Validation of deuterium-labeled fatty acids for the measurement of dietary fat oxidation during physical activity

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Abstract  Measurement of $^{13}$C-labeled fatty acid oxidation is hindered by the need for acetate correction, measurement of the rate of CO$_2$ production in a controlled environment, and frequent collection of breath samples. The use of deuterium-labeled fatty acids may overcome these limitations. Herein, $d_{31}$-palmitate was validated against $[1-^{13}$C]palmitate during exercise. Thirteen subjects with body mass in

Stable isotopes have been used to quantify plasma and dietary fat oxidation in the past (1–3). When a $^{13}$C-labeled fatty acid is dosed orally or by constant infusion, the $^{13}$CO$_2$ in breath can be used to measure the tracer oxidized. The $^{13}$C liberated during oxidative metabolism, however, is partly sequestered in the intermediates of the tricarboxylic acid (TCA) cycle (~40%) and the bicarbonate pool (10%), causing fat oxidation to be underestimated. To correct for this sequestration, an additional dose of $[1-^{13}$C]acetate is administered. Acetate is converted to acetyl-CoA and is oxidized in the TCA cycle (4). Unfortunately, sequestration is variable and depends on the conditions under which it is measured; hence, estimation of acetate sequestration is essential (5). In addition, the use of $^{13}$C-labeled fatty acid is further constrained by the need for frequent sampling of breath and the use of a metabolic cart or respiratory chamber to quantify the flux of CO$_2$ to calculate $^{13}$C recovery accurately. These factors increase subject burden; thus, an alternative method to quantify fat oxidation in the body would be useful.

One such method for the measurement of fat oxidation is the use of deuterium-labeled fatty acids (3, 6). When oxidized, $^3$H-labeled fatty acid is metabolized to acetyl-CoA, releasing NADH molecules. The $^3$H label is released as water, in part, when NADH molecules are oxidized in the respiratory chain. Oxidation of acetyl-CoA in the TCA cycle releases the rest of the deuterium label in the form of $^3$H-labeled water. This $^3$H$_2$O mixes with the body water and can be sampled in the urine (7). Urinary and insensible water losses are minimal (8); hence, the enrichment of label in urine can be used effectively to calculate the cumulative recovery of the label and hence the fat oxidized. Consequently, the need for measurement of CO$_2$ and flux is also eliminated.

Deuterium-labeled palmitic acid has been validated against acetate-corrected $^{13}$C-labeled palmitic acid during rest in humans by Votruba, Zeddun, and Schoeller (6), who dosed subjects at rest with $^{13}$C- and $d_{31}$-labeled palmitic acid and demonstrated that the cumulative recoveries for

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both tracers were highly correlated ($y = 1.045x - 0.47; r^2 = 0.88; P < 0.0002$). More importantly, the mean difference in percentage recovery of the labels was $0.5 \pm 2.8\%$ when $^{13}$C data were corrected for acetate fixation. This method raises interesting possibilities for use under free-living conditions; however, this method has not been validated under nonresting conditions. Herein, we compared the metabolic fate of orally ingested $d_{31}$-palmitate and $[1-^{13}]$C palmitate during physical exercise of varying durations at moderate intensity. The objective was to ensure the validity of $^{13}$H-labeled fatty acids as an accurate tool to measure dietary fat oxidation under a range of free-living conditions.

**METHODS**

**Materials**

Labeled fatty acids were obtained from Cambridge Isotope Laboratories (Andover, MA). $[1-^{13}]$C palmitic acid and Na salt of $[1-^{13}]$C acetate were 99 atom% $^{13}$C. The $d_{31}$-palmitic acid was 98 atom%, and Na salt of $d_{3}$-acetate was 99 atom% $^{3}$H. The $^{18}$O was obtained as water (Isotec, Inc., Miamisburg, OH) and was 10.8 atom%.

**Subjects**

Healthy volunteers (six males and six females) were recruited, and their characteristics are summarized in Table 1. Two additional subjects were recruited for the measurement of natural isotope abundances and daily natural variability of isotopes in the body (body mass index $= 22.8 \pm 3.2$ kg/m$^2$; mean $\pm$ SD). The experimental protocol was thoroughly explained to the subjects before their recruitment into the study, and a signed informed consent was obtained from each of the subjects. The protocol was approved by the Institutional Review Board at the University of Wisconsin, Madison. Subjects completed a Physical Activity Readiness Questionnaire (9) to screen out those at risk.

**Protocol**

The study included two visits 3–4 weeks apart. The protocol for both visits was similar for a given subject except for the tracers given. The subject reported to the General Clinical Research Center GCRC at 6:00 PM on the evening before the test. During this day, the subjects were allowed to engage in normal daily activities, with the exception of any vigorous activities or structured exercise. At 7:00 PM, they were asked to void, and a urine sample was stored to determine the basal body enrichment in $H_2^{18}$O. After being weighed, a 0.4 g/kg estimated total body water dose of 10% $H_2^{18}$O was given to the subjects. Dinner (a standard meal) was provided at 6:00 PM. At 11:00 AM, another urine sample was collected for the determination of the total body water through $H_2^{18}$O enrichment of bodily fluids at isotopic equilibration, achieved 4 h after dose.

The 24 h energy requirements of the subjects were calculated from the World Health Organization (WHO) equation (10) and were divided into 40% at dinner, 30% at breakfast, and 30% at lunch. An activity factor of 1.5 for the previous day and 1.7 for the test day were added to the WHO calculated energy expenditure. The macronutrient distribution of each standard meal consisted of 50% carbohydrates, 35% fat, and 15% protein.

On the day of the test, subjects were awakened at 6:30 AM, and a urine sample was collected at 6:45 AM. Urine and breath samples were collected at 8:00 AM to measure the natural abundance of $^{13}$H in the body water and the natural $^{13}$CO$_2$ abundance in breath, respectively. The first visit consisted of an oral load of $[1-^{13}]$C acetate at 2 mg/kg body weight and $d_{31}$-acetate at 10 mg/kg body weight mixed in a liquid replacement meal (Boost high protein; Mead Johnson Nutritional) during breakfast. The second visit consisted of an oral load of $[1-^{13}]$C palmitate at 10 mg/kg body weight and $d_{31}$-palmitate at 15 mg/kg body weight, mixed in the same liquid replacement meal during breakfast.

Subjects exercised at 10:00 AM for 2 h (2 hr-ex) or 4 h at a light intensity (25% VO$_{2max}$) on a cycle ergometer. Within the 4 h exercise group ($n = 8$), three subjects started exercise at 10:00 AM [45 min after dose; early exercise (4 hr-ex)] and five subjects started exercise at 1:00 PM [3 h, 45 min after dose; late exercise (4 hr-ex)]. Measurements of CO$_2$ flux were taken for 20 min every hour. Respiratory gas exchange (RGE) was measured for 20 min every hour during the rest of the stay including at rest, during exercise, and after exercise using a Deltatrac I metabolic cart (SensorMedics). The O$_2$ and CO$_2$ analyzers were calibrated with a standard gas containing a 96% O$_2$ and 4% CO$_2$ mixture. The subjects breathed through a mouthpiece with their noses clipped to ensure complete respiratory gas monitoring. The valve is connected to a canopy system, which acts as a mixing chamber, and is exhausted into the Deltatrac system to measure the rate of oxygen consumption and carbon dioxide production. The first 5 min of measurement was excluded, and the hourly RGE was calculated assuming that the subsequent 15 min measurements per hour were representative of the whole hour.

$[1-^{13}]$C palmitate oxidation rates were calculated using hourly breath samples collected when subjects blew through straws into 15 ml additive-free Vacutainers$^\text{TM}$ (BD, Franklin Lakes, NJ), and $d_{31}$-palmitate oxidation rates were calculated from the spot urine samples collected every hour and stored in cryogenically stable tubes (Corning, Inc.). The recovery correction factor for $[1-^{13}]$C palmitate and palmitate was determined using the $[1-^{13}]$C acetate and $d_{31}$-acetate recovery rates.

**Sample analysis**

To measure the ratio of $^{13}$CO$_2$ to $^{12}$CO$_2$ in breath, a sample of breath was injected into a continuous-flow isotope ratio mass spectrometer (IRMS; Delta S; Finnigan MAT). The sample was injected into a continuous helium stream onto a Chromosorb-Q for separation of CO$_2$, which is directed into the source of the IRMS. Injections were made in duplicate, and the average standard deviation was 0.3%. Postdose enrichments within a subject were calculated using baseline predose breath samples (7).

**TABLE 1.** Subject characteristics (mean $\pm$ SD)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Women (n)</th>
<th>Men (n)</th>
<th>All (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>58.7 $\pm$ 7</td>
<td>81.5 $\pm$ 11</td>
<td>70.1 $\pm$ 15</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.7 $\pm$ 0.1</td>
<td>1.8 $\pm$ 0.1</td>
<td>1.8 $\pm$ 0.1</td>
</tr>
<tr>
<td>Body mass index</td>
<td>21.3 $\pm$ 4</td>
<td>24.3 $\pm$ 2</td>
<td>22.8 $\pm$ 3</td>
</tr>
<tr>
<td>Total body water (kg)</td>
<td>32.8 $\pm$ 5</td>
<td>49.0 $\pm$ 10</td>
<td>41.2 $\pm$ 12</td>
</tr>
</tbody>
</table>
To measure the ratio of $^2$H to $^1$H in urine, a sample (5 ml) of urine was mixed with 200 mg of carbon black to reduce impurities and was passed through a 0.45 μm filter (11). One milliliter of decolorized urine was placed in a 3 ml autosampler vial and analyzed for $^2$H/$^1$H ratios using the Delta plus IRMS (Finnigan MAT). A 0.8 μl aliquot was injected into a chromium-packed quartz tube held at 850°C to reduce water to hydrogen gas. Each sample was injected three times with independent analysis. Data were corrected for $^1$H and memory errors. Results were corrected to the standard mean ocean water (SMOW) scale.

Total body water was estimated from $^{18}$O enrichments in urine samples collected at baseline and 4 h after dosing with $^{18}$O-labeled water. One milliliter of decolorized urine sample was allowed to equilibrate with CO$_2$ at 25°C for 48 h in a water bath. The $^{18}$O enrichment was measured using a continuous-flow IRMS as detailed by Schoeller and Luke (12). Total body water was calculated from the $^{18}$O data using the dilution method as described by Schoeller and van Santen (8).

**Label calculations**

$^2$H and $^{13}$C label recoveries were calculated every hour for 9 h after dose. Baseline enrichment of the individual subject was subtracted from hourly recoveries to derive the increase in label recovery above baseline. Two subjects were made to follow the protocol without dosing, and the average enrichments per hour of these subjects were used to correct for natural variations in abundance of the isotopes attributable to meals and exercise. Thus, the final recovery values ($\Delta$δ) represent individual recoveries per hour corrected for baseline abundance of individual subjects and for natural variation during the day. Rate of CO$_2$ production ($V_{CO_2}$) was obtained from RGE measurements (6).

\[
\% \text{ }^{13}\text{C recovery} = 100 \times \left(\frac{V_{CO_2} \times \Delta \delta \times R_{STD} / 1,000}{D \times P \times n / MW \times 100}\right)
\]

where $V_{CO_2}$ is measured in milliliters per minute; $R_{STD}$ = $^{13}$C/$^{12}$C of standard CO$_2$; $D$ = dose in grams; $P$ = $^{13}$C isotope atom%; $n$ = number of labeled atoms per molecule of tracer; $MW$ = molecular weight of the tracer ($[^{13}$C]acetate Na salt δ = 88; [^{13}$C]palmitic acid δ = 257); and isotopic enrichment above the baseline (Del per mille ($\delta$) = ($R_2$/$R_{STD}$ - 1) × 1000.

Breath samples were collected for 9 h after dose on both visits to correct for hourly acetate sequestration of acetate label in the TCA cycle. Deuterium recovery was calculated assuming that it is equally distributed across the total body water (TBW).

\[
\% \text{ }^2\text{H recovery} = 100 \times \left(\frac{V_{CO_2} \times \Delta \delta \times R_{STD} / 1,000}{D \times P \times n / MW \times 100}\right)
\]

where TBW (moles) is multiplied by a factor of 1.035 to get a $^2$H dilution space from the TBW obtained by $^{18}$O dilution space; $R_{STD}$ = $^2$H/$^1$H of SMOW; $D$ = dose in grams; $P$ = $^2$H isotope atom%; $n$ = number of labeled atoms per molecule of tracer; $MW$ = molecular weight of the tracer ($d_3$-acetate Na salt δ = 88; $d_{31}$-palmitic acid δ = 287). The hours after dose represents the midpoint between voids for $^2$H recovery calculations.

Because the mean difference in body weight between visits was 0.39 ± 1.5 kg, the TBW in these subjects was assumed to be constant.

**Statistical analysis**

A Student’s paired t-test was used to compare both tracers. Regression analysis was done to correlate the two tracers. ANOVA was used to identify the effects of exercise on the correlation of the tracers. $P \leq 0.05$ was required for statistical significance. All values are presented as means ± SD. All statistical analyses were performed using STATVIEW version 5.0.1 (SAS Institute, Inc., Cary, NC).

**RESULTS**

ANOVA's of the ratios of [^{13}$C]palmitate to d$_3$-palmitate recoveries did not show a significant difference between exercise groups (mean difference = 0.03%; $P = 0.9$). Hence, exercise groups were combined for this analysis.

**Acetate recovery**

Cumulative $^{13}$CO$_2$ recovery from [^{13}$C]acetate plateaued at ~6 h after dose in all exercise groups, as shown in Fig. 1. Cumulative $^{13}$CO$_2$ recoveries 3, 6, and 9 h after dose were 36.3 ± 9%, 52.6 ± 8%, and 56.8 ± 9%, respectively, indicating that most of the label was recovered at 6 h after dose in all exercise groups. $^2$H$_2$O recovery from d$_3$-acetate peaked at 45 min after dose in all three exercise groups, as shown in Fig. 1. Cumulative d$_3$-acetate recoveries 3, 6, and 9 h after dose were 92.1 ± 8%, 90.6 ± 4%, and 88.4 ± 4%, respectively, indicating that most of the label was recovered at 3 h after dose. Compared with [^{13}$C]acetate, d$_3$-acetate showed greater cumulative recovery (mean difference = 31%; $P < 0.005$) (Table 2). Instantaneous d$_3$-acetate recovery was significantly higher than $^{13}$C at any time during the measurement period.

<table>
<thead>
<tr>
<th>Exercise</th>
<th>[^{13}$C]acetate</th>
<th>$^2$Hacetate</th>
<th>[^{13}$C]palmitate</th>
<th>$^2$Hpalmmitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h</td>
<td>51.2 ± 14</td>
<td>89.8 ± 4</td>
<td>6.1 ± 2</td>
<td>12.0 ± 3</td>
</tr>
<tr>
<td>Early 4 h</td>
<td>53.0 ± 7</td>
<td>84.6 ± 3</td>
<td>7.5 ± 2</td>
<td>13.0 ± 1</td>
</tr>
<tr>
<td>Late 4 h</td>
<td>58.7 ± 5</td>
<td>89.9 ± 5</td>
<td>4.5 ± 1</td>
<td>8.8 ± 2</td>
</tr>
</tbody>
</table>
3.4 h after dose in all exercise groups alike. The d31-palmitate tracer in the TCA cycle, d31-palmitate recovery at each 3%, and 10.6% recovery 3, 6, and 9 h after dose was 1.5 as a percentage of dose per hour (n = 12) uncorrected for acetate sequestration. The mean [2H]palmitate recovery was 10.3 ± 2.8% in all exercise groups together, and that of [13C]palmitate was 5.8 ± 1.8% in all exercise groups together. Uncorrected [2H]palmitate recovery was significantly different from uncorrected [13C]palmitate recovery (mean difference = 4.7%; P < 0.0001). Closed symbols represent [2H]palmitate recoveries and open symbols represent [13C] palmitate recoveries.

**Palmitate recovery**

Instantaneous [13CO2] recovery from [1-13C]palmitate oxidation peaked at 4.25 h after dose in the 4 hr-eEx group and at 6 h after dose in the 2 hr-eEx and 4 hr-lEx groups. Cumulative [1-13C]palmitate recovery at 3, 6, and 9 h after dose was 0.6 ± 1%, 2.9 ± 1%, and 5.6 ± 2% (Fig. 2), respectively, without acetate correction. When [1-13C]palmitate recovery was corrected for sequestration using average group acetate recovery correction factor, the mean 3, 6, and 9 h postdose recoveries were 1.1 ± 1%, 5.7 ± 3%, and 10.7 ± 4%, respectively. Corrected [1-13C]palmitate using each individual subject’s acetate recovery was 1.3 ± 1%, 5.7 ± 3%, and 10 ± 3% at 3, 6, and 9 h after dose (Table 3).

[2H2O] recovery from d31-palmitate oxidation peaked at 3.4 h after dose in all exercise groups alike. The d31-palmitate recovery 3, 6, and 9 h after dose was 1.5 ± 1%, 7.3 ± 3%, and 10.6 ± 3%, respectively. To correct for loss of tracer in the TCA cycle, d31-palmitate recovery at each time point was divided by exercise group mean acetate recovery at the same time point. The 3, 6, and 9 h postdose d31-palmitate recovery was 1.7 ± 2%, 8.3 ± 4%, and 12.0 ± 4%, respectively (Table 3). These values are not significantly different from uncorrected d31-palmitate recoveries (mean difference = 1.76%; P = 0.55), indicating little need for acetate corrections when [3H]-labeled fatty acids are used.

**Validation**

Correlation analysis showed that cumulative d31-palmitate recovery uncorrected for acetate fixation was correlated with [1-13C]palmitate recovery group corrected for [1-13C]acetate fixation (r2 = 0.51; y = 0.55x + 4.6; SEM = 0.19; P < 0.02) (Fig. 3). Because the y-intercept was not significant (P = 0.58), it was removed from the regression model (r2 = 0.2; y = 0.96x; SEM = 0.07; P = 0.0001). When d31-palmitate recovery was corrected for acetate sequestration using group average d3-acetate recovery, the correlation r2 was 0.56 (y = 0.74x + 4.6), and when an individual subject’s d3-acetate recovery was used to correct his/her own d31-palmitate recoveries, the correlation r2 was 0.54 (y = 0.69x + 4.9). The intercept in both of these instances was significantly different from zero (P = 0.02). The mean difference between the two tracers was lowest when uncorrected d31-palmitate recoveries were used (mean difference = 0.4 ± 3%; P = 0.64).

Votruba, Zeddun, and Schoeller (6) validated the use of d31-palmitate for the measurement of dietary fatty acid oxidation against [1-13C]palmitate under rest conditions. Mean cumulative recovery of [1-13C]acetate at 10 h after dose was 53.7 ± 10.4%. Mean cumulative [1-13C]palmitate recovery corrected for acetate sequestration at 10 h after dose was 12.9 ± 8.5% and that of d31-palmitate was 13.2 ± 7.7%. Acetate corrected [13C] data correlated well with d31-palmitate recovery in these subjects at rest (r2 = 0.88). These data, when combined with our data, showed a strong correlation (r2 = 0.79) between [1-13C]palmitate recovery corrected for acetate recovery and d31-palmitate recovery, suggesting that [2H] label can be used independent of acetate correction under conditions of rest and exercise alike (Fig. 4). Also, the exercise subjects showed no significant difference in their label recoveries compared with the recoveries of subjects at rest reported by Votruba, Zeddun, and Schoeller (6) (P = 0.11).

**DISCUSSION**

The present study demonstrates the validity of deuterium-labeled fatty acids to measure the oxidation of dietary fatty acids during exercise, expanding on the previ-
viously validated rest condition. The $^2$H label not only offers an effective alternative to $^{13}$C labeling, it also has the advantage of eliminating the need for an acetate correction, because acetate hydrogen sequestration in the TCA is minimal. The needs for frequent sampling and $V_{CO2}$ measurements are also eliminated by the use of $^2$H label; hence, it can be used under free-living conditions without hindering the subject’s normal daily activities.

Although this validation was performed using stable isotope-labeled fatty acids, radioisotope-labeled fatty acids have also been used to measure dietary fatty acid oxidation under rest and exercise conditions. Romanski, Nelson, and Jensen (13) dosed their subjects with $[^3]$H-triolein and $[^14]$C-triolein along with their meal to study the trafficking of dietary fat toward oxidation or storage for 24 h after dose. Although acetate corrections for both radioisotopes were not measured, the cumulative $^3$H and $^{14}$C recoveries were correlated; thus, our results should apply equally well to these radioisotopes.

Carbon labeling of fatty acids appears as a natural choice for the measurement of oxidative processes, but it is subject to sequestration in the TCA, leading to reduced yield (14). Since Sidossis et al. (4) proposed the use of a correction factor for acetate sequestration in the TCA cycle intermediates, $^{13}$C-labeled fatty acids have been used for the measurement of substrate metabolism, including fatty acid oxidation rates, but their accuracy has been debated. Acetate exits the path of the labeled substrate metabolism before entry into the TCA cycle and hence can account for label sequestration occurring in the TCA cycle. The accuracy of correction for sequestration depends on the position of the label in the fatty acid and the physiological conditions under which its metabolism is measured (15).

Unlike labeled carbons, most of the $^2$H label from fatty acids is rapidly released as reducing power ($NADH\cdot H^+\$ and $FADH_2$) during $\beta$-oxidation and then again in the TCA cycle (4). Thereafter, the $^2$H is oxidized to water and released into the total body water pool, which can be sampled in the urine. Because most of the NADH is formed during $\beta$-oxidation, only $\sim 25\%$ of the label enters the TCA cycle and only $\sim 10\%$ of the label is expected to be sequestered (6). This estimate is supported by our data, with $\sim 88\%$ (SEM = 1.4%) of $^2$H label recovered when subjects were dosed with $d_3$-acetate. Hence, $^2$H-labeled fatty acids have the further advantage of not requiring an individual acetate recovery measurement and can be used independently of an acetate correction factor. An added advantage is that the time taken by the $^2$H label to metabolize through the intermediate pools of the body is reduced. This is shown by the more rapid appearance of $^2$H in urine than $^{13}$C in breath CO$_2$ after acetate administration. This is probably attributable to the time required by $^{13}$C to pass through the TCA cycle and internal bicarbonate pools, which is eliminated when $^2$H label is used, because it mixes very rapidly with the body water pool (16).
Deuterium-labeled fatty acids when oxidized release the label in the body water pool, and sampling of urine measures the abundance of the isotope in the body water pool, whereas measurement of the abundance of $^{13}$CO$_2$ in breath is an instantaneous measure of oxidation. Hence, administration of $^2$H label does not require the frequent measurement of $V_{CO_2}$ that is needed when using $^{13}$C label for the calculation of recovery. The use of hydrogen-labeled fatty acids virtually obviates the need for a controlled environment during the collection of a subject’s samples. Moreover, because the tracer accumulates in body water, it reduces the potential error of missed peak oxidation with carbon labeling should the peak excretion occur between breath samples, a particularly troublesome issue with rapidly oxidized substrates such as acetate. The use of hydrogen labeling, however, requires the measurement of body water for recovery calculation, correction for water turnover for long recovery times, and is subject to dilution in the large body water pool.

Correction of $^2$H fatty acid oxidation measures for acetate sequestration did not yield any significant difference against uncorrected data compared with group corrected $^{13}$C fatty acid oxidation. Because the coefficient of variation for $d_3$-acetate recovery was only 5% between individuals of all exercise groups, correction using group acetate recovery versus individual recovery did not make a discernible difference in our data. This further emphasizes that deuterium-labeled fatty acids can be used to measure dietary fat oxidation without the need for measurement of acetate correction factor.

In conclusion, we demonstrated that $^2$H-labeled fatty acids can be used to accurately measure the oxidation of dietary fat during exercise. Minimal sequestration of $^2$H label in the TCA eliminates the need for an acetate oxidation measure and hence an additional stay in the hospital. Also, compared with $^{13}$C label, $^2$H label can be used without the need for frequent sampling, $V_{CO_2}$ measurement, and restricted environment for measurements. Furthermore, high correlation between $^{13}$C-labeled fatty acids (corrected for acetate sequestration) and $^2$H-labeled fatty acids (uncorrected) suggests the accuracy of the measurement of fat oxidation using either method.

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REFERENCES