TRANFORMATION OF THE STREPTOCOCCUS LACTIS R FACTOR TO "FOLIC ACID" BY RESTING CELL SUSPENSIONS OF ENTEROCOCCI

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Although relatively few microorganisms, primarily various lactic acid bacteria, are known to require an exogenous supply of "folic acid" for growth (Snell and Peterson, 1940; Hutchings, Bohonos, and Peterson, 1941; Niven and Sherman, 1944; Stokes, Keresztesy, and Foster, 1944), the presence of this growth factor has been demonstrated in numerous organisms which develop without added folic acid (Hutchings et al., 1941; Thompson, 1942). It appears likely, therefore, that folic acid plays a vital, although as yet unknown, role in the metabolism of all microorganisms. The complex problems of nomenclature, chemistry, and activity for microorganisms and animals of this relatively new growth factor and vitamin and its related forms have been comprehensively reviewed recently by Wieder (1944). For the purposes of the present paper it is sufficient to note that several folic acids, or more correctly Lactobacillus casei factors, have been isolated which vary greatly in activity for Lactobacillus casei and Streptococcus lactis R,¹ the two organisms most commonly used in measuring their microbiological activity. Thus the L. casei factor from liver, which is identical, apparently, with the chick antianemia factor, vitamin B₉ (Pfiffner et al., 1943), is highly potent for both bacteria, whereas another factor isolated from yeast is only half as active as the latter for S. lactis R, although fully as active as the latter for L. casei (Stokstad, 1943). A third factor has been isolated (Hutchings, Stokstad, Bohonos, and Slobodkin, 1944) which is almost as potent for L. casei as the liver factor but only 6 per cent as active as the latter for S. lactis R. A fourth factor isolated by Keresztesy, Rickes, and Stokes (1943) and named Streptococcus lactis R factor (SLR factor) differs from the other three in that it does not support growth of L. casei, although it is highly active for S. lactis R. Thus the factors range in potency from little or no growth-promoting activity for one or the other of the two test organisms to high activity for both.

That the various growth factors are closely related is clearly evident from their complete interchangeability for the growth of either L. casei or S. lactis R or both. Additional evidence for this close relationship is the fact that streptococci that can utilize the SLR factor invariably convert it during growth into a substance active for L. casei (Stokes, Keresztesy, and Foster, 1944). This was interpreted to mean that growth is due to the ability of those organisms to transform the SLR factor into L. casei activity, and that, therefore, the latter form is the one more directly involved in the metabolism of both types of microorganisms.

¹Now properly classified as Streptococcus faecalis (Gunsalus, Niven, and Sherman, 1944).
(L. casei and S. lactis R). It is noteworthy that the antianemia activity for chicks of the various factors tends to parallel L. casei rather than S. lactis R activity (Hutchings et al., 1944). The present investigation is concerned with the following aspects of the conversion of the SLR factor to folic acid (L. casei activity) by resting cell suspensions of enterococci: (a) efficiency of different strains, (b) effect of growth medium, pH, age, and concentration of cells, addition of carbohydrates, and other environmental factors, and (c) extraction of the folic acid from the bacterial cells.

METHODS

The streptococci were grown in a substantially synthetic medium (Mitchell et al., 1941) consisting of glucose, casein hydrolyzate, cystine, tryptophane, sodium acetate, growth factors, minerals, and 60 millimicrograms of the SLR factor per liter. It will be referred to as medium A. The cells were harvested in the centrifuge after incubation for 16 hours at 30°C, washed once with water, and resuspended in sufficient phosphate buffer, M/15 at pH 7.0 or M/7.5 at pH 8.0, to read 15 in an Evelyn photoelectric colorimeter, at 520 mμ wave length. Such suspensions contained 1.6 mg of dry cells per ml.

Each conversion was carried out in a 1" x 8" test tube, which usually received 9 ml of cell suspension, 2 μg to 20 μg of SLR factor dissolved in 20 per cent ethanol, and sufficient phosphate buffer to bring the volume to 10 ml. In some experiments a small amount of glucose or other carbohydrate was added. In most experiments the mixture was stirred with a slow stream of nitrogen gas during incubation. The specific conversion conditions for each experiment are described later. After incubation, generally for 3 hours, the whole suspension was assayed for folic acid with L. casei (Landy and Dicken, 1942). A 7.7 per cent concentrate of folic acid, kindly supplied by Dr. R. J. Williams, was used as the standard in the assays. The quantities of folic acid in the tables are expressed in terms of millimicrograms of folic acid of "potency 40,000" (Mitchell and Snell, 1941) per 10 ml of suspension. Since folic acid has not been isolated as yet in pure form, and in view of the existence of a number of compounds with related microbiological activity, the term, as used here, refers to growth factor activity for L. casei and not to a specific substance. "Folic acid" is used in place of other and perhaps preferable terms, such as "L. casei factor," because a concentrate of folic acid served as the standard for measuring activity.

CONDITIONS AFFECTING ACTIVITY OF CELLS

Organism, medium, age of culture, etc. In addition to S. lactis R, four other streptococci, namely, S. faecalis F24 and 732, S. zymogenes 5Cl, and S. durans 98A, can satisfy their need for folic acid with the SLR factor. This is due to their ability to convert the SLR factor to folic acid (Stokes, Keresztesy, and Foster, 1944). Their relative conversion efficiencies are shown in table 1. S. lactis R, S. faecalis F24, and S. zymogenes 5Cl formed more folic acid and in roughly similar amounts, whereas S. faecalis 732 and S. durans 98A produced only approximately 1/3 as much folic acid. S. lactis R was used in all subsequent experiments.
Media containing yeast extract, tryptone, brain heart infusion, or proteose peptone supported more abundant growth of *S. lactis* R than did medium A. Cells from those media, however, formed much less folic acid than cells from medium A (table 2). The greater activity of the bacteria from the latter medium is probably due to their previous contact with the SLR factor, which, as will be shown later, stimulates folic acid formation. Growth in the complex organic media was supported by the folic acid contained in them (Stokes, Gunness, and Foster, 1944).

**TABLE 1**

*Formation of folic acid from SLR factor by resting cell suspensions of streptococci*

Suspensions were adjusted to 15 on the galvanometer and contained 9 ml cells in M/7.5 phosphate buffer at pH 8, 0.1 ml SLR factor (5 µg) and 0.9 ml M/5 glucose. Aerated for 3 hr at 37 C with N₂.

<table>
<thead>
<tr>
<th>MICROORGANISM</th>
<th>FOLIC ACID (millimicrograms)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. lactis</em> R</td>
<td>3,200</td>
</tr>
<tr>
<td><em>S. faecalis</em> F24</td>
<td>2,800</td>
</tr>
<tr>
<td><em>S. faecalis</em> 732</td>
<td>500</td>
</tr>
<tr>
<td><em>S. zymogenes</em> 5Cl</td>
<td>3,600</td>
</tr>
<tr>
<td><em>S. durans</em> 98A</td>
<td>600</td>
</tr>
</tbody>
</table>

**TABLE 2**

*Influence of growth medium on conversion of SLR factor to folic acid*

<table>
<thead>
<tr>
<th>MEDIUM*</th>
<th>GROWTH†</th>
<th>FOLIC ACID‡ (millimicrograms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>49</td>
<td>100</td>
</tr>
<tr>
<td>Tryptone</td>
<td>50</td>
<td>1,300</td>
</tr>
<tr>
<td>Brain heart infusion</td>
<td>48</td>
<td>400</td>
</tr>
<tr>
<td>Proteose peptone</td>
<td>48</td>
<td>500</td>
</tr>
<tr>
<td>A</td>
<td>72</td>
<td>3,500</td>
</tr>
</tbody>
</table>

* Consisted of 0.5% of the bacto product, salts A and B (Mitchell and Snell, 1941), 0.6% sodium acetate, and 1% glucose.
† Measured in an Evelyn photometer as percentage of light transmission; uninoculated medium = 100.
‡ Conditions for the conversion were those outlined in table 1.

*S. lactis* R grown in medium A was harvested after 10, 12, and 24 hours and allowed to act on the SLR factor under the conditions described above. A progressive increase in folic acid, ranging from 2,600 to 4,500 millimicrograms, was obtained with increase in age of the culture except that the 18- and 24-hour cells were about equally active; at 24 hours the culture had passed the logarithmic growth period. Cultures of from 16 to 18 hours were used routinely.

The concentration of SLR factor in the growth medium in the range of 6 to 40 millimicrograms per 100 ml of medium did not significantly affect the conversion
activity of the \textit{S. lactis} R cells, although more abundant growth was obtained with the larger amounts of the factor.

\textbf{CONDITIONS INFLUENCING THE CONVERSION PROCESS}

\textit{Time, pH, cell concentration, etc.} The equal growth rates of \textit{S. lactis} R in media containing folic acid or SLR factor had suggested previously that conversion of the latter to folic acid is very rapid (Stokes, Keresztesy, and Foster, 1944). This was confirmed with resting cell suspensions. Conversions carried out for 2, 4, 7, and 24 hours with \textit{S. lactis} R cells suspended in M/15 phosphate (pH 7.0) without glucose or agitation revealed that the maximum amount of folic acid was formed within 2 hours. Shorter periods were not tried.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Influence of pH on conversion of the SLR factor to "Folic Acid" by resting cell suspensions of \textit{Streptococcus lactis} R.}
\end{figure}

The effect of pH is indicated in figure 1. The conversion conditions were the same as those for the time experiment except that the cells were suspended in M/15 PO$_4$ at the pH's indicated and the incubation period was 3 hours. The optimum pH was 8. Higher pH's were not tested. Little folic acid was formed at pH 5.

The amount of folic acid produced is also related to cell concentration. Suspensions containing the equivalent of 1.6 mg (usual concentration), 3.2 mg, 4.8 mg, and 6.4 mg of dry cells per ml gave folic acid values of 4,700, 7,600, 9,500, and 12,800 millimicrograms, respectively. The same holds for concentration of the SLR factor; a progressive increase in folic acid, ranging from 30 to 610 millimicrograms, was obtained as the concentration of the factor was raised from 0.02 \(\mu\text{g}\) to 2.0 \(\mu\text{g}\) per ml of suspension.

Agitation of the conversion mixture with nitrogen during incubation resulted in somewhat greater folic acid formation than stationary conditions or stirring with air.
Carbohydrates. The addition of 8 mg of glucose to the conversion mixture prior to incubation produced a 4- to 20-fold increase in folic acid. Sodium pyruvate, sodium lactate, and ether extracts of liver had no effect. The last stimulates synthesis of folic acid by rat liver suspensions under certain conditions (Wright and Welch, 1943). A survey of 12 additional carbohydrates revealed that fructose and d-ribose also increased formation of folic acid (table 3). The explanation of why only those three sugars were stimulatory was obtained from an investigation of the constitutive and adaptive nature of the carbohydrate-

<table>
<thead>
<tr>
<th>CARBOHYDRATE</th>
<th>FOLIC ACID*</th>
<th>METHYLENE BLUE REDUCTION†</th>
<th>GROWTH‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>180</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>l-Arabinose</td>
<td>180</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>l-Xylose</td>
<td>210</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>d-Ribose</td>
<td>960</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucose</td>
<td>2,400</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Galactose</td>
<td>310</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>3,000</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>170</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>60</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>230</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>240</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dulcitol</td>
<td>160</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Salicin</td>
<td>80</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>130</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Conversion mixture contained 9 ml cells in M/15 phosphate buffer (pH 7), 2 μg SLR factor, and 0.9 ml of M/20 solution of carbohydrate. Incubated without aeration.
† Small test tubes received 1 ml each of methylene blue (1:4000 dilution) phosphate buffer (M/15, pH 7.0), and M/20 carbohydrate and 2 ml of S. lactis cells (equivalent to 3.2 mg of dry cells) suspended in M/15 phosphate at pH 7.0. The tubes were sealed with vaseline and incubated at 37 C for 3 hours; + indicates complete reduction of methylene blue; - indicates no visible reduction.
‡ Recorded after 3 days of incubation in medium A.

fermenting enzymes of S. lactis R. Although many of the nonstimulatory carbohydrates are available to S. lactis R for growth, only the stimulatory carbohydrates, glucose, fructose, and d-ribose were fermented by the resting cells, as indicated by methylene blue reduction tests (table 3). It will be recalled that medium A, from which the resting cells were obtained, contained glucose. Reduction of methylene blue in the presence of fructose or d-ribose indicated that their respective dehydrogenases were present in the S. lactis R cells grown with glucose. Therefore, those enzymes are constitutive. The inactivity of the other sugars, namely, l-xylose, galactose, sucrose, lactose, and maltose, and the glucoside, salicin, which are available for growth, is due to the absence of their

TABLE 3
Stimulatory effect of carbohydrates on folic acid formation: correlation with methylene blue reduction and availability for growth
respective enzymes in the cells grown with glucose, indicating that those enzymes are adaptive. Proof of this fact is that cells grown in medium A containing sucrose in place of glucose reduced methylene blue in the presence of sucrose and were stimulated to form folic acid by the addition of sucrose to the conversion mixture (table 4). Maltose failed to stimulate the sucrose-grown cells as predicted from the foregoing data. It appears, therefore, that any carbohydrate which is available for the growth of S. lactis R can increase folic acid formation on addition to the conversion mixture provided it can be fermented by the resting cells. In the case of a sugar which is fermented by an adaptive enzyme, the cells must be grown in the presence of that particular sugar prior to use in the conversion in order to obtain stimulation of folic acid formation.

The yields of folic acid were not significantly raised by increasing the amount of glucose in the conversion mixture from 8 mg to 32, 64, or 162 mg. Determinations of residual glucose indicated that the cells had fermented all of the 8 mg quantity, and 23, 24, and 17 mg respectively, of the larger amounts of glucose. The suspension with 8 mg of glucose had a final pH of 6.5, whereas with the larger amounts of glucose sufficient acid had been formed to lower the pH to about 5, which is considerably below the optimum for conversion. Use of M/7.5 phosphate buffer at pH 8, in place of the customary M/15 buffer at pH 7, maintained the conversion mixture with 32 mg of glucose at pH 7 to 8. However, no appreciable increase in folic acid was obtained except when more of the SLR factor was used. The carbohydrate appears to stimulate formation or activity of the enzyme(s) involved in the transformation of the SLR factor to folic acid, rather than to supply some compound necessary for the conversion, since water- or enzyme-treated extracts of S. lactis R cells incubated with glucose failed to stimulate folic acid formation when added to the conversion mixture in place of glucose.

**Extraction of folic acid.** After conversion practically all of the folic acid is within the bacterial cells. Difficulty was encountered in extracting the growth factor without extensive destruction. Eighty-seven per cent was destroyed by autoclaving the cells with water, although the remainder was obtained in the liquid phase (table 5). The autoclaving of the cells in the original phosphate buffer at pH 7 was only somewhat less harmful. However, no destruction of
folic acid occurred when the cells were autoclaved in the folic acid assay medium. Tests of the various components of the medium revealed that no one substance was so effective as the complete medium, but that a combination of the casein hydrolyzate, glucose, and salts A (phosphates), or casein hydrolyzate, salts A, and salts B, afforded full protection; the purines, pyrimidines, vitamins, and sodium acetate were not essential.

The folic acid in the cells could also be extracted with water without loss by autoclaving the cells in the presence of certain reducing agents. Thus 0.5 per cent sodium thioglycollate or 3.0 per cent ascorbic acid fully protected the growth factor; smaller quantities were proportionally less effective. However, cysteine, reduced iron, NaHSO₃, and Na₂SO₄ in 0.25 or 0.5 per cent concentration did not prevent loss of activity on autoclaving. It is possible that the protective

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>FOLIC ACID (millimicrograms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unextracted cells</td>
<td>11,600</td>
</tr>
<tr>
<td>Autoclaved in water*</td>
<td>1,400</td>
</tr>
<tr>
<td>Autoclaved in phosphate buffer†</td>
<td>3,300</td>
</tr>
<tr>
<td>Autoclaved in assay medium‡</td>
<td>12,200</td>
</tr>
<tr>
<td>Autoclaved in 0.5% sodium thioglycollate solution‡</td>
<td>11,900</td>
</tr>
<tr>
<td>Digested with clarase‡</td>
<td>10,400</td>
</tr>
</tbody>
</table>

* Thirty minutes at 15 pounds' pressure.
† Suspension was assayed. Additional experiments showed that after autoclaving all of the folic acid was in the liquid phase.
‡ Ten ml of the conversion mixture was adjusted to pH 4.6 and 20 mg of clarase added. After incubation for 16 hours at 37 C under benzene, the mixture was neutralized, steamed to remove the benzene, and adjusted to 15 ml with water prior to assay.

action of the assay medium, as noted above, may be due to its reducing properties.

Digestion of the cells with clarase also was effective in liberating most of the folic acid without reduction of activity.

Typical folic acid, i.e., the concentrate used as the standard, can be autoclaved in aqueous solution without loss of activity. The necessity of adding reducing substances to prevent, under the same conditions, destruction of folic acid formed from the SLR factor suggests that the latter differs from typical folic acid in that it is more susceptible to oxidation. It is possible that other substances in the streptococcus cells may be responsible for the greater lability of microbial folic acid. This appears unlikely, however, since the activity of the folic acid standard was not altered when autoclaved with S. lactis R cells which had been treated exactly as in a conversion experiment except that the SLR factor was omitted from the conversion mixture. It is noteworthy that the
residual folic acid present in the liquid phase, after autoclaving the cells in water, is resistant to further autoclaving.

Substitution of 1 μg or 10 μg of standard folic acid (Williams concentrate) for the SLR factor in the conversion, to determine whether it is transformed to the "labile" form, resulted in an unaccountable disappearance of 60 to 80 per cent of the added folic acid, i.e., it could not be recovered by assay. The loss was not due to the formation of "labile" folic acid since the same small amount was recovered irrespective of whether or not the cells were autoclaved in water prior to assay. Apparently most of the folic acid was metabolized to inactive material by the resting cells.

Twenty-five per cent of the conversion activity of the S. lactis R cells in M/15 phosphate buffer (pH 7) was destroyed at 50 C in 30 minutes. Complete inactivation occurred at 60 C in 30 minutes and at 100 C in 15 minutes. Also, the enzyme system involved in the transformation of the SLR factor to folic acid is of the constitutive type since conversion was obtained regardless of whether the cells were cultivated in media containing folic acid, thymine (Stokes, 1944), or the SLR factor. However, cells grown with the SLR factor formed the most folic acid.

SUMMARY

Resting cell suspensions of enterococci requiring the Streptococcus lactis R factor (SLR factor) for growth vary, quantitatively, in ability to convert the SLR factor to "folic acid." S. lactis R cells grown in media containing the SLR factor are more active than those grown with folic acid or thymine. Within the range of 0.02 μg to 2.0 μg per ml of suspension, the concentration of SLR factor in the growth medium does not affect the conversion significantly. Cells from cultures nearing the end of the log phase form more folic acid than younger cells. The transformation is rapid, maximum folic acid values being obtained within two hours, and is proportional to cell and SLR factor concentration. Fermentable carbohydrates added to the conversion mixture increase folic acid formation, 4- to 20-fold. Within the range studied, the optimum pH is 8, higher pH's not being tested. Activity is destroyed completely on heating the cells, suspended in phosphate buffer, at 60 C for 30 minutes.

After conversion practically all of the folic acid is within the bacterial cells. It can be extracted by autoclaving the cells in the folic acid assay medium or with certain combinations of the medium constituents. It can also be obtained free of the cells by digestion with clarase. Extraction by autoclaving with water results in the destruction of most of the folic acid. This destruction is completely prevented by sodium thioglycollate or ascorbic acid.

REFERENCES


Wieder, S. 1944 The present status of Lactobacillus casei factor. Lederle Laboratories, Inc., Pearl River, N. Y.