Partial Purification and Characterization of Deoxyguanosine Kinase from Pig Skin

By F. John GREEN and Roger A. LEWIS

*Division of Biochemistry, College of Agriculture and School of Medical Sciences,
University of Nevada–Reno, Reno, NV89557, U.S.A.*

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Deoxyguanosine kinase, which catalyses the phosphorylation of deoxyguanosine to form deoxyguanosine 5'-monophosphate, was purified 1024-fold from extracts of newborn-pig skin. This activity requires the presence of a bivalent cation and a nucleoside triphosphate, which functions as a phosphate donor, ATP being twice as effective as CTP or GTP and 4 times as effective as UTP. The enzyme appears to have a molecular weight of 58 500 as determined by Sephadex-column chromatography. Optimal enzymic activity was observed at pH 8.0; however, the enzyme remained active over a broad pH range of 5.5–9.0. Several deoxyribonucleoside and ribonucleoside monophosphates and triphosphates were tested as effectors of catalytic activity. Effective inhibitors were dGMP [$K_{i(\text{app.})} = 7.6 \times 10^{-5} \text{ M}$] and dGTP [$K_{i(\text{app.})} = 2.1 \times 10^{-5} \text{ M}$]. Both of these inhibitors acted in a competitive manner. A $K_{m(\text{app.})}$ of $3.2 \times 10^{-7} \text{ M}$ was measured for deoxyguanosine and a $K_{m(\text{app.})}$ of 3.3 mM was determined for MgATP. Of the four major deoxynucleosides tested, this catalytic activity appears to phosphorylate only deoxyguanosine; thus the enzyme is a specific deoxyguanosine kinase.

There is little information in the literature about purine deoxynucleoside kinases from mammalian sources. In particular, detailed studies on deoxyguanosine kinase are primarily limited to partially purified preparations, which possess other deoxynucleoside kinase activities, and the chemical and kinetic properties of deoxyguanosine kinase activity in these preparations have been only marginally defined (Tarr & Comer, 1964; LePage, 1968; Peery & LePage, 1969; Schrecker, 1970; Durham & Ives, 1970a, 1971; Krygier & Momparker, 1971a; Nakai & LePage, 1972; Krenitsky *et al.*, 1974; Meyers & Kreis, 1976). For each of the activities reported, there seems to exist a generalized non-specificity for substrate preference. Durham & Ives (1970a) found that a partially purified enzyme from calf thymus phosphorylated deoxycytidine, deoxyguanosine and deoxyadenosine. Krygier & Momparker (1971a), also working with calf thymus, concluded that their preparation would phosphorylate deoxyadenosine and deoxyguanosine, but not deoxycytidine. Using crude preparations of L1210 cells resistant to inhibition by arabinofuranosylcytosine, Schrecker (1970) found that this activity would phosphorylate deoxycytidine and deoxyguanosine, but not deoxyadenosine. In P815 mouse neoplasm preparations, Meyers & Kreis (1976) have demonstrated the chromatographic separation of deoxycytidine kinase activity from a second activity which phosphorylates

both deoxyguanosine and deoxyadenosine. Baxter *et al.* (1977) report the separation of deoxyguanosine kinase from both deoxycytidine kinase and deoxyadenosine kinase activities in extracts of calf thymus. Neither of the latter two deoxyguanosine kinases has been characterized with respect to their catalytic properties.

In this investigation, deoxyguanosine kinase (NTP-deoxyguanosine 5'-phosphotransferase) has been partially purified from pig skin and some of its chemical and kinetic properties have been determined. This enzyme is specific for deoxyguanosine as its substrate and is distinctly separate from enzymic activities that phosphorylate deoxyadenosine and deoxycytidine.

Experimental

Materials

[G-³H]Deoxyguanosine (1.6 Ci/mmol) and [G-³H]-thymidine (7.8 Ci/mmol) were supplied by ICN Pharmaceuticals; [G-³H]deoxyadenosine (11.0 Ci/mmol) and [G-³H]deoxycytidine (15.1 Ci/mmol) were purchased from New England Nuclear Corp.

The sodium salts of dGMP, dTMP, dCMP, ATP, CTP, dATP, deoxyguanosine (all Sigma grade), and DEAE-Sephadex A-50-120 were supplied by Sigma. AMP, UMP, CMP, GMP, deoxyadenosine, dTTP,

dCTP, GTP, UTP (all supplied as the sodium salt), haemoglobin, cytochrome *c* and bovine serum albumin were obtained from Calbiochem. All other chemicals were reagent grade.

Methods

Assay procedures. The reaction solution (0.25 ml) contained 10 mM-ATP, 10 mM-MgCl₂, 100 mM-Tris/acetate (pH 8.0 at 37°C), 10 mM-2-mercaptoethanol, 2.0 μCi of [³H]deoxyguanosine (1.6 Ci/mmol) and 0.05 ml (20 μg) of enzyme solution. After a 10 min reaction time at 37°C, the assay solution was placed in a boiling-water bath for 3 min to stop the reaction, frozen to facilitate protein coagulation and centrifuged for 5 min at room temperature at 1500g. The unchanged radioactive substrate was removed from the product by applying a 10 μl portion of the supernatant to a polyethyleneimine-impregnated (PEI) cellulose t.l.c. plate (Brinkmann Instruments) and washing the unchanged substrate from the origin with butan-1-ol/methanol/water/NH₃ (sp.gr. 0.880) (60:20:20:1, by vol.). The origin, which contained the product, was cut out and counted for radioactivity by liquid-scintillation techniques (Mompalmer & Fischer, 1968). One unit of enzymic activity is defined as that amount of enzyme catalysing the formation of 1.0 pmol of product in 10 min under the existing assay conditions. All data points represent the mean of either duplicate or triplicate samples.

Protein determination. The method developed by Lowry *et al.* (1951) was used to determine protein concentration. To remove 2-mercaptoethanol, protein samples were freeze-dried and redissolved in water. Standards of bovine serum albumin were prepared in the same buffer used for the enzyme preparation and treated identically with those solutions.

Results

Enzyme purification

Homogenate. The temperature throughout the purification process was maintained at 0–4°C unless otherwise specified. The skins from killed piglets (approx. 1 week old) were shaved, weighed, minced and mixed, 1:1 (w/v) with 0.1 M-Tris/acetate buffer, pH 8.0 at 4°C, containing 10 mM-2-mercaptoethanol (buffer A). A VirTis blender with a turbo shear blade was used to homogenize this mixture and the supernatant was collected by centrifugation of the crude homogenate for 30 min, 4°C at 27000g. This crude homogenate (fraction I) had a protein concentration of 45 mg/ml and a specific activity of 0.33 unit/mg of protein. In the presence of 2-mercaptoethanol, this solution could be frozen and stored for several months without loss of activity.

pH fractionation. After centrifugation, the supernatant was placed in an ice bath and, with constant stirring, adjusted to pH 5.5 with cold 0.5 M-acetic acid containing 10 mM-2-mercaptoethanol (fraction II). This solution was immediately centrifuged (27000g for 10 min at 4°C) and the supernatant adjusted to pH 8.0 with 2.0 M-NaOH containing 10 mM-2-mercaptoethanol. Recovery of activity was 95%.

Precipitation with (NH₄)₂SO₄. Step (1). To the supernatant obtained from pH fractionation was added, with stirring and during a 30 min interval, 43 ml of saturated (NH₄)₂SO₄ per 100 ml of supernatant, giving a final concentration of 30% satn. The mixture was allowed to equilibrate for 8 h, centrifuged (29000g for 30 min at 4°C), decanted, and an additional 95 ml of saturated (NH₄)₂SO₄ solution (to give the 30–57% satn. fraction)/100 ml of mixture was introduced into the stirred supernatant over a period of 120 min. After overnight equilibration, the mixture was centrifuged (29000g for 60 min at 4°C), the supernatant discarded and the precipitate dissolved in 9 ml of buffer A (fraction III).

Step (2). Over a period of 20 min, 4.9 ml of saturated (NH₄)₂SO₄ solution was added to the protein solution. After 10 h equilibration, the mixture was centrifuged (29000g for 60 min at 4°C) and the supernatant was discarded. The precipitate was dissolved in 4.7 ml of buffer A (fraction IV), and the resulting solution had a specific activity of 15.5 units/mg.

DEAE-Sephadex ion-exchange column chromatography. DEAE-Sephadex A-50-120 was converted from the chloride into the acetate form by exhaustively washing the resin with 1 M-Tris/acetate (pH 7). After the effluent contained no Cl[−] ions as indicated by the AgNO₃ test, a 2.5 cm × 14 cm column was prepared and 96 mg of fraction IV was loaded on the column. Protein elution was accomplished by stepwise increases in buffer strength (Tris/acetate, pH 8.0): 0.1 M (70 ml), 0.15 M (60 ml) and 0.4 M (70 ml). The flow rate was maintained at 0.5 ml/min and 5 ml fractions were collected. A portion of each fraction was assayed for protein content and deoxyguanosine kinase activity (Fig. 1). This chromatographic procedure resulted in the separation of protein into two areas, one in the 0.1 M-Tris/acetate eluate and one in the 0.4 M-Tris/acetate eluate. Most of the deoxyguanosine kinase activity appeared in the 0.15 M-Tris/acetate eluate, with only a minor peak appearing in the 0.1 M-Tris/acetate eluate. Specific activity in the major enzyme peak (fraction V) was calculated to be 350 units/mg, with 58% recovery.

Concentration of DEAE-Sephadex column eluate. In order to concentrate protein and facilitate handling, protein in the major peak of activity was pooled and made 70% satd. with respect to (NH₄)₂SO₄. The precipitated protein was collected by centrifugation (29000g for 60 min at 4°C) and redissolved in buffer

Fig. 1. DEAE-Sephadex column chromatography of deoxyguanosine kinase

After loading with 96 mg of protein from fraction IV, the column was eluted in a stepwise fashion (indicated by arrows) with increasing concentrations of Tris/acetate, pH 8.0: 0.1 M (70 ml), 0.15 M (60 ml) and 0.4 M (70 ml). Samples of each fraction (5 ml) were assayed for enzymic activity (●) and protein content (○) as described in the Experimental section.

Fig. 2. Effect of pH on deoxyguanosine kinase activity. Enzymic activity was assayed as described in the Experimental section, except that the assay solutions were buffered by a solution which contained Tris/acetate, potassium phosphate and glycine, all at 0.1 M concentrations. Individual samples were assayed over the pH range 4.5–9.5. In this buffer, reaction rates at pH 8.0 were identical with that measured in the standard buffer (100 mM-Tris/acetate).

A (fraction VI). The resulting specific activity was 338 units/mg, recovery was 45% and the protein was purified 1024-fold. The purification procedures and results obtained are summarized in Table 1.

Product identification

Dowex 50 (200–400 mesh) was equilibrated with 0.05 M-ammonium formate buffer (pH 3.2) and used to prepare a 1 cm × 27 cm column by the method of Doctor *et al.* (1973). Authentic deoxynucleoside monophosphates were added to a 0.5 ml solution containing the enzymic reaction product (from fraction VI). This solution of standards and reaction product was applied to the column, fractionated and the identities of each of the eluted peaks were determined by a comparison of their elution profiles with those found by Doctor *et al.* (1973). Nucleotide identities were verified by their A_{260}/A_{280} ratios. The location and amount of reaction product in each fraction were determined by quantifying the radioactivity contained in each fraction by using scintillation techniques, and the radioactive reaction product was observed to co-chromatograph with authentic dGMP. Recovery of radioactivity in the dGMP peak was calculated to be 92% and no other radioactivity was detected in any of the other three deoxynucleoside monophosphates.

Dependence on time and protein concentration

With the most highly purified preparation (fraction VI), the reaction catalysed by this enzyme was linear with respect to time over a period from 2.5 to 30 min and over a protein-concentration range of 10–50 μ g per assay (at a reaction time of 10 min).

Temperature optimum

The effect of temperature variation on deoxyguanosine kinase activity was tested for temperatures ranging from 20 to 65°C. Assay solutions without enzyme were preincubated at the desired temperature

Table 1. Enzyme purification

One unit of enzyme is defined as that amount of protein required to catalyse the formation of 1.0 pmol of product in 10 min under existing assay conditions.

Fraction	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
I. Homogenate	1570	4800	0.33	100	1
II. pH	1490	4200	0.35	95	1
III. 30–57% -satd.-(NH ₄) ₂ SO ₄ precipitate	1410	360	3.9	12	12
IV. 0–35% -satd.-(NH ₄) ₂ SO ₄ precipitate	1490	96	15.5	95	47
V. DEAE-Sephadex 0.15 M-Tris eluate	910	2.6	350	58	1061
VI. 70% -satd.-(NH ₄) ₂ SO ₄ precipitate	710	2.1	338	45	1024

for 5 min, after which protein was added and the timed assay initiated. The optimum temperature was 37°C with a 17% loss of activity at 50°C. There was a 50% loss of activity at 30 and 53°C.

pH optimum

When enzymic activity was assayed over the pH range 4.5–9.5, the pH/activity profile exhibited a broad plateau from 6.0 to 7.5 and an optimum pH of 8.5 (Fig. 2). Deoxyguanosine kinase retained 55% of its activity at both pH 5.5 and 9.0.

Metal ion requirement

Selected metal ions (chloride salts) were examined for their ability to act as a substitute for the Mg^{2+} cofactor in the deoxyguanosine kinase reaction. In all cases the metal salts were added in a 1:1 ratio with 10 mM-ATP. Mg^{2+} , a common metal-ion cofactor of kinases, was the most effective ion tested. Mn^{2+} easily substituted for Mg^{2+} (92% activity), whereas other bivalent ions were not good substitutes: Ca^{2+} , 67%; Fe^{2+} , 13%; Zn^{2+} , 0% activity.

Phosphate-donor effectiveness

ATP, UTP, GTP and CTP, all at 10 mM and added in a 1:1 ratio with $MgCl_2$, were tested to determine their relative abilities to act as phosphate donors in the deoxyguanosine kinase reaction. ATP was twice as effective as either CTP or GTP. UTP was relatively ineffective as a phosphate donor (25% of activity with ATP).

In a separate experiment no significant change in catalytic activity was observed when the $[Mg^{2+}]/[ATP]$ ratio was varied from 1 to 0.5 at an ATP concentration of 10 mM. The apparent K_m for MgATP was calculated to be 3.3 mM (Fig. 3).

Fig. 3. Lineweaver-Burk plot of MgATP as substrate. Assay conditions are as described in the Experimental section.

Fig. 4. DEAE-Sephadex column chromatography of deoxyguanosine kinase, deoxycytidine kinase, deoxyadenosine kinase and thymidine kinase.

The column was prepared and eluted as described in the text. Samples of each fraction were assayed for deoxynucleoside kinase activity by using, separately, $[^3H]$ thymidine ($\circ$), $[^3H]$ deoxyadenosine ($\square$), $[^3H]$ deoxyguanosine ($\bullet$) and $[^3H]$ deoxycytidine ($\blacksquare$) as substrates.

Phosphate-acceptor specificity

When fraction IV was assayed before further purification (under reaction conditions similar to those stated for the deoxyguanosine kinase assay), this fraction was observed to catalyse the phosphorylation of deoxyguanosine, deoxyadenosine, thymidine and deoxycytidine with specific activities of 15.5, 4.4, 4.0 and 1.1 units/mg of protein respectively. However, DEAE-Sephadex column chromatography of fraction IV resulted in the separation of these four catalytic activities (Fig. 4). Thymidine kinase and deoxycytidine kinase activity appeared in the 0.1 M-Tris/acetate eluate fraction, deoxyguanosine kinase in the 0.15 M-Tris/acetate eluate and deoxyadenosine kinase in the 0.4 M-Tris/acetate eluate. The peak area containing deoxycytidine kinase activity also contained a second kinase activity for both deoxyadenosine and deoxyguanosine.

Molecular weight

A Sephadex G-75 column, prepared by the method of Andrews (1965), was calibrated by applying separately a 0.3 ml sample containing 5 mg each of bovine serum albumin (mol.wt. 67000), haemoglobin (mol.wt. of dimer 32000) and cytochrome *c* (mol.wt. 13000) and eluting with buffer A at a flow rate of 12 ml/h. A protein solution containing deoxyguanosine kinase activity (0.3 ml of fraction VI) was

Fig. 5. Inhibition of deoxyguanosine kinase

Conditions of assay were as described in the Experimental section: ○, no additions; ●, +0.1 mM-deoxyadenosine; □, +0.1 mM-dGMP; ■, +0.01 mM-dGTP.

Table 2. Inhibition of deoxyguanosine kinase by nucleoside monophosphates

The enzyme was assayed as outlined in the Experimental section, except that the reaction solution was supplemented with nucleotide to a final concentration of 0.1 mM. Data are reported as the means $\pm$ S.E.M.

Additions to assay solution	Activity (units)	Inhibition (%)
None	7.2 $\pm$ 0.2 (3)	0
dTMP	6.5 $\pm$ 0.9 (3)	10
dCMP	6.5 $\pm$ 0.4 (3)	10
dAMP	7.2 $\pm$ 0.1 (3)	0
dGMP	4.0 $\pm$ 0.2 (3)	44
UMP	5.5 $\pm$ 1.1 (3)	24
CMP	6.5 $\pm$ 0.9 (3)	10
AMP	6.5 $\pm$ 0.3 (3)	10
GMP	6.6 $\pm$ 1.1 (3)	11

applied and eluted in a manner identical with that for the standards. Standards were monitored spectrophotometrically, and the location of deoxyguanosine kinase was determined by assaying samples of each fraction of eluate. Void volume was determined by addition and elution of Blue Dextran 2000. By this procedure an apparent molecular weight of 58 500 was measured.

Deoxyguanosine kinase inhibitors

Deoxyadenosine. This metabolite was found to be a poor competitive inhibitor of deoxyguanosine kinase activity. By using data from a Lineweaver–

Table 3. Inhibition of deoxyguanosine kinase by nucleoside triphosphates

The enzyme was assayed as outlined in the Experimental section, except that the reaction solution was supplemented with nucleoside triphosphate (sodium salts) to a final concentration of 0.1 mM. Data are reported as the means $\pm$ S.E.M.

Additions to assay solution	Activity (units)	Inhibition (%)
None	7.2 $\pm$ 0.2 (3)	0
dTTP	5.0 $\pm$ 0.1 (3)	30
dCTP	2.7 $\pm$ 0.2 (3)	62
dATP	3.7 $\pm$ 0.4 (3)	48
dGTP	0.0 (3)	100
UTP	5.0 $\pm$ 0.3 (3)	30
CTP	8.0 $\pm$ 0.4 (3)	–11
ATP	6.7 $\pm$ 0.4 (3)	7
GTP	4.8 $\pm$ 0.3 (3)	33

Burk plot (Fig. 5), a $K_{i(\text{app.})}$ of 3.4×10^{-4} M was calculated. This value is 10^3 times the $K_{m(\text{app.})}$ for deoxyguanosine (3.2×10^{-7} M).

Nucleoside monophosphates. Inhibition studies were conducted in which enzymic activity was measured in the presence of the eight common nucleoside monophosphates, all at a concentration of 0.1 mM (Table 2). An inhibition of 44% was observed for dGMP and 24% for UMP. The remainder of the monophosphates tested inhibited the reaction by 10% or less.

The inhibition of deoxyguanosine kinase by dGMP was examined further by Lineweaver–Burk analysis and the data are shown in Fig. 5. The $K_{i(\text{app.})}$ for dGMP was calculated to be 7.6×10^{-5} M and inhibition was of the competitive type.

Nucleoside triphosphates. Ribonucleoside triphosphates and deoxyribonucleoside triphosphates were tested to determine their inhibitory effect on the deoxyguanosine kinase reaction (Table 3). At 0.1 mM all of the triphosphates tested except ATP and CTP were inhibitory to some degree. dCTP and dATP inhibited the reaction by 62 and 48% respectively, and dTTP, UTP and GTP caused a 30–33% decrease in activity. The most notable effect was that of dGTP; at 0.1 mM it caused total inhibition. The dGTP concentration was subsequently varied to determine the effectiveness of this compound as an inhibitor; 50% inhibition of the reaction was obtained at 0.01 mM. Lineweaver–Burk studies at this concentration gave a calculated $K_{i(\text{app.})}$ of 2.1×10^{-5} M, with the inhibition being competitive (Fig. 5).

Discussion

An enzyme capable of specifically phosphorylating deoxyguanosine has been partially purified

from pig skin. The molecular weight of the enzyme was estimated by gel-filtration techniques to be 58500. This value is in agreement with molecular weights reported for other deoxynucleoside kinases (Durham & Ives, 1970a, 1971; Her & Momparler, 1971; Krygier & Momparler, 1971a).

As with other deoxyribonucleoside kinases, skin deoxyguanosine kinase exhibits no absolute requirement for ATP as the phosphate donor (Kessel, 1968; Schrecker, 1970; Durham & Ives, 1970a, 1971; Her & Momparler, 1971; Krygier & Momparler, 1971a; Nakai & LePage, 1972), but there is a preference for this compound, with the order of reactivity being $\text{ATP} > \text{CTP} \approx \text{GTP} > \text{UTP}$. Of the cations tested, Mg^{2+} and Mn^{2+} were effective.

Deoxyguanosine was shown to be the only deoxynucleoside that served as a substrate for skin deoxyguanosine kinase. A brief report by Baxter *et al.* (1977) documented the presence of a specific deoxyguanosine kinase in calf thymus. However, that deoxyadenosine and/or deoxycytidine were not phosphorylated by deoxyguanosine kinase is in contrast with results observed by Durham & Ives (1970a), Schrecker (1970) and Krygier & Momparler (1971a). Although not necessarily in absolute agreement, these investigators all concluded that a more general deoxynucleoside kinase was responsible for the phosphorylation of deoxyguanosine. In the present study, the enzymic activity in fraction IV, a less purified preparation, demonstrated appreciable activity for deoxycytidine, deoxyadenosine, thymidine and deoxyguanosine as substrates, although there were indications that the activity with thymidine was being purified away from the other three during previous fractionation procedures (F. J. Green & R. A. Lewis, unpublished work). However, subsequent fractionation demonstrated that skin tissue contains several separate deoxynucleoside kinase activities. Four major activities were found, one for each of the deoxyribonucleosides tested. In the chromatographically separated fraction which contained the activity for deoxycytidine kinase, minor activities for a second deoxyguanosine kinase and a second deoxyadenosine kinase were also found. No data were collected to suggest that this preparation, which possesses a more generalized substrate specificity, is in fact a single enzyme. However, Durham & Ives (1970a) indicate that a general deoxynucleoside activity of this type exists in calf thymus. Thus pig skin seems to have two enzymes capable of phosphorylating deoxyguanosine, one of which is specific for deoxyguanosine, whereas the other is at present not separated from deoxycytidine kinase and deoxyadenosine kinase activities. This conclusion agrees well with the information of Nakai & LePage (1972), who suggested the presence of two enzymes capable of phosphorylating deoxy-

thioguanosine, one of which is associated in some fashion with deoxycytidine. Multiple deoxyguanosine kinases have also been noted by Grower & Ives (1978) to exist in rat liver and thymus, HeLa cells, mouse and hamster liver and calf thymus.

The $K_{m(\text{app.})}$ for deoxyguanosine of $3.2 \times 10^{-7} \text{ M}$ found in the present work agrees more with the value of $5 \times 10^{-6} \text{ M}$ reported by Deibel & Ives (1977) for a bacterial enzyme than with values of $3.1 \times 10^{-4} \text{ M}$ and $1.1 \times 10^{-3} \text{ M}$ found by Durham & Ives (1970b) and Krygier & Momparler (1971b) respectively.

Both dGTP and dGMP are significantly inhibitory to deoxyguanosine phosphorylation, whereas dATP and dCTP were somewhat less effective as inhibitors. All the other nucleotides studied caused slight or no inhibition. Thus the general pattern of inhibition is that deoxyribonucleoside triphosphates are more potent than the ribonucleoside triphosphates or the nucleoside monophosphates, except dGMP. This is consistent with literature reports of other preparations regarding this enzymic activity (Durham & Ives, 1970a, 1971; Her & Momparler, 1971; Krygier & Momparler, 1971a; Nakai & LePage, 1972).

Durham & Ives (1970a, 1971) observed greater inhibition of deoxyguanosine kinase with dCTP than was measured in the present study. However, their enzyme preparation was able to phosphorylate deoxycytidine and deoxyadenosine as well as deoxyguanosine, with deoxycytidine being the preferred substrate. They suggest that a single enzyme in their preparation had the ability to utilize all three of the deoxynucleosides as a substrate. Thus the stronger inhibition by dCTP may be a reflection of the control of deoxycytidine metabolism more than one of the deoxyguanosine metabolism.

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