STUDIES ON DEXTRANASES

PART I. ISOLATION OF EXTRACELLULAR, BACTERIAL DEXTRANASES

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ABSTRACT

A bacterium isolated from soil has been shown to yield, inductively, at least two extracellular dextranases (and also some intracellular dextranase activity) when grown on a medium containing dextran as sole carbon-source. Purification by ammonium sulphate precipitation and gel-permeation chromatography yielded a dextranase (D_1), which behaved as a single protein on electrophoresis at two different pH values, and a different fraction (D_2) containing dextranase activity, which gave two protein bands on electrophoresis. Both enzymes yielded mixtures of oligosaccharides from dextran and are regarded as endo-enzymes. Effects of temperature and pH on the activity of the enzymes were investigated.

INTRODUCTION

Dextranases (E.C. 3.2.1.11) are capable of degrading the α-(1→6)-D-glucopyranosyl linkage in dextrans. The latter are elaborated by several species of bacteria, *e.g. Leuconostoc mesenteroides*, and are a heterogeneous group of glucans which vary in their degree of branching and the type of branch points. There has been a recent increase in interest in dextranases in part, at least, because of the probable implications of dextran in dental caries[1] and in the production of sugar cane. Dextran is often produced by *Leuconostoc* on sugar cane after burning and chopper-harvesting and before milling. This process is of considerable technological importance because it results in losses of sucrose, and particularly because the dextran so produced in the juice can cause major problems in sugar milling. These problems have been shown to result from the effect of dextran on sucrose crystallisation, whereby the rate of crystallisation on certain faces is slowed and the crystal shape is changed[2,3]. There is currently much interest in the potential use of dextranases both in the removal of dextran from the juice of deteriorated sugar cane[4] and in the prevention of dental caries. For the study of such systems, we anticipate that the isolation of pure dextranases will be very desirable and should also yield powerful tools for the further study of the detailed chemical structure of dextrans.

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Dextranase production has been induced in a number of fungi and bacteria, and such enzymes have been found in some mammalian tissues, such as the spleen and liver[5], and also in coleoptiles of *Avena*[6,7]. However, in comparison, for example, with the extensive studies of amylases, only a limited amount of work has been done on the enzymes from any of these sources. Extracellular dextranase preparations from *Penicillium lilacinum* and *P. funiculosum* were used extensively by Bourne *et al.*[8,9] for studies of substrate specificity and of degradation products from linear and branched dextrans and short-chain oligosaccharides, but no attempt was reported to determine whether more than one dextranase was present. Recently, Chaiet *et al.*[10] have succeeded in purifying an extracellular dextranase produced by *P. funiculosum*. The fungus was grown on a different dextran from that used in the previous study and, as no results on action patterns were given, it is difficult to correlate the results of the two laboratories.

There has been conflicting evidence for the presence of intracellular dextranases in these species of *Penicillium*. Hultin and Nordström[11] had reported their occurrence, whereas Tsuchiya *et al.*[12] had failed to find any evidence for their production. Hutson and Weigel[13], however, conclusively demonstrated the presence of intracellular enzyme or enzymes with an extract which was not further purified but which was quite different from the extracellular enzymes in its action on isomaltosaccharides. The most complete study on dextranases from a *Penicillium* has been published by Fukumoto *et al.*[14] who obtained a pure enzyme from *P. luteum* and studied its substrate specificity. Although this dextranase was induced by a type of dextran different from those used for the enzymes previously mentioned, it exhibited similar modes of attack on linear oligosaccharides.

Less work has been reported on bacterial dextranases than on the fungal enzymes. An extracellular dextranase[15–17] produced by *Lactobacillus bifidus* has been described, together with a cell extract containing an α-(1→6)-D-glucosidase[18] which degrades isomaltodextrins. A dextranase has been purified from the cell-wall surface of *Cytophaga*[19] and, although no extensive studies were done on its mode of action, the preliminary results suggested that it was an endo-enzyme. Other intracellular dextranases, which were apparently exo-enzymes, have also been extracted from two species of *Bacillus*[20]. These latter enzymes and one obtained from a cell-free culture of *Bacteroides*[21] are the only bacterial exo-dextranases described.

EXPERIMENTAL

Materials. — The dextran, Type 100-C, in the molecular weight range of 100,000 to 200,000, and the bovine serum albumin, fraction V, were obtained from the Sigma Chemical Co.

General methods. — (*a*) *Assay for dextranase activity.* Dextranase activity was assayed in 7.7mM citrate buffer (1.3 ml, pH 5.5) containing 0.4% of dextran. The solutions were incubated at 33°, usually for 1 h, and samples removed for assay of reducing sugars by the Nelson–Somogyi method[22], using anhydrous D-glucose as the

reference standard. One unit of dextranase activity is defined as that amount of enzyme which produces 1 μgequivalent of D-glucose per min under the above conditions, and specific activity as the units of activity per mg of total protein.

(*b*) *Protein assays.* Protein, eluted during chromatography on BioGel P-200, was monitored directly by absorbance at 280 nm. Protein was also determined by the Folin–Ciocalteau method[23] as modified by Oyama and Eagle[24], using bovine serum albumin as reference standard.

(*c*) *Carbohydrate assay.* Carbohydrate was determined by the phenol–sulphuric acid method[25], using anhydrous D-glucose as reference standard.

(*d*) *Chromatography.* Oligosaccharides were separated and identified on Whatman No. 1 paper by using ethyl acetate–pyridine–water (10:4:3, v/v) and silver nitrate–sodium hydroxide[26]. Samples from dextranase digests were deionised prior to chromatography with Amberlite IR-45(HO^-) and IR-120(H^+) resins.

Isolation and growth of bacterium. — (*a*) *Isolation of bacterium.* The bacterium was isolated from a sample (14 g) of a surface soil from a second ratoon field of Pindar variety sugar-cane and grown at 33° in a shaking water-bath in the following medium: basal mineral medium (50 ml), 2% KNO_3 (25 ml), 0.5% K_2HPO_4 (50 ml), dextran (10 g), and distilled water (925 ml). The basal mineral medium consisted of $MgSO_4$ (1.3 g), NaCl (2.5 g), $FeSO_4$ (0.05 g), $MnSO_4$ (0.05 g), and Na_2MoO_4 (0.05 g) made up to 1 litre with distilled water. The purity of the bacterial culture was periodically checked on agar plates consisting of the above medium plus 1.5% of agar. Bacterial growth was followed by measuring the absorbance of culture samples at 645 nm, using sterile medium as the blank.

On agar plates, after 2 days, the bacterium produced cream-coloured colonies, 0.1 to 0.2 cm in diameter, which had a glistening, smooth surface and a smooth perimeter. Gram stains performed on bacteria from a day-old slope culture showed Gram negative rods, approximately 0.5 by 1.0 μm, either singly or in chains. After 4 days, another morphological type of rod predominated. These were much larger, approximately 1.0 by 2.0–4.0 μm, with rounded ends, and were often elongated ovoid in shape. These cells showed irregular staining patterns, with some being almost devoid of stain whilst others had a distinct, beaded appearance. Single cells and also long chains were apparent, although it was difficult to distinguish the individual cells in a chain.

The bacterium has been lodged with Professor V. B. D. Skerman of the Queensland University for characterization and classification, and has been given the code number UQM 733.

(*b*) *Dextran content of bacterial cultures.* The amount of dextran remaining in samples of the culture medium during bacterial growth was measured after removal of the cells by centrifugation at 12,000 *g* for 30 min at 4°. The dextran was precipitated from each sample by addition of an equal volume of 95% ethanol and allowing to stand overnight at 4°. The precipitates were centrifuged, washed three times with 50% ethanol, and finally dissolved in water for assay of carbohydrate content. Results are shown in Fig. 1.

(*c*) *Change in extracellular dextranase activity during growth of bacterium.* Samples (10 ml) of culture were removed at various times, for measurement of dextranase activity after precipitation of the enzyme with ammonium sulphate as described under "Purification of dextranases (*a*)". Results are shown in Fig. 1.

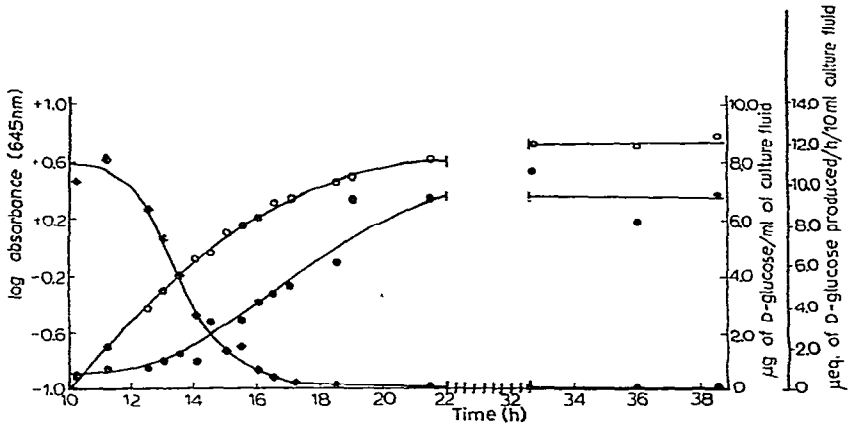


Fig. 1. Production of dextranase during growth of bacterium. O, growth curve (Log absorbance at 280 nm); ●, dextranase activity (μeq. of D-glucose produced/h/10 ml of culture fluid); ◆, dextran concentration (mg of D-glucose/ml of culture fluid).

(*d*) *Measurement of intracellular dextranase activity.* After harvesting, the bacterial cells were washed twice in 0.2M citrate buffer (pH 5.5) and dispersed with the aid of a syringe in the same buffer to give a dense suspension. This was then passed through a French Press at 8 tons/in.^{2}, and an aliquot of the slurry was used for dextranase assay. Under these conditions, dextranase activity corresponding to 6 units/litre of original culture was found, in comparison with an extracellular activity of 24 units/litre of cell-free culture fluid.

Purification of dextranases. — (*a*) *Precipitation by ammonium sulphate.* Six 1-litre cultures were grown in 3-litre conical flasks with shaking at 33°. The bacterial cells were harvested by centrifugation at 12,000 *g* for 30 min at 4° when the absorbance at 645 nm (after 10-fold dilution of an aliquot portion) had reached 0.35–0.4. The cell-free culture fluid was then brought to 60% saturation by the gradual addition of solid ammonium sulphate and the solution was kept at 4° for 15 h (all subsequent operations in the isolation of the enzymes were carried out at 4°). The fine precipitate was collected by centrifugation at 12,000 *g* for 30 min and re-suspended in 0.2M citrate buffer (pH 5.5) prior to dialysis against several changes of the same buffer. The solution was then vacuum-dialysed for concentration to ~4 mg of protein/ml.

(*b*) *Chromatography on BioGel P-200.* A BioGel P-200 column (91.5 × 3.5 cm) was equilibrated with 0.2M citrate buffer (pH 5.5). A sample (4.0 ml) of the concentrated solution of protein was applied to the top of the column and eluted with the

same buffer at the rate of 7 ml/h. Fractions were assayed for dextranase activity, carbohydrate, and protein, and the results are shown in Fig. 2.

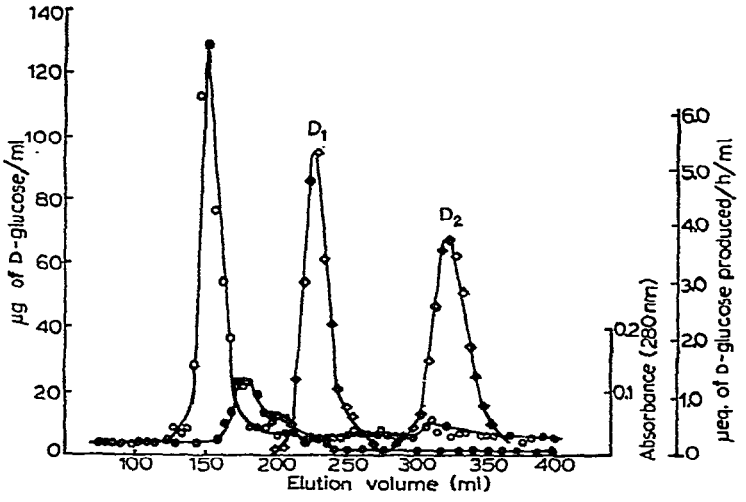
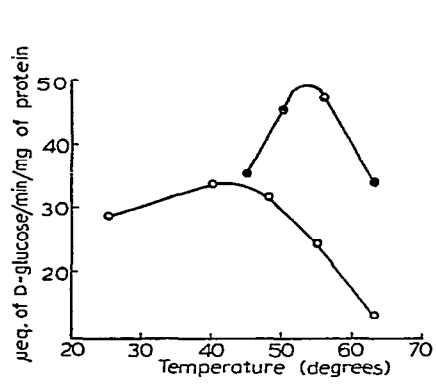


Fig. 2. Separation of dextranases on BioGel P-200. ○, carbohydrate (μg of D-glucose/ml); ●, protein (absorbance at 280 nm); ◇, dextranase activity (μeq of D-glucose produced/h/ml).

Electrophoresis of enzymes. — Electrophoresis was performed on cylinders (3 × 50 mm) of 5% polyacrylamide gel made up in two separate buffer systems, *viz.* borate–hydrochloric acid (pH 8.3) and borate–sodium hydroxide[27] (pH 9.3). D_1 and D_2 fractions (210 to 240 ml, and 305 to 340 ml, respectively) from BioGel chromatography were each dialysed against each of the borate buffers and then concentrated by vacuum dialysis to a final volume of ≤1 ml. Samples (30 μl) were then mixed with an equal volume of 68% sucrose and applied to the gel cylinders before a further, short sample (15 mm) of acrylamide was polymerised on top of this. The gels were run at 4° for 4 h (at pH 8.3) and 2 h (at pH 9.3) with a current of 2 mamp per cylinder. The cylinders were then fixed in 15% trichloroacetic acid (TCA) for 1 h and stained with 0.1% Coomassie Brilliant Blue in 15% TCA overnight[28], before being de-stained with further 15% TCA.

Gel chromatography of D_1 *and* D_2. — Fractions, 210 to 250 ml, (D_1) and 305 to 350 ml (D_2), from the gel chromatography (Fig. 2) were combined and concentrated by vacuum dialysis. Concentrated D_1 (4.0 ml) was applied to the column previously used and eluted with 0.2M citrate buffer (pH 5.5). The fractions were collected and assayed for dextranase activity, as previously described, except that the sensitivity of the dextranase assay was increased by incubating the digests for 4 h at 33°. The experiment was repeated with concentrated D_2 (6.0 ml).

General properties of the dextranase. — (*a*) *Effect of temperature on activity.* Dextranase activity was assayed [see General methods (*a*)] during incubation at different temperatures for 1 h (Fig. 3).



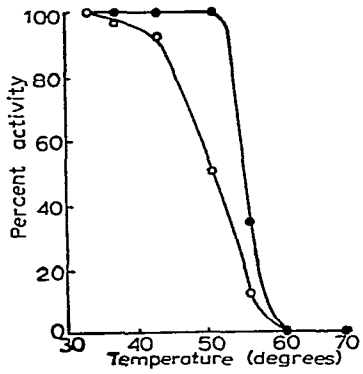


Fig. 3. Effect of temperature on dextranase activities. ●, D_1; ○, D_2.

Fig. 4. Effect of temperature on dextranase stability (the enzyme activity is expressed as % of the activity at 33°). ●, D_1; ○, D_2.

(*b*) *Temperature stability.* Enzyme in citrate buffer (7.7mM, pH 5.5) was incubated for 1 h at a series of temperatures (Fig. 4) and then transferred to a water bath at 33°. Dextran solution was added to a final concentration of 0.4% and, after incubation for a further 1 h at 33°, samples were removed for assay of reducing sugars.

(*c*) *Effect of pH on activity.* Dextranase activity was measured [see General methods (*a*)] at different pH values (Fig. 5) by replacing citrate buffer with acetate buffer (77mM, pH 3.15–5.5) and phosphate buffer (77mM, pH 5.5–8.0).

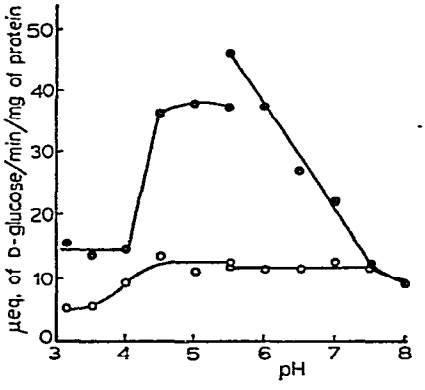


Fig. 5. Effect of pH on dextranase activities. Acetate buffers, pH 3.0 to 5.5; phosphate buffers, pH 5.5 to 8.0. ●, D_1; ○, D_2.

RESULTS AND DISCUSSION

In sugar-cane fields, the juxtaposition of *Leuconostoc* bacteria from soil and an abundance of sucrose is likely to produce an unusually high content of dextran in the

soil. Thus, the soil from a cane field seemed a likely source for dextranase-producing bacteria. Both top soil and deeper soil samples were taken, but only the top-soil samples gave a prolific supply of organisms when grown on dextran as the sole carbon-source. Three or four bacteria and several fungi were isolated from these samples. The organism used for this study was the most hardy of the bacteria and had the most vigorous growth under the conditions used. The optimal temperature for growth was found to be ~33°.

During bacterial growth, extracellular dextranase activity was found to increase and levelled off at a maximum value with the onset of stationary phase of growth. At this stage, the dextran content of the medium was reduced to a very low level (Fig. 1). Dextranase was found intracellularly at this stage, although the activity was only 25% that of the extracellular enzyme. However, the full activity of the intracellular enzyme may not have been apparent under the conditions used for solubilisation. The purification of this enzyme will be the subject of a further paper. The bacteria were usually harvested at the beginning of the stationary phase, in order to minimise contamination of the preparation with protein released during bacterial lysis. A similar experiment, in which dextran was replaced with 1% of D-glucose as the sole carbon-source and using bacteria previously adapted to growth on a D-glucose medium, indicated that no extracellular dextranase was produced although there was some (<0.5% of that found when grown on dextran) present intracellularly. Thus, it would seem that both extra- and intra-cellular dextranases are induced in the presence of dextran.

Most of the extracellular dextranase could be precipitated from the cell-free culture medium by addition of ammonium sulphate to 60% saturation (Table I). Part of the precipitate was normally obtained as an aerated scum. Solutions of the precipitate were surprisingly viscous, both before and after dialysis, presumably because of a polysaccharide component (*cf.* Fig. 2). This high viscosity represented a limiting factor to the extent of concentration possible in vacuum dialysis and in loading the column for gel chromatography. However, the carbohydrate was removed from the dextranase fractions during chromatography (Fig. 2) and may have been

TABLE I

PURIFICATION OF DEXTRANASES FROM CELL-FREE CULTURE MEDIUM

Procedure	*Specific activity (units/mg of protein)*	*Yield (%)*	*Purification*
Cell-free culture medium	0.44	100	1
Precipitation by 60% $(NH_4)_2SO_4$	2.50	85	5.74
Concentration by vacuum dialysis	2.59	66.2	5.92
Separation on BioGel P-200 column:			
Peak tube, D_1	21.7		49.5
Peak tube, D_2	16.9		38.7
Total D_1 and D_2		63.5	

dextran which co-precipitated with the protein. No greater-than-background quantities of carbohydrate were eluted with the two dextranase peaks.

Gel chromatography also resulted in the separation of two distinct dextranase peaks, named D_1 and D_2 (Fig. 2). D_1 was eluted first, shortly after the void volume, and was well separated from major polysaccharide and protein components. D_2 was eluted last and was always the smaller of the two dextranase peaks. In both cases, considerable purification was obtained (see Table I), D_1 having a specific activity of 22 units/mg of protein, a 50-fold purification, and D_2 a specific activity of 17 units/mg of protein, representing a 39-fold purification. In both calculations for specific activity, the low proportion of protein in the peak fractions necessitated the concentration of these fractions by vacuum dialysis prior to protein assay by the Folin–Ciocalteau method.

These results may be compared with those of Eriksson and Pettersson[29] who reported two peaks of cellulase activity on chromatography. However, when their original preparation was dialysed prior to chromatography, a high proportion of carbohydrate could be removed. The second peak of cellulase activity was then eliminated during chromatography and was considered to be a protein–carbohydrate complex of the first peak. Our results cannot be due to this type of effect, because insignificant quantities of carbohydrate were removed on dialysis of the crude preparation although practically all of this carbohydrate was separated from D_1 and D_2 during chromatography.

Both of the peaks reported by Eriksson and Petersson also showed homogeneity on re-chromatography as did D_1 and D_2. On re-chromatography, D_1 and D_2 emerged at the same elution volume as the original D_1 and D_2 peaks, respectively, and there was no indication of dextranase activity in any other fractions. However, when the total activity emerging from the column on re-chromatography of either D_1 or D_2 was compared with the total activity applied, a considerable variation was found on repeated runs. Sometimes the total activities were equal, but often more activity, reaching twice that applied in some instances, was eluted. The cause of this effect is not immediately apparent but will be the subject of further investigation.

The purity of the D_1 and D_2 dextranases was monitored by polyacrylamide electrophoresis. D_1 behaved as a single protein during eletrophoresis at two different pH values. At pH 8.3, the protein migrated at 55 mm/h, and at pH 9.3 at 120 mm/h. D_2, however, gave two bands of protein at pH 8.3; one migrated at 60 mm/h and the other, a less-distinct band, at 56 mm/h. At pH 9.3, the two bands ran almost together at 115 mm/h. When both D_1 and D_2 were applied to the same gel column for electrophoresis at pH 8.3, only two protein bands were apparent. One corresponded to the D_1 sample and the other corresponded to the faster of the two D_2 bands and was slightly ahead of the D_1 band. Evidently, however, each of the protein components of the D_2 fraction differs from D_1 on the evidence of gel chromatography, supported by the different products of enzymic action on dextran (Table II). The two components of D_2 may, of course, be isoenzymes, although we have no significant evidence for this possibility.

D_1 and D_2 differ in their physico-chemical properties. D_1 has optimal activity at a much higher temperature (~55°) than D_2 which shows optimal activity in the range of 40–45° (Fig. 3). D_1 is also the more thermostable during a one-hour period than D_2 (Fig. 4), the former being stable up to 51°, whereas the stability of D_2 decreased above 43°. Similarly, the two enzymes differ in pH optima (Fig. 5). D_1 has optimal activity between pH 4.5 and 5.5, whereas D_2 shows optimal activity over a very broad pH range between 4.5 and 7.5, an effect which is perhaps associated with the presence of two proteins in D_2. The break in the D_1 curve (Fig. 5) is associated with a change in buffer and indicates inhibition by acetate.

Although D_2 had only a slightly lower specific activity than D_1, when measured against dextran as the substrate, the relative yields of oligosaccharides from dextran degradation by the two enzymes were very different. Samples from digests were removed at various time intervals for paper-chromatographic identification of the degradation products (Table II). D_1 produced isomaltotetraose and the pentaose within 30 min, and isomaltose and the triose were also apparent after 4 h. After 15 h, these four oligosaccharides, particularly isomaltose, had increased in concentration, whereas those having d.p. greater than six were present in very small proportion, and no material of high molecular weight was apparent at the origin of the chromatogram. The D_2 degradation products, however, showed a completely different pattern. After 30 min, no oligosaccharides of d.p. lower than six were detected, but some tetraose and pentaose were apparent within 4 h. These increased slightly in concentration during 15 h of digestion, but only after prolonged incubation for 30 h was any triose evident and there was still no isomaltose produced.

TABLE II

DEXTRAN DEGRADATION PRODUCTS AFTER INCUBATION WITH D_1 AND D_2

Oligosaccharide[a]		D_1			D_2		
	Time (h)	*0.5*	*4*	*15*	*0.5*	*4*	*15*
IM2		0[b]	2	5	0	0	0
IM3		0	3	5	0	0	0
IM4		1	5	6	0	1	1
IM5		1	4	4	0	4	4
IM6 to IM10		3	5	3	5	3	3
At origin		1	0	0	3	3	3

[a]IMx = linear isomaltodextrin of d.p. = x. [b]The figures represent relative amounts of oligosaccharide present, as judged visually on paper chromatograms.

D_1 therefore produces very much greater yields of oligosaccharides of low molecular weight from dextran than does D_2 in the early stages of degradation, despite the fact that the specific activities of the two enzymes do not differ greatly. This is probably due to the fact that the specific activities are measured over a short time-period and represent the rate of attack on dextran of high molecular weight.

Apparently, D_1 is capable of subsequently extending its attack to smaller oligosaccharide fragments than is D_2. Such differences in substrate specificity will be the subject of further studies.

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