Glucose and Triiodothyronine Both Induce Malic Enzyme in the Rat Hepatocyte Culture

EVIDENCE THAT TRIIODOTHYRONINE MULTIPLIES A PRIMARY GLUCOSE-GENERATED SIGNAL

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ABSTRACT We have simulated in a cultured hepatocyte system the synergistic interaction between triiodothyronine (T_3) and dietary carbohydrate in the induction of malic enzyme (ME). Kinetic studies revealed that isolated hepatocytes equilibrate with media T_3 within 5 min; nuclei equilibrate with media T_3 by 45 min; and the half-time of T_3 metabolism was 10 h in 10% serum. We demonstrated nuclear T_3 receptors in isolated hepatocytes and the induction of ME by T_3 in physiological concentrations. However, in the complete absence of T_3 glucose could still induce ME. At all concentrations of glucose (100–1,000 mg/dl), T_3 (0.3 nM free T_3) resulted in a relatively constant (1.4- to 1.7-fold) increase in ME response. The augmentation in ME activity was paralleled by an enhanced rate of enzyme synthesis as determined by [^{3}H]leucine incorporation into immunoprecipitable ME. Cells cultured in serum free media also demonstrated a glucose-dependent increase in ME. Insulin greatly stimulated the glucose induction of ME, whereas dexamethasone had very little influence on ME induction. These studies demonstrate the usefulness of an adult hepatocyte tissue culture model for the study of the effects of T_3 on gene expression in cells that are not derived from tumor. They clearly demonstrate that well established effects of T_3 can be simulated in such a system at levels of free hormone that approximate those in extracellular body fluids. Our results indicate that an increased concentration of glucose per se can induce the formation of ME in the absence of alterations in extrahepatic hormones or factors. Moreover, our findings confirm inferences from in vivo studies that T_3 acts as a multiplier of a glucose-induced signal.

Received for publication 9 April 1981 and in revised form 12 August 1981.

INTRODUCTION

We have previously demonstrated a synergistic interaction between triiodothyronine (T_3)[1] and a high-carbohydrate fat-free diet in the induction of malic enzyme (ME) (EC 1.1.1.40) as well as other lipogenic enzymes (1). Such interaction occurs at a pretranslational level (2) since an increase in ME activity is paralleled by an increase in the specific messenger (m)RNA for ME. The T_3 nuclear complex appears to act by multiplying a signal generated in response to the diet (3). Of potential physiologic and clinical interest was the finding that a diminution in this signal may be responsible for the decline in responsivity of ME to T_3 in the aging rat (3, 4).

Although previous in vivo studies had suggested that the interaction between T_3 and carbohydrate was not mediated by fluctuation of insulin levels (5), this possibility could not be rigorously excluded. Moreover, the role of other hormones or extrahepatic metabolic factors in the induction of lipogenic enzymes remained possible. Accordingly, we have developed an in vitro hepatocyte culture system in order to study this problem under defined conditions. This model demonstrated that glucose per se is the responsible factor in the carbohydrate induction of ME. Insulin appears to play only a permissive role in ME induction and T_3 acts to multiply the carbohydrate signal. The system thus appears to simulate well-established hormonal effects at physiological concentrations of glucose in the bathing medium and with a physiologic intranuclear T_3 content.

METHODS

Male Sprague-Dawley rats, 180–250 g, were used. Collagenase (Type II) was obtained from Worthington Biochemical

[1] *Abbreviations used in this paper*: ME, malic enzyme; MEM, Minimal Eagles Medium; STTM, Sucrose-Tris-Triton-Mg buffer; T_3, triiodothyronine.

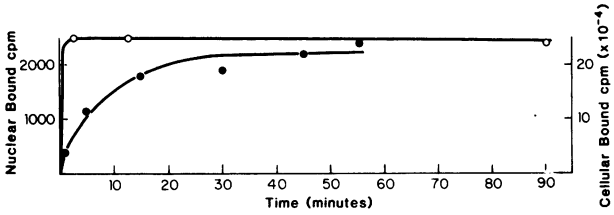

FIGURE 1 Rate of equilibration between medium, cells, and nuclei. Freshly isolated hepatocytes were incubated in medium containing 1.9 nM T_3. At the times indicated 8×10^5 cells were removed in duplicate for determination of counts per minute bound to whole cells (open circles) or nuclei (closed circles).

Co., Freehold, N. J. and nylon mesh (Nytex 40/42) from Northwestern Graphics, Minneapolis, Minn. Williams Medium E was purchased without glucose, pyruvate, and oleic acid from Gibco Laboratories, Grand Island Biological Co. (Grand Island, N. Y.). [^{125}I]T_3 was obtained from Abbott Laboratories, Inc., Chicago, Illinois, and at least 90% purity was assured by paper chromatography (6).

Hepatocytes were obtained by the collagenase perfusion techniques of Berry and Friend (7) as modified by Seglen (8). At least $2-3 \times 10^8$ cells were harvested from each liver with a viability estimated by trypan blue exclusion of >85%. Collagen gel meshes were prepared as described by Sirica et al. (9) with minor modifications. 3.5 ml of collagen solution were placed on sterile, washed, nylon mesh circles and neutralized with 1 ml of 10 × concentrated Ham's F-10 media: 0.34 N NaOH (2:1). Once the gel was set, 7 ml of Williams Medium E was added to the petri dish and allowed to incubate 24 h before plating the cells.

The culture system used is unique in that once the cells are attached they remain viable and function normally for at least 3 wk (9, 10). The few cells that die during the culture period detach from the collagen and are removed with the media changes. 5×10^6 cells were plated onto each gel in fresh Williams Medium E. The medium was supplemented with insulin (100 mU/ml), dexamethasone (1 μM), penicillin-streptomycin (1 U/ml-1 μg/ml), glutamine (2 mM), and 10% calf serum, either unmodified or rendered T_3-free (11). After 4 h, the nonattached cells and medium were aspirated and fresh medium was replaced. In some experiments the fresh medium was serum free. T_3 was added to the desired concentrations and the medium was replaced every 24–48 h. Cells were harvested by incubation in minimal Eagles' medium (MEM) containing 75 mg/dl collagenase at 37°C for 15–20 min. The released cells were centrifuged at 500 *g* for 5 min, washed twice in MEM, and resuspended in 1 ml of 0.32 M sucrose, 0.003 M $MgCl_2$, 0.01 M Tris pH 7.4, 1% Triton X-100 (STTM). Cells were broken by homogenization with a teflon pestle. Cytosol was obtained by centrifugation of homogenates at 100,000 *g* for 45 min.

T_3-nuclear receptor binding was quantitated in freshly isolated hepatocytes (8×10^5 cells) by incubating cells at 37°C for 45 min with tracer [^{125}I]T_3 and increasing concentrations of either ^{125}I[T_3] or unlabeled T_3 in a volume of 2 ml. Reactions were stopped by cooling on ice for 5 min and centrifuging at 500 *g* for 5 min. The cell pellet was resuspended in 1 ml of ice-cold STTM, homogenized, layered over 0.5 ml of 50% glycerol, and centrifuged at 5,000 *g* for 10 min. Nuclei were washed twice in 1 ml of ice-cold STTM. The final pellet was assayed for radioactivity. Scatchard plots were used to determine affinity and binding capacity after subtraction of nonspecifically bound T_3. Nonspecific binding was <15% of tracer bound T_3.

The rate of T_3 metabolism was determined in cells cultured for 3 d in 100 or 500 mg/dl glucose. After a preliminary culture for 1 d in unlabeled medium (0.10 nM T_3) fresh medium and tracer [^{125}I]T_3 was added. Samples of medium were withdrawn at predetermined intervals. The [^{125}I]T_3 remaining in the medium was determined by trichloracetic acid (TCA) precipitation with outdated human serum (12).

ME, protein, and DNA were assayed as previously described (1). ME is expressed as nanomoles of NADP reduced per minute per milligram cytosolic protein. Glucose was analyzed by the glucose oxidase method with a Beckman glucose analyzer (Beckman Instruments, Inc., Fullerton, Calif.). We calculated the free T_3 concentration from the product of the total T_3 concentration and the dialysis fraction determined by equilibrium dialysis (12).

ME synthesis was determined by specific immunoprecipitation of cytosol after 2 h of hepatocyte incubation in leucine-free MEM with 100 μCi [^{3}H]leucine. ME radioactivity was quantitated from SDS gel electrophoresis of the immunoprecipitated products (2) and related to total protein radioactivity determined from TCA precipitation.

RESULTS

Because induction of hepatic ME by T_3 in the intact animal is believed to be mediated by nuclear receptors for T_3 (13), we were interested in identifying such sites in isolated hepatocytes and establishing the relationship between T_3 in the medium and the cellular and nuclear uptake of T_3. Fig. 1 demonstrates that the isolated hepatocyte achieves equilibrium with tracer T_3 in the medium in <5 min. Furthermore, the exchange between nuclear and medium T_3 appears to be complete in 45 min. Therefore, displacement analyses were performed with 45-min incubations to assure complete exchange.

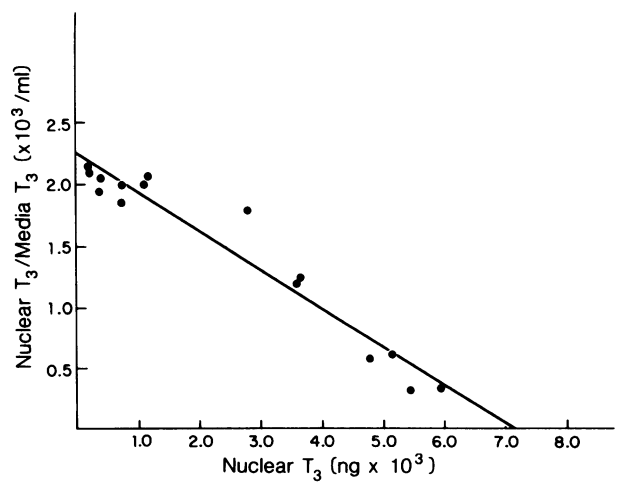

FIGURE 2 Scatchard plot of T_3-nuclear receptors. Freshly isolated hepatocytes were incubated in medium containing 10% calf serum for Scatchard analysis as described in Methods. Each point represents 8×10^5 cells. Nonspecifically bound counts, which were 10% of the bound counts at the lowest concentration of T_3 used, have been subtracted from each point. The line represents the least-squares best fit ($r = 0.96$).

We measured the affinity of specific nuclear binding sites for T_3 in freshly isolated cells, both in the presence (Fig. 2) and absence of 10% T_3-free serum in the medium. The dissociation constant (k_d) for T_3 was 56 pM in serum-free medium, and 2.0 nM in medium containing 10% calf serum (mean of three experiments). Equilibrium dialysis of the 10% calf serum yielded a dialyzable fraction of 3% both at concentrations of 1 μM and 0.1 nM total T_3. Thus, the free T_3 concentrations with a total T_3 concentration of 2 μM was 60 pM, nearly identical to the k_d determined in serum-free medium. This correspondence supports our assumptions about the role of free T_3 in the equilibration of hormone between cells, nuclei, and medium. Furthermore, the binding capacity per cell in six experiments was 12,000±3,000 sites (SD) or 0.68 ng T_3/mg DNA, a value similar to that previously found in whole liver homogenates, 0.6 ng/mg DNA (14). We concluded that the nuclear sites in freshly isolated hepatocytes were identical to those demonstrated in liver homogenates and in vivo.

The metabolism of T_3 by hepatocytes was determined from the disappearance of T_3 from the medium as determined by TCA precipitation of [^{125}I]T_3. The $t_{1/2}$ of disappearance of T_3 from the medium at 100- and 500-mg/dl glucose concentrations was ~10 h (Fig. 3). This value, together with the known dialysis fraction (3%) allowed us to calculate the free T_3 concentration at any time. With the knowledge of the k_d, we could also calculate the instantaneous nuclear occupancy as a function of time. A total initial concentration of 0.1 μM (3 nM free) maintained T_3 occupancy >90% for a 48-h period. At this concentration we demonstrated a progressive increase in ME activity with a two- to threefold increase over basal levels at 5 d and a four- to fivefold increase between the 7th and 10th d of incubation. In most experiments we harvested our cultures 5–6 d after initial plating.

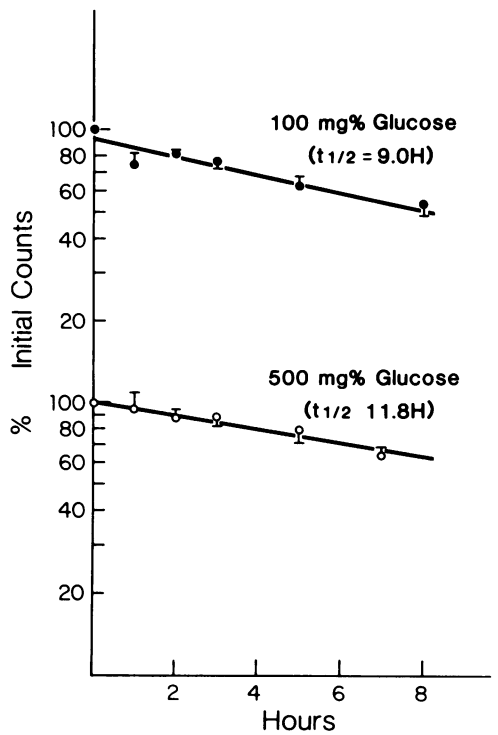

FIGURE 3 T_3-disappearance. Hepatocytes were incubated as described in Methods. Each point represents the mean and standard deviation of four individual plates. The line drawn represents the least-squares best fit. There were no significant differences between the slopes at the two concentrations of glucose utilized ($P > 0.05$).

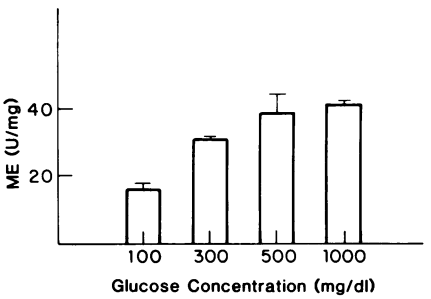

FIGURE 4 Induction of malic enzyme by glucose. Hepatocytes were cultured for 5 d in T_3-free medium as described in Methods. Each bar represents a mean and range of duplicate plates from a representative experiment. Malic enzyme activity is expressed as units per milligram protein.

Studies were also initiated to determine whether glucose per se was capable of inducing ME. The results (Fig. 4) demonstrate a progressive augmentation of ME activity with increasing initial glucose concentration in medium containing T_3-free serum. The contribution from the endogenous concentration of T_3 in the cell was considered to have been insignificant since cellular T_3 would have been both rapidly diluted by the medium and metabolized over the period of the 5 d of incubation. Thus, we conclude that glucose per se can induce ME in the absence of any extrahepatic signals and in the absence of T_3.

We also investigated the interaction of T_3 with glucose in the induction of ME (Fig. 5). In the presence of 10% calf serum and a total endogenous T_3 concentration of 0.2 nM we observed a significant accumulation of ME with increasing concentrations of glucose. Addition of T_3 to a final total concentration of 10 nM (0.3 nM free T_3) resulted in a 50–70% increase in ME accumulation at all concentrations of glucose. The calculated mean nuclear occupancy in hepatocytes incubated with T_3-supplemented serum was 50%, whereas in the absence of such supplementation the mean occupancy was estimated to be <10%. Three other experiments yielded essentially similar results. T_3 thus appears to be effective under conditions that approximate physiological concentrations of hormone in the extracellular fluid and under conditions of nuclear

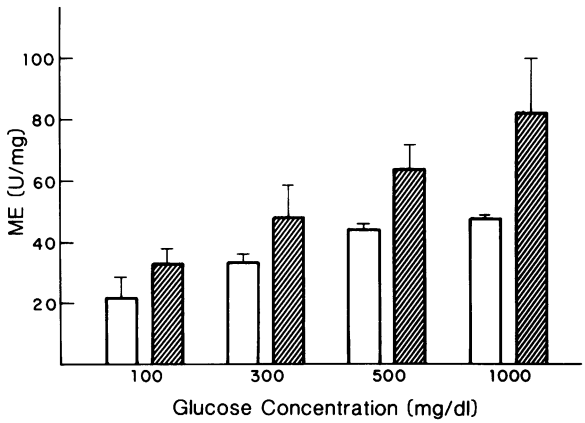

FIGURE 5 Glucose-T_3 interaction in malic enzyme induction. Hepatocytes were incubated for 5 d in medium containing 0.1 nM T_3 (clear bars) or 10 nM T_3 (hatched bars). The mean and range of a representative experiment are presented. Malic enzyme activity is expressed as unit per milligram protein.

occupancy similar to those observed in the intact animal (15). Moreover, these findings support our previous inference from in vivo data that T_3 acts as a constant multiplier of a carbohydrate-induced stimulus (1, 3).

To be certain that ME activity is a valid index of ME synthesis, we measured the relative rate of ME synthesis under various experimental conditions in which the levels of T_3 and glucose were altered both in the presence and in the absence of serum. Fig. 6 demonstrates a highly significant correlation ($r = 0.9, P < 0.001$) between the relative rate of ME synthesis and the specific activity of ME in 5-d cultured hepatocytes. Since over

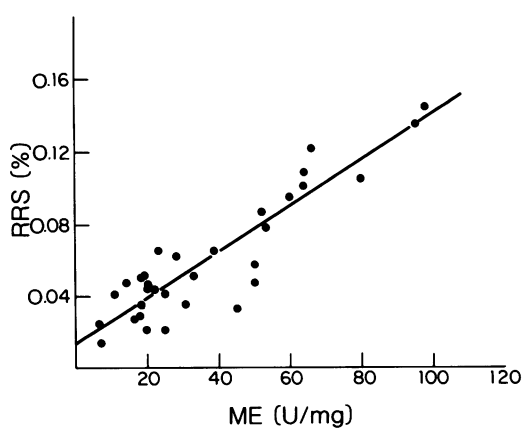

FIGURE 6 Relative rate of malic enzyme synthesis. The relative rate of synthesis (%RRS) was determined as described in Methods. The RRS is plotted against simultaneously determined malic enzyme activity (units per milligram protein). Each point represents an individual plate cultured for 5 d. The data are from several experiments in which both T_3 concentration and glucose concentration were varied. The line was generated by linear regression analysis.

TABLE I
Influence of Serum on Malic Enzyme

10% serum	100 mg/dl glucose	500 mg/dl glucose	
+	15.4±3.0 (6)	43.7±6.8 (6)	$P < 0.01$
−	16.5±2.6 (10)	93.8±12.4 (6)	$P < 0.001$
	NS	$P < 0.01$	

5-d cultured hepatocytes were grown in the presence or absence of 10% calf serum as indicated. Malic enzyme was assayed as described in Methods and expressed as units per milligram protein. Each value represents the mean malic enzyme activity ±SEM and the number of cultures are in parenthesis. Significant differences were determined by two-tailed Student's t test and P values are indicated for the respective column or row in the table.

81% of the variation in ME activity can be accounted for by a change in ME synthesis, ME activity reflects the degree of enzyme induction rather than changes in the fractional rate of enzyme degradation or simple activation of preexisting enzyme.

In order further to exclude the possibility that glucose or T_3 increases ME artifactually, we examined several indices of cell viability. There were no significant differences ($P > 0.05$) in DNA content per plate among the various glucose and T_3 treatments. The DNA content averaged 52±3 μg/plate (mean±SEM). Moreover, the rate of protein synthesis, as determined by [^{3}H]leucine incorporation into total cytoplasmic protein, was also uninfluenced by alterations in media glucose or T_3. In any individual experiment there was only 16% variation in [^{3}H]leucine incorporation among different treatment groups. These data indicate that with the concentrations used neither T_3, nor glucose, influence the viability or rate of overall protein synthesis.

Since serum contains many proteins and peptides that may influence glucose metabolism, we also investigated the ability of glucose to induce ME in completely chemically defined media. In serum-free medium 500 mg/dl glucose is more effective in inducing ME than it is in the presence of 10% serum (Table I). Thus, serum does not contain factors essential for glucose stimulation of ME induction. On the contrary, 10% calf serum significantly inhibits the ability of glucose to induce ME by 50%. Similar results were found when two other lots of serum were tested, as well as with 2% rat serum.

We were also interested in determining whether insulin or dexamethasone were required for glucose induction of ME. The results in Table II demonstrate that dexamethasone has only a minor influence on the induction of ME. On the other hand, in the absence of insulin the ability of glucose to induce ME is markedly reduced. Insulin, therefore, appears to play a permissive role in the carbohydrate induction of ME.

TABLE II
Influence of Insulin and Dexamethasone on Malic Enzyme

Insulin	100 mg/dl glucose	500 mg/dl glucose	Dexamethasone
+	12.9	70.0	+
−	8.8	14.6	+
+	15.5	50.2	−
−	8.2	19.8	−

Representative experiment of 6-d cultured hepatocytes grown in the absence of serum. Each point represents the average malic enzyme activity (units per milligram protein) of two individual plates with <10% variation between plates.

DISCUSSION

We have shown that the primary hepatocyte culture is an appropriate and useful model for studying T_3 action in a cell type that is not derived from a tumor. These studies clearly indicate that freshly isolated hepatocytes have nuclear binding characteristics that are identical to those observed after standard subcellular fractionation of total liver homogenates and that the cultured cells respond in an appropriate fashion to physiologic concentrations of thyroid hormone. Furthermore, the rapid exchange between media, hepatocyte, and nucleus is characteristic of the kinetics of T_3 exchange in vivo (14).

Our studies show that graded concentrations of glucose are capable of increasing the rate of induction of ME. Thus, the synthesis of lipogenic enzymes by a high carbohydrate, fat free diet in vivo is due in part, if not entirely, to the direct stimulation of lipogenic enzymes by glucose. In previous studies with intact and diabetic animals we, and others, have suggested that insulin may not be necessary for the dietary induction of lipogenic enzymes (5, 16). Nevertheless, these studies were not conclusive inasmuch as it was impossible to be absolutely certain that administration of carbohydrate to animals rendered diabetic with streptozotocin had not elicited a minimal increase in insulin concentration, or required the presence of a minimal amount of insulin, especially in the hepatic portal system. Moreover, these experiments did not rule out the possibility that other hormones or metabolites of extrahepatic origin might have acted as a dietary-generated stimulus. The present results make it unnecessary to postulate the action of such agents. Indeed, our findings suggest there are as yet unidentified factors in serum that act to inhibit the ME response to carbohydrate induction. This finding is not surprising since serum contains factors such as glucagon, known to inhibit the induction of ME in vitro (17), and lipids that inhibit ME induction in vivo (18).

The lack of a major effect of dexamethasone on the glucose induction of ME lends further support to the physiologic characteristic of the hepatocyte culture model. Others have shown in vivo that lipogenic enzymes are easily inducible in adrenalectomized rats (19). Adrenalectomy only appears to prevent the enzyme overshoot phenomenon that occurs following refeeding after 48 h of fasting.

On the other hand, insulin plays an important permissive role in the induction of ME by glucose. In the absence of insulin, 500 mg/dl glucose resulted in only minimal induction, whereas in the presence of insulin, ME rose five- to sixfold. The same concentration of insulin in the presence of a lower glucose concentration (100 mg/dl) exerted only a modest effect on ME induction (Table II). Insulin thus facilitates the metabolism of glucose and the generation of an as yet unidentified product or products which acts as the proximate signal of lipogenic enzyme induction.

Our finding of a significant increase in the rate of induction of ME between 100 and 500 mg/dl glucose may have particularly important physiological implications since similar excursions are found in hepatic portal blood glucose after meals (unpublished observations). Thus, it appears entirely conceivable that the induction of ME and other lipogenic enzymes is mediated in large part by the portal vein glucose and insulin concentrations.

The finding that the addition of T_3 results in a constant proportional increase in ME parallels in vivo results and supports our suggestion that this hormone acts as a simple multiplier of carbohydrate generated signals (1, 3). The possibility should be considered that T_3 may serve an analogous multiplying function when it acts in concert with other metabolic and hormonal stimuli. The expression of thyroid hormone action at a cellular level may be conditioned in large measure by the intensity of such stimuli, thus making it difficult to predict the physiological impact of a given level of plasma or specifically bound nuclear T_3. This may explain the dilemma posed by the "low T_3 syndrome" in patients with nonthyroidal disease but without any evidence of clinical hypothyroidism (20). The hepatocyte model should be helpful in studying the interaction of T_3 with other factors involved in the regulation of hepatic gene expression.

ACKNOWLEDGMENTS

We wish to thank Colleen Jahnel and Nancy Rutledge for their excellent secretarial assistance.

This work supported by National Institutes of Health Clinical Investigator Award AM00800, American Diabetes Association Research grant, National Institutes of Health grant AM19812, and AM26919.

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