THE EFFECT OF VITAMIN DEFICIENCIES UPON THE METABOLISM OF CARDIAC MUSCLE IN VITRO

II. THE EFFECT OF BIOTIN DEFICIENCY IN DUCKS WITH OBSERVATIONS ON THE METABOLISM OF RADIOACTIVE CARBON-LABELED SUCCINATE*

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The gross effects of biotin deficiency in bacteria, animals, poultry, and man have been studied in detail by a number of investigators. They range from growth failure in all species to dermatitis, alopecia, neuromuscular imbalance, anemia, spectacle eye, and thymus and testis atrophy in rats (1-4), poor feathering, dermatitis, and perosis in poultry (5-9), and changes in skin color, somnolence, glossitis, and electrocardiographic changes in man (10, 11). Data bearing on the underlying biochemical defects in biotin deficiency, however, have only recently been forthcoming.

In 1942 Pilgrim, Axelrod, and Elvehjem (12) showed that homogenates of liver from biotin-deficient rats oxidized pyruvate more poorly than did those from normal controls and, in 1944, Summerson, Lee, and Partridge (13) reported that the addition of biotin to liver slices from biotin-deficient rats in vitro resulted in increased lactate utilization. In 1943 Burk and Winzler (14) suggested that biotin might be concerned with carbon dioxide transfer. Koser, Wright, and Dorfman (15) and Stokes, Larsen, and Gunness (16) have reported that aspartate synthesis by bacteria is depressed in the absence of biotin, and Lardy, Potter, and Elvehjem (17) and Shive and Rogers (18) have presented data which account for this depressed aspartate synthesis on the basis of a failure to fix carbon dioxide in oxalacetate. Lichstein and Umbreit (19) have demonstrated recently the need for biotin in the decarboxylation of oxalacetate by Escherichia coli, and Ochoa et al. (20) have shown that the content of triphosphopyridine nucleotide-specific malate dehydrogenase-decarboxylase is lowered in biotin-deficient turkey liver.

Recent studies of the metabolism of cardiac muscle have shown that pyruvate utilization in vitro is depressed by thiamine deficiency in rats and


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ducks (21) and by pantothenic acid deficiency in rats (22). It occurred to us that if biotin functions in metabolism to facilitate the reversible $\beta$-carboxylation of pyruvate to oxalacetate the utilization of pyruvate as well as other members of the tricarboxylic cycle might be depressed in tissues from biotin-deficient birds. To test this hypothesis we have determined the rate of oxygen consumption of slices of heart muscle from biotin-deficient and control ducks without added substrate and with added pyruvate and succinate, the rate of pyruvate disappearance in the presence of added pyruvate, and the rate of carbon dioxide production from succinate. The effect of pair-feeding normal controls, the effect of adding biotin to deficient tissues in vivo and in vitro, and the comparative effects of deficiency upon the respiration of auricle and ventricle were also studied. A preliminary report of these experiments has been made (23).

**EXPERIMENTAL**

White Pekin ducklings 2 days old, averaging 50 gm. in weight, were divided into experimental and control groups and placed in heated, raised bottom cages. The control groups were fed the following diet: casein 18 per cent, gelatin 10 per cent, Cellu flour 3 per cent, dextrose 50 per cent, corn oil 10.2 per cent, cod liver oil 2.0 per cent, salts (24) 5 per cent, CaHPO$_4$ 1 per cent, choline chloride 0.3 per cent, $\alpha$-tocopherol in corn oil (10 mg. per ml.) 0.5 per cent. Supplementary crystalline vitamins were added in the following quantities, expressed in micrograms per 100 gm. of ration: thiamine 400, riboflavin 800, pyridoxine 400, nicotinic acid 4000, calcium pantothenate 2000, folic acid 100, menadione 100, and biotin 20. The experimental series was fed the same diet supplemented with 20 per cent dried raw egg white at the expense of carbohydrate and no biotin. To evaluate the effect of the partial inanition of biotin deficiency, several groups of control ducks were pair-fed either a deficient diet with parenteral biotin or the control diet. Other groups of control ducks were fed the control diet ad libitum.

Deficient ducks were taken for respiration studies after their growth had been arrested and some weight loss had occurred. The birds were killed by decapitation, the heart quickly excised, chilled on cracked ice, and then immersed in iced, oxygenated saline-phosphate solution. The auricles and ventricles of the chilled heart were removed, sliced to a thickness of 0.5 to 0.7 mm., placed in standard Warburg flasks containing potassium hydroxide in the center well, gassed with oxygen, equilibrated at 37°, and the rate of oxygen consumption determined. Each flask contained a total volume of 3.0 ml. of phosphate-saline at pH 7.4 of the following composition: NaCl 0.119 M, KCl 0.004 M, MgCl$_2$ 0.0005 M, and Na$_2$HPO$_4$-NaH$_2$PO$_4$ 0.020 M. When biotin was added in vitro it was present in the medium initially and
allowed to react with tissue components for 30 minutes before the addition of substrate. For study of the effects of biotin \textit{in vivo}, the vitamin was injected intraperitoneally into deficient ducks in amounts of 45 \textmu g per bird per day.

Substrates were added from the side arm at zero time. Sodium pyruvate was prepared from redistilled pyruvic acid which was neutralized to pH 6.5 with bicarbonate and then crystallized from ethanol. The final concentration of pyruvate in the Warburg flask after dumping was 5 mM per liter. The non-isotopic succinate was chemically pure sodium succinate. Isotopic succinic acid containing C\textsuperscript{14} distributed between its two carboxyl groups was prepared from lithium acetylide by carbonation with C\textsuperscript{14}O\textsubscript{2} and subsequent reduction. The final product melted at 189—190\degree C and contained about 440,000 counts per minute per mm. For respiration studies this was diluted with non-isotopic succinate to yield an activity of about 10,000 counts per minute per mm. The final concentration of succinate in the Warburg flask was 10 mM per liter.

Oxygen uptake was measured for 1 hour. At the end of this period tissue reactions were stopped by addition of 0.2 ml. of 100 per cent trichloroacetic acid. In the case of the flasks containing radioactive succinate this addition was made in a closed system so that the C\textsuperscript{14}O\textsubscript{2} liberated from the buffer and tissue would be absorbed by the alkali of the center well. For determinations of the amount of succinate oxidized the contents of the center well were pipetted with washings into a 15.0 ml. centrifuge tube (a filter paper in the center well was generally \textit{not} used in the succinate experiments because it was shown that even assiduous washing of the alkali-soaked filter paper would leave 5 to 10 per cent of the carbon dioxide in the paper) and precipitated with BaCl\textsubscript{2}. The barium carbonate was centrifuged and washed with water until free of alkali and finally with ethanol and then plated on a tared, round, stainless steel cup having a central depression 1.5 mm. deep and 1.6 sq. cm. in area. The samples were counted with an end window Geiger counter and corrected for background, self-absorption, and counter sensitivity. The accuracy of the counting as determined by checks with samples of known activity was \pm 5 per cent.

Pyruvate was determined by the direct method of Friedemann and Haugen (25) and lactate by the method of Barker and Summerson (26) on trichloroacetic acid filtrates of the Warburg fluid after incubation. Final dry weight of the tissue slices was determined by heating to constant weight in an oven at 110\degree C. In the protocols, the rate of oxygen consumption has been expressed as $Q_{O_2}$ (microliters of O\textsubscript{2} used per mg. of dry weight of tissue per hour), pyruvate disappearance as $-Q_{\text{pyruvate}}$ (microliters of pyruvate disappearance per mg. of dry weight of tissue per hour, 1 micromole of pyruvate being equivalent to 22.4 microliters), and lactate formation as $Q_{\text{lactate}}$. 
The net pyruvate disappearance, i.e. the conversion of pyruvate to non-lactate products, is expressed as net $-Q_{\text{pyruvate}}$ (21).

Results

About 70 ducklings were used in this study. Typical growth curves for deficient, pair-fed controls, and controls fed ad libitum are shown in Fig. 1. Each point on the growth curve represents the mean of ten ducks. In this series of experiments it was invariably found that the experimental group receiving egg white grew more rapidly for the first 6 to 7 days and then lagged behind the control groups, reaching a plateau at about 200 gm. The food consumption, which is also plotted for all groups in Fig. 1, dropped to 30 gm. per day for the experimental group after the 1st week and remained at or about that level for the duration of the experiment. Poor feathering and baldness were noticed as early as 4 to 6 days in the experimental groups and occurred in some 90 per cent of the deficient ducks. Perosis was observable between the 8th and 24th days and occurred in 70 per cent of the deficient ducks. Mortality in the deficient group ranged from 20 to 30 per cent. The pair-fed control groups began to lag in growth after 10 days on their regimen and generally reached a plateau at a body weight of 300 to 350 gm. These birds were continually famished and consumed their food allowance in 30 to 45 minutes daily. Either pair-feeding the control diet or pair-feeding the deficient diet plus intraperitoneal injections of 20 $\gamma$ of biotin per duck per day gave the same growth curve. In general, the hearts of the deficient ducks were paler, larger, and the myocardium thicker than in those from controls fed ad libitum or pair-fed.

The effect of biotin deficiency upon the respiration of slices of ventricle is shown in Table I. Mean data on oxygen consumption, lactate formation, and pyruvate utilization for deficient, cured, and control ducks are presented. All values are depressed in biotin deficiency. When pair-fed controls are used as the standard of comparison, the $Q_0$, of heart ventricle from biotin-deficient ducks was reduced 43 per cent without added substrate and 35 per cent with added pyruvate. Pyruvate utilization was decreased 48 per cent. When compared to slices from pair-fed ducks the lactate accumulation expressed as $Q_{\text{lactate}}$ was 15 per cent of normal in the absence of added substrate and 25 per cent of normal in the presence of pyruvate at 5 mM per liter. Compared with values from ducks fed ad libitum, the above values become 39 per cent and 52 per cent respectively. The elevation of lactate formation in the pair-fed birds is reminiscent of our findings in normal ducks fasted for 48 hours (21) and suggests that the pair-fed ducks in this series are effectively fasted by the feeding of suboptimal food allowances which are almost immediately consumed. Time studies of
Fig. 1. Growth curves for biotin-deficient, pair-fed normal control, and control ducks fed *ad libitum*. The mean body weight in gm. for groups of ten biotin-deficient (●), pair-fed control (○), and controls (●) fed *ad libitum* is plotted against the time in days. The crosses indicate death. Perosis was observable after the 8th day. The food intake in gm. per day per duck is plotted for these groups across the bottom.
the rate of lactate formation in ventricular muscle from normal ducks and those fed *ad libitum* indicate that the lactate accumulation is a product of initial glycolysis and subsequent oxidation in slices without added pyruvate and the product of initial glycolysis plus further reduction of pyruvate in the tissues with added pyruvate (27). The data obtained with biotin deficiency would seem to indicate that the initial glycolysis is decreased.

When deficient birds are injected with biotin intraperitoneally at a level of 45 γ per bird per day, there is restoration of respiration to normal within 24 hours of the injection. The data on cured ducks shown in Table I were obtained from three deficient ducklings showing all the features of the biotin deficiency syndrome and injected with biotin from 1 to 7 days. One

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
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<tbody>
<tr>
<td><strong>Effect of Biotin Deficiency upon Oxygen Consumption, Pyruvate Utilization, and Lactate Formation in Heart Ventricle Slices from Ducklings</strong></td>
</tr>
<tr>
<td>Group</td>
</tr>
<tr>
<td>Deficient</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Controls fed <em>ad libitum</em></td>
</tr>
<tr>
<td>Controls fed <em>ad libitum</em></td>
</tr>
<tr>
<td>Deficient + biotin intraperitoneally</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

* Duplicate determinations made on each duck.  
† All deviations are the standard error of the mean.

bird was killed after 1 day, one after 2 days, and one after 7 days. The one allowed to go for 7 days showed resumption of growth at a slope paralleling the normal controls fed *ad libitum* and refeathering. It may be seen that the oxygen consumption with and without added pyruvate and pyruvate utilization in these cured ducks are restored to levels not significantly different from those of normal controls.

Table II shows the results of studies on the oxidation of succinate by these same groups of biotin-deficient, cured, and control ducks. The oxygen consumption by ventricle from biotin-deficient ducks in the presence of succinate is decreased below the values found for control birds and is restored by injections of biotin. Column 4 of Table II lists the radioactivity recovered in the center well of the Warburg vessels after incubation.
Column 5 of Table II gives the rate of oxidation of succinate in terms of $Q_{\text{CO}_2}$ (succinate carboxyl). These values were calculated from the initial total quantity and activity of the succinate carboxyl $\text{CO}_2$ and the final activity of the respiratory $\text{CO}_2$ collected from the center well; viz., each flask contained 3.0 ml. of buffer having a succinate concentration of 10 mM per liter. The total succinate per flask was thus 30 micromoles and the total succinate carboxyl $\text{CO}_2$, 60 micromoles. The total radioactivity of the succinate carboxyl was 300 counts per minute per flask. The period of incubation was 1 hour. From this it follows

\[
\text{Activity of center well, } \text{CO}_2, \text{ counts per min. per flask} \times \frac{1}{\text{dry weight of tissue, mg.}} = Q_{\text{CO}_2} \text{(succinate carboxyl)}
\]

The $Q_{\text{CO}_2}$ (succinate carboxyl) is thus the number of microliters of succinate carboxyl carbon dioxide produced per mg. of dry weight of tissue per hour. Since only the carboxyl groups of the isotopic succinate are labeled, and since the known pathways for succinate oxidation make possible removal of one carboxyl without the other, the $Q_{\text{CO}_2}$ (succinate carboxyl) values obtained in the above manner are not indicative of the number of moles of succinate undergoing oxidation or the extent of the oxidation. If one wished to assume for the sake of an approximation that the liberation of one carboxyl $\text{CO}_2$ insured the complete combustion of succinate under our conditions,

### Table II

**Comparative Rates of Succinate Oxidation in Biotin-Deficient, Cured, and Control Duck Ventricle As Measured with Isotopic Succinate Labeled in Carboxyl with $^{14}C$**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of determinations</th>
<th>$Q_{\text{CO}_2}$ succinate, 10 mM per liter</th>
<th>$Q_{\text{CO}_2}$ produced during incubation (1 hr.)</th>
<th>Counts per min. per 100 mg. dry weight tissue per hr.</th>
<th>$Q_{\text{CO}_2}$ succinate carboxyl</th>
<th>Per cent of values obtained in pair-fed controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficient</td>
<td>18</td>
<td>13.04 ± 0.49</td>
<td>56 ± 4</td>
<td>4.255 ± 0.18</td>
<td>72 ± 2</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>Pair-fed controls</td>
<td>11</td>
<td>18.01 ± 0.68</td>
<td>126 ± 7</td>
<td>7.74 ± 0.31</td>
<td>100 ± 4</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Controls fed <em>ad libitum</em></td>
<td>14</td>
<td>17.13 ± 0.41</td>
<td>90 ± 4</td>
<td>4.17 ± 0.20</td>
<td>95 ± 2</td>
<td>73 ± 4</td>
</tr>
<tr>
<td>Deficient + biotin intraperitoneally</td>
<td>6</td>
<td>15.76 ± 0.45</td>
<td>132 ± 10</td>
<td>6.04 ± 0.47</td>
<td>88 ± 3</td>
<td>105 ± 8</td>
</tr>
</tbody>
</table>

* All deviations are the standard error of the mean.
then the rate of succinate combustion in microliters (micromoles \times 22.4)
per mg. of dry weight per hour would equal one-half the \( Q_{\text{CO}_2 \text{ succinate carboxyl}} \).

It is of interest to note (Table II, Columns 6 and 7) that biotin deficiency depresses \( \text{CO}_2 \) production from succinate in heart muscle more than it lowers oxygen consumption in the presence of succinate. Conversely, the administration of biotin to deficient ducks restores \( \text{CO}_2 \) production from succinate more completely than it restores oxygen consumption in the presence of succinate. These data are in support of the idea that the activity of biotin is concerned with decarboxylation reactions. If one calculates the \( -Q_{\text{succinate}} \) values for normal and deficient hearts from the \( Q_{\text{CO}_2 \text{ succinate carboxyl}} \) values on the basis of assumptions outlined in the preceding paragraph, and then calculates the equivalent oxygen consumption from \( -Q_{\text{succinate}} \) by multiplying by 3.5, it would appear that, while

<table>
<thead>
<tr>
<th>Ventricle slices</th>
<th>( Q_{\text{O}_2} ) no substrate</th>
<th>( Q_{\text{O}_2} ) pyruvate, 5 mm per liter</th>
<th>( Q_{\text{O}_2} ) succinate, 10 mm per liter</th>
<th>( Q_{\text{O}_2} ) pyruvate, 5 mm per liter</th>
<th>Pyruvate, 5 mm per liter</th>
<th>Lactate formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficient</td>
<td>12.34</td>
<td>0.43</td>
<td>0.87</td>
<td>3.58</td>
<td>3.14</td>
<td></td>
</tr>
<tr>
<td>( + ) biotin, 5 γ per ml.</td>
<td>11.38</td>
<td>0.32</td>
<td>0.71</td>
<td>3.97</td>
<td>3.56</td>
<td></td>
</tr>
<tr>
<td>Normal controls</td>
<td>17.37</td>
<td>0.75</td>
<td>1.75</td>
<td>6.01</td>
<td>5.01</td>
<td></td>
</tr>
</tbody>
</table>

\footnote{All deviations are the standard error of the mean.}

succinate combustion in normal heart ventricle accounted for 50 per cent of the observed oxygen consumption, in heart slices from biotin-deficient ducks it accounted for only 34 per cent. It is probable that the succinooxidase system which oxidizes succinate to fumarate (and other systems which oxidize fumarate to oxalacetate) accounts for a fair proportion of the extra oxygen consumption in both the normal and deficient tissues. It is of some interest that the succinate utilization of heart ventricle from control ducks fed \textit{ad libitum} is below that of pair-fed controls.

Attempts to remedy the respiration defects in ventricle from biotin-deficient ducks by adding the vitamin \textit{in vitro} and incubating 30 minutes before adding substrates failed, as indicated by Table III. There were no significant changes in oxygen consumption in the absence and presence of added pyruvate, in the presence of succinate, in pyruvate utilization, or in lactate formation. The only positive change was in the oxygen con-
sumption in the presence of pyruvate, a change from 5.36 ± 0.68 in the absence of biotin to 6.78 ± 0.89 in the presence of biotin, a difference of 1.42 for which \( t = 1.3 \), which is not significant. Normal control slices from ducks fed *ad libitum* run with this series showed normal values not different from the previous series of controls.

Table IV presents a comparison of the effects of biotin deficiency upon the respiration of auricle and ventricle slices taken from the same heart in a series of ducks. The same decreases in \( Q_02 \) and pyruvate utilization were observed for both auricle and ventricle. In general, the changes were parallel in both auricle and ventricle for all of the measurements made with the exception of oxygen consumption in the presence of pyruvate, which seemed to be depressed in auricle more than in ventricle. The degree of change from normal in this series of deficient ducks was not quite as marked as in earlier series, although the results are still significantly different from normal. This group of deficient birds was older and it has been found in two or three cases that birds which survive more than 24 days on the deficient diet give values for oxygen consumption and pyruvate utilization for heart ventricle which are closer to normal than are those obtained on ducks showing gross deficiency signs at 12 to 16 days. The nature of this accommodation to deficiency disease is being explored.

**DISCUSSION**

The finding of a deranged pyruvate and succinate metabolism in cardiac muscle slices from biotin-deficient ducks adds to the growing weight of evidence assigning to biotin the rôle of a respiratory catalyst. As early as 1933, Allison, Hoover, and Burk (28) showed that a heat-stable water-
soluble factor from yeast which they named coenzyme R and which was later identified with biotin (29, 30), would stimulate the respiration of the Rhizobium trifolii from legume nodules 100-fold. More recently, Burk, Winder, and du Vigneaud (31) have pointed out that the respiration of biotin-deficient yeast is markedly depressed and the addition of biotin stimulates both fermentation and respiration. The finding of a depressed endogenous oxygen consumption in ventricle slices from biotin-deficient ducks is, therefore, not unexpected. The utilization of pyruvate by both ventricle and auricle has been found to be depressed in biotin-deficient ducks. This effect has been previously observed in liver tissue from biotin-deficient rats by Pilgrim, Axelrod, and Elvehjem (12) and by Summerson, Lee, and Partridge (13).

Recent studies mentioned previously (15-18) have implicated biotin in the reversible β-carboxylation of pyruvate to oxalacetate. Although the Wood-Werkman reaction has not been shown to occur in heart muscle, fixation of CO₂ in oxalosuccinate by heart muscle extracts has been shown by Ochoa (32). Presumably, fixation of CO₂ in oxalacetate also occurs in heart muscle and experiments to verify this are under way at present. The production of CO₂ from succinate is significantly slowed in ventricle from biotin-deficient ducklings. The conversion of pyruvate to non-lactate products is decreased to almost the same quantitative extent, i.e. 50 per cent. If the fixation of CO₂ by pyruvate is a prerequisite for the maintenance of adequate levels of oxalacetate for condensation of C₂ or C₃ radicals from pyruvate and their ultimate oxidation by way of the tricarboxylic acid cycle, a loss of β-carboxylase activity would explain both the decreased CO₂ production from succinate and the decreased pyruvate utilization in cardiac ventricle slices from biotin-deficient ducks. The decreased lactate accumulation observed in these slices is more difficult to explain. That biotin has a single function in metabolism is doubtful in view of the studies of Lichstein and Umbreit (33) on the deaminase content of biotin-deficient Escherichia coli, of McHenry and Gavin (34) on synthesis of fatty acids in rats supplemented with biotin, and of Potter and Elvehjem (35) on oleic acid-biotin interrelationships in Lactobacillus arabinosus.

The mechanism of the action of biotin is not clear. Although biotin appears to influence the reversible β-carboxylation of pyruvate, other cofactors are also involved. Vennesland, Evans, and Altman (36) found stimulation of CO₂ fixation by pigeon liver β-carboxylase in the presence of adenosine triphosphate but stimulation of the reverse reaction in the presence of triphosphopyridine nucleotide. Cheldelin et al. (37) report that biotin is more firmly bound in tissue combination than any other vitamin. Our studies with the addition of biotin to deficient tissues in vitro indicate that the vitamin is not easily converted to an active form. The relative efficacy of biotin upon deficient ducks in vitro and in vivo parallels, however,
the effects of pantothenic acid (22) which is conjugated to a relatively complex coenzyme. Ochoa et al. have found no biotin by a microbiologic technique in their preparations of malate dehydrogenase-decarboxylase and entertain the possibility that biotin may in some way stimulate enzyme formation. The resolution of the mechanism of biotin activity awaits further investigation.

SUMMARY

1. The oxygen consumption of heart ventricle slices from biotin-deficient ducks in the presence and absence of pyruvate (5 mM per liter) and in the presence of succinate (10 mM per liter) was decreased 35, 43, and 28 per cent respectively from that of ventricle slices from pair-fed normal controls. The values for ventricle from controls fed ad libitum were not significantly different from those of the pair-fed controls.

2. The accumulation of lactate in ventricle slices from biotin-deficient ducks in the presence and absence of pyruvate was decreased to about 20 per cent of the values from pair-fed controls and to about 45 per cent of those from controls fed ad libitum. The higher lactate formation in pair-fed ducks appears to be related to their state of partial inanition.

3. The conversion of pyruvate to non-lactate products was reduced 48 per cent in ventricle slices from biotin-deficient ducks.

4. \(^{14}C\)O\(_2\) production from carboxyl-labeled succinate in heart ventricle from biotin-deficient ducks was decreased 55 per cent from that of pair-fed controls.

5. The prior intraperitoneal administration of biotin to deficient ducks restored the respiration and pyruvate utilization of heart ventricle slices essentially to normal.

6. The incubation of deficient heart ventricle slices with biotin in vitro had no significant effect upon their respiration or pyruvate utilization.

7. The respiration and pyruvate utilization of auricular and ventricular heart muscle, in general, were depressed to the same extent in biotin-deficient ducks.

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