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Effects of temperature shifts on the activities of *Neurospora crassa* glycogen synthase, glycogen phosphorylase and trehalose-6-phosphate synthase

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Abstract **Conidiospore germlings of *Neurospora crassa* submitted to a heat shock at 45°C accumulate trehalose and degrade glycogen. The opposite occurs upon reincubation at a physiologic temperature (30°C). These observations suggest a temperature-dependent mechanism for the preferential synthesis of one or the other sugar reserve. Here we show that concomitant with these shifts of temperature, occurred reversible changes in the activities of glycogen synthase and phosphorylase. Glycogen synthase was inactivated at 45°C while phosphorylase was activated. The reverse was true when the cells were shifted back to 30°C. Addition of cycloheximide did not prevent the reversible enzymatic changes, which remained stable after gel filtration. Apparently, the effects of temperature shifts occurred at the level of reversible covalent enzymatic modifications. Trehalose-6-phosphate synthase properties were also affected by temperature. For instance, the enzyme was less sensitive to in vitro inhibition by inorganic phosphate at 50°C than at 30°C. Fructose-6-phosphate partially relieved the inhibitory effect of phosphate at 30°C but not at 50°C. These effects of the assay temperature, inorganic phosphate, and fructose-6-phosphate, on trehalose-6-phosphate synthase activity, were more evident for crude extracts obtained from heat-shocked cells. Altogether, these results may contribute to explain the preferential accumulation of trehalose 45°C, or that of glycogen at 30°C.**

Key words: Glycogen; Trehalose; Heat-shock; Glycogen synthase; Phosphorylase; Trehalose-6-phosphate synthase; *Neurospora crassa*

1. Introduction

Previous studies from our laboratory demonstrate that temperature shifts induce rapid changes on the levels of glycogen and trehalose, the two principal reserve carbohydrates of *N. crassa*. Accumulation of trehalose concomitant with degradation of glycogen is triggered by increasing the temperature of incubation from 30°C to 45°C, and the opposite takes place when heat-shocked cells are returned to 30°C [1]. Accumulation of trehalose at 45°C occurs in media supplemented either with glucose or with glycerol, as carbon sources. Under those conditions, a control step which contributes to the channeling of the flux of carbon from these substrates toward trehalose biosynthesis, is the interconversion of fructose-6-phosphate and fructose-1,6-bisphosphate. Phosphofructokinase-1 reaction is partially inhibited at 45°C, while that of fructose-1,6-bisphosphatase seems activated, probably as a consequence of a fall of fructose-2,6-bisphosphate concentration [2]. Therefore, at high temperatures, a large part of hexoses-phosphate, either derived from glucose uptake or gluconeogenic metabolism, is converted into UDPGlc, a common precursor for trehalose and glycogen synthesis. Nevertheless, in *N. crassa* trehalose is actively synthesized at 45°C while glycogen is even degraded. This fact suggested the existence of an additional metabolic cross-point at the level of UDPGlc utilization. Therefore, the present study was undertaken to explain how the pool of UDPGlc was preferentially used for trehalose synthesis at 45°C. The results suggested that heat shock triggers reversible changes of glycogen synthase and phosphorylase activities, reminiscent of those mediated by in vitro phosphorylation/dephosphorylation reactions [3,4]. It was also observed that incubation at high temperatures might stimulate trehalose-6-phosphate synthase activity.

2. Materials and methods

2.1. Neurospora crassa *strains and culture conditions*

The *N. crassa* strains used for this study were: FGSC 424 (wild type), obtained from the Fungal Genetics Stock Center (Kansas City, KS, USA), and a glycogen-deficient (*glc*) mutant, MAN 60 *cot-1*(allele c-102t); *inl*(allele 89601); *nic-3*(allele Y 31881) isolated in our laboratory by UV mutagenesis (Noventa-Jordão, unpublished results) and selection by colony staining with iodine [5]. The glycogen-deficient mutant had less that 10% of wild type glycogen synthase activity even in the presence of high concentrations of G-6-P and UDPGlc. On the other hand, it was fully normal in temperature shifts-induced responses of trehalose accumulation and degradation (Noventa-Jordão, unpublished results). By using such mutant we wanted to minimize the interference of glycogen synthase activity in the assay of trehalose-6-phosphate synthase which measures production of UDP [6]. All strains were maintained by weekly transfers on slants of Vogel's [7] medium solidified with 1.5% agar and supplemented with 2% sucrose, and inositol (200 μg/ml) plus nicotinic acid (50 μg/ml), when required.

2.2. *Temperature shifts conditions*

Conidiospores harvested from 8–10-day-old slants were suspended in liquid Vogel's medium ($1–3 \times 10^7$/ml, final concentration) supplemented with the carbon source indicated for each experiment. The culture was incubated at 30°C with agitation (120 rpm) for 5 h, and then submitted to temperature shifts protocols as described in [2].

2.3. *Extraction and quantification of trehalose, glycogen and protein*

For the determination of trehalose and glycogen, cell samples were extracted in 1.5 ml of 0.25 M Na_2CO_3 and processed as described [1]. Glycogen was quantitated with amyloglucosidase [8]. Trehalose was enzymatically degraded with a partially purified trehalose preparation from the fungus *Humicola grisea* [9] and glucose was determined by the glucose oxidase/peroxidase procedure [10]. Protein was assayed by the method of Lowry et al. [11] using bovine serum albumin as a standard.

2.4. *Preparation of cell extracts and enzymatic determinations*

All operations were carried out at 0–4°C. Cell extracts were prepared

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by homogenization with glass beads (0.5 mm diameter) [12]. For determination of glycogen synthase and glycogen phosphorylase activities, cell-free extracts were prepared in 50 mM HEPES, pH 7.1, containing 200 mM KCl, 5.0 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.5% glycogen, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 2 μg/ml of each antipain, chymostatin, leupeptin and pepstatin (extraction buffer). The extracts were then centrifuged for 20 min at 484 × *g* and supernatants were used for enzyme assays. Glycogen synthase (EC 2.4.1.11), G-6-P dependent and independent forms, was measured by the method described by François et al. [13] using 0.25 mM UDP-[U-^{14}C]Glc; 1.8 mCi/mmol, as a substrate. Phosphorylase (EC 2.4.1.1) was determined in the direction of glycogen synthesis using [U-^{14}C]Glc-1-P 5.0 mM; 1.0 mCi/mmol as a substrate [14]. Reactions were terminated as in the assay for glycogen synthase. Protein concentration in the assay systems of glycogen synthase and phosphorylase was 0.15–0.30 mg/ml and the reactions were terminated before 15 min of incubation at 30°C. Under the conditions of the assays less than 10% of the substrate was consumed and the rate of incorporation of radioactivity into glycogen was linear for at least 30 min. One unit is the amount of enzyme that catalyses the incorporation of 1 μmol of glucose into glycogen in one minute, under the conditions of the assays. Trehalose-6-phosphate synthase (EC 2.4.1.15) activity was assayed in crude extracts prepared in 60 mM HEPES, pH 7.1 (optimum of pH), containing 200 mM KCl, 5 mM EDTA, 1 mM DTT, 2 mM NaF, 1 mg/ml bovine serum albumin, and protease inhibitors as described above. The enzyme activity was assayed at the temperatures indicated for each experiment, and protein concentrations which insured linear initial rates, by colorimetric [15] or spectrophotometric [12] quantification of the amount of UDP produced in the reaction. One unit is the amount of enzyme which produces 1 μmol of UDP per min under the conditions of the assay.

All experiments were repeated at least three times with consistent results. Representative data are shown.

2.5. Reagents

Enzymes, vitamines, sugars, enzyme substrates and protease inhibitors were from Sigma Chem. Co. (USA). UDP-[U-^{14}C]glucose and [U-^{14}C]glucose-1-phosphate were from Amersham International plc (UK). Agar and culture media reagents were from Difco (USA). All other chemicals were of AR grade.

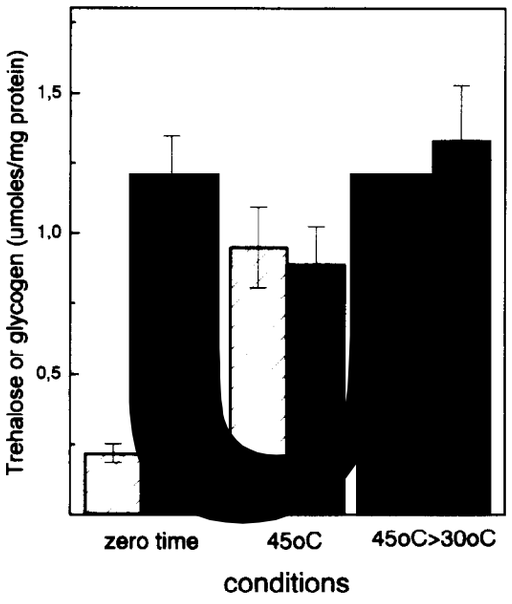

Fig. 1. Effects of temperature shifts on the levels of trehalose and glycogen of conidiospore germlings of the wild type. Conidiospores were germinated for 5 h at 30°C in liquid minimal Vogel's medium supplemented with 2% (w/v) glucose. At that time (zero time) the culture was harvested and resuspended in fresh medium of the same composition pre-warmed at the temperature of 45°C (45°C). After 30 min the culture was rapidly cooled to the temperature of 30°C (45°C > 30°C), and incubated at that temperature for 30 min. Aliquots were removed at zero time and 30 min after each temperature shift, and processed for the determination of total protein, trehalose and glycogen as described in section 2. Bars represent the average ± S.D. of seven independent experiments. Hatched bars = trehalose; filled bars = glycogen.

3. Results and discussion

3.1. Effects of temperature shifts on the activities of glycogen synthase and phosphorylase

Conidiospore germlings of a wild type strain of *N. crassa* contained glycogen as the principal reserve carbohydrate. On the other hand, under heat stress conditions, the amount of trehalose increased several-fold while that of glycogen tended to decrease. The opposite phenomenon was observed for heat-shocked cells reincubated at the physiologic temperature (Fig. 1).

The temperature shifts also affected significantly the activity of glycogen synthase (Fig. 2). After 5 min of incubation at 45°C the enzyme activity, measured in the absence (form *a*) or in the presence of 10 mM G-6-P, was reduced five- and three-fold, respectively. The temperature shock affected the ratio of G-6-P independence, which decreased from 0.71 to 0.49. Thereafter, the activity of both G-6-P-dependent and -independent forms of glycogen synthase increased, and after 30 min approximated the initial values. Upon reincubation of the heat-shocked cells at the temperature of 30°C, glycogen synthase was further activated, doubling its activity. In this case, the enzyme was almost fully independent of G-6-P. The pattern of temperature-induced responses was the same for cells incubated in the presence of 100 μg/ml cycloheximide (Fig. 2), except that glycogen synthase inactivation was more pronounced (G-6-P independence ratio dropped to 0.29), and its reactivation was less effective. The reason for this effect of cycloheximide is not known, nevertheless it was quite clear that the temperature-induced enzymatic changes were independent of de novo synthesis of protein. Phosphorylase was affected by temperature shifts just in the opposite way (Fig. 3). This enzyme was initially activated by the heat shock, and then its activity decreased. After the shift at 30°C phosphorylase activity decreased even more, but raised to its initial value by the end of the experiment. Cycloheximide had no effect on the changes of phosphorylase activity. All these enzymatic changes remained stable after Sephadex G-50 gel filtration (not shown).

The reversible effects of temperature shifts on the activities of glycogen synthase and phosphorylase in *N. crassa* were comparable to the effects of glucose, or nitrogen sources, on the same enzymes of *Saccharomyces cerevisiae* [13]. For the *N. crassa* enzyme the heat shock also diminished V_{max} (determined in the presence or the absence of G-6-P). On the other hand, the K_m (1.35 ± 0.31 mM; pooled results of crude extracts for all experimental conditions) was not significantly affected (data not shown). The reason for the reversible inactivation of *N. crassa* total glycogen synthase activity is not clear at the present time. The same results was obtained in vitro, for crude enzyme incubated with Mg-ATP [3]. Considering that the in vivo inactivation was reversible in the absence of de novo protein synthesis, a proteolytic mechanism appears unlikely.

3.2. Effects of temperature shifts on the activity of trehalose-6-phosphate synthase

Trehalose accumulated in the cells incubated at 45°C, and was consumed upon reincubation at 30°C (Fig. 4A). In the presence of cycloheximide trehalose accumulation or its hydrolysis was not affected. The heat shock activated about 30% trehalose-6-phosphate synthase (Fig. 4B). This activation was not observed for cycloheximide-treated cells. In this case, trehalose-6-phosphate synthase activity decayed with a half-life of about 50 min, both for control and heat-shocked cells. Considering that cycloheximide-treated cells still accumulated trehalose (Fig. 4A), the initial activation of trehalose-6-phosphate synthase, as well as de novo synthesis of protein, did not appear to be a pre-requisite for trehalose accumulation. Similar results have been reported for *S. cerevisiae* [16,17].

It has been reported that changes of temperature affect the kinetic characteristics of the trehalose synthase complex of *S. cerevisiae*, as well as its sensitivity to inhibition by inorganic phosphate [16,18]. Furthermore, fructose-6-phosphate partially relieves the inhibitory effect of inorganic phosphate in vitro [18]. In view of these precedents, we decided to investigate the influence of temperature, inorganic phosphate and fructose-6-phosphate, on the activity of the trehalose-6-phosphate synthase of *N. crassa*. Preliminary assays demonstrated that the activity of *N. crassa* trehalose-6-phosphate synthase increased about 80% when the assay temperature was raised from 30°C to 55°C, and dropped sharply at 60°C (data not shown). Therefore, the enzyme activity of heat-shocked and control cells was determined simultaneously at 30°C and 50°C, in the presence of increasing concentrations of potassium phosphate, and with

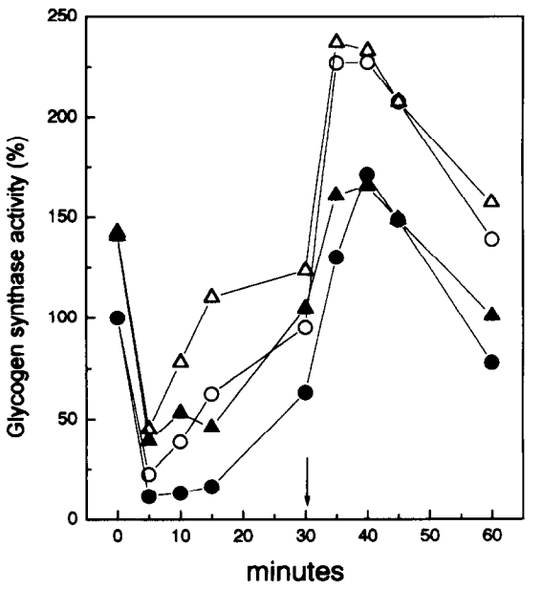

Fig. 2. Effects of temperature shifts and cycloheximide on G-6-P- dependent and G-6-P-independent forms of glycogen synthase. Conidiospore germlings were submitted to temperature shifts as indicated in the legend to Fig. 1, in the absence (open symbols) or presence (closed symbols) of 100 μg/ml cycloheximide. At the times indicated in the abscissa samples were withdrawn and processed for the determination of glycogen synthase activity in the presence (△, ▲) or absence (○, ●) of 10 mM glucose-6-phosphate. Zero time specific activity was 1.7 mU/mg protein. The arrow marks the shift from 45°C to 30°C. Other details as in section 2.

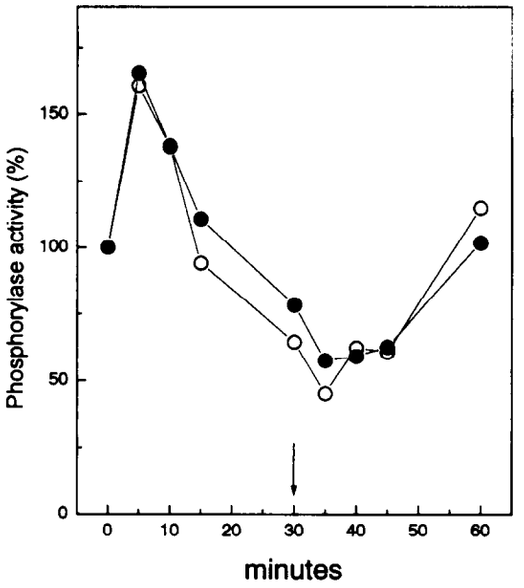

Fig. 3. Effects of temperature shifts on phosphorylase activity. Conidiospore germlings were submitted to temperature shifts as indicated in the legend to Fig. 1, in the absence (○) or presence (●) of 100 μg/ml cycloheximide. At the times indicated in the abscissa samples were withdrawn and processed for the determination of spontaneously active phosphorylase. Zero time specific activity was 15.4 mU/mg protein. The arrow marks the shift from 45°C to 30°C. Other details as described in section 2.

or without addition of 5.0 mM fructose-6-phosphate (Fig. 5A and B). Phosphate inhibition was more pronounced at 30°C than at 50°C, but this difference was more evident for extracts of heat-shocked cells (Fig. 5B). Addition of fructose-6-phosphate into the assay system was without effect when the activity was assayed at 50°C, but inhibited further the enzyme of control samples assayed at 30°C (Fig. 5A). In contrast, fructose-6-phosphate significantly relieved from phosphate inhibition the enzyme of heat-shocked cell extracts assayed at 30°C (Fig. 5B). These results resembled those reported for the yeast enzyme [16,18]. Interestingly, the effect of temperature was more pronounced for extracts of cells which had been submitted to a heat shock. In principle, this result might imply that heat shock promoted postranslational modifications on the enzyme. However, a definitive answer must await further studies.

Altogether, our results suggest that the changes of activities of glycogen synthase, glycogen phosphorylase and trehalose-6-phosphate synthase, elicited by temperature shifts may have contributed to the preferential utilization of UDPGlc, either for trehalose synthesis at 45°C, or that of glycogen 30°C. Yeast cells submitted to a heat shock also accumulate trehalose. In an early study [19] it was reported that at 45°C *S. cerevisiae* cells accumulate trehalose rather than glycogen. This fact was attributed to the differences in vitro optima of temperature of the enzymes of synthesis of glycogen and trehalose. More recent studies in yeast (for instance, [20–22]) have examined the effects of heat shock on trehalose metabolism, and its relationship with the acquisition of thermotolerance but, as far as we are aware, the effects of heat shock on glycogen metabolism have not been considered further. This is a point which might deserve clarification.

The activity of trehalose-6-phosphate synthase of *N. crassa*, as that of yeast, might be favoured by incubation at high temperatures. In the case of yeast, the increased synthesis of trehalose at high temperatures has been attributed to kinetic changes of the trehalose synthase system [16,18]. In other study [17], the increased synthesis of trehalose observed in heat-shocked yeast cell is also attributed to the drastic increases in the concentration of substrates (UDPGlc and G-6-P), and to a fall in the concentration of inorganic phosphate. This might be true for *N. crassa*, in which heat shock elicits a sudden and transient rise in the concentration of G-6-P, although UDPGlc concentration remains more or less constant [2]. The lack of information about the trehalose synthase system of *N. crassa* precludes further speculations. Up to date, there are no published studies about the properties or the biochemical organization of the trehalose synthesizing enzymes of *N. crassa*. In view of the increasing evidence demonstrating the relationship of the trehalose synthase complex with glycose-sensing mechanisms and other important signal transduction pathways in yeasts, and probably in other organisms (reviewed in [23]), a detailed study of these enzymes in *N. crassa* seems necessary.

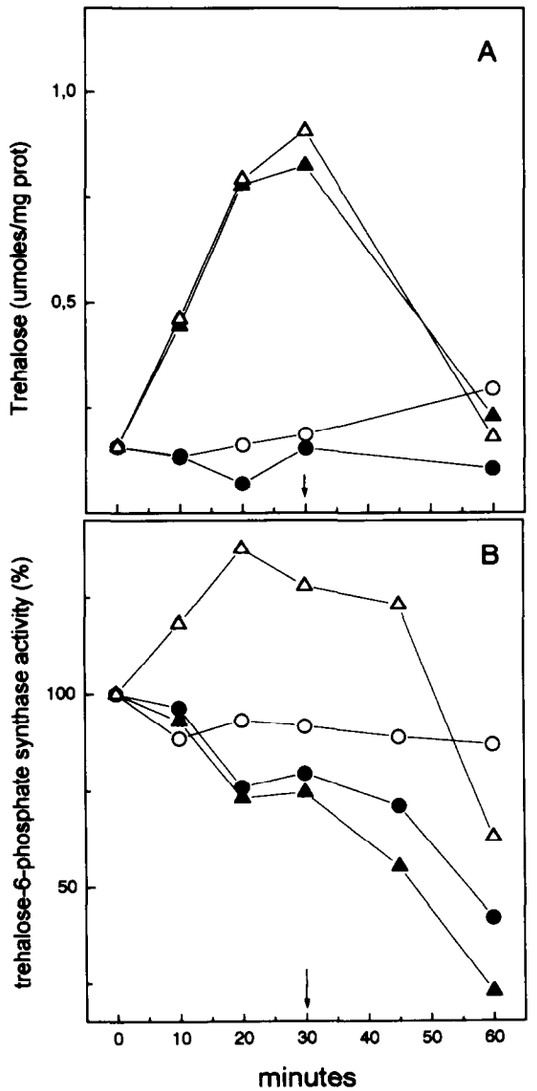

Fig. 4. Effects of temperature shifts and cycloheximide on the accumulation of trehalose (A) and trehalose-6-phosphate synthase activity (B). Conidiospore germlings were submitted to temperature shifts as indicated in the legend to Fig. 1, in the absence (△) or presence (▲) of 100 μg/ml cycloheximide. The arrow marks the shift from 45°C to 30°C. A control culture was maintained at 30°C throughout the experiment in the presence (●) or absence (○) of cycloheximide. At the times indicated in the abscissa samples were withdrawn and processed for the determination of trehalose, trehalose-6-phosphate synthase and total protein as described in section 2. Zero time specific activity was 263 mU/mg protein.

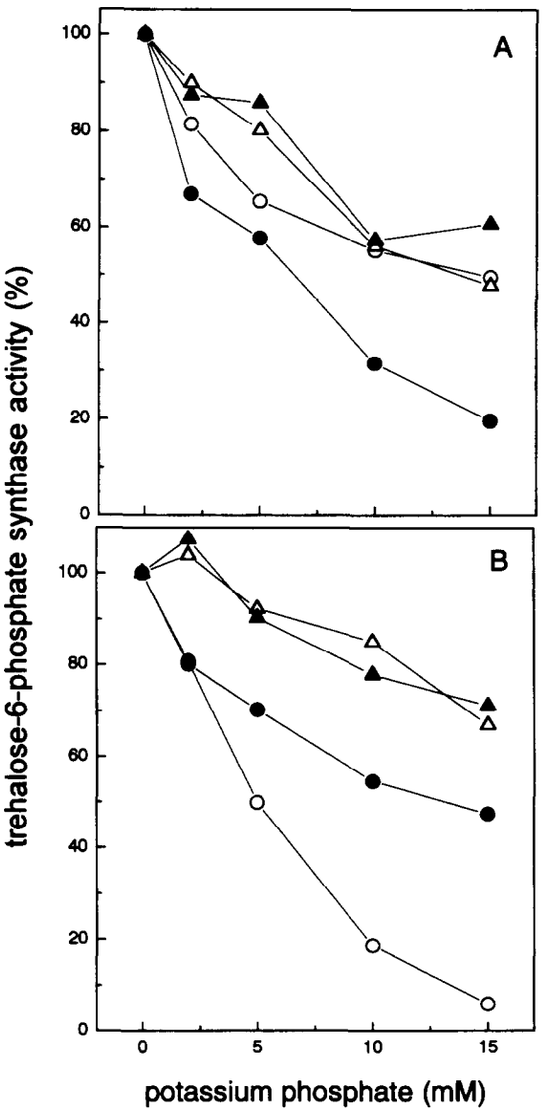

Fig. 5. Effect of inorganic phosphate and fructose-6-phosphate on trehalose-6-phosphate synthase activity of control (A) and heat-shocked cells (B). The enzyme was assayed at 30°C (○, ●) or 50°C (△, ▲) in the presence of different concentrations of potassium phosphate as indicated in the abscissa, and with (closed symbols) or without (open symbols) addition of 5.0 mM fructose-6-phosphate in the assay. Zero time specific activity was 229 mU/mg protein.

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