Antihypertensive effect of *heshiko*, a fermented mackerel product, on spontaneously hypertensive rats

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**ABSTRACT:** During the processing of *heshiko*, a fermented mackerel product, a rapid increase in peptide content in the extract and a remarkable decrease in the IC₅₀ (the inhibitor concentration to inhibit 50% of enzyme activity) as an index of the angiotensin I-converting enzyme (ACE) inhibitory activity were observed. Systolic blood pressure (SBP) in spontaneously hypertensive rats (SHR) decreased between 2 and 4 h after single oral administration of *heshiko* extract at a dose of 10 mg/kg as a peptide, and SBP recovered its initial level by 8 h. For single doses of extract at three different levels (5, 10 and 50 mg/kg), SBP similarly decreased after between 2 and 4 h. The decreased SBP at 50 mg/kg was almost equal to that at 10 mg/kg, indicating a low dose dependency for *heshiko* extract. Through successive administration of *heshiko* extract or its desalted extract at 10 mg/kg for 10 days, SBP decreased 7 days after the start of administration and it recovered its initial level 5 days after stopping administration. During these periods, the change in ACE activity in blood plasma from SHR administered the extract roughly corresponded to that of SBP, suggesting that ACE inhibition was related to a decrease in SBP. For long-term administration of the extract to 5-week-old SHR for 70 days, SBP decreased 28 days after the start of administration. The decreased SBP remained low for 28 days after stopping administration, whereas the decreased ACE activity recovered its initial level. These results suggest that *heshiko* extract influences not only ACE inhibition, but also other systems that regulate blood pressure.

**KEY WORDS:** angiotensin I-converting enzyme inhibition, antihypertensive effect, fermented mackerel, *heshiko*, peptide, spontaneously hypertensive rat.

**INTRODUCTION**

Hypertension is thought to be one of the principal factors that triggers circulatory organ diseases such as ischemic heart disease and cerebral apoplexy, which are the predominant causes of death among the Japanese population. Angiotensin I-converting enzyme (ACE, EC.3.4.15.1 [the ‘enzyme number’ according to the International Union of Biochemistry and Molecular Biology, used to classify enzymes systematically]) catalyzes the formation of the potent vasoconstrictive octapeptide angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) from the decapeptide angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) by liberating a dipeptide (His-Leu) in the renin-angiotensin system and it also inactivates the vasodilative peptide bradykinin in the kallikrein-kinin system. Therefore, ACE inhibitors can be expected to reduce high blood pressure and to regulate blood pressure. Recently, the physiological functions of food components have drawn considerable attention from the viewpoint of interrelationships between eating habits and human health. Since the isolation of several kinds of ACE inhibitory peptides from snake toxin by Ondetti *et al.*, such as Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (Pyr: L-pyroglutamic acid),¹ much research has concentrated on the separation of ACE inhibitory peptides from foodstuffs such as sardine,²–⁴ salmon²–⁴ and various other food proteins.⁶–¹⁵ Several kinds of ACE inhibitory peptides isolated have been demonstrated to have an antihypertensive effect in vivo by administration to spontaneously hypertensive rats (SHR).²,⁵,⁶,⁷,⁹–¹⁵ However, most of this research has focused on enzymatic protein hydrolysates¹¹–¹³ rather than processed foods.¹⁴,¹⁵

*Heshiko* is a traditional fermented Japanese fishery product made from chub mackerel, and is produced and consumed in large quantities within the coastal areas of the Sea of Japan, especially around Wakasa Bay. As *heshiko* is delicious and stimulates...
a good appetite, it is customarily taken in several 10-g quantities at a time as a daily dish. As heshiko usually contains a relatively high NaCl content (7–10%), much effort in the manufacturing process has been devoted to reducing its salt content as far as possible from a health standpoint. In contrast, it has been reported previously that large quantities of peptides, taste-active free amino acids and organic acids are produced in mackerel meat during the processing of heshiko. Consequently, it was expected that ACE inhibitory peptides might exist in heshiko, and that heshiko would have as yet unrecognized health value if it could be proved to exhibit an antihypertensive effect in vivo.

In the present study, attempts were made to clarify whether ACE inhibitory activity increases in water extractive components from mackerel meat during the processing of heshiko, and to determine whether an antihypertensive effect in vivo on SHR occurs with orally administered heshiko extract.

MATERIALS AND METHODS

Preparation of heshiko

Fresh raw mackerel (chub mackerel, Scomber japonicus) caught in the Sea of Japan were obtained from a wholesale market in Obama city and immediately brought to the laboratory. Heshiko samples were prepared according to a method previously described. Briefly, 100 raw mackerel, gutted and washed to remove blood, were treated with 23% NaCl relative to the fish weight by evenly scattering the salt on them, and they were salt-pickled in a 100-L plastic barrel with a 40-kg weight on the lid for 1 week at room temperature. Then, the salt-pickled mackerel were taken out of the barrel and rinsed in brine secreted from the fish during the pickling and again placed in another plastic barrel with rice bran (45% of the fish weight), which contained suitable amounts of crushed red pepper. The barrel was filled with brine to the top to avoid exposing the materials to air. The mackerel were pickled for 7 months at room temperature with a 50-kg weight on the lid.

Preparation of hot-water and perchloric acid extracts

Raw, salted and pickled mackerel were periodically taken out of the barrel and used for the preparation of hot-water and perchloric acid (PCA) extracts. Approximately 10-g dorsal white muscle was homogenized with 100-mL distilled water, and the homogenate was heated at 100°C for 5 min. After cooling to room temperature, it was centrifuged at 10 000×g for 20 min at 4°C. After separating the supernatant solution, 50 mL of distilled water was added to the precipitate to obtain a second supernatant solution in the same manner. The two supernatants were mixed and filtrated by filter paper (No. 2; Toyo, Tokyo, Japan) and made up to 200 mL with distilled water. Perchloric acid solution was added to 5 mL of the hot-water extract so as to make a 5% final concentration. After stirring, the solution was centrifuged under the same conditions as above, and the precipitate was re-extracted with 1 mL of 5% PCA solution. The supernatants thus obtained were mixed and neutralized to pH 7.0 ± 0.1 with NaOH. Distilled water was added to the mixture to make up 10 mL of PCA extract. The peptide content of the hot-water extract (total peptides) and PCA extract (PCA peptides) were measured by the method of Lowry et al. using bovine γ-globulin as a standard.

Desalting of hot-water extract of heshiko

Hot-water extract of heshiko was desalted by an electric dialysis apparatus (Micro Acilyzer G3; Asahi-kasei, Osaka, Japan) installed with a filter cassette with a 100-Da molecular weight cut-off. The dialysis was carried out under the automatic mode by setting the initial voltage at 14.5 V and applying 3% potassium nitrate as the electrode solution.

Measurement of angiotensin I-converting enzyme inhibitory activity

Angiotensin I-converting enzyme inhibitory activity was measured by the method of Saito et al. with some modifications. Aliquots of 60 μL of the sample solution with 30 μL of 100 mU/mL ACE (from rabbit lung; Sigma, St. Louis, MO, USA) were preheated at 37°C for 3 min. To this mixture, 250 μL of substrate solution containing 6.5 mM hippuryl-L-histidyl-L-leucine (HHL; Nacalai, Kyoto, Japan), 500 mM NaCl and 100 mM borate buffer, pH 8.3, was added and the mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 500 μL of 1 M HCl and then hippuric acid liberated from HHL by ACE was extracted by 1.5 mL of ethyl acetate with stirring. A 1-mL aliquot of the ethyl acetate layer was transferred to another test tube. After removal of ethyl acetate by drying in a vacuum desiccator, the residue containing the hippuric acid was redissolved in 3.2 mL of distilled water and the optical density (OD) was measured at 228 nm (UV-1200; Shimadzu, Kyoto, Japan).
The ACE inhibition rate was calculated as follows:

\[
\text{ACE inhibition rate}(\%) = \frac{(C - S)}{(C - B)} \times 100 \quad (1)
\]

where \(S\) is the OD value when the sample solution was added to the assay media, \(C\) is the control OD value when distilled water was added to the media as a substitute for the extract, and \(B\) is the blank OD value when 500 \(\mu\)L of 1 M HCl was added to the assay media beforehand to inactivate ACE.

The ACE inhibitory activity of the extract was expressed as the IC\(_{50}\) value, which corresponds to the concentration of peptides that inhibits 50% of the ACE activity in the assay media.

Total inhibitory activity (TIA), another index of ACE inhibitory activity, was expressed as the number of ACE units inhibited by peptides in 1 g of mackerel meat. TIA was calculated as follows:

\[
\text{TIA (mU/g)} = 1.5 \times \frac{\text{peptide content in the fish meat (mg/g)}}{\left(\text{IC}_{50} \ (\text{mg/mL}) \times 0.34 \ (\text{mL})\right)} \quad (2)
\]

One unit (U) is defined as the amount of ACE that could release 1.0 \(\mu\)mol of hippuric acid per 1 min from the substrate under the above conditions.

**Measurement of angiotensin I-converting enzyme activity in blood plasma of spontaneously hypertensive rats**

Spontaneously hypertensive rats were placed under anesthesia by intraperitoneal injection of Somnopentyl (Takeda-Schering Blough Animal Health, Tokyo, Japan) at 0.1 mL/100 g body weight and venous blood was collected by making incisions in the tail 2 cm distal to the body. To avoid blood coagulation, 1 part of 3.2% sodium citrate solution was added to 9 parts of the blood and stirred well. The plasma, which was separated from this mixture by centrifuging at 735 \(\times g\) (3000 r.p.m.) for 10 min at 5\(^\circ\)C, was kept at −135\(^\circ\)C until measurement of ACE activity. ACE activity in the plasma was determined by the method of Masuda et al. with some modifications. \(^{14}\) Aliquots of 50 \(\mu\)L of blood plasma pre-incubated at 37\(^\circ\)C for 3 min were added to 150 \(\mu\)L of substrate solution (6.5 mM HHL, 500 mM NaCl, 100 mM borate buffer, pH 8.3) and the mixture was incubated at 37\(^\circ\)C for 30 min. The amount of hippuric acid liberated was determined as described above. ACE activity was expressed as mU/mL plasma.

**Breeding of spontaneously hypertensive rats**

Three- and seven-week-old SHR (Charles River Japan, Yokohama, Japan) were obtained; the former were used for long-term administration and the latter were used for single or short-term administration. They were pre-bred for 2 weeks before the experiments at 23 ± 2\(^\circ\)C, with a relative humidity of 55 ± 5%, under a 12 : 12 h light : dark cycle (light from 08.00–20.00 hours). They were fed an artificial diet (CE-2; Clea Japan, Osaka, Japan) and sterilized tap water ad libitum.

**Oral administration to spontaneously hypertensive rats**

The body weights of SHR were measured by an electronic balance (MC1; Sartorius, Tokyo, Japan) prior to oral administration. The hot-water extract and its desalted extract from raw mackerel or heshiko were compulsorily administered using a stainless steel sonde. The extracts, which were stored at −30\(^\circ\)C, were used after thawing and warming to 23\(^\circ\)C. The amounts of these extracts used for administration were adjusted as peptide to 5, 10 or 50 mg/kg in the rat, and the administration volume was fixed at 1 mL/100 g body weight. Equal volumes of distilled water or NaCl solution equivalent to the same amount of NaCl in the hot-water extract were used as controls.

For single administration, the rats were prevented from eating for 24 h before administration and 4 h after. For daily administration for 10 days (short-term) and 10 weeks (long-term), they were given feed and water ad libitum.

**Measurement of blood pressure of spontaneously hypertensive rats**

Systolic blood pressure (SBP) was measured at least four times by the tail-cuff method with a programmable electrosphygmomanometer (MK-1030; Muromachi-kikai, Tokyo, Japan) after warming the rats in a chamber maintained at 37\(^\circ\)C for 15–20 min. For single administration, SBP measurement was carried out just before administration and 2, 4, 6 and 8 h afterward. For short-term administration, SBP measurement was carried out just before administration and 1, 4, 7 and 10 days afterward. The measurement was also carried out 5 days after stopping administration. For long-term administration, the measurement was carried out 1 week before the start of administration (4-week-old SHR), just before the start of administration (5-week-old SHR) and every week for 10 weeks (15-week-old SHR). The measurement was also carried out 4 weeks after stopping administration.
RESULTS AND DISCUSSION

Changes in peptide content and angiotensin I-converting enzyme inhibitory activity during processing of heshiko

Figure 1 shows the changes in peptide content and IC$_{50}$ values for the hot-water extract of mackerel meat after 7 days of salt-pickling and after 7 months of fermentation with rice bran during the processing of heshiko. It was necessary to examine the ACE inhibitory activities of low molecular peptides (PCA peptides) in heshiko because many kinds of ACE inhibitory peptides with low molecular weights have been reported in other studies. The IC$_{50}$ of the total peptides of raw mackerel decreased from 0.4 to 0.2 mg/mL after 7 days of salt-pickling, and during fermentation it slightly decreased to 0.1 mg/mL until 4 months and then decreased little afterward. In contrast, the IC$_{50}$ of the PCA peptides of raw mackerel decreased from 2.1 to 1.5 mg/mL during salt-pickling and to 0.1 mg/mL for the 4 months of fermentation followed by little change afterward. For raw and salted mackerel, the IC$_{50}$ of the PCA peptides was higher than that of the total peptides. This could have been because of the inhibition of high molecular weight peptides, which can be insolubilized by PCA.

It has been reported previously that total peptide and PCA peptide contents increase throughout the processing period of heshiko. A similar tendency can also be seen in Figure 1. These results suggest that some parts of the peptides that are produced abundantly during processing can inhibit ACE.

Figure 2 shows the changes in TIA of hot-water and PCA extracts during processing. The TIA of raw mackerel increased from 82 to 354 mU/g for the 7 days of salt-pickling and up to 1122 mU/g for the 4 months of fermentation followed by a gradual increase until 7 months. The TIA of PCA extracts of raw mackerel increased from 8 to 24 mU/g during salt-pickling and up to 490 mU/g after 4 months of fermentation.

The TIA value is the product of the peptide content and reciprocal of the IC$_{50}$ value. Therefore, the TIA increases with increasing ACE inhibitory peptide content and/or decreasing IC$_{50}$. The increase in TIA during the processing period from the raw mackerel state until 4 months of fermentation was the result of a decrease in IC$_{50}$ and an increase in peptide content. The increase in TIA after 4 months of fermentation was mostly the result of a gradual increase in peptide content rather than a change in IC$_{50}$, because the IC$_{50}$ value changed little during this period (Fig. 1).
Antihypertensive effect of heshiko

Effects of single administration of heshiko and raw mackerel extracts on blood pressure in spontaneously hypertensive rats

It was expected that the heshiko extract would induce an antihypertensive effect in vivo, because it inhibited ACE in vitro more than the raw mackerel extract did. Figure 3 shows the change in SBP caused by single oral administration of heshiko and raw mackerel extracts to SHR at 10 mg peptide/kg in the rats. Saline as a positive control had negligible effects on SBP. Raw mackerel extract slightly lowered the SBP from 4 to 6 h after administration, although the effect was not significant, and it recovered its initial level after 8 h. For heshiko extract, the SBP decreased significantly from 2 to 4 h after administration, and it recovered its initial level after 8 h. The sample weights of raw mackerel and heshiko, which contained 10 mg of peptide, were 1.6 g and 0.4 g, respectively, because the peptide content of raw mackerel is 6.5 mg/g and that of heshiko is 26.0 mg/g. The maximum decrease in the SBP induced by the raw mackerel extract was 12 mmHg and that for heshiko extract was 36 mmHg. These results indicate that the antihypertensive effect of heshiko is stronger than that of raw mackerel, and that antihypertensive components, such as antihypertensive peptides, must be produced during the processing of heshiko. Crushed salmon head hydrolysate at 2 g peptide/kg in rats decreases the SBP by 20 mmHg at 24 h after administration, and 10 mg peptide/kg of sardine muscle hydrolysate decreases the SBP by 15 mmHg between 4 and 8 h after administration. Thus, heshiko has a considerably stronger antihypertensive effect on SBP compared with these preparations.

Effects of single administration of heshiko extract and its desalted extracts on blood pressure in spontaneously hypertensive rats

Heshiko possesses a relatively high content of NaCl, as much as 7–10%, which is derived from salt added to the mackerel during processing. A high intake of NaCl is thought to induce hypertension. Thus, in the present study, the effect of NaCl in heshiko on the SBP in SHR was examined by administering heshiko extract and its desalted extract. As shown in Figure 4, the administration of 0.32% NaCl solution as a control did not have a significant effect on SBP. The heshiko extract and its desalted extract lowered SBP significantly from 2 to 4 h after administration in the former and 4 h after in the latter, and the maximum decrease in SBP caused by the desalted extract was larger than that...
caused by the *heshiko* extract. This result suggests that the exclusion of NaCl from the *heshiko* extract was related to the decreasing effect on SBP. However, the presence of any significant effects on SBP could not be distinguished, because the *heshiko* and desalted extracts induced different individual patterns in SBP changes.

**Single administration of *heshiko* extract to spontaneously hypertensive rats at different dose levels**

Although *heshiko* extract lowered the SBP in SHR significantly by oral administration at 10 mg peptide/kg in the rats, the minimum SBP observed after 4 h recovered its initial level 8 h after administration. The reason why the decreased SBP continued for a relatively short time seemed to be the low administration level of 10 mg/kg. Single administrations were carried out at three different doses (5, 10 and 50 mg/kg) to confirm the dose dependence of *heshiko* extract. As shown in Figure 5, the maximal SBP decreases were observed 4 h after administration for all doses and the SBP recovered its initial level after 8 h. Although the maximal SBP decrease for 5 mg/kg was smaller than that for 10 mg/kg, that for 10 mg/kg was almost the same as that for 50 mg/kg. The observation that the reduction in the SBP did not depend on doses higher than 10 mg/kg suggests that the administration of larger amounts of *heshiko* extract would not bring about an extreme decrease in SBP.

Sardine hydrolysate at three different doses (10, 100 and 1000 mg peptide/kg) similarly decreases the SBP in SHR after oral administration, and the change in SBP in SHR administered sardine protein hydrolysate for 2 g protein/kg is similar to that for 10 g/kg. From these data, the low dose dependence on SBP might be common to fish hydrolysate and fermentation products.

**Effects of short-term successive administration for 10 days on blood pressure in spontaneously hypertensive rats**

As mentioned above, *heshiko* extract decreased the SBP in SHR for low dose administration, but it had a relatively short antihypertensive effect lasting less than 8 h. It was expected that successive administration of *heshiko* extract to SHR would induce a prolonged duration of decreased SBP. Therefore, short-term successive administration for 10 days was conducted using raw mackerel extract, *heshiko* extract and its desalted extract (Fig. 6). Administration of 0.32% NaCl solution as a
control showed no significant effect on SBP for 10 days, and that of raw mackerel extract (10 mg/kg) showed the tendency, although not significant, to a slight decrease in SBP between 7 and 10 days. For heshiko extract and its desalted extract, the SBP was lowered significantly between 7 and 10 days and recovered its initial level 5 days after stopping administration. Desalted heshiko extract, although not significant, had a tendency to reduce the SBP more than heshiko extract, suggesting that the amount of NaCl in heshiko extract would not have an intense effect on SBP. As seen in Figure 6, the SBP in SHR administered heshiko extract or its desalted extract remained low from 7 to 10 days during the administration period. This indicates that the successive administration of heshiko extract has an effect in decreasing the basic SBP in SHR.

The reason why heshiko extract exerts such an antihypertensive effect is not clear. However, the large amount of peptides produced in mackerel (Fig. 1) and the increase in its ACE inhibiting effect (Figs 1,2) during the processing of heshiko indicate that ACE inhibitory peptides in heshiko should contribute to the antihypertensive effect.

Yoshii et al. reported that the administration of oligopeptides derived from egg decreased the SBP in SHR and serum ACE activity simultaneously.11 Xu et al. also reported that ACE activity in the serum of SHR, whose SBP was decreased by the administration of proteinase hydrolysate from zein, was significantly lower than in the control group.12 However, Wu and Ding reported that ACE activity in the serum of SHR administered soy protein hydrolysate from zein was almost the same level as the control group.13 Therefore, in the present study, the ACE activity of plasma from SHR administered mackerel and heshiko extracts at 10 mg peptide/kg per a day for 10 days and that from SHR 5 days after stopping administration were determined.

Figure 7a shows the ACE activities (mU/mL) of plasma from SHR 10 days after the start of administration and Figure 7b shows the ACE activities (mU/mL) of plasma from SHR 5 days after stopping administration. As shown in Figure 7a, the plasma ACE activity of the heshiko extract group was significantly lower than that of the control (0.32% NaCl solution). However, it was almost the same as the control 5 days after stopping administration (Fig. 7b). As shown in Figure 6, the SBP in SHR administered heshiko extract for 10 days was significantly lower than that of the control SHR, and the SBP of the former 5 days after stopping administration was equal to that of the latter. These results suggest that some kinds of peptides abundantly produced in heshiko have roles in decreasing SBP by inhibiting plasma ACE after absorption from the alimentary canal into the blood with or without digestive modification.

Long-term administration for 10 weeks

As shown in Figure 6, successive administration of heshiko extract effectively decreased the SBP in SHR 7 weeks old or older. Thus, it is necessary to confirm the antihypertensive effect of heshiko extract on young SHR during the growth stage, for the prevention of hypertension through long-term administration. Figure 8 shows the changes in body weight in SHR with continuous administration of raw mackerel, heshiko and desalted heshiko extracts for 10 weeks (70 days) and the change following 4 weeks without administration. The changes in body weight were similar among all SHR groups and significant differences were not observed, suggesting that heshiko extract does not prevent the growth of SHR.

Figure 9a shows the change of SBP in SHR administered a 1.6% NaCl solution (control), raw mackerel extract (10 mg/kg) and two levels of heshiko extract (10 and 50 mg/kg). During the predominant growth stage from 5 to 9 weeks old, corresponding to 28 days after the start of administration, the SBP in every SHR group rapidly and equally increased with increasing body weight as shown in Figure 8. During the 6 weeks from 9 to 15 weeks old, corresponding to the stopping point of administration, the SBP in every SHR group rapidly and equally increased with increasing body weight as shown in Figure 8. During the 6 weeks from 9 to 15 weeks old, corresponding to the stopping point of administration, the SBP in SHR administered 1.6% NaCl solution (control) and that in SHR administered mackerel extract tended to increase.
slightly, whereas the SBP in SHR administered *heshiko* extract remained low and was mostly constant, especially for a dose of 50 mg/kg of *heshiko* extract. Thus, *heshiko* extract effectively decreases SBP in SHR through successive intake, although it cannot suppress the rapid SBP increase seen in the young SHR during the growth stage. Even 4 weeks after stopping administration, the SBP in SHR administered 50 mg/kg of *heshiko* extract remained significantly lower than that of the control.

Figure 9b shows the changes in the SBP in SHR with distilled water only (control), 1.6% NaCl solution (positive control), 50 mg/kg of *heshiko* extract and 50 mg/kg of its desalted extract. During the 6 weeks between 9 and 15 weeks old, the SBP in SHR administered 50 mg/kg of *heshiko* extract and 50 mg/kg of its desalted extract remained at a lower and constant level. Kabir et al.\(^{19}\) and Kabir and Kimura\(^{20}\) reported that controlling body weight was a valid method for suppressing blood pressure if done through the administration of *maitake*. The antihypertensive effect seen in the present study was considered to be unrelated to the changes in body weight. For 4 weeks after stopping administration, the SBP of SHR administered *heshiko* extract and its desalted extract remained significantly lower than that of the controls, in spite of the interruption of intake of these *heshiko* extracts. As shown in Figures 6 and 7, however, the decreased SBP and ACE activity of blood plasma in SHR administered *heshiko* extract for 10 days returned to the control level 5 days after stopping administration. In addition, no differences could be found between the plasma ACE activity for SHR administered *heshiko* extract and that of the control 4 weeks after stopping administration (data not shown). These results present a new problem: some factors effectively decrease the SBP by means other than ACE inhibition, which could be related to the antihypertensive effects seen in the long-term administration of *heshiko* extract (10 weeks).

In conclusion, it was confirmed by single, short-term and long-term administration that *heshiko* extract at low doses decreases SBP in SHR. ACE inhibitory peptides derived from *heshiko* extract are closely involved in the antihypertensive effect. However, the possibility of the participation of factors affecting blood vessels other than ACE cannot...
be excluded. Further research will address these questions in a subsequent paper.

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