstream sequences analyzed. GRE consensus sequences also occur in the upstream region of *CYP2C18* and human *CYP2C8* (9,10). A 15 base sequence was identified in the upstream regions of both *CYP2C18* and *CYP2C9* which had high homology (7-8 of 15 bases including the prerequisite AAAG at bases 5 to 8) to a 5'-flanking sequence responsible for barbiturate-inducible expression of P450_{BM-3} in *Bacillus megaterium* (19). A similar sequence has been identified in the 5'-flanking region of a number of phenobarbital inducible genes including *CYP2B1* and *CYP2B2* (20). The presence of these sequences in the upstream regions of human *CYP2C18* and *CYP2C9* suggests the possibility that these cytochromes may be inducible by glucocorticoids and phenobarbital.

A comparison of the regulatory regions of the three human P450C genes using the COMPARE/DOTPLOT program (Genetics Computer Group, Madison, WI) shows that the 5'-flanking region of *CYP2C9* is highly similar (75%) to that of *CYP2C8* through most of the available 2200 bp sequence, with only one stretch of *CYP2C8* between -684 and -739 showing no homology to *CYP2C9* (Fig. 3). We reexamined the sequence of 2C9 in this area and found no comparable sequence in this area. However, comparison of the sequence of *CYP2C8* with itself in the reverse orientation indicated that this area was almost an exact reverse repeat of bases -54 to -112, suggesting that this sequence may have been accidentally inserted twice in *CYP2C8* during the sequence compilation. In contrast, the upstream region of *CYP2C18* showed homology with *CYP2C9* and *CYP2C8* for only the first 200 bases. The position of one TATA box (57 bp upstream from the ATG start codon) is conserved in all three human 2C genes, but

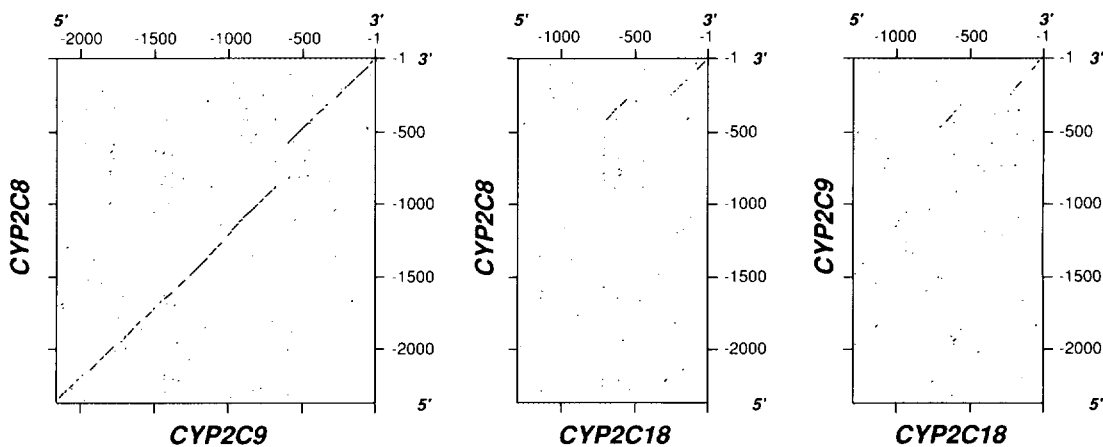


Fig. 3. Comparison of the 5'-flanking regions of human *CYP2C* genes. Sequences were compared by dot matrix analysis (COMPARE/DOTPLOT, GCG) in which the best match is plotted at each position for a sequence window of 21 nucleotides. The number -1 denotes the first nucleotide prior to the ATG coding for the initiation codon.

CAAT boxes are located at different positions: at -147 bp for *CYP2C18*, at -110 bp for *CYP2C9* (in reverse orientation) and at -113, -118 and -123 bp for *CYP2C8*. The transcription start site for *CYP2C8* (9) has been reported to be located 23 bp upstream of the ATG, consistent with those of the rabbit and rat *CYP2C* genes. Interestingly, *CYP2C18* contains an additional TATA box, located at -674 bp and an additional CAAT box at -935 bp. This may indicate two start sites for *CYP2C18* transcription, and could explain the isolation of a *CYP2C18* cDNA clone containing 200 bases of 5'-noncoding leader sequence (12,13).

Promotor regions of *CYP2C18* and *CYP2C9* contained a motif which agreed with the consensus sequence for the HepG2-specific factor 1 (HPF-1)(21) at positions -650 and -150 bp, respectively. This sequence, which is similar to the consensus sequence for HNF-4, has been found to be essential for the expression of the rabbit *CYP2C* genes in HepG2 cells but not for expression in COS-1 cells. It appears to be conserved in the *CYP2A*, *CYP2C*, and *CYP2D* genes, and it may be important for the liver-specific expression of these genes. Sites for other liver-enriched transcription factors common to the two genes include: C/EBP, HNF-1, and AP-1 (Figure 2). *CYP2C18* also contains sites for AP-2, Sp1, and DBP, that are not found in *CYP2C9* (Figure 2). DBP is a liver transcription factor which has been shown to play a role in the developmental regulation of expression of the rat *CYP2C6* (22). Additional studies will define the role of these regulatory elements in the expression of the human *CYP2C* genes. The determination of the sequence of the 5'-upstream regions and the exon-intron junctions of *CYP2C9* and *CYP2C18* will allow analysis of these regions in humans differing in their ability to express *S*-mephenytoin 4'-hydroxylase.

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