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Role of pepsin in modifying the allergenicity of Bhetki (*Lates calcarifer*) and Mackerel (*Rastrelliger kanagurta*) fish

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The effect of pepsin digestion on the allergenicity of raw and thermally processed (boiled and fried) fish muscle extracts of two widely consumed fishes bhetki (*Lates calcarifer*) and mackerel (*Rastrelliger kanagurta)* was studied. Sere were collected from 110 patients who were hypersensitive to fish, as evidenced by their clinical history, symptoms and positive skin-prick test results. The various extracts after digestion with pepsin at different times of incubation were tested for specific IgE-binding activity by ELISA and immunoblotting with patients' sera. All the extracts of both the fishes retained their allergenicity as evidenced by ELISA and immunoblotting. In bhetki, maximum allergenicity was found in the pepsin-digested fried extract, whereas similar treatment decreased the allergenicity in fried mackerel. Results showed that raw as well as thermally processed allergens of both the fishes maintained strong allergenicity, even after digestion with pepsin for different time periods. The study revealed that the fish proteins played an important role in manifestation of allergy, due to their stable structure, which was retained even after pepsin and heat treatment.

Keywords: Allergen, Bhetki, Mackerel, Pepsin, IgE-reactivity, ELISA, Immunoblotting

Fish is one of the common food allergens and fish allergy is quite high in coastal population where it is consumed as a staple diet. Fish hypersensitivity resulting from contact and consumption of fish products or even by inhalation of fish vapor is common in Eastern and North-eastern India. This results in severe health problem ranging from dermatitis, urticaria to angioedema, diarrhoea, asthma and in severe cases systemic anaphylactic reaction and death. The protein stability is considered as the most important factor, while examining the characteristic of food allergens[1,2].

Food allergens, in general tend to be resistant to heat, acidic condition as well as to proteolytic digestion[3]. Hence, for evaluation of allergenicity of food protein, effect of *in vitro* proteolytic digestion on allergic protein by pepsin is important. Several food allergens, such as peanut, soybean, mustard, egg and milk have been found to be highly resistant to proteolytic action by gastrointestinal enzyme, simulated gastric fluid (SGF), as well as purified pepsin than the non-allergenic food proteins. The crude extracts of most latex and vegetable food proteins are digested by SGF within 4 min, but a 28 kDa latex allergen and the allergen hevein (4.7 kDa) are not completely digested even at 1 h. Most cross-reactive allergens from the fruits are digested with the pepsin within 4 min. In contrast, the potato protein is stable in SGF, even after 1 h digestion. In contrast, many cross-reactive allergens from the pollens, fruits and vegetables are found to be sensitive to SGF digestion like Bet v1-related protein and profilins that are heat labile and susceptible to enzyme digestion[4-7].

Recently, we identified allergens in two highly consumed Indian fishes *viz.*, bhetki (*Lates calcarifer*) and mackerel (*Rastrelliger kanagurta)* and examined the effect of thermal processing on them[8,9]. We found that both fishes maintain strong reactivity even after thermal treatment. Thus, to reduce the allergenicity, such allergenic food proteins require alteration. In the present study, the effect of pepsin digestion to alter the allergenicity of these fish proteins has been investigated in raw, as well as thermally processed fish extract using ELISA and immunoblotting.

Materials and Methods

Patients

One sixty-one patients, who exhibited allergic symptoms with different foods visited the Allergy and Asthma Research Clinic, Kolkata. Among them, 110 patients who had clinical history of fish hypersensitivity, as well as reported of their allergic

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symptoms after ingestion of fish and showed positive skin-prick test (SPT) with any of the two fish allergens were recruited in this study with their informed consent. Ninety-two of them were found hypersensitive to both fishes. To make the sample size substantial, 50 patients were chosen from 92 for this study by random selection. Sera from these patients were used and those from non-allergic individuals served as negative control. The Human Ethical Committee of our Institute approved this study.

Preparation of bhetki and mackerel fish extracts

Fresh bhetki and mackerel fish were purchased from city municipal market. The raw extract of each fish was prepared by homogenization of boneless muscles in 0.1 *M* PBS (*p*H 7.2), followed by stirring overnight at 4°C. The slurry of each extract was centrifuged at 10,000 rpm for 20 min, and the supernatant was concentrated by YM 10 membrane filtration (Amicon). Similarly, raw boneless muscles of each fish was boiled with 0.1 *M* PBS for 10 min at 90°C to obtain the extract and the supernatant was collected and concentrated as above. Also, the extract of each fried fish was prepared by lightly frying the muscles with mustard oil for 5 min, placed on filter paper to remove the oil, homogenized in 0.1 *M* PBS and centrifuged as above. All the extracts were preserved at –20°C for further experiments. Protein concentration of the extracts was determined by the method of Lowry *et al*[10].

Skin prick tests (SPTs)

The SPTs were performed with the raw fish extracts at Allergy and Asthma Research Clinic, Kolkata by an expert clinical technician under the supervision of Dr. Arijit Das, Director of the Clinic. The extract was prepared in PBS-glycerol (1:20 v/v) and sterilized by Millipore filtration (0.22 μm). A drop (10 μl) of the extract was placed on the volar aspect of the forearm with a disposable (26 gauge) hypodermic needle and scratched. The skin reaction was measured after 20 min and a wheal diameter of ≥ 3 mm surrounding the erythema was regarded as a positive response. Fifty percent glycerol in PBS was taken as negative control and histamine (1 mg/ml) as positive control.

Pepsin digestion

The extracts of both fish species were dialyzed in 10 m*M* HCl (*p*H 2.5) and pepsin was added to a final concentration of 1 mg/ml (2200 units/mg, Sigma) for optimization of enzyme activity[6] and the digestion was performed at 37°C. The aliquot (1 ml) from each extract was withdrawn at an interval of 1, 15, 30 min, 1 h and 2 h respectively and the reaction was stopped by the addition of Tris-HCl (1 *M*, *p*H 8). The digested proteins were analyzed by SDS-PAGE, stained with Coomassie brilliant blue G 250 and immunoblot was done on nitrocellulose membrane for IgE reactivity. The 1 ml aliquot of each extract, similarly done without pepsin treatment served as the control.

Enzyme-linked immunosorbant assay (ELISA)

The specific IgE reactivity of various fish extracts which were prepared as before, was measured by ELISA. Each well of a microtiter plate was coated with 100 μl of different fish extract (2 μg/well) in 10 m*M* carbonate buffer (*p*H 9.6) and incubated overnight at 4°C, washed with PBS-T (10 m*M* PBS, *p*H 7.2 containing 0.05% Tween 20) and incubated again with 1% BSA in PBS at 37°C for 1 h. After usual washing, the wells were incubated with patients' sera (100 μl, 1:20) for 1 h at 37°C and then with 100 μl of goat-antihuman IgE-HRP (1:1000) in PBS for 1 h at 37°C. After washing, the wells were incubated with 100 μl of *o*-phenylenediamine (1 mg/ml in 0.05 *M* citrate phosphate buffer with 0.01% H_2O_2, *p*H 5) for 20 min in the dark at 25°C. The absorbance was measured at 492 nm after addition of 3 *N* H_2SO_4 in an ELISA reader (Flow Laboratory, UK). At each step washing was performed thrice, each of 5 min duration. Absorbance ≥ 0.08 suggested hypersensitivity. A panel of non-allergic individuals' serum was used as negative control. The mean of these values was regarded as control value, and thus threshold sensitivity was established. Sera from non-allergic subjects gave O.D. values, ranged between 0.04 and 0.07 and were used as a background control. The ELISA readings were the average of triplicate assays[11].

SDS-PAGE and immunoblotting

The fish extracts after pepsin treatment at different time periods were subjected to SDS-PAGE (10%) according to Laemmli[12]. Protein bands were visualized with 0.2% Coomassie brilliant blue G-250, followed by 50% acetic acid. Immunoblotting was performed by electrophoretic transfer of proteins of each fish extract from polyacrylamide gel to nitrocellulose membrane (0.45 μ pore size) in Tris-glycine buffer containing 25% methanol[13]. The membranes were cut into strips and free sites were

Table 1—Clinical summary of 50 fish hypersensitive patients taken by random selection

Subject No.	Age	Sex	Symptoms	SPT*		Clinical history
				B	M	
1	18	M	Skin rash	+	++	Nil
2	65	F	Breathlessness	+++	+++	Nil
3	36	M	Skin rash	++	++	Nil
4	19	F	Skin rash	+	+	Asthma
5	53	M	Breathlessness, Diarrhoea	+++	+++	Asthma
6	43	F	Breathlessness	++	++	Nil
7	50	M	Severe skin rash	+	+	Nil
8	46	F	Breathlessness, Skin rash	++	+++	Asthma
9	36	F	Breathlessness	+++	++	Asthma, Eczema
10	35	F	Breathlessness, Diarrhoea	++	+	Eczema
11	65	M	Asthma, Reddening of eyes	+++	+++	Asthma, Urticaria, Eczema
12	40	F	Urticaria, Vomiting	++	+	Nil
13	45	M	Reddening of eyes, Sneezing, Skin rash	++	+	Nil
14	33	F	Skin rash, Reddening of eyes	++	++	Nil
15	11	M	Breathlessness, sneezing	++	++	Asthma, Eczema
16	23	F	Diarrhoea, Vomiting	+	+	Asthma. Urticaria
17	14	M	Skin rash, Asthma	+++	++	Asthma, Rhinitis, Urticaria
18	40	M	Breathlessness, Skin rash	+	+++	Eczema, Urticaria, Asthma
19	49	F	Diarrhoea, Urticaria	+++	+	Asthma
20	24	M	Sneezing, Urticaria	++	+	Urticaria
21	14	M	Skin rash	++	+	Rhinitis
22	16	M	Breathlessness, Diarrhoea	+++	+++	Urticaria, Asthma
23	85	F	Reddening of eyes	++	++	Rhinitis, Asthma
24	2	F	Sneezing, Breathlessness	+	+++	Urticaria, Eczema
25	20	M	Skin rash, Diarrhoea	++	++	Eczema, Urticaria
26	45	M	Skin rash, Reddening of eyes	+	+	Nil
27	29	M	Skin rash	++	++	Asthma, Urticaria
28	43	M	Urticaria	++	+++	Nil
29	51	F	Sneezing, Skin rash	++	++	Nil
30	9	M	Skin rash	++	+	Urticaria, Asthma
31	10	F	Breathlessness, Vomiting	+	+	Asthma
32	31	M	Urticaria, Diarrhoea	++	++	Nil
33	14	F	Sneezing	+	++	Astmma, Eczema
34	32	F	Breathlessness, Reddening of eyes	++	+++	Asthma, Eczema Urticaria
35	40	M	Breathlessness, vomiting	++	+	Nil
36	18	F	Breathlessness, Sneezing, Skin rash	++	++	Asthma, Eczema Urticaria
37	30	F	Reddening of eyes, Diarrhoea	+	++	Asthma
38	30	F	Skin rash	+	+	Asthma, Eczema Urticaria
39	35	F	Breathlessness, Vomiting	+++	++	Asthma, Eczema Urticaria
40	7	F	Skin rash	++	+++	Asthma Eczema
41	47	F	Skin rash	+	+	Nil
42	28	M	Diarrhoea, Urticaria	+++	++	Asthma
43	16	M	Diarrhoea	+	++	Nil
44	20	F	Vomiting, Urticaria	++	++	Nil
45	37	M	Skin rash, Diarrhoea	++	+++	Urtcaria
46	35	M	Sneezing, Diarrhoea, Reddening of eyes	+	+	Eczema, Urticaria
47	3	M	Skin rash, Breathlessness, Diarrhoea	+	++	Asthma
48	21	M	Skin rash, Reddening of eyes	++	+	Asthma
49	15	F	Skin rash	+++	+++	Urticaria, Eczema
50	42	M	Skin rash, Reddening of eyes, Diarrhoea	+++	+	Nil

*Wheal diameter: + = 3.5 mm, ++ = >6 mm, +++ = > 6 with pseudopodia.
SPT, Skin prick test; B, Bhetki raw extract; M, Mackerel raw extract

blocked with 1% BSA. Each strip was incubated with pooled patients' sera (1:20) for 1 h at room temperature. The strip was again washed with PBS-T and incubated with anti-human IgE-HRP (1:1000) for 1 h at room temperature. After washing, the protein bands were developed with diaminobenzidine and 0.01% H_2O_2 in sodium acetate buffer (*p*H 5). Washing at each step was done thrice with saline-Tween 20, each of 5 min duration.

Results

Skin prick test and specific IgE of patients

The clinical history and SPT results of 50 fish hypersensitive patients are summarized in Table 1. Among them, 48% showed skin rash, 32% breathlessness, 20% reddening of eyes, 14% sneezing, 8% asthma, 28% diarrhoea, 14% urticaria and 8% vomiting. Of them, 28 patients reported allergic symptoms within 30 min, 17 patients within 30-60 min and 5 patients after 1 h, following the ingestion of fish.

ELISA was performed to measure specific IgE levels in patients who had a positive SPT. The absorbances were compared with those of the control individuals[14] and the patients showing values (min ± 2 SD) greater than the controls were considered positive (cut-off value being 0.08). Fig. 1 shows the ELISA results of two fish extracts under different conditions with pooled patients' sera. The results were in concordance with the clinical symptoms and SPT results of the patients. No significant increase in IgE level was detected in the control subjects.

SDS-PAGE of fish extracts

In bhetki, the raw control extract and pepsin-digested samples (Fig. 2) showed three major bands of molecular mass 34, 150 and 250 kDa, of which the 34 kDa band was most intense. In addition, some minor bands appeared in the range of 15-25 kDa, and interestingly among them, the 25 kDa band disappeared after 1 min digestion. The boiled control as well as the digested samples displayed many bands in the range of 15-250 kDa and 37 kDa band appeared to be the most prominent in all the lanes. In fried extract, 250 kDa band disappeared, whereas 150 kDa band intensified as compared to raw extract. Another band at 37 kDa was present in the control and the pepsin-digested samples. No prominent change in band intensity in the control as well as pepsin-digested samples was observed in three different conditions. Some low molecular mass faint bands were present in the range of 17-30 kDa.

The raw control mackerel extract showed (Fig. 2) seven major polypeptides of 17, 25, 27, 30, 37, 46 and 50 kDa respectively, however, they gradually faded away with increase of incubation period. Most of the polypeptides seen in raw control extract lost their intensities upon boiling, except the bands of 27, 37 and 50 kDa, which were intensified. The most prominent band at 27 kDa was present in the control as well as pepsin-digested extract. After 1 min digestion, the 75 kDa band became prominent. The fried control extract showed some very faint polypeptides and no band appeared in the extract, when digested for different time periods with pepsin.

Immunoblot study

Fig. 3 shows the immunoblot results of allergenic band profiles of two fish extracts, prepared under different conditions and digested with pepsin for different times. The raw control and pepsin-digested samples showed a IgE-reactive band at 34 kDa. The boiled control and pepsin-digested extracts showed a

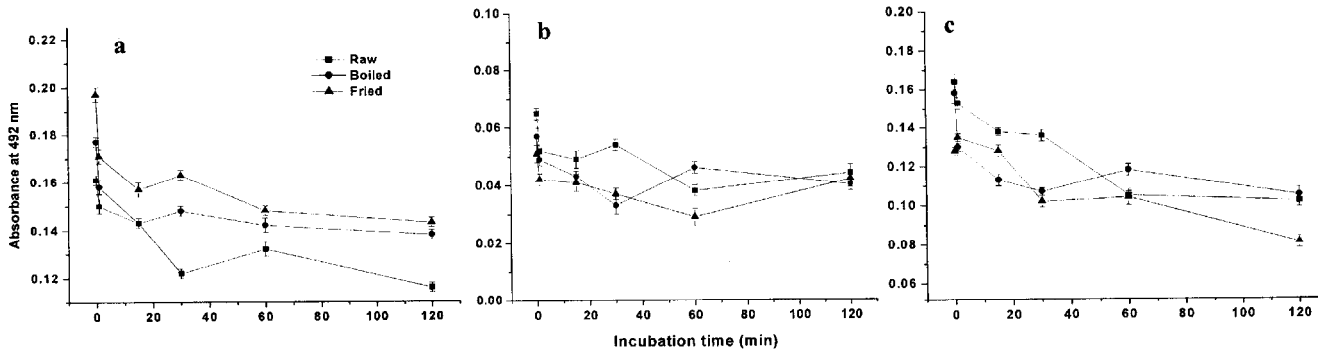

Fig. 1—Specific IgE-binding reactivity of pepsin-treated raw, boiled and fried extracts of bhetki and mackerel with pooled patients' sera [Time period '0' indicates the control (extracts without pepsin digestion). Incubation of pepsin-treated different fish extracts with (a) non-allergic individuals sera; (b) patients sera with bhetki extract; and (c) patients sera with mackerel extract (n =10, $p < 0.001$ in all cases)]

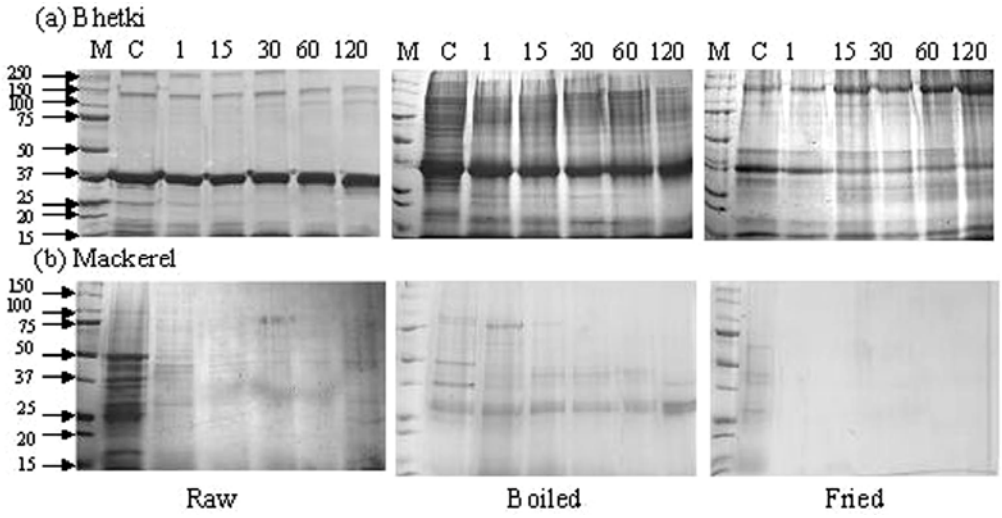

Fig. 2—10% SDS-PAGE profiles of raw, boiled and fried fish extracts digested with pepsin for 1, 15, 30, 60 and 120 min [(a) bhetki (b) mackerel. M, precision plus protein^{TM} standards (BIO-RAD); and (c), control extract (without pepsin-treated). Each well was loaded with 10 µg of protein]

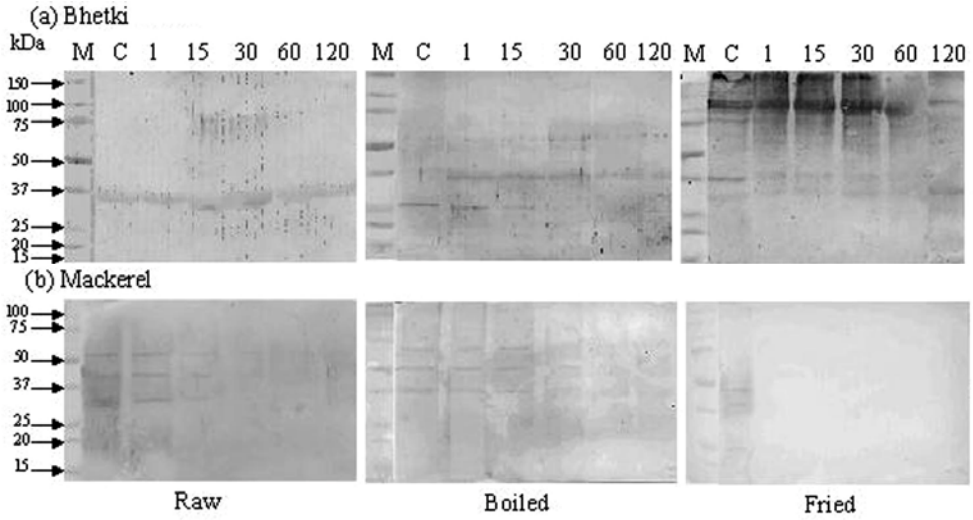

Fig. 3—Identification of IgE-reactive polypeptides by immunoblot of pepsin-digested raw, boiled and fried fish extracts for 1, 15, 30, 60 & 120 min [(a) bhetki (b) mackerel. M, Precision plus protein^{TM} standards (BIO-RAD); and (c), control extract (without pepsin treated)]

37 kDa moderate binding IgE-reactive band (not observed in without pepsin digestion extract) and another IgE-binding polypeptide 30 kDa band, which gradually faded away with increase of pepsin digestion time. The fried control and digested preparations showed maximum IgE-binding activity, since they produced IgE-binding bands at 30, 37 and 125 kDa respectively. However, all the bands gradually became faint with increase of the incubation time.

In mackerel, the maximum IgE-binding was observed in the raw extract and the prominent IgE-binding bands in raw control and digested samples were at 30, 46, and 52 kDa respectively. However, the intensity of the bands decreased gradually with the increase of incubation period with pepsin. The boiled control and pepsin-digested extracts showed IgE-reactive bands at 37, 50, 65 kDa respectively, which faded away with increase of incubation period. In the fried extracts, there was a complete disappearance of all the immunoreactive bands, except the presence of very few faint bands in the control.

Discussion

The present investigation was focused on the effect of pepsin treatment on the allergenicity of proteins of

two commonly consumed Indian fish namely bhetki and mackerel. Many of the food allergens are proteins that contain intramolecular disulfide bonds that may be important for their allergenicity. This observation leads to the assumption that protein structure may be a critical factor for resistance of an allergen towards denaturation[15]. The characteristics of known food allergens may help to predict the allergenicity of novel proteins. Peanut allergens Ara h1 and Ara h2 contain highly ordered structures that are responsible for their resistance to the enzymatic digestion and hence persistence of their allergenicity. Ara h1 forms stable homotrimers, maintained by hydrophobic interaction between amino acids at the monomer-monomer contact points, the same region where the majority of the IgE-binding epitopes are clustered. Whereas the resistance of Ara h2 to proteolysis is imparted by the compact nature of this small protein, enhanced by di-sulfide linkages between the molecules of eight cysteine residues[16]. In cockroach allergen Bla g2 the enzyme activity, especially proteolytic activity has been found to be a contributor to the allergenicity[17].

Immunoblot study showed that the number of allergenic bands and their intensities were increased in the bhetki fish extract after pepsin digestion, as compared to raw extract. Also, in some fish extracts many immunoreactive bands were more intensified, as the digestion time with pepsin increased. Our result indicated that possibly the structural changes in the proteins upon heating offered some degree of protection from the enzymatic digestion. Surprisingly, many people in India are hypersensitive to bhetki and mackerel fish, though they consume after cooking (boiling or frying). Possibly thermal processing generated new allergenic epitopes that were pepsin stable. This might explain the fact that people develop allergic symptoms, even after consuming cooked fish, due to action of pepsin present in the gastrointestinal fluid during food digestion. Earlier[9], we showed that bhetki and mackerel extracts containing allergenic protein, were thermally stable. Immunoblot profile of raw bhetki fish showed that the number of immunoreactive bands decreased on digestion with pepsin, when compared with the thermally processed (boiled and fried) extract, before pepsin digestion[9]. Interestingly, unlike the pepsin-digested boiled mackerel extract, very faint immunoreactive polypeptides were found in the pepsin-digested fried extract, suggesting that different forms of heating might alter the IgE-binding epitopes of a protein differently. Consequently, IgE-reactive sites showed different stability towards pepsin digestion.

The protease activity present in gastric fluid is due to the proteolysis by pepsin, hence, simple peptic hydrolysis could be used as model for *in vitro* digestion[15]. Therefore, the results thus obtained may reasonably extrapolate to human gastric digestion. The present study demonstrated that most of the thermally-treated fish extracts after pepsin digestion retained or showed enhanced allergenicity, except the fried mackerel extract, where allergenicity decreased after pepsin digestion. The raw extract of mackerel had shown strong allergenicity. In some cases, the intensity of the IgE-binding proteins was also decreased with increase in the time of incubation with pepsin. The allergenicity of fish proteins might be due to conformation-dependent IgE recognition. Though the allergens in their native state were somewhat labile towards pepsin digestion, similar treatment on the heated allergen, in general, increased their allergenicity.

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