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CONCENTRATIONS OF VARIOUS EFFECTORS AND SUBSTRATES OF PHOSPHOFRUCTOKINASE IN THE JEJUNUM OF RAT AND THEIR RELATION TO THE LACK OF PASTEUR EFFECT IN THIS TISSUE*

GOPI A. TEJWANI**, JASMINDER KAUR§, M. ANANTHANARAYANAN*** and ABBURI RAMAIAH§§

Department of Biochemistry, All-India Institute of Medical Sciences, New Delhi-110016 (India)

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SUMMARY

1. The concentrations of various substrates and effectors of phosphofructokinase were estimated in the intact jejunum of rat. The concentrations of ATP, ADP, AMP, fructose 6-phosphate, NH_4^+ and P_i were, respectively, 1.25, 0.65, 0.19, 0.014, 1.65 and 9.5 μmoles/g wet weight of jejunum.

2. The activity of partially purified rat mucosal phosphofructokinase (specific activity 0.8) when measured under these conditions was not affected by the inclusion of citrate or an additional concentration of positive effectors in the assay mixture suggesting that the enzyme was present in an uninhibited state under these conditions.

3. It is suggested that the lack of the Pasteur effect may be due to the favourable ratio of positive effectors to negative effectors of phosphofructokinase in this tissue which keeps the enzyme active even under aerobic conditions.

4. It is argued that the regulation of phosphofructokinase in this tissue is mainly affected by alterations in the concentrations of fructose 6-phosphate and is thus consistent with the additional function of glycolysis in this tissue, i.e. transport of sugars as lactate in addition to other modes of their transport.

INTRODUCTION

Phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) is identified as the locus for the Pasteur effect based on the studies of the properties of this enzyme from a number of tissues and micro-organisms [1, 2]. Properties of phosphofructokinase from the mucosa of rat jejunum where the Pasteur effect was not observed [3–6], were studied [7]. The properties of phosphofructokinase from this tissue were found to be similar to those of the enzyme from other sources

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Present address: Department of Physiological Chemistry, Institute of Molecular Biology, Nutly, N. J., §Department of Neurology, San Francisco Medical Center, California University, Calif., ***Department of Biochemistry, Lady Hardinge Medical College, New Delhi, India, §§Biochemistry Department, Michigan State University, Mich. 48823.

except that this enzyme was very sensitive to activation by NH_4^+. It was argued that the reasons for the lack of the Pasteur effect in this tissue may be due to the favourable ratio of activators to inhibitors of the enzyme [7] even under aerobic conditions.

In the present communication, the actual concentration of various effectors and substrates of phosphofructokinase in the rat jejunum are described. The partially purified phosphofructokinase activity of jejunal mucosa was estimated at the observed concentrations of effectors and substrates of this enzyme in the intact jejunum.

Phosphofructokinase was not inhibited under these conditions suggesting that the favourable ratio of activators to inhibitors of the enzyme in this tissue under aerobic conditions may be the explanation for the lack of the Pasteur effect.

It is suggested that phosphofructokinase and thus glycolysis is controlled simply by substrate concentration (fructose 6-phosphate) which is well suited in the mucosa where glycolysis plays a role in the transport of sugars [8] (for reviews see refs 9 and 10).

MATERIALS AND METHODS

Animals

Female albino rats of the All-India Institute of Medical Sciences strain, weighing 150–200 g were used.

Chemicals and enzymes

The following chemicals and enzymes were obtained from Sigma Chemical Co., St. Louis, Mo, U.S.A. ADP, phosphoenolpyruvate, α-ketoglutarate, imidazole (fluorescence free), dithiothreitol and $(NH_4)_2SO_4$ suspensions of aldolase, triose phosphate isomerase, α-glycerophosphate dehydrogenase, hexokinase, pyruvate kinase, adenylate kinase and a glycerol suspension of L-glutamate dehydrogenase. The source of all the other chemicals used is given in the previous paper [7].

Preparation of tissue extracts

Unless otherwise mentioned, all the operations were performed at 0–4 °C. Five rats (fed ad libitum) were used in each experiment. They were anaesthetized with ether. The abdomen was cut open and the jejuna were removed and within 30 s transferred and pooled in a preweighed test tube containing a mixture of 99% methanol–0.1 M HCl, maintained at −20 °C in an ice–salt mixture. The weight of the jejuna was obtained by the difference in the weight of the test tube before and after the addition of the jejuna. The jejuna were then homogenized (15 ml of methanol, HCl mixture for every 5 g of jejuna) in a polytron homogenizer for 1 min by keeping the test tube at −20 °C in the ice–salt mixture. To the resulting homogenate was added 30 ml of a cold mixture of 0.45 M $HClO_4$ and 1.5 mM EDTA. It was again homogenized in a polytron homogenizer for 1 min. The homogenate was centrifuged for 20 min at 20 000 × *g*. The precipitate was discarded. The supernatant was adjusted to pH 7.0 by the addition of 7.5 ml of 1 M K_2CO_3. The $KClO_4$ which precipitated was removed by decantation and the supernatant was stored at −70 °C.

Analytical methods

Aliquots of this extract were used for the fluorometrical analysis of various

metabolites according to the procedures of Lowry et al. [11]. An Aminco spectrophoto-fluorimeter was used with an excitation wavelength at 340 nm, and an emission wavelength at 450 nm for monitoring the NADH or NADPH concentration.

General procedure

All the measurements were done in a total volume of 1.0 ml. The details of the analytical procedures are given in Table I. The reaction period ranged from 15 to 30 min. Standards of appropriate strength were used in each case. Ammonium was estimated spectrophotometrically using a Perkin–Elmer spectrophotometer following NADH oxidation in presence of α-ketoglutarate, glutamate dehydrogenase, ADP and NADH.

TABLE I

ANALYTICAL CONDITIONS FOR VARIOUS METABOLITES

Analyses were conducted (unless noted) in a total volume of 1 ml containing 0.01% bovine serum albumin in a fluorimeter cuvette. The neutralized ClO_4^- aliquot equivalent to the amount of jejunum indicated (wet weight) was added.

Metabolite	Buffer	Enzymes	Other additions	Jejunum used (mg)
ATP	50 mM Tris–HCl, pH 7.5	Yeast hexokinase, 5.8 μg; yeast glucose-6-*P* dehydrogenase, 1.8 μg.	Glucose, 2 mM; $NADP^+$, 1 mM; $MgCl_2$, 2 mM	2
ADP	50 mM imidazole·HCl, pH 7.5	Skeletal muscle lactate dehydrogenase, 50 μg; skeletal muscle pyruvate kinase, 4 μg.	NADH, 0.015 mM; ATP, 0.02 mM; Phosphoenolpyruvate, 0.02 mM; $MgCl_2$, 3 mM	2
AMP	50 mM imidazole·HCl, pH 7.5	Same plus skeletal muscle adenylate kinase, 5 μg.	Same	Same sample
Glucose 6-*P*	50 mM Tris–HCl, pH 7.5	Yeast glucose-6-*P* dehydrogenase, 1.8 μg.	$NADP^+$, 1 mM	20–30
NH_4^+*	50 mM Tris–HCl, pH 8.0	Bovine liver L-glutamate dehydrogenase, 100 μg	NADH, 0.15 mM; ADP, 0.1 mM; α-Ketoglutarate, 5 mM	10–40

* Measured spectrophotometrically.

Inorganic phosphate. P_i was measured by the method of Taussky and Shorr [12]. To an aliquot of ClO_4^- extract 2 ml of $FeSO_4$–molybdate reagent was added. The absorbance was read 10 min later using a glass filter 66 in a Klett–Summerson colorimeter.

Fructose 6-phosphate. The concentration of fructose 6-phosphate was determined indirectly. Its concentration was calculated from the concentration of glucose

6-phosphate, on the assumption that the ratio of glucose 6-phosphate to fructose 6-phosphate would be in the equilibrium proportion of 3:1 in the intact tissue. For each set of analysis the course of the reaction was followed at room temperature (25–27 °C) for one standard, a tissue sample and the reagent blank. The time required for the completion of the reaction was noted. Each analysis was done in duplicate or triplicate.

Assay of phosphofructokinase

The phosphofructokinase was measured spectrophotometrically at room temperature (25–27 °C) as described by Tejwani and Ramaiah [7] earlier except that 50 mM imidazole—HCl, pH 7.2, was used instead of Tris–HCl, pH 7.5. The concentration of ATP, fructose 6-phosphate and various effectors are given in the legends to the figures and tables.

The reaction was initiated by adding the enzyme. The reaction velocity is directly proportional to the enzyme concentration.

RESULTS

After the publication of the paper on the partial purification of phosphofructokinase from the jejunal mucosa of rat [7], an improved method of purification of this enzyme was described by Ho and Anderson [13]. They purified the enzyme to a specific activity of about 0.8. We used the homogenizing solution described by them and all other steps were performed as described in [7] except in the following details. After Step II in ref. 7, the solution was treated with propan-2-ol to a final volume of 0.3. It was stirred occasionally for 15 min at −8 °C and then centrifuged for 15 min at −8 °C and at 20 000 × *g*. The precipitate thus obtained was dissolved in the homogenizing solution. The specific activity of enzyme at this stage was 0.8 and was used in the experiments described in this paper. The enzyme when stored at −70 °C under these conditions is stable for several months.

Concentrations of effectors and substrates of phosphofructokinase in the jejunum

The concentration of various effectors and substrates of phosphofructokinase in the jejunum of rat are presented in Table II. The activity of the partially purified phosphofructokinase of rat jejunal mucosa was tested at varying concentrations of ATP and at the concentration of its effectors as given in Table II (Fig. 1). $MgCl_2$ was used at a concentration of 1.7 times the concentration of ATP found in the intact jejunum similar to their proportion found in resting frog muscle [32]. The enzyme under these conditions is uninhibited by 1.69 mM ATP, the level obtained in the intact jejunum, as judged by the lack of increase in its activity in the presence of higher concentrations of positive effectors which are known to deinhibit the enzyme completely. This conclusion is further supported by the fact that the $(\text{Fru-6-}P)_{0.5}$ value* (apparent K_m) under these conditions is lowest as obtained from a double-reciprocal plot which is linear (Fig. 2). If the enzyme were to be inhibited by ATP under these

* $(\text{Fru-6-}P)_{0.5}$ value: the concentration of fructose 6-phosphate required to give half-maximum velocity under any particular condition of assay.

TABLE II

THE CONCENTRATIONS OF VARIOUS METABOLITES IN THE INTACT JEJUNUM OF RAT

All values are expressed as μmoles/g wet weight. In each experiment the jujuna from five animals were pooled. The values given are the average values of two or more analyses done for each metabolite, in each experiment. The duplicates varied by 0–5% with each other.

Metabolite	Experiment 1	Experiment 2	Average of 1 and 2
ATP	1.18	1.32	1.25
ADP	0.63	0.66	0.645
AMP	0.18	0.20	0.19
Glucose-6-*P*	0.039	0.044	0.0415
NH_4^+	1.58	1.73	1.65
P_i	10.35	8.65	9.50
Energy charge*	0.75	0.75	0.75

* Energy charge = (ATP) + $^1/_2$ (ADP)/(ATP) + (ADP) + (AMP).

conditions the double-reciprocal plots would have been non-linear and concave upward and the $(\text{Fru-6-}P)_{0.5}$ value would have not been the lowest, since one of the effects of ATP inhibition was to increase the $(\text{Fru-6-}P)_{0.5}$ value. The addition of

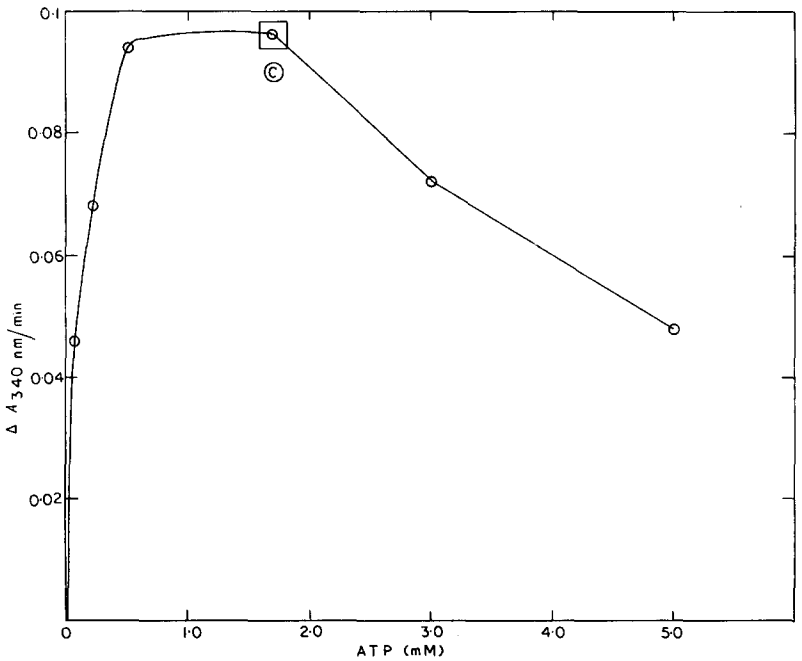

Fig. 1. Effect of an increasing concentration of ATP on the phosphofructokinase activity in the presence of concentrations of metabolites as described in Table II. The concentrations of various metabolites described in Table II are converted to their concentration in cellular water assuming that the water content of the tissue is 75% of its wet weight. The assay mixture in a total volume of 1 ml contained 50 mM imidazole·HCl, pH 7.2, 3 mM $MgCl_2$, 0.15 mM NADH, 2.2 mM NH_4Cl, 12.6 mM Na_2HPO_4, 0.02 mM fructose 6-phosphate, 0.86 mM ADP and 0.25 mM AMP. □, activators: 10 mM $(NH_4)_2SO_4$, 5 mM K_2HPO_4 and 0.38 mM AMP. ©, 0.25 mM citrate.

0.25 mM citrate, which is in the range of citrate concentration in the small intestine as estimated by Steenbock and Bellin [14], did not cause an appreciable inhibition under the above conditions of assay (Fig. 1).

The activity of partially purified phosphofructokinase was determined in the presence of various effectors and substrates and at an adenylate energy charge of 0.9 and 0.75 with a total nucleotide pool fixed at their concentrations as given in

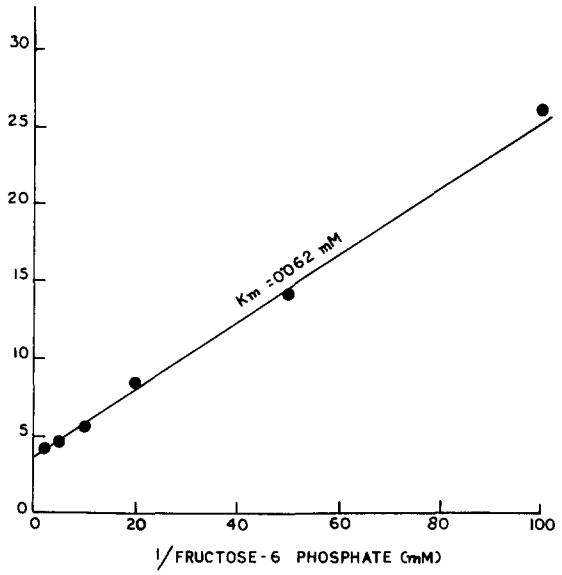

Fig. 2. Apparent K_m value of phosphofructokinase for fructose 6-phosphate as obtained by a double-reciprocal plot. The conditions of the assay are as described for Fig. 1, except that citrate was always present at a concentration of 0.25 mM and the ATP concentration was fixed at 1.69 mM.

Table II. In view of the discrepancy between our results and those of Lamers and Hulsmann [15] the activity of the enzyme was also determined at an adenylate energy charge of 0.9 and at a nucleotide concentration as described by them. These results are given in Table III. Since citrate is also an inhibitor of phosphofructokinase from various sources (for review see ref. 2) we decided to see the effect of citrate on the activity of partially purified phosphofructokinase under various conditions as described in Table III. The phosphofructokinase activity is not inhibited to a significant extent at an adenylate energy charge of either 0.9 or 0.75 with a total nucleotide concentration at 2.7 or 1.65 mM and other conditions as described in Table III. It also made no difference if P_i content was lowered from 12.6 to 3.9 mM as determined by Lamers and Hulsmann [15]. Addition of citrate at a 0.25-mM concentration which is in the range of the citrate concentration in the small intestine as described by Steenbock and Bellin [14], did not inhibit further under the above conditions of assay. However, the phosphofructokinase is inhibited by citrate and has an apparent K_i value of 0.25 mM when assayed at 1 mM fructose 6-phosphate and 0.078 mM ATP; and 3.25 mM when assayed under the conditions described in Fig. 3. The extent of inhibition of phosphofructokinase by citrate was about the same even at

TABLE III

EFFECT OF P_i AND CITRATE ON THE PHOSPHOFRUCTOKINASE ACTIVITY AT AN ENERGY CHARGE OF 0.9 OR 0.75

The energy charge of 0.75 with a total nucleotide concentration of 2.774 mM contained 1.66 mM ATP, 0.86 mM ADP and 0.254 mM AMP. The energy charge of 0.9 with a total nucleotide concentration of 2.785 contained 2.3 mM ATP, 0.43 mM ADP and 0.055 mM AMP or 1.37 mM ATP, 0.253 mM ADP and 0.036 mM AMP when the total nucleotide concentration was 1.659. Other conditions of the assay are as described in Fig. 1. Activators: 10 mM $(NH_4)_2SO_4$, 5 mM K_2HPO and 0.38 mM AMP.

Various effectors			Total nucleotide concentration		
Activators	Citrate (mM)	P_i (mM)	2.774 mM		1.659 mM
			Energy charge		
			0.75	0.9	0.9
			ΔE_{340}/min	ΔE_{340}/min	ΔE_{340}/min
—	0	12.66	0.12	0.10	0.10
—	0	3.9	0.12	0.09	0.14
—	0.25	12.66	0.12	0.08	0.09
—	0.25	3.9	0.12	0.07	0.12
+	0	0	0.11	0.11	0.12

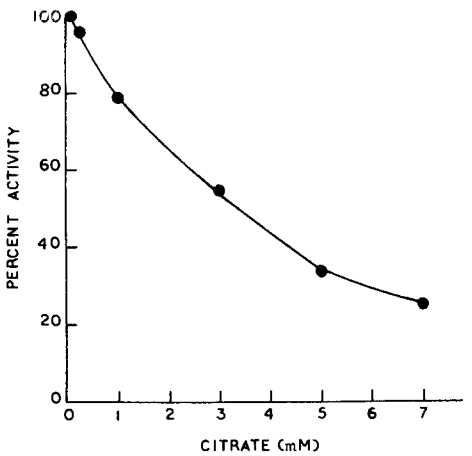

Fig. 3. Effect of increasing the concentration of citrate on the phosphofructokinase activity in the presence of concentrations of metabolites as described in Table II. The conditions of assay are as described in Fig. 1, except that the ATP concentration was fixed at 1.69 mM.

an inhibitory concentration of ATP (3 mM), under the conditions described in Fig. 3 suggesting no synergism in their effects on phosphofructokinase in contrast to their synergistic inhibition of phosphofructokinase from other sources [16, 17].

DISCUSSION

As predicted earlier [7] the NH_4^+ concentration in rat jejunum is considerable and is about four times higher than the concentration of NH_4^+ in brain (0.41 μmole/g

wet weight [18]; and in liver (0.36 μmole/g wet weight tissue [19]; and at least 6–100 times the NH_4^+ concentration in the skeletal muscle of rat at rest (0.011 μmole/g wet weight tissue [18]; 0.275 μmole/g wet weight tissue [20]).

The concentration of fructose 6-phosphate in the jejunum calculated from the value of glucose 6-phosphate assuming that the isomerase reaction is in equilibrium under in vivo conditions is in the range of the values observed for mouse brain [11]. This is in close agreement with the value obtained by Lamers and Hulsmann [15] although the ratio of glucose 6-phosphate to fructose 6-phosphate in their determination is 5:1 rather than 3:1 which was used for the computation of the fructose 6-phosphate level in the rat jejunum in the present study. This concentration is increased 2-fold by fructose loading [15] but not as much as reported by Papadopoulos and Roe [21]. The concentrations of ATP, ADP and 5'-AMP estimated in the jejunum are in agreement with the data obtained by Parsons [22] but differ from the values reported by Lamers and Hulsmann [15] and those for mucosa reported by Iemhoff et al. [23]. The values of the adenine nucleotides reported by Iemhoff et al. [23] for the jejunal mucosa of rat are not likely to be the true in vivo values of these metabolites since the adenylate energy charge as defined by Atkinson and Walton [24] will be only 0.46 which may not be compatible with the maintainance of life [25]. The adenylate energy charge based on the levels of adenine nucleotides in the freeze-clamped jejunum determined by Lamers and Hulsmann [15] is 0.9 in contrast to 0.75 observed by our analysis as presented in Table II. The low adenylate energy charge in jejunum based on our analysis may reflect a certain amount of hydrolysis of ATP to ADP or AMP during the time of isolation of the jejunum and its freezing in the methanol–HCl mixture maintained at −20 °C, but the total pool of adenine nucleotides is likely to be the correct estimate of the levels under in vivo conditions. The total adenine nucleotide pool as determined by Lamers and Hulsmann [15] is 1.24 μmoles/g wet weight of tissue as compared to 2.08 μmoles/g wet weight of tissue in our analysis. This difference may be partly due to the possibly higher water content in their tissues since they froze the tissue after cannulation and rinsing with warm saline and possibly also due to the leakage of adenine nucleotides from the cells into the perfusion medium similar to the leakage of adenine nucleotides from the mucosal cells into the medium during their isolation [6].

Similarly the P_i content of jejunum as determined by us is 9.5 μmoles/g wet weight and is much higher than the value obtained by Lamers and Hulsmann [15] which is about 3.0. The reason for such a wide discrepancy is not clear at present. The phosphofructokinase is not inhibited to any significant extent at either the concentration of effectors and substrates of the enzyme determined by Lamers and Hulsmann [15] or as described in this paper. The extrapolation of this result to in vivo conditions requires an assumption that the intracellular concentration of metabolites described in Table II reflects the concentration that surrounds phosphofructokinase, and that all phosphofructokinase in the cell is localized in such an environment. This assumption is not entirely true but may be taken as an approximation. On this basis, therefore, it appears that the lack of the Pasteur effect and the high aerobic glycolysis in this tissue [3] confirmed repeatedly over the years [4–6, 26] with one exception [27], may be due to the favourable ratio of activators to inhibitors of phosphofructokinase.

The glycolytic rate in the tissue is thus controlled mainly by variation in the

fructose 6-phosphate concentration. This very simple control, inadequate in other tissues, is probably well suited to the additional function of glycolysis in this tissue, i.e. to transport some glucose and fructose into the portal blood as lactate. Conversion of fructose or glucose to lactate could be important in maintaining a concentration gradient between mucosal cells and the lumen. That glycolysis may play a role in the transport of sugars is further supported by the fact that the glycolytic enzymes in this tissue are adaptable. The activities of hexokinase, glucokinase, fructokinase, phosphofructokinase and pyruvate kinase decrease on fasting and increase on feeding glucose or fructose [28–30]. It may appear unreasonable that so many activators and inhibitors are evolved to regulate the phosphofructokinase of this tissue only to be cancelled out under physiological conditions. In most of the tissues, the function of glycolysis is to provide ATP and biosynthetic intermediates which are then stringently controlled to suit the instantaneous demands of the cell while in the case of mucosal cells, functions of glycolysis in addition to the above mentioned, may include the transport of sugars which occurs to a significant extent by way of lactate. Consequently the glycolytic rate should be correspondingly altered by the substrate level in the diet. Therefore, the properties of phosphofructokinase evolved in cells for the stringent regulation of glycolysis which come in the way of the function of the mucosal cells of the jejunum are cancelled out rather than completely eliminated. The favourable ratio of activators to inhibitors of phosphofructokinase in the jejunum of rat may be correlated with a decreased oxygen tension in the intestinal fluid surrounding the mucosa of the jejunum. In addition, fructokinase and fructose 1-phosphate aldolase decrease on fasting and increase on feeding fructose or glucose [31]. These reactions together with triokinase which is present in the intestinal mucosa lead to the formation of glyceraldehyde phosphate and ultimately to pyruvate or lactate and are not under the regulatory control of the hexokinase pathway as they do not involve hexokinase or phosphofructokinase. This could be the additional reason for the high lactate production and the lack of the Pasteur effect in the jejunum or mucosa.

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