THE DENATURATION OF EGG ALBUMIN BY ULTRAVIOLET RADIATION

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It is a well known fact that exposure to ultraviolet radiation will denature a protein solution. Egg albumin, after such an exposure, shows a loss of solubility in water and will precipitate at the isoelectric point or, on half saturation with ammonium sulfate, at a pH somewhat removed from the isoelectric point (1, 2). Previous work on light denaturation has been largely of a qualitative nature and no satisfactory attempt has been made to separate the two steps involved in the process although Bovie (3) stated in 1913 that there are two steps in the coagulation of proteins by light; the first, the denaturation of the protein molecule, and the second, the flocculation of the denatured protein as a visible coagulum. At a pH removed from the isoelectric point only the first step takes place and the degree of denaturation can only be determined by precipitating the denatured molecules with ammonium sulfate or by bringing the radiated solution to the isoelectric point. If solutions are radiated at the isoelectric point flocculation follows denaturation more or less rapidly depending on the temperature of the solution.

This investigation was planned as a study of the temperature coefficient of the first step of the reaction and it was found that, by controlling the temperature at which the denaturation and subsequent flocculation were carried out, a quantitative analysis of the steps involved in the process could be made.

Method

The intensity of the Tyndall beam from the opalescent solutions was used to measure their degree of aggregation. This method has been frequently used (4, 5) and gives accurate results if the conditions are controlled. For very small par-

ticles the strength of the Tyndall beam = $T = kcd^3$ and for large particles $T = k' c/d$, where c is the concentration and d is the diameter of the particles, so that the strength of the Tyndall beam is only proportional to the concentration if the size of the particles remains constant. Mecklenburg found that in a sulfur suspension of constant concentration the intensity of the Tyndall beam increased with the diameter of the particles from $d = 0.05 \times 10^{-5}$ cm. to $d = 0.9 \times 10^{-5}$ cm. but did not change appreciably between $d = 0.9 \times 10^{-5}$ cm. and $d = 2.5 \times 10^{-5}$ cm. Tolman, working with a silica suspension of larger particle size, found that the intensity of the beam decreased with increasing diameter for particles larger than $d = 10 \times 10^{-5}$ cm. It would seem, therefore, that the region between 10^{-5} and 10^{-4} cm. diameter is the region in which the law of scattering changes from that characteristic of small particles to that characteristic of large particles and that for this region the intensity of the Tyndall beam is practically independent of particle size. Emulsions have a diameter of the order of 10^{-4} to 10^{-5} cm. and it has been found (6) that 4×10^{-5} cm. is a

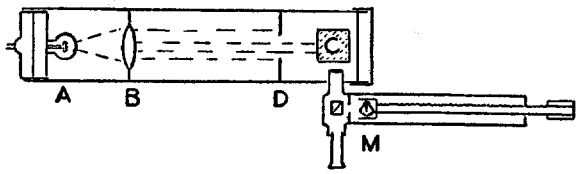

FIG. 1. Diagram of Tyndallmeter. A = lamp, B = lens, D = diaphragm, C = cell (2 cm. square), M = Macbeth illuminometer.

critical size for equilibrium in oil emulsions, whether the emulsion is obtained by breaking down larger masses or by the coagulation of smaller particles. When Tyndall beam readings are made on an albumin solution during the process of flocculation the intensity of the beam increases at first, owing to increasing size of the particles, but finally reaches a constant value. When this condition is reached the particles begin to settle out but, if redispersed by shaking, the same constant value for the Tyndall beam is obtained. It would seem therefore that this constant value represents the reading obtained when the particles reach a critical size (10^{-4} to 10^{-5} cm. diameter) and that in this region the intensity of the Tyndall beam is independent of the size and proportional to the concentration of aggregated protein.

Apparatus.—An adaptation of the Tyndallmeter of Tolman and Vliet (4) was used and is shown in Fig. 1. The meter consists of a 10 watt 120 volt Mazda bulb inserted at A in a metal tube and a 10 diopter lens placed at B, 10 cm. from A, to give a beam of parallel light which passes through a diaphragm D and falls on the solution in the cell at C. The cell is a rectangular absorption cell 2 cm. square (outside dimension). A Macbeth illuminometer is inserted at a hole opposite the side of this absorption cell to read the intensity of the Tyndall beam in

apparent foot-candles. This instrument reads, with great accuracy, illuminations as low as 0.02 foot-candles. Solutions of undenatured, isoelectric egg albumin had a Tyndall beam reading of 0.03 to 0.04 apparent foot-candles. Solutions were exposed in small quartz test tubes, 1 cm. diameter, placed inside and against the side of a quartz jar packed with ice and water, or filled with water at 14°C. The source of radiation was a quartz mercury Uviarc at a distance of 6 inches.

Crystalline egg albumin was prepared as described in a previous publication (1). After four recrystallizations with ammonium sulfate the crystals were dissolved in distilled water and dialyzed 2 days to remove the ammonium sulfate. This gave a solution of undenatured isoelectric egg albumin of pH 4.8. To determine

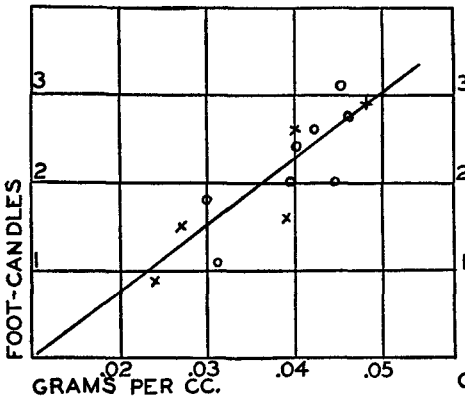

FIG. 2

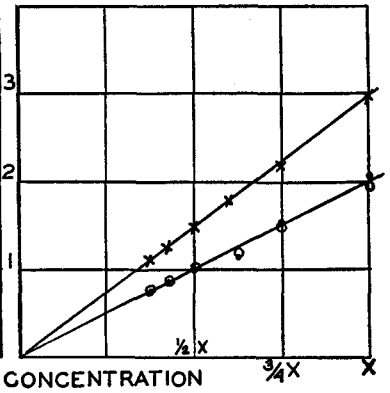

FIG. 3

FIG. 2. Standardization of Tyndall beam. Ordinates, Tyndall beam in apparent foot-candles. Abscissae, grams of aggregated protein per cc. (X = results with Solution 1, o with Solution 2).

FIG. 3. Standardization of Tyndall beam. Ordinates, Tyndall beam in apparent foot-candles. Abscissae, dilutions of original concentration of X grams per cc. of aggregated protein (o and • = results with two samples of Solution 1 and X with Solution 2).

the concentration of the solution 10 cc. were boiled and the coagulum filtered and weighed. The whole series of experiments was carried out with two separately prepared solutions, the first a 1.1 per cent and the second a 1.01 per cent solution. The results of the two series were in complete agreement.

Standardization of Tyndall Beam.—Two methods were used to prove that the intensity of the Tyndall beam is proportional to the concentration of the aggregated material after critical particle size is reached, in solutions where part of the albumin had been denatured and flocculated. First, solutions of known Tyndall beam reading were filtered on weighed filter papers and the grams of aggregated material determined by drying to constant weight. To check the results the

filtrates were boiled and the coagulum filtered and weighed to see that the combined weight of albumin in the precipitate and filtrate checked with the known concentration of the original solution. The results are given in Fig. 2 and show that for both solutions used the Tyndall beam reading was proportional to the concentration, after critical particle size was reached, up to a beam of 3 apparent foot-candles which was equivalent to a concentration of 0.05 gm. per cc. Above this point the solutions become so opaque that they absorb an appreciable fraction of the incident light and the Tyndall beam readings are no longer proportional to the concentration of aggregated protein.

The second method by which the intensity of the beam was shown to be equal to the concentration was by taking a solution of concentration X gm. per cc. and measuring the intensity of the Tyndall beam with progressive dilution. The results of three such experiments are given in Fig. 3 and show that the concentration is proportional to the Tyndall beam up to 3 apparent foot-candles.

The Tyndall meter can therefore be used quantitatively to measure concentrations of aggregated albumin up to 0.05 gm. per cc. provided the aggregated particles have reached the critical size at which they begin to settle out.

RESULTS

Heat Denaturation.—Chick and Martin (7) working with hemoglobin and egg albumin, found that heat denaturation is a reaction between protein and water which takes place at any temperature but has an extraordinarily high temperature coefficient. The velocity of the reaction is also influenced by the pH of the solution and the presence of salts. Heat coagulation involves two processes (1) the denaturation of the protein and (2) the separation of the denatured protein in flocculated form. They determined the velocity constant of the denaturation and calculated the temperature coefficient. In Fig. 4(*a*) the logarithm of K, the velocity constant, is plotted against temperature from their results for hemoglobin. Their results for egg albumin are at a higher temperature range where the temperature coefficient is apparently increasing with increasing temperature. In order to compare heat and light denaturation with the same material isoelectric egg albumin, unradiated, was put in baths of 40°, 50°, 60°, and 65°C. At the isoelectric point flocculation proceeds so rapidly that the rate of aggregation may be taken as a measure of the rate of denaturation. The rate of aggregation was followed by measuring the strength of the Tyndall beam and it was assumed that a given Tyndall reading indicated the same condition of aggregation in all solutions. Taking

the velocity as $1/t$ where t is the time necessary to reach a certain Tyndall beam reading one finds the results for velocity given in Table I. At 40° the reaction between protein and water proceeds so slowly that it is practically negligible. Between 50° and 60° the temperature coefficient determined by the ratio of the velocities is 14.3 for a 10° rise in temperature. In Fig. 4(*b*) the logarithm of the velocity of heat denaturation of egg albumin is plotted against the temperature from these results.

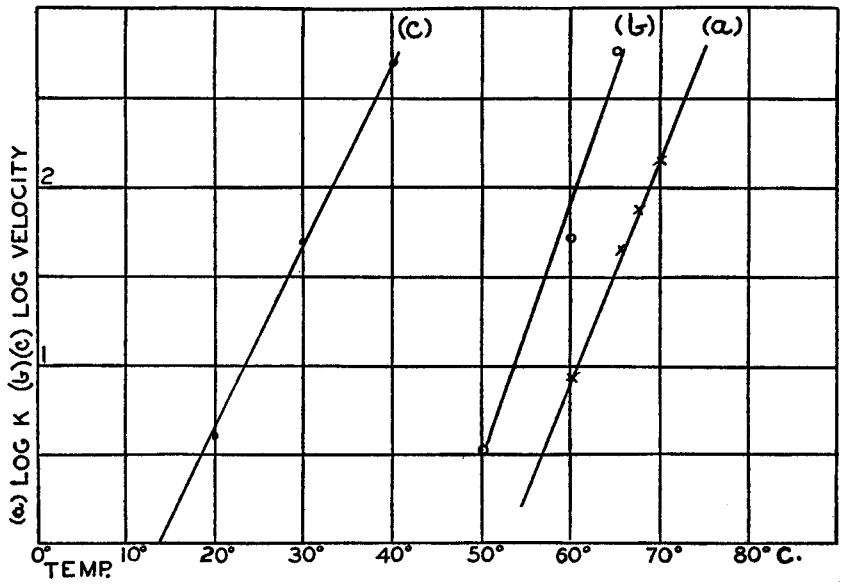

FIG. 4 (*a*) Log K (velocity constant) plotted against temperature for heat denaturation of hemoglobin (Chick and Martin). (*b*) Log velocity of heat denaturation of egg albumin. (*c*) Log velocity of flocculation after light denaturation of egg albumin.

As flocculation only follows denaturation at the isoelectric point, in salt free egg albumin, solutions were heated to 90° for 10 minutes at a pH of 4.4 and 6.4 where they are denatured without flocculation. After heating they were cooled to 4° and then brought to the isoelectric point. There was immediate flocculation at this temperature showing that after heat denaturation the subsequent flocculation, although dependent on pH, occurs at a low temperature.

Denaturation by Ultraviolet Radiation.—Isoelectric egg albumin (pH 4.8) exposed to ultraviolet radiation in quartz tubes, 6 inches from a quartz mercury Uviarc, and kept at a temperature of 4°C. during radiation, showed only a trace of opalescence after 30 minutes radiation. When solutions radiated in this way were subsequently put in a constant temperature bath at 40°C. they became opalescent and the rate of increase in opalescence, determined by Tyndall beam readings, is shown in Fig. 5. The Tyndall readings reached a practically constant value at the end of 1½ to 2 hours. By this time the flocculated material was settling out so the critical particle size had been reached and the Tyndall beam reading 2 hours after immersion in a 40° bath was taken as a measure of the amount of material denatured by the radiation as unradiated albumin does not flocculate at an appreciable

TABLE I

Velocity of Heat Denaturation of Egg Albumin

Temperature	t = min. to reach Tyndall beam = 1.0	Velocity = $1/t \times 1000$	$\log_{10}$ velocity
°C.			
40	5760		
50	285	3.5	0.54
60	20	50.0	1.7
65	1.7	588.0	2.77

rate at 40° (see Table I). In Fig. 6 the final Tyndall beam readings are plotted against the time of radiation, and these Tyndall readings give the concentration of denatured and flocculated material by comparison with Fig. 2. These results show that after denaturation with ultraviolet radiation the denatured material will flocculate rapidly at a temperature much below that necessary for rapid flocculation of unradiated albumin.

In Figs. 5 and 6 the circles are Tyndall readings obtained after radiation at 6 inches from the arc at 14° and the crosses after radiation at 6 inches at 4°C. As the rate of flocculation after radiation and the final Tyndall beam reading reached at the end of 2 hours is the same the temperature coefficient of the light denaturation is 1. It is interesting in this connection to note that Gates (8) found a temperature coefficient of 1.02 for the inactivation of pepsin by ultraviolet radiation.

However, the process leading to flocculation after light denaturation has a high temperature coefficient. In Fig. 7 the results are given for

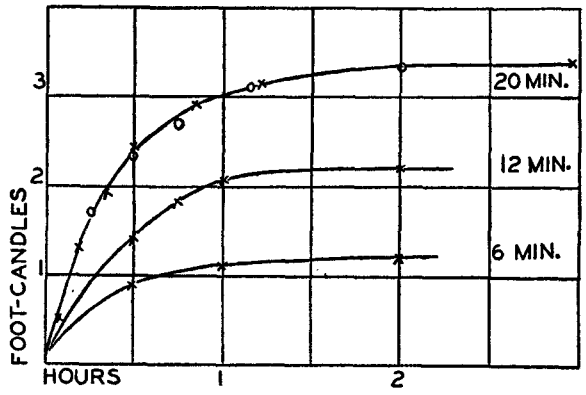

FIG. 5. Increase in Tyndall beam on immersion in bath at 40°C. for solutions radiated 6, 12, and 20 minutes at 6 inches from the arc (X = results for solutions radiated at 4°C. and o for solutions radiated at 14°C.). Ordinates = Tyndall beam in apparent foot-candles. Abscissae = time of immersion in bath.

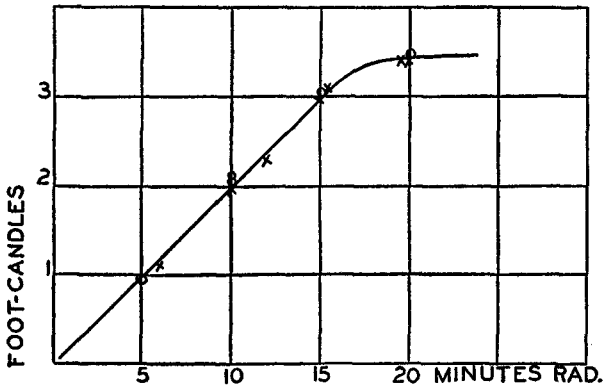

FIG. 6. Final Tyndall beam readings after 2 hours in 40°C. bath. Abscissae = time of radiation (X = results for solutions radiated at 4°C. and o for solutions radiated at 14°C.). Ordinates = Tyndall beam in apparent foot-candles.

the rate of flocculation at 20°, 30°, 40° for solutions radiated 20 minutes at 4°C. at 6 inches from the arc. Some flocculation occurs even

at 5° though the rate is slow and after a week in the ice box a radiated solution showed a Tyndall reading of 0.72 apparent foot-candles. From Fig. 7 the temperature coefficient can be calculated by finding the velocity required to reach a certain degree of opalescence. In Table II the opalescence reached in a certain time is given for 20°, 30°, and 40° baths. From these results the temperature coefficient of flocculation after radiation was found to be 10.3 and in Fig. 4(*c*) the logarithm of the velocity to reach a certain degree of opalescence is plotted against temperature and shows, by comparison with Fig. 4(*b*) (unradiated albumin), that after radiation albumin flocculates

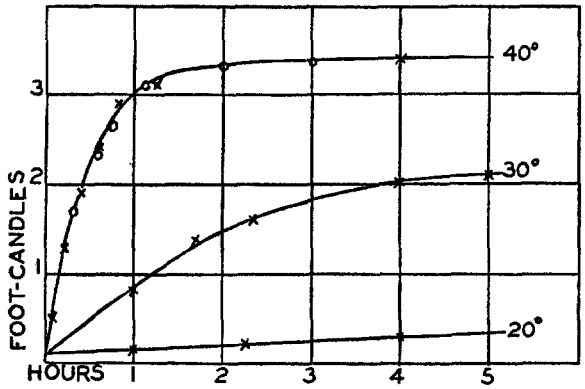

FIG. 7. Increase in Tyndall beam in solutions radiated for 20 minutes at 6 inches on immersion in baths at 20°, 30°, and 40°C. (X = solutions radiated at 4°C. and o = solutions radiated at 14°C.). Ordinates = Tyndall beam in apparent foot-candles. Abscissae = time of immersion.

at an appreciable rate at a temperature as low as 12°, and that the rate of flocculation increases rapidly with temperature, whereas unradiated albumin shows no appreciable flocculation at temperatures below 45°C.

In coagulation by ultraviolet radiation therefore the first part of the process, the denaturation of the protein molecules, takes place at the same rate at any temperature. It has been shown in previous work (1) that it occurs over a wide pH range but the rate of light denaturation has not been investigated except at the isoelectric point and may be found to vary with pH as has been found for the photoinactivation

of pepsin (9). The subsequent flocculation of the denatured molecules only occurs at the isoelectric point in salt-free albumin and although it occurs slowly at temperatures as low as 5°C., the rate of flocculation increases rapidly with temperature (temperature coefficient = 10^{+}).

It has been previously noted (2, 3) that the temperature of coagulation is lowered after radiation but the process had not been studied in detail. The very high temperature coefficient of heat denaturation and of flocculation after light denaturation shows that for these two processes the molecule must be put into an active state by the absorption of a large amount of energy before the process can take place.

TABLE II

Flocculation after Light Denaturation

Time	Tyndall readings			Temperature coefficient $\frac{\text{Vel. } 40°}{\text{Vel. } 30°}$	Temperature	Velocity = $1/t$ to reach 0.6	$\log_{10}$ velocity × 1000
	20°	30°	40°				
min.					°C.		
2			0.6	10.0			
14			1.5	9.4	40	0.5	2.7
20		0.6			30	0.05	1.7
24			2.0	11.5	20	0.004	0.6
132		1.5					
240	0.6						
272		2.0					

The high temperature coefficient of heat denaturation is thought to be associated with a chemical reaction between the protein molecule and water which is followed by the physical process of flocculation. The high temperature coefficient of flocculation after light denaturation would lead one to suppose that the process is, in part at least, a chemical one and it is probable that the entire process of protein coagulation with ultraviolet radiation involves not two but three steps. The first is a physical process which produces a permanent change in the molecule which we may call light denaturation. It is independent of temperature and occurs over a wide pH range and unpublished observations by the author indicate that it occurs in the absence of water. The second is a chemical reaction between the light denatured

molecule and water, with a high temperature coefficient, which may be similar to the first step in heat denaturation but occurs at a lower temperature. The third, the flocculation of the light and heat altered molecules, is a physical change similar to flocculation after heat denaturation. This conception of three steps in the process is borne out by the following observation.

A solution radiated at pH 6.4 has passed through step 1 but not steps 2 and 3. If this solution is heated to 40°C. for 2 hours there is no increase in opalescence, owing to the pH, but it has now passed through step 2. If this solution is then cooled to 4°C. and brought to pH 4.8 there is a rapidly developing opalescence which reaches a Tyndall beam reading of 1.95 apparent foot-candles at the end of $1\frac{1}{2}$

TABLE III

Rate of Denaturation by Ultraviolet Radiation

Time of radiation	Opalescence 1.5 hrs. at 40°	Concentration of denatured protein	Denaturation	C = concentration unchanged protein	$\text{Log}_{10}C$	K Velocity constant
min.		*gm. per 100 cc.*	*per cent*			
0				100	2.0	
5	1.0	0.024	21	79	1.892	
10	2.0	0.037	32	68	1.8325	0.0384
15	3.0	0.051	45	55	1.74	0.0391
20	3.4	0.057	50	50	1.699	0.0345
						0.0373

hours while a solution radiated at pH 6.4 and kept at 4°C. (step 1 but not step 2) shows only a slight opalescence (0.6 foot-candles) when brought to pH 4.8. Therefore, heating to 40°C., at a pH where no flocculation occurs, produces a permanent change in the light denatured molecule, a change which is necessary before the final step of flocculation can take place.

When the amount of material denatured by different lengths of exposure is calculated from the Tyndall beam readings in Fig. 6 the logarithm of the concentration of unchanged albumin plotted against time of radiation (see Table III and Fig. 8) indicates that the process of denaturation is a unimolecular one, the velocity varying with the concentration of unchanged material.

At 6 inches from the Uviarc a 1 per cent albumin solution is half denatured in 20 minutes and the denaturation is complete in about 2 hours. The Uviarc has an ultraviolet radiation intensity of 9 ZnS units a minute at 6 inches. Therefore a radiation of 20 minutes gives 180 ZnS units or approximately 1.3×10^8 ergs per sq. cm.

Velocity Constant and Critical Increment.—The velocity of the second part of the coagulation process, the heat change leading to flocculation of the denatured albumin, could only be determined by the reciprocal of the time to reach a certain degree of opalescence. It is possible, however, to calculate the velocity constant of the first part of the

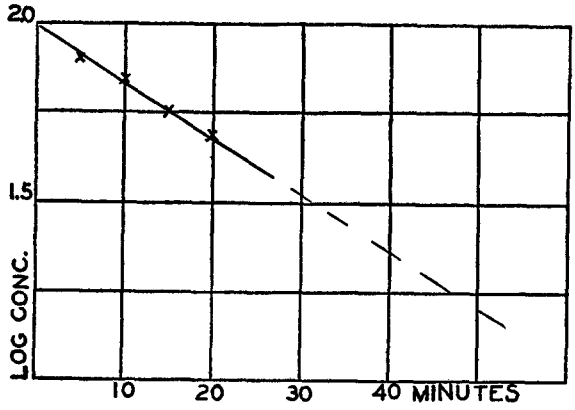

FIG. 8. Log of concentration of undenatured albumin plotted against time of radiation.

process, the light denaturation of the albumin molecules, and the results are given in the last column of Table III. In calculating K which is equal to $\frac{\log_e C_o - \log_e C_n}{t_n - t_o}$ the logarithms to base 10 are multiplied by 2.3 to convert them to natural logarithms. As the process of denaturation is independent of temperature and K_1 (velocity constant at absolute temperature T_1) = K_2 (velocity constant at absolute temperature T_2) the critical increment $E = 0$ since $\log_e K_1 - \log_e K_2 = \frac{E}{R}\left(\frac{T_1 - T_2}{T_1 T_2}\right)$. In other words the protein molecule is in a condition favorable for light denaturation without addition of energy.

Heat denaturation has a very large critical increment given as 130,000 calories (10). The high temperature coefficient of the second part of the light coagulation process, the heat change leading to flocculation of the light denatured molecules, indicates a large critical increment for this step in the coagulation process.

Freezing.—It has been found (11) that the proteins in muscle juice are partially denatured by very prolonged freezing at about −2°C. Solutions of isoelectric egg albumin frozen at −6°C. and kept at −2°C. for 3 hours showed no opalescence when thawed out and brought to 40° for 2 hours. Freezing for a period of 3 hours, therefore, does not appreciably denature isoelectric egg albumin.

CONCLUSIONS

The coagulation of isoelectric egg albumin solutions, on exposure to ultraviolet radiation, involves three distinct processes, (1) the light denaturation of the albumin molecule, (2) a reaction between the light denatured molecule and water which may be similar to heat denaturation but occurs at a lower temperature, and (3), the flocculation of the denatured molecules to form a coagulum. The light denaturation is unimolecular, independent of temperature, and occurs over a wide pH range. The reaction between the light denatured molecule and water has a temperature coefficient of 10^+ and occurs rapidly at 40°C., a temperature at which heat denaturation is inappreciable.

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