NMR indirect detection of glutamate to measure citric acid cycle flux in the isolated perfused mouse heart

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Abstract ^{13}C-edited proton nuclear magnetic resonance (NMR) spectroscopy was used to follow enrichment of glutamate C3 and C4 with a temporal resolution of ~20 s in mouse hearts perfused with ^{13}C-enriched substrates. A fit of the NMR data to a kinetic model of the tricarboxylic acid (TCA) cycle and related exchange reactions yielded TCA cycle (V_{tca}) and exchange (V_x) fluxes between α-ketoglutarate and glutamate. These fluxes were substrate-dependent and decreased in the order acetate (V_{tca} = 14.1 μmol g^{-1} min^{-1}; V_x = 26.5 μmol g^{-1} min^{-1}) > octanoate (V_{tca} = 6.0 μmol g^{-1} min^{-1}; V_x = 16.1 μmol g^{-1} min^{-1}) > lactate (V_{tca} = 4.2 μmol g^{-1} min^{-1}; V_x = 6.3 μmol g^{-1} min^{-1}). © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Indirect detection; ^{1}H nuclear magnetic resonance; Tricarboxylic acid cycle kinetics; Cardiac metabolism

1. Introduction

Although genetic manipulations in mice are now common [1–3], metabolic characterization of a resulting phenotype is not easily implemented in the mouse due to size limitations and tissue availability. Cardiac physiology is particularly challenging [4,5] yet some early reports suggest there may be significant metabolic differences between the mouse heart and hearts from larger mammals [4,6,7]. Various methods, including high field nuclear magnetic resonance (NMR) spectroscopy, have been used to study metabolism in isolated perfused hearts of genetically altered and wild type mice [5–8]. ^{31}P NMR spectroscopy has proven valuable for monitoring energetics in isolated mouse hearts [9–11]. Although ^{13}C NMR is particularly useful for studying intermediary metabolism [12,13], the low sensitivity of this nucleus often limits this technique to larger tissues. ^{13}C NMR has been used to measure tricarboxylic acid (TCA) cycle kinetics in perfused rat [14–16] and rabbit hearts [17,18], but this tool has not been applied in the mouse heart.

Entry of ^{13}C-enriched acetyl-CoA into the TCA cycle results in temporal enrichment of all intermediates to steady-state values. Such kinetic data provides a direct measure of TCA cycle flux (V_{tca}) as well as flux through exchange reactions related to cycle intermediates [14–18]. Temporal enrichment of glutamate is typically monitored because this metabolite is relatively abundant in heart tissue and exchanges directly with the cycle intermediate, α-ketoglutarate. Enrichment kinetics of α-ketoglutarate and glutamate could diverge if exchange between these two pools is comparable to or slower than cycle turnover. Thus, glutamate enrichment curves can provide an accurate measure of V_{tca} only if an exchange flux (V_x) is also considered. This is especially true in hearts because V_x has been shown to be comparable to V_{tca} in the perfused heart [14–17].

Indirect detection of ^{13}C via a directly attached ^{1}H can substantially increase the sensitivity of this experiment [19–20]. Proton-observed carbon-edited spectroscopy has been applied in the in vivo rat [21] and human brain [22], but not in isolated perfused tissue. In this study, we combined WET [23] (to eliminate the bulk water signal) and 1D HMQC [24] sequences to obtain ^{13}C-edited ^{1}H spectra of ^{13}C-enriched glutamate in isolated perfused mouse hearts. The improved sensitivity of this indirect detect WET–HMQC method resulted in ~10-fold decrease in temporal measures of glutamate enrichment. The improved temporal resolution allowed an accurate measure of V_{tca} and V_x in mouse hearts perfused with acetate, octanoate, or lactate. This substrate comparison was designed to test the hypothesis that both V_{tca} and V_x are sensitive to generation of reducing equivalents external to the TCA cycle. V_{tca} determined by NMR was also compared to a direct measure of O_2 consumption (MVO_2) measured with an oxygen electrode. Hearts perfused with acetate and the transaminase inhibitor aminooxyacetate (AOA) are also studied to validate measures of V_x.

2. Materials and methods

2.1. Heart perfusions

Mouse hearts were rapidly excised from 10–12 week old female wild type mice (Charles River Laboratories, Wilmington, MA, USA) weighing 18–22 g. Hearts were perfused using conventional Langendorff methods (retrograde) at 100 cm H_2O with Krebs–Henseleit bicarbonate buffer (KH) containing 118 mM NaCl, 4.7 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 0.7 mM Na_2EDTA and 5.6 mM unenriched glucose [11]. 250 ml of recirculating KH buffer was bubbled continuously with a 95/5 mixture of O_2/CO_2. Four different substrates were investigated using four hearts each (n = 4): (A) 3 mM [2-^{13}C]acetate, (B) 3 mM [3-^{13}C]lactate, (C) 0.75 mM [2,4,6,8-$^{13}C_4$]octanoate, and (D) 3 mM [2-^{13}C]acetate with

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0.2 mM AOA. A water-jacketed, all-glass perfusion apparatus equipped with two chambers and maintained at 37°C fit entirely into the bore of a 14.1 T magnet. Hearts were positioned in an 8 mm NMR tube and heart rate was monitored with an open-ended cannula positioned in the left ventricle and connected to an external pressure transducer. Hearts were allowed to beat spontaneously at 300–350 bpm, rates comparable to those reported by others for the isolated perfused mouse heart [4,5,7,11]. Each heart was allowed to stabilize for 15–20 min, during which time the NMR probe was tuned, and field homogeneity was optimized. The probe temperature was maintained at 37°C using a flow of warm air. Flow to the heart was switched from the first chamber containing natural abundance substrate to the second chamber containing the same concentration of ^{13}C-enriched substrate to initiate collection of kinetic data.

Oxygen consumption was measured at the completion of each kinetic experiment. The perfusion apparatus was removed from the magnet, and perfusate samples collected into gas-tight syringes from just above the aorta and below the pulmonary artery were immediately injected into a blood gas analyzer for measurement of pO_2 (Instrumentation Laboratory, Lexington, MA, USA). The pO_2 difference and a separate measure of coronary flow were used to evaluate MVO_2. Hearts were then freeze-clamped using aluminum tongs precooled in liquid nitrogen and the frozen tissue was stored at −70°C until extraction.

2.2. Tissue measurements

Tissue dry weight was determined by weighing each individual heart (20–29 mg) after prolonged lyophilization of the frozen tissue. The freeze-dried tissue was then ground to a fine powder and extracted with 1 ml of 3.6% ice-cold perchloric acid. The extract was neutralized with KOH and centrifuged at 10 000 rpm. The resulting supernatant was freeze-dried and dissolved in 600 μl of D_2O for ^{13}C and ^{1}H analysis. A portion (2×50 μl) of the sample was removed for the enzymatic assay of glutamate. Other metabolites associated with the TCA cycle (aspartate, malate, succinate, citrate) were not detected in the ^{13}C NMR spectra, so the tissue content of these metabolites was estimated from literature data [15]. Given the low concentration of other TCA cycle intermediates relative to glutamate, small errors in these estimates do not significantly affect flux values determined from the kinetic data [15].

2.3. NMR methods

WET–HMQC NMR spectra of isolated perfused mouse hearts were collected on a Varian INOVA 14.1 T spectrometer equipped with an 8 mm inverse detection probe equipped with pulse field gradient coils (Nalorac, Martinez, CA, USA). The FID and line-shape of the water signal was used to optimize field homogeneity (a water line-width of 15–20 Hz was typical). The pulse sequence consisted of two parts: a WET sequence [23] for water suppression, followed by an HMQC sequence [24] for ^{13}C editing. The WET water suppression used a 2 ms Gaussian-shaped pulse, followed by a 2 ms homospoil gradient. This suppression scheme was repeated four times with the gradient strength decreasing by 50% on each cycle. The HMQC sequence used a $\tau = 1/2J = 3.9$ ms ($J = 128$ Hz) and GARP [25] decoupling during acquisition. The first increment of the HMQC provided a ^{13}C-edited ^{1}H spectrum of the heart. Spectra were collected with 2048 points (zero filled to 4096) over 4000 Hz, and a Gaussian function was applied before Fourier transformation. A 1 s delay was used between each acquisition, and 16–32 FIDs (depending upon the amount of glutamate in the heart) were averaged for each spectrum, giving a temporal resolution of 20–40 s. Peak areas were measured using the line-fitting routine of NUTS (Acorn NMR, Fremont, CA, USA).

^{1}H-decoupled ^{13}C NMR spectra of heart extracts were acquired in a 5 mm Varian broad-band probe using a 45° pulse, 1.5 s acquisition time and a 1.5 s post-acquisition delay. A bi-level WALTZ-16 [26] sequence was used for broadband proton decoupling. ^{13}C NMR multiplets were deconvoluted using NUTS, and multiplet ratios were used to determine the fractional contribution of ^{13}C-enriched substrate to acetyl-CoA (F_{C2}) and the relative anaplerotic flux (y) [27].

2.4. Kinetic analysis

The individual peak areas of glutamate H4 and H3 obtained in the WET–HMQC experiments were normalized to fractional enrichment using F_{C2} (percentage of labeled acetyl-CoA utilized by the heart) values determined by isotopomer analysis [27] of the ^{13}C NMR spectrum of each heart extract at steady-state. The kinetics of C3 and C4 enrichment and the experimentally determined tissue glutamate content (and estimated pool sizes for aspartate and other intermediates) were used as input for tcaFLUX [15] to obtain values of V_{tca} and V_x. V_{tca} and V_x are reported as the average and standard deviation for four hearts in each substrate group.

3. Results

3.1. Indirect detection

The low natural abundance of ^{13}C (1.1%) makes it an excellent tracer for metabolism studies since the metabolic fates of ^{13}C-enriched substrates can be followed with little to no interference from endogenous ^{13}C. However, low natural abundance coupled with a relatively low gyromagnetic ratio ($\gamma_H/\gamma_{13C} \approx 4$) makes ^{13}C observation much less sensitive than ^{1}H. The WET–HMQC experiment combines the sensitivity of ^{1}H spectroscopy with the tracer advantages of ^{13}C. Fig. 1 compares an unedited ^{1}H spectrum of a mouse heart perfused with [2-^{13}C]acetate with an edited spectrum obtained using the 1D WET–HMQC sequence. The ^{1}H–^{12}C signals were efficiently filtered by the HMQC sequence, leaving behind a relatively simple spectrum showing the H3 and H4 resonances of

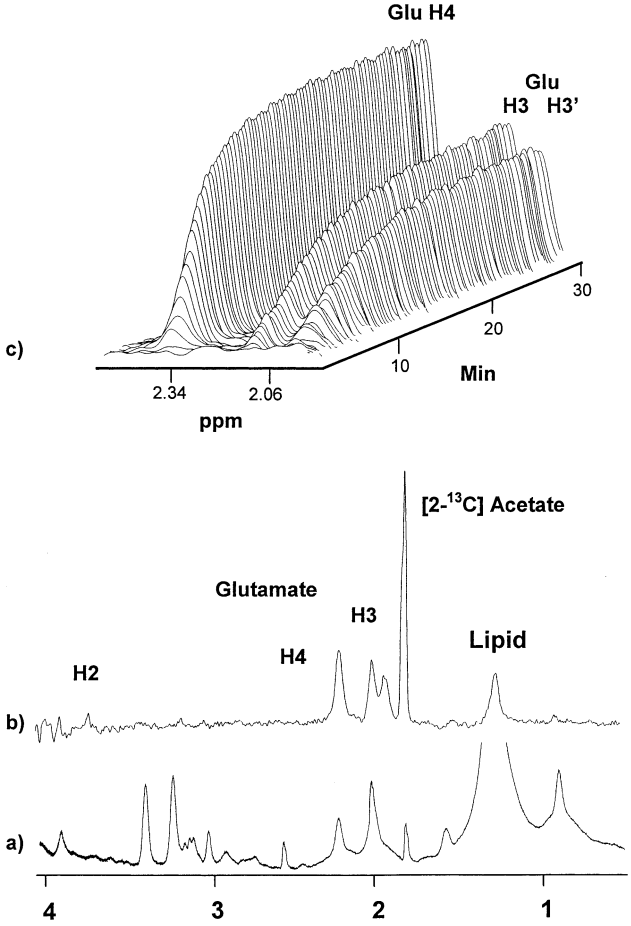

Fig. 1. a: ^{1}H spectrum of an acetate-perfused heart acquired with water pre-saturation. b: 1D WET–HMQC spectrum of a [2-^{13}C]-acetate-perfused heart. The glutamate H2 signal is small due to its proximity to the suppressed water signal. c: Enrichment of the glutamate C3 and C4 in a [2-^{13}C]acetate-perfused heart over time as monitored by 1D WET–HMQC. Spectra were averaged over 20 s intervals.

^{13}C-enriched glutamate (the H2 resonance is close to the bulk water resonance and is destroyed by the WET sequence) and resonances from the large ^{1}H–^{13}C natural abundance lipid signal and [2-^{13}C]acetate. The spectrum shown represents an average of 16 scans collected over 20 s.

3.2. Metabolic parameters

The contribution of each enriched substrate (acetate, octanoate, or lactate) makes to total acetyl-CoA entering the TCA cycle (F_{C2}) and y relative to V_{tca} were determined by isotopomer analysis of the ^{13}C NMR spectrum of each heart extract. While measurements of this type have been reported for larger rodent hearts [28–30], they have not been reported for the perfused mouse heart. Table 1 lists F_{C2}, y, and total tissue glutamate for each perfusion condition. Acetate contributes most to acetyl-CoA, followed by octanoate and lactate (the F_{C2} values), while y was 6% or less for all three substrates.

3.3. TCA cycle kinetics

Fig. 1c shows a stacked plot of NMR spectra (glutamate H3 and H4 only) of an isolated mouse heart perfused with [2-^{13}C]acetate collected every 20 s over 40 min. This perfusion period was sufficient for the heart to reach isotopic steady-state as indicated by the maxima in the enrichment curves. Spectra of octanoate- and lactate-perfused hearts were averaged over 40 s due to the lower levels of glutamate in these hearts and the lower enrichments achieved under these conditions (as indicated by the F_{C2} values of Table 1). Typical kinetic data sets for hearts perfused with acetate, octanoate and lactate are compared in Fig. 2. These data (plus F_{C2} and y values and total tissue glutamate obtained from extract data) were fit to a kinetic model of the TCA cycle using tcaFLUX as previously described [15]. The dashed lines through these data show enrichment curves predicted by the kinetic model. Interestingly, both V_{tca} (14.1 ± 3.6 μmol g^{-1} min^{-1}) and V_x (26.5 ± 7.5 μmol g^{-1} min^{-1}) were significantly higher in hearts perfused with acetate than with octanoate (V_{tca} 6.0 ± 1.4; V_x 16.1 ± 4.9) or lactate (V_{tca} 4.2 ± 1.1; V_x 6.3 ± 4.3).

It has been shown in perfused rat hearts that the transaminase inhibitor AOA slows the rate of glutamate enrichment from a ^{13}C-enriched substrate by slowing exchange between tissue glutamate and α-ketoglutarate [31–33]. We observed similar alterations in mouse hearts perfused with [2-^{13}C]-acetate and AOA (stacked plots not shown). An analysis of those kinetic data indicated that V_{tca} was unchanged (17.2 ± 2.5 μmol g^{-1} min^{-1}) in the presence of AOA, while V_x was 9.1 ± 2.6 μmol g^{-1} min^{-1}, approximately ~3 times lower than without AOA.

3.4. Oxygen consumption

The primary function of the TCA cycle in the heart is to provide reducing equivalents (NADH, $FADH_2$) for electron transport and subsequent synthesis of ATP. Since V_{tca} is proportional to MVO_2 and independent of possible uncoupling of the electron transport chain, MVO_2 can be estimated from the V_{tca} values [28]. These were 27.1 ± 6.8, 17.1 ± 2.0, and 13.1 ± 2.1 μmol g^{-1} min^{-1} for acetate-, octanoate-, and lactate-perfused hearts, respectively. Measured MVO_2 values, 25.2 ± 3.8, 18.2 ± 1.2, and 14.6 ± 1.2 μmol g^{-1} min^{-1}, respectively, were not significantly different.

Table 1
TCA cycle parameters and tissue glutamate concentrations

Substrate[a]	F_{C2}	y	[Glu][b]
3 mM Acetate	0.92 ± 0.02	0.03 ± 0.01	34.6 ± 1.3
0.75 mM Octanoate	0.78 ± 0.01	0.05 ± 0.02	17.8 ± 2.8
3 mM Lactate	0.54 ± 0.01	0.06 ± 0.03	8.1 ± 4.0

$n = 4$.
[a]In addition to 5.6 mM unenriched glucose.
[b]μmol g^{-1} dry wt.

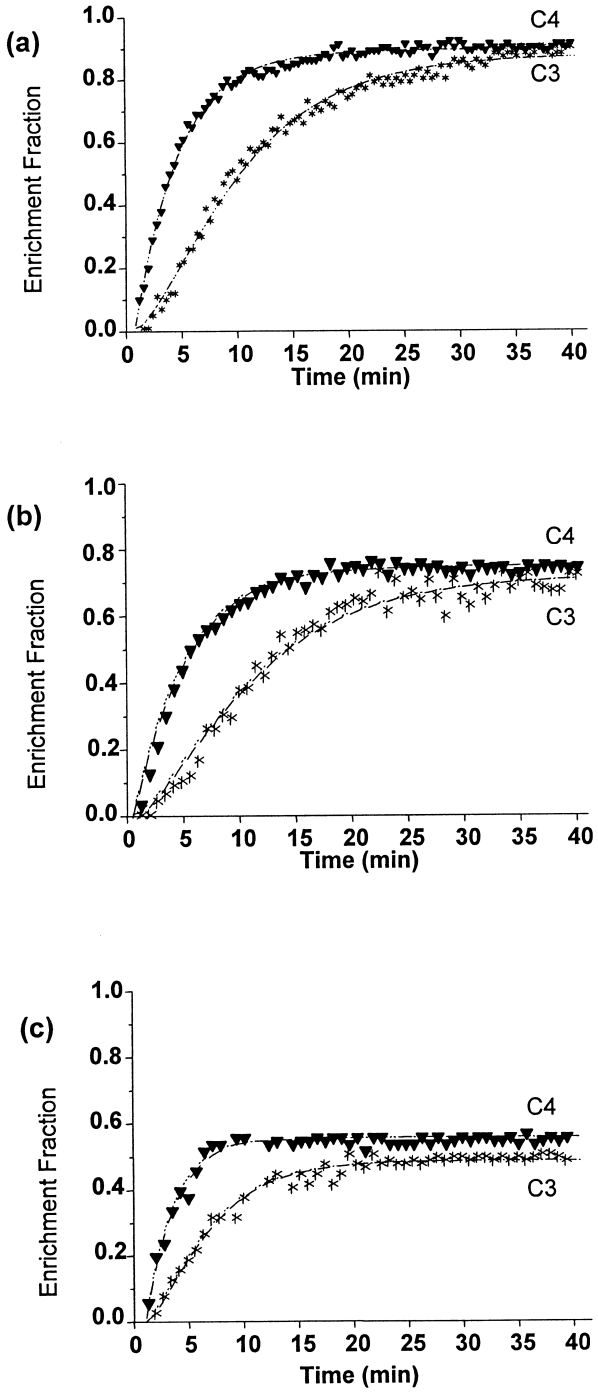

Fig. 2. Kinetic curves for acetate- (a), octanoate- (b), and lactate- (c) perfused hearts. The dashed lines represent a best fit of the experimental data to a kinetic model of the TCA cycle using the program, tcaFLUX, written in-house.

4. Discussion

Advances in transgenic mouse technologies have placed an increased emphasis on non-invasive methods for monitoring metabolism in small animal tissues. NMR spectroscopy has been widely used to detect metabolic events in larger animal tissues, but many of these same techniques are sample-limited in the mouse. The present study demonstrates that the WET–HMQC experiment provides ample sensitivity to perform kinetic experiments in isolated perfused mouse hearts (~25 mg dry tissue). Based on direct comparison of S/N, we estimate that the WET–HMQC experiment (using an 8 mm ID probe) provides a ~6–10-fold time-saving over a similar kinetic experiment performed in a 10 mm broad-band probe tuned to ^{13}C (unpublished).

Using a previously reported isotopomer analysis [27], we found that the contribution of acetate to acetyl-CoA in the perfused mouse heart (92%) was similar to that reported earlier for the perfused rat heart (96%) [15]. However, octanoate contributed 78% of the total acetyl-CoA in mouse hearts (compared to 92% in the rat hearts under similar conditions [29]) and lactate contributed 58% of the acetyl-CoA in mouse heart (compared to 75% in guinea pig hearts [30]). Similarly, Belke et al. [7] recently measured glucose and palmitate oxidation in the working mouse heart by using ^{14}C and ^{3}H tracers, and found that palmitate tended to contribute less to acetyl-CoA in mouse hearts (0.73) compared to typical rat hearts (0.80).[1] These combined data suggest there may be a limitation in the ability of the mouse heart to handle more highly reduced substrates (palmitate, octanoate, lactate) compared to hearts from larger rodents.

The substrates in this study were chosen to provide variations in cellular redox, and thus, in TCA cycle flux (V_{tca}). Acetate has a single metabolic fate in the heart (to contribute to acetyl-CoA), so the energy requirements in an acetate-perfused heart largely come from reducing equivalents generated in the TCA cycle. This is consistent with our observation that the highest value of V_{tca} was found in mouse hearts perfused with acetate. In contrast, octanoate generates NADH via β-oxidation (external to the TCA cycle) and indeed V_{tca} was significantly lower in hearts perfused with octanoate. Similarly, lactate generates even more NADH (2 moles per mole of lactate) external to the cycle, consistent with the lowest value of V_{tca} observed for mouse hearts perfused with lactate. These correlations support the hypothesis that V_{tca} (as measured by NMR) is a good indicator of oxygen consumption in hearts [15]. Further confirming evidence came from an independent measure of MVO_2 using an oxygen electrode (MVO_2 also decreased in the order, acetate > octanoate > lactate). As would be expected for the smaller rodent, V_{tca} (and MVO_2) was ~40% higher in mouse hearts perfused with acetate compared to rat or rabbit hearts perfused with this same substrate. Belke et al. [7] also reported that fatty acid and glucose utilization was 49% higher in the isolated mouse heart compared to the rat.

The rate limiting step associated with V_x is not well understood, but ^{13}C-enriched mitochondrial α-ketoglutarate can be converted to glutamate via a number of pathways. α-Ketoglutarate could be converted to glutamate via glutamate dehydrogenase (GDH) in heart tissue [34]. We found that the non-specific transaminase inhibitor AOA had a similar effect on V_x in the mouse heart compared to previous reports in rat hearts [31–33]. In the presence of 0.2 mM AOA, V_x decreased ~three-fold. This is similar to the two-fold reduction reported by Weiss et al. [33] using 0.1 mM AOA in the rat heart. Thus, GDH is likely not an important contributor to V_x in the heart.

Glutamate and α-ketoglutarate can also exchange via the ubiquitous transaminase reactions. In particular, aspartate aminotransferase, an essential component of the malate–aspartate shuttle, is quite active in heart tissue. This pathway is important for transporting NADH from the cytosol (generated during glycolysis) to the mitochondria for ATP synthesis. White et al. [18] found that V_x was elevated in rabbit hearts perfused with lactate compared to acetate, and ascribed this to increased malate–aspartate shuttle activity brought about by conversion of lactate to pyruvate. This was not observed here in isolated mouse hearts. We found that V_x was consistently about two-fold greater than V_{tca} for all three substrates examined here. As shuttle activity is expected to be highest in hearts perfused with lactate, and lactate is avidly oxidized (it contributed 54% of the total acetyl-CoA entering the TCA cycle in hearts perfused with lactate alone, Table 1), this observation suggests that V_x is not proportional to malate–aspartate shuttle activity in the mouse heart. This is again different from that found in rabbit hearts perfused with lactate [18].

In summary, we have demonstrated that metabolic fluxes can be reliably measured in isolated perfused mouse hearts using a ^{13}C tracer and the indirect detection NMR method, WET–HMQC. This allows NMR detection of enriched glutamate with a ~10-fold higher temporal resolution than typically used in rat or rabbit heart experiments by ^{13}C direct observation. Given this increased sensitivity, it is likely that kinetic NMR methods can be extended to even smaller tissue samples (i.e. cell cultures).

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