Relationship of Electrochemical Oxidation of Catechins on Their Antioxidant Activity in Microsomal Lipid Peroxidation

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The oxidation potentials of catechins were measured by employing flow-through column electrolysis. The oxidation potentials of catechins were shown to depend on their structures. At the same time, the antioxidant activity of catechins on NADPH-dependent lipid peroxidation in rat liver microsomes was evaluated. Catechins showed a 50% inhibition of lipid peroxidation in the concentration range of 10—51 μM. Among those studied, galloylated catechins exhibited stronger antioxidant activities than those of nongalloylated catechins. A quantitative relationship has been obtained to describe the antioxidant activity of catechins: $\log IC_{50}$ (μM)$=1.56+2.49E_{1/2}$ (V)$-0.29 \log P$ ($r=0.907$), where IC_{50} represents the concentration for 50% inhibition of lipid peroxidation, $E_{1/2}$ represents the half-wave potential of the first oxidation wave, and P represents the octanol/water partition coefficient. This relationship suggested two important characteristics determining catechin antioxidant activity, namely the ease of oxidation and the lipophilicity.

Key words catechin; oxidation potential; microsomal lipid peroxidation; lipophilicity

Catechins (Fig. 1), the main constituents in green tea, have attracted lasting interest because of their wide biological effects. Recently, the antioxidant activity (AA) of catechins has been widely reported. Catechins effectively suppressed lipid peroxidation (LPO) in biological tissues and subcellular fractions such as mitochondria, microsomes, liposomes, and low-density lipoproteins (LDL).[1—5] On the other hand, they exhibited scavenging activity on free radicals, such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, superoxide anion, hydroxyl radicals, and singlet oxygen.[6—11] However, absolute as well as relative efficiency of catechins seems to vary from one assay to another, thus obscuring the structure–activity relationship. The specific mode of inhibition of LPO by catechins is not clear; they may act by chelating transition metals to inhibit the decomposition of lipid hydroperoxide (LOOH), or by scavenging chain-propagating peroxyl radicals, because the reduction potential of catechin radicals was reported to be lower than those of alkylperoxyl and hydroperoxyl radicals.[12]

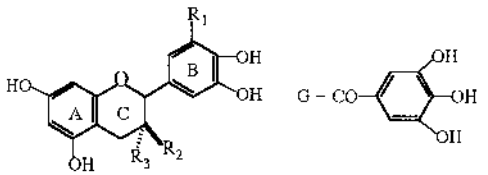
In the present work, we evaluated the AAs of catechins on reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent LPO in rat liver microsomes. A quantitative relationship was established, linking the AAs of catechins to their oxidation potentials and lipophilicity.

In our previous work,[13] we determined the oxidation potentials of flavonoids using cyclic voltammetry and flow-through column electrolysis, and found that variation existed between the half-wave potential ($E_{1/2}$) obtained from hydrodynamic voltammograms and the midpoint potential (E_{mid}) obtained from cyclic voltammograms. When using cyclic voltammetry, some compounds were irreversibly oxidized along with the adsorption or kinetic reaction, resulting in the inaccurate data. In the case of flow-through column electrolysis, the large surface area of the working electrode made high efficiency and rapid electrolysis possible, and thus less interference reaction was expected to affect the electrode process compared to cyclic voltammetry. In this paper, the oxidation potentials of catechins were determined using flow-through column electrolysis instead of conventional cyclic voltammetry.

Experimental

Materials (+)-Epicatechin [(+)-EC] (>98%) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). (−)-Epicatechin [(−)-EC] (>98%), (−)-gallocatechin [(−)-GC] (>98%), (−)-gallocatechin gallate [(−)-GCg] (>98%), (−)-catechin gallate [(−)-Cg] (>98%), (−)-catechin [(−)-C] (>98%), (−)-epigallocatechin [(−)-EGC] (>98%), (−)-epicatechin gallate [(−)-ECg] (>98%), (−)-epigallocatechin gallate [(−)-EGCg] (>98%), (+)-catechin [(+)-C], and polyphenol extract from tea were obtained from Kurita Industrial Co. (Tokyo, Japan). All these chemicals were used as received. Water was purified through a Nanopure Q-plus purification train (Nanopure II, Sybron Branstead, U.S.A.). NADPH, adenosine 5′-diphosphate monosodium salt (ADP), $FeCl_3$ and other reagents were from Wako Pure Chemical Industries, Ltd.

Voltammetry and Flow-Through Column Electrolysis The half-wave potential of the catechins and the number of electrons transferred in the oxidation process were determined using flow-through column electrolysis. The system consisted of a Jasco PU-980 pump (Jasco, Tokyo, Japan), a Jasco DG-980-50 Degasser (Jasco, Tokyo, Japan), an 8125 injector fitted with a 5-μl injection loop (Reodyne, Cotati, U.S.A.), a Potentiostat/Galvanostat HAB-151 (Hokuto Denko Ltd., Tokyo, Japan) and an LR 4110 Recorder



Catechins	Abbreviations	R_1	R_2	R_3
(-)-(2S,3R)-Catechin	(-)-C	H	H	OH
(+)-(2R,3S)-Catechin	(+)-C	H	OH	H
(-)-(2R,3R)-Epicatechin	(-)-EC	H	H	OH
(+)-(2S,3S)-Epicatechin	(+)-EC	H	OH	H
(-)-(2S,3R)-Gallocatechin	(-)-GC	OH	H	OH
(-)-(2R,3R)-Epigallocatechin	(-)-EGC	OH	H	OH
(-)-(2R,3R)-Epigallocatechin gallate	(-)-EGCg	OH	H	OG
(-)-(2S,3R)-Gallocatechin gallate	(-)-GCg	OH	H	OG
(-)-(2S,3R)-Catechin gallate	(-)-Cg	H	H	OG
(-)-(2R,3R)-Epicatechin gallate	(-)-ECg	H	H	OG

Fig. 1. Structures of Catechins

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(Yokogawa, Japan). The working electrode was carbon fiber threads (Nihon Carbon GF-20-P7 carbon cloth) packed in a Vycor glass cylinder (4 mm inner diameter and 10 mm length). The reference electrode was an Ag/AgCl electrode. The counter electrode was a platinum wire. The carrier solution was methanol: 0.1 M phosphate buffer (pH 7.5) (1 : 1, v/v). The peak current on a current–time curve obtained by column electrolysis was measured and plotted against the applied potential to obtain the hydrodynamic voltammogram, from which the half-wave potential was measured.

At the same time, cyclic voltammetry was used to observe roughly the redox behavior of the catechins. Cyclic voltammograms of the catechins were recorded with an HAB-151 potentiostat (Hokuto Denko Ltd., Tokyo, Japan) provided with a 3036 X-Y Recorder (Yokogawa, Tokyo, Japan), in methanol: 0.1 M phosphate buffer (pH 7.5), (1 : 1, v/v). A plastic formed carbon disk electrode served as a working electrode, an Ag/AgCl electrode was used as the reference electrode, and a platinum electrode was used as a counter electrode. To prevent possible air oxidation of the flavonoids in the experimental pH range, oxygen-free nitrogen was used to thoroughly purge the solution being analyzed before the measurement and to pass above the solution during the recording of the voltammograms. Before each measurement, the working electrode was polished thoroughly with a polishing cloth and sonicated in water.

Microsomal Preparations Wistar rats (♂, *ca.* 300 g, 13 weeks) were decapitated. Livers were excised immediately after being washed with 1.15% KCl *via* the portal vein, and stored at −80 °C for later use. The livers were thawed in ice-cold water and homogenized in ice-cold phosphate buffer [1.15% KCl, 10 mM KH_2PO_4–K_2HPO_4 buffer, pH 7.4, 1 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 0.5 mM dithiothreitol (DTT), 3 ml/g tissue]. The homogenate was centrifuged at 10000×***g*** (20 min at 4 °C). Subsequently, the supernatant was centrifuged twice at 105000×***g*** (60 and 20 min). The microsomal pellet was resuspended in phosphate buffer (50 mM KH_2PO_4–K_2HPO_4 buffer, pH 7.4, 0.1 mM EDTA), and stored at −80 °C. The protein concentration was determined by the Bradford method using the Bio-Rad protein assay (Bio-Rad Laboratories, U.S.A.).

LPO Assay LPO was monitored by detecting the formation of malondialdehyde (MDA), using the thiobarbituric acid (TBA) assay.[14] Briefly, 20 μl of liver microsomes (26.8 mg of protein/ml) in 0.05 M Tris–HCl solution (pH 7.4) were incubated at 37 °C for 30 min in a water bath with 30 μl of 9.2 mM NADPH, 20 μl of a solution containing 94.7 mM ADP and 1.6 mM $FeCl_3$ ($ADP–Fe^{3+}$), and 10 μl of catechins at different concentrations in 50% methanol aqueous solution, through which oxygen-free nitrogen was passed for 20 min. The total volume of the incubation mixture was 1 ml. All substances were added on ice. Incubations were quenched by the addition of 50 μl of an aqueous solution of trichloroacetic acid (TCA) (100 w/v%). Upon cooling on ice and subsequent centrifugation (10000 rpm, 10 min), the supernatant solution was mixed with TBA solution. The reaction mixture was heated in a water bath at 80 °C for 60 min. After cooling, the absorbance at 532 nm was read by a Jasco V-550 UV/Vis spectrophotometer (Jasco, Tokyo, Japan). The AA value was calculated as percent inhibition relative to control using the following equation:

$$\mathrm{AA}\ (\%)=\frac{(A_{c(30)}-A_{c(0)})-(A_{s(30)}-A_{s(0)})}{(A_{c(30)}-A_{c(0)})}\times 100 \quad (1)$$

where $A_{c(30)}$ and $A_{c(0)}$ is the absorbance of the control at $t=30$ min and $t=0$ min, respectively; and $A_{s(30)}$ and $A_{s(0)}$ is the absorbance of the sample at $t=30$ min and $t=0$ min, respectively. The A values used were the average data of three measurements. The IC_{50} values were determined by interpolating the 50% inhibition point on a straight line fitted through concentrations that resulted in 10 to 90% inhibition.

The octanol/water partition coefficients of the catechins were calculated using a computerized version called PrologP 5.1 (CompuDrug Chemistry Ltd.). The multiparameter regression analysis was performed using NLRAna software (version 4.1f).

Results and Discussion

Electrochemical Oxidation The cyclic voltammograms and the hydrodynamic voltammograms of the catechins representing the different types of electrode processes are shown in Figs. 2 and 3, respectively. Although the oxidation peaks (I_a—III_a) in the cyclic voltammograms corresponded to the oxidation waves (I_a—III_a) in the hydrodynamic voltammograms, the peak separation was not clear and it was difficult to determine the $E_{1/2}$ value of each peak. On the other hand, in the hydrodynamic voltammograms, the oxidation wave was evident. Accordingly, the oxidation waves for different types of catechins could be classified as waves I_a—III_a. I_a (E_p was *ca.* 0.000 V) may correspond to the oxidation of the 3′,4′,5′-trihydroxyl substituent on the B ring. This oxidation was not observed for catechins without an *ortho*-trihydroxyl substituent on the B ring. II_a (E_p was *ca.* 0.200 V) was re-

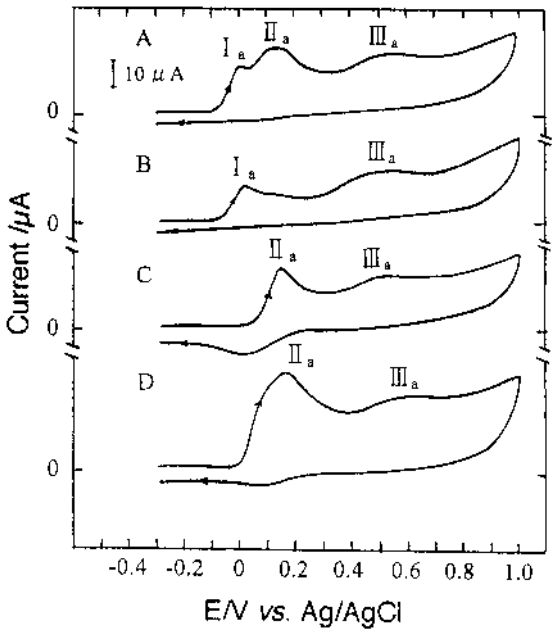

Fig. 2. Cyclic Voltammograms of Catechins in Methanol: 0.1 M Phosphate Buffer (pH 7.5) (1 : 1, v/v) with a Scan Rate of 100 mV/s

A, (−)-EGCg (0.48 mM); B, (−)-GC (0.56 mM); C, (−)-C (0.52 mM); D, (−)-ECg (0.62 mM).

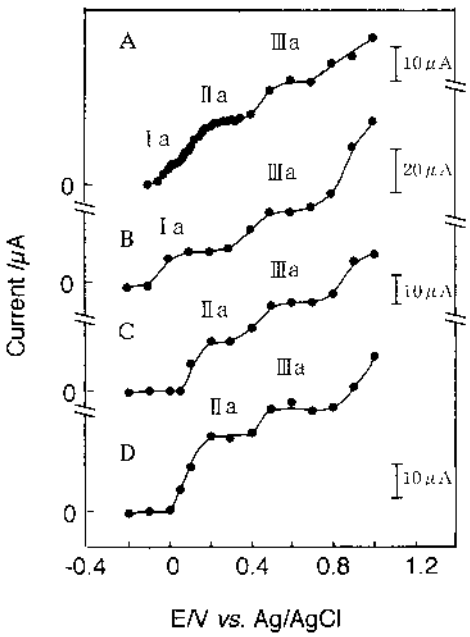

Fig. 3. Hydrodynamic Voltammograms of Catechins in Methanol: 0.1 M Phosphate Buffer (pH 7.5) (1 : 1, v/v) Using Flow-Through Column Electrolysis

A, (−)-EGCg (0.14 mM); B, (−)-GC (0.31 mM); C, (−)-C (0.18 mM); D, (−)-ECg (0.11 mM).

versible or quasi-reversible due to the oxidation of the 3′,4′-dihydroxyl substituent on the B ring, or/and the oxidation of the galloyl moiety at the C-3 position. The final oxidation may correspond to the oxidation of the 5,7-dihydroxyl substituent on the A ring.[15)]

The half-wave potential of the first oxidation potential (I_a or II_a depending on the substrate) of the catechins obtained from the hydrodynamic voltammograms is shown in Table 1. The range was from −0.035 V [(−)-EGC] to 0.102 V [(+)-C]. The number of electrons (n) transferred in the oxidation was calculated from the electrolytic charge Q, determined from the peak area under the current–time wave, at a potential of *ca.* 0.1 V more positive than $E_{1/2}$, and the Faraday equation written as:

$$Q=Fcvn \quad (2)$$

where n is the number of electrons, c is the concentration (mol/l) of catechins, v is the solution volume (l), and F is the Faraday constant (96500 coulomb/eq). The results are summarized in Table 1.

AA The catechins showed obvious AA on NADPH-dependent lipid peroxidation in rat liver microsomes (Table 1). Good linear relationships between concentration and activity were found for 0—75 μM (+/−)-C and (+/−)-EC; for 0—50 μM (−)-GC; for 0—30 μM (−)-GCg, (−)-EGC and (−)-Cg; and for 0—15 μM (−)-EGCg. In the case of (−)-ECg, when the concentration was 15 μM, its inhibitory effect reached 98.7% inhibition. In Table 1, IC_{50} values were determined by interpolating the 50% inhibition point on a straight line fitted through concentrations which result in 10 to 90% inhibition. The IC_{50} values of the catechins were shown to be in the range of 10—51 μM. Their order of effectiveness was (−)-ECg>(−)-EGCg>(−)-GCg=(−)-Cg>(−)-EGC>(+)-EC>(−)-GC>(−)-EC>(−)-C>(+)-C. It was shown clearly that the inhibitory activities of galloylated catechins were stronger than those of nongalloylated catechins. A similar tendency in the scavenging effects of catechins on DPPH radical was previously observed.[6—9)]

The AAs of catechins on an LPO system are of interest because of the chiral structure of catechins. It is known that drug enantiomers can have qualitatively or even quantitatively different physiological actions.[16)] In our experiments, there were various differences between the AAs of catechins [(+)-C, (−)-C, (−)-GC, (−)-Cg, (−)-GCg] and their corresponding epimers [(+)-EC, (−)-EC, (−)-EGC, (−)-ECg, (−)-EGCg]. The greatest difference was observed between (+)-C and (+)-EC, followed in decreasing order by (−)-GC and (−)-EGC, and (−)-C and (−)-EC. The differences between the galloylated catechins and their corresponding epimers were rather small. Considering the chiral structures, the substituents at C3 and the B ring take the *cis* form in the cases of catechins [(+)-C, (−)-C, (−)-GC, (−)-Cg, (−)-GCg]. However, for their epimers, the substituents at C3 and the B ring take the *trans* form. This steric change may result in the deviations in AA. The presence of the freely rotating gallate ring[9)] may diminish this steric difference, resulting in the corresponding small variation of AA between galloylated catechins and their epimers. Evaluating the scavenging effects of catechins and their derivatives on DPPH radical, Nanjo *et al.* suggested that the scavenging effects of tea catechins depended on their chemical structures but not their steric structures.[10)] This discrepancy can be explained in that the experimental system they used is not a biological system, in which the effect of steric hindrance may not strongly affect the experimental data. Further investigation is needed to elucidate the effect of steric structures of catechins on AA in an LPO system.

Table 1. Electrochemical Characteristics of Catechins and Their AAs on Microsomal LPO

Catechin	$E_{1/2}$ [a)] (V)	n [b)]	log P [c)]	IC_{50} [d)]
(+)-C	0.102	1.81±0.06	0.86	51
(+)-EC	0.082	2.32±0.07	0.86	25
(−)-C	0.092	2.06±0.07	0.86	38
(−)-EC	0.082	2.29±0.05	0.86	30
(−)-GC	−0.030	1.69±0.02	0.43	28
(−)-EGC	−0.035	1.95±0.03	0.43	16
(−)-Cg	0.040	3.36±0.16	2.1	13
(−)-ECg	0.080	3.54±0.20	2.1	10
(−)-GCg	−0.010	1.20±0.04	1.67	13
(−)-EGCg	−0.020	1.43±0.06	1.67	11

a) The half-wave potentials for the first oxidation wave (V *vs.* Ag/AgCl) in MeOH: 0.1 M phosphate buffer (pH 7.5) (1 : 1, v/v). *b*) The number of electrons transferred in the oxidation process. *c*) The calculated octanol/water partition coeffecients. *d*) The concentrations (μM) of catechins needed to inhibit the reaction by 50%. Tested once performed in triplicate.

Correlation between Half-Wave Potential and AA Although various biological or nonbiological assays have been used to evaluate the AA of catechins, the results were not always consistent, perhaps because using different systems means dealing with different oxidation mechanisms and corresponding antioxidant mechanisms. In the present work, we evaluated catechin AA on NADPH-dependent LPO in rat liver microsomes. When investigating the flavonoid effects on metal-induced lipid hydroperoxide-dependent LPO, Sugihara *et al.* mentioned that the ability of flavonoids to terminate the chain reaction of LPO by the lipid radical scavenging activity may overweigh the ability of flavonoid–metal complexes to decompose LOOH, particularly for catechol-containing flavonoids.[17)] After analyzing the differences between IC_{50} data obtained from an iron-dependent LPO assay and an iron-independent LPO assay, van Acker *et al.* came to the conclusion that for most flavonoids, iron chelation did not play a role in the AA in microsomal LPO.[18)] In contrast, the effect of catechins in scavenging chain-propagating peroxyl radicals was demonstrated.[2,4)]

To investigate the chelate formation of catechins with the ADP–Fe^{3+} complex, the UV/Vis absorption spectrum of catechins with ADP–Fe^{3+} was compared with that of catechin and ADP–Fe^{3+}, respectively. The characteristic absorbance peaks of (−)-EGCg in Tris–HCl solution were at 275 nm (a main absorption peak) and 375 nm (a shoulder for the main absorption peak). The addition of (−)-EGCg to the ADP–Fe^{3+} complex (the same concentration as used in the LPO assay) resulted in a small absorption wave around 500 nm and a very small increase in absorbance at 375 nm, which was probably due to the formation of (−)-EGCg–ADP–Fe^{3+} complexes. A similar phenomenon was observed in the case of (+)-C, *i.e.*, a rather small absorption peak appeared around 500 nm after the addition of (+)-C to the ADP–Fe^{3+} complex. These small spectral changes indicated that the equilibrium constant for the chelating reaction between catechins and ADP–Fe^{3+} might be small. Accordingly,

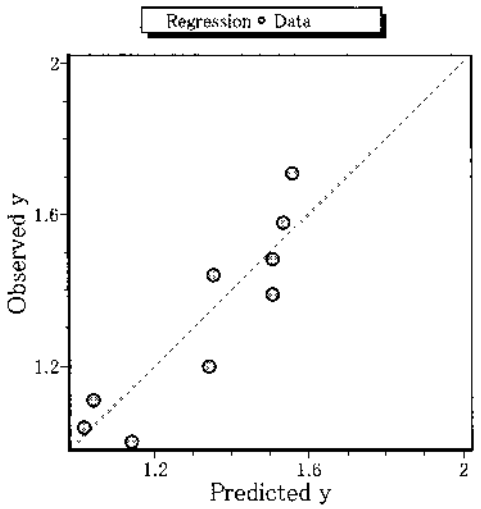

Fig. 4. Plot of Observed log IC_{50} Values against Calculated log IC_{50} Values Using Eq. 3 (r=0.907)

we presumed that catechins acted mainly as chain-breaking antioxidants by scavenging chain-propagating peroxyl radicals. Because the reduction potential of catechin radical was lower than that of peroxyl radicals as mentioned earlier. One may suppose that the AA of catechins may increase with easier oxidation, *i.e.*, the more negative the potential, the higher the AA. The results showed that catechins exhibited strong AAs in this system. However, the correlation between IC_{50} and $E_{1/2}$ of the catechins was extremely poor, with r=0.554, which meant other factors operated in this system. Because chain-propagating lipid peroxyl radicals were located within the membrane, evidently, catechins had to interact with the membrane before exerting their effects. Consequently, the localization of catechins should be taken into account in understanding the effectiveness of their AAs. We thus calculated the values of log P for each catechin investigated using version 5.1 of CompuDrug's log P prediction program. Prolog P estimates the log P values of organic compounds in an octanol/water system based on their chemical structures. The predicted results are the combined data based on three different estimation methods: Rekker's fragmental method and the atomic approaches of Broto *et al.* and Ghose *et al.* This method is commonly used as an accurate method for predicting log P values.[19] Subsequently, we observed a good description of the AAs of the catechins shown by the following multiparameter equation (Fig. 4):

$$\log IC_{50}\ (\mu M) = 1.56 + 2.49E_{1/2}\ (V) - 0.29 \log P\ (r = 0.907) \quad (3)$$

This means that a fundamental requisite for the expression of AA by catechins appears to be, together with their redox properties, the ability to interact with biomembranes. This finding is consistent with what other workers have reported, in which they observed that the interaction of flavonoids with a model membrane, including their incorporation rate into cells and their orientation in a biomembrane, affected their AA or other pharmacological effects.[20—24]

AA of Tea Extract The study of catechins is particularly important for the understanding of the antioxidant property of tea. In this paper, the AA of tea extract (mainly containing polyphenols) was tested. Table 2 shows the relationship between the content of each catechin contained and its corresponding activity. In Table 2, the concentrations of catechins in tea extract were used to calculate their predicted AAs, according to their measured dose–activity relationships, the total amount of predicted data (117.9% inhibition) was reasonably consistent with the measured data (99.8%) of tea polyphenon. This means that each catechin contained in the tea extract contributed to the total AA of the extract.

Table 2. Contribution of Catechins to the AA of Tea Extract

	% Composition of tea extract[a)]	Contribution to AA (calculated)[b)]
(−)-EGCg	53.9	92.2
(−)-GCg	2.9	2.5
(−)-ECg	1.7	0.7
(−)-EGC	13.4	19.5
(−)-EC	9.4	3.4

a) The catechin composition of the tea extract as percentage weight. *b*) The calculated contribution (except for EGCg, the measured data) of each catechin to the AA of tea extract.

In conclusion, catechins showed obvious AAs on NADPH-dependent microsomal LPO. The antioxidant effects of galloylated catechins were higher than those of nongalloylated catechins. The oxidation potential and lipophilicity of the catechins play important roles in their AAs.

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