Dried yeast has found wide acceptance as a rich source of protein and vitamin B, and has been successfully incorporated into many foods. Up to 2.5% dried yeast is frequently used in cereals and canned vegetables for infants and children. Lesser amounts are added to breads, cereals, soups, and other enriched products for adult consumption for both flavoring and nutritional purposes.

The National Formulary (1) sets minimum specifications on both brewers’ dried yeast and primary dried yeast for protein at 45%, niacin at 300 μg per g, riboflavin at 40 μg per g, and thiamine at 120 μg per g. Nutritional significant quantities of pantothenic acid, pyridoxine, biotin, and folic acid may also be present in 10 g, a portion of reasonable size. The average total folic acid content of brewers’ dried yeast is reported to be about 20 μg per g (2). However, not all of the chemical forms of folic acid (folates) that comprise the total content (determined microbiologically) are equally potent for animals. A proper evaluation of the various derivatives of folic acid and their conjugated forms, each contributing some biological activity, and their added or cumulative potency for humans is of continuing importance.

No single chemical or microbiological method for the assay of total natural folates exists. Such a method, to be useful for evaluation of folic acid activity in human nutrition, should provide for the quantitative determination of each of the biologically active compounds. Chemical methods have not yet been applied. Microbiological methods, which are inherently dependent on the variable growth-promoting activity of the many derivatives for assay organisms, give results that are difficult to assess quantitatively.

Various substituted forms of naturally occurring folates have been characterized by applying special procedures during extraction, chromatography, and assay to protect the labile forms from oxidative and heat destruction. For example, appreciable quantities of the labile N⁵-formyltetrahydrofolate and N⁵-methyltetrahydrofolate have been isolated and identified in chicken liver extract (3). In earlier studies in which protective techniques were not used, it is possible that only the relatively stable N⁵-formyltetrahydrofolate conjugates had been properly identified (4). Naturally occurring folates from various other sources such as bacterial cells, human blood, horse liver, mouse liver, plant materials, yeast, and gas glands of Physalia physalis L. have been reported (4–10).

The majority of the naturally occurring folates, previously identified by procedures designed to provide complete protection from oxidative degradation, have been the tetrahydro forms. The oxidized forms (dihydrofolates and folates) have been referred to as artifacts resulting from nonenzymatic oxidation of the natural tetrahydro forms.

The purpose of the present investigation is to characterize the pattern of folate acid derivatives in primary dried yeast. A chromatographic procedure similar to that employed by Noronha and Silverman (3) was used to fractionate a yeast extract. The fractions were subsequently assayed by differential microbiological techniques before and after digestion with a γ-glutamyl conjugase preparation from chicken pancreas. Antioxidants were used throughout the procedure, and autoclaving temperatures above 100° were avoided to protect the labile yeast folates from oxidative degradation. A characterization of the folates in the separate fractions is presented, and their approximate degree of conjugation and type of substitution are given. A tabulation of total activities of all fractions is included.

**EXPERIMENTAL PROCEDURE**

The yeast sample (50 g) was extracted by stirring for 30 min at 70° in 1 liter of an aqueous 1% solution of sodium ascorbate (Merck), pH 6. After the addition of 25 g of Hydro Super-Cel (Fisher) with stirring, the suspension was filtered by suction through a Buchner funnel. The filter cake was washed with 100 ml of the ascorbate solution, and the filtrate and wash were combined.

A column was prepared from a 24-inch length of glass tubing (5-cm outside diameter) by sealing on an outlet and plugging with glass wool. Approximately 60 g of DEAE-cellulose (Esstman No. 7392) were suspended in distilled water and packed into the column by gravity to a height of approximately 19 cm (column will settle about 4 cm during elution), and covered with a 1-inch layer of glass wool. At no point in the procedure was the solution allowed to drain below the surface of the column. The total yeast extract, followed by 1 liter of 0.2% 2-mercaptoethanol, was passed through the column. An additional 500 ml of the latter solution were added to the tube to form a mixing chamber above the column. Gradient elution was achieved by allowing a solution of 0.2% 2-mercaptoethanol in 0.5 M phosphate buffer (pH 7) to drip from an overhead container into the mixing chamber at 5 ml per min while the eluate left the column at the same rate. Fifty 100-ml fractions were collected and frozen for storage.

Portions of such eluted fractions were assayed microbiologically, first, as originally eluted from the column, and second, after treatment of a portion of the original eluted fractions with chicken pancreas conjugase preparation. The assays were based on responses of Streptococcus faecalis, ATCC 8043, Lactobacillus

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1 Kindly supplied by Robert F. Light, Standard Brands Incorporated, as Fleischmann’s pure dry yeast (Saccharomyces cerevisiae), and described as a natural product, primary, grown in pure media.
casei, ATCC 7469, and Pediococcus cerevisiae, ATCC 8081, and data are reported in terms of an L-leucovorin standard.2

The conjugase was prepared by extracting 0.6 g of dehydrated chicken pancreas (Difco No. 0459-12) with water and diluting the extract to 50 ml. One milliliter of this preparation was added to a 10-ml aliquot of each fraction, and the reaction mixture was incubated overnight in a water bath at 34° in preparation for assay.

Aliquots from these enzyme-treated and untreated samples were taken for assay and diluted with 0.5% sodium ascorbate solution (pH 6) to volumes the “folate activity” of which approximated those of the standards, which were as follows: for L. casei and P. cerevisiae, 0.2 µg per ml and for S. faecalis, 0.5 µg per ml.

Stock cultures of all assay organisms were carried as stab cultures in test tubes containing 10 ml of agar culture medium. This medium for L. casei contains 5 g of sodium acetate trihydrate and 13.7 g of Difco yeast dextrose agar No. 0029-02 per 500 ml of aqueous solution as recommended by Flynn.3 For carrying S. faecalis and P. cerevisiae, the present A.O.A.C. universal agar culture medium was used (11).

The inocula for all assays were grown in 10 ml of the appropriate liquid culture medium by transferring them from agar stock cultures and incubating them overnight at 34°. The final assay tubes were inoculated dropwise directly from these cultures, which consisted of suspensions of the organism. The liquid culture medium for L. casei contained 0.2 µg of folic acid and 5 mg of solubilized liver extract (Eli Lilly and Company, 1 g = approximately 20 g of fresh liver) in 500 ml of half-strength L. casei basal medium.3 The S. faecalis and P. cerevisiae inocula were grown in the present A.O.A.C. universal liquid culture medium (11). Washing of the organisms in a special suspension medium was usually unnecessary.

The basal medium used for L. casei and P. cerevisiae assays in the present study was that recommended for the 1962 collaborative study.4 This is, with minor modifications, the formula used in the 1948-1949 A.O.A.C. collaborative studies on folic acid and is also similar to the formula used by Toepfer et al. (2) for L. casei. This medium, as used here for L. casei or P. cerevisiae, includes the ingredients for the present A.O.A.C. medium for folic acid (11), with the following exceptions for each 500 ml. In place of the acid-hydrolyzed casein solution, 5 g of powdered acid-hydrolyzed casein (Difco vitamin-free Cassimino acids No. 0288-02) were used; 5 µg of cyanocobalamin were added; 100 µl of vitamin Solution III (double strength in all vitamins) were used; 10 ml of salt Solution A were added in addition to salt Solution B; 20 g of anhydrous sodium acetate were added; and the anhydrous KH₂PO₄ and sodium citrate dihydrate were omitted.

The basal medium used for S. faecalis was a dehydrated product (Difco folic acid assay medium No. 0318-15). Another formula (Difco folic A.O.A.C. medium No. 0967-15) was also found to be satisfactory but was not used here.

The assay procedure followed conventional Association of Official Agricultural Chemists methods regarding tube arrangement, addition of samples, and basal medium. Assay tubes containing basal media and samples were sterilized in flowing steam at 100° for 20 min prior to inoculation. After incubation in a water bath for 16 to 24 hours at 34°, the tube contents were transferred to matched test tubes (18 × 150 mm) and the growth was measured by turbidimetric readings at 550 nm.

The original “folate standard” contained 3 mg of leucovorin2 per ml. This synthetic product is a mixture of d and l diastereoisomers, of which only the l form occurs naturally and is biologically active, having approximately twice the microbiological activity of the synthetic dl products (12). Hence, the stock leucovorin solution contained 1.5 mg of active standard per ml.

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1 Sold by American Cyanamid Company as leucovorin, the synthetic calcium salt of N5-formyl-dL-5,6,7,8-tetrahydropteryl-1-glutamic acid.
2 Dr. Laura M. Flynn, Department of Agricultural Chemistry, University of Missouri, Columbia, Missouri, Associate Referee on Bound Folic Acid, for the Association of Official Agricultural Chemists (A.O.A.C.), 1962.
Folic Acid Derivatives

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AFTER CHICK PANCREATIC CONJUGASE DIGESTION

Fig. 2. Same as Fig. 1 except that folate activities refer to growth responses observed after treatment of the eluted fractions with a chicken pancreatic conjugase.

TABLE I
Activity of folic acid analogues for various organisms

<table>
<thead>
<tr>
<th>Glutamic acids</th>
<th>Analogue</th>
<th>Organism*</th>
<th>Chick</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SF</td>
<td>LC</td>
</tr>
<tr>
<td>0</td>
<td>Pteric acid</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>1</td>
<td>Pteroyl-L-glutamic acid (folic acid)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>Tetrahydrofolic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>N\textsuperscript{2}-Methyltetrahydrofolic acid</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>N\textsuperscript{8}-Formyl and N\textsuperscript{8}-formyltetrahydrofolic acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Pteroyl diglutamates</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Pteroyltetraglutamates</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>7</td>
<td>Pteroylheptaglutamates</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* SF, S. faecalis; LC, L. casei; PC, P. cerevisiae. Symbols are as follows: +, active; ±, partially active; −, inactive. Data were obtained from the literature (13-19).

A freshly prepared solution of 0.5% sodium ascorbate, pH 6, was used as the diluent for standards and samples. Stock solutions of the standard were frozen during storage, as were the chromatographic fractions of the yeast extract.

RESULTS AND CONCLUSIONS

Two chromatograms represent folate activity in successive chromatographic fractions as determined with the three test organisms both before and after treatment of each fraction with chick pancreatic conjugase (Figs. 1 and 2). Based on these data and the biological activity of known folate derivatives (Table I), a classification has been made of the probable degree of conjugation and type of folic acid derivative represented by each chromatographic peak (Table II). Total activities recovered in each chromatogram are given in Table III.

It should be noted that, with untreated eluates (Fig. 1), the only significant peak of activity for each organism occurred between tubes 5 and 15. Tubes 25 through 50 produced no response greater than 0.1 µg per tube. After treatment with the conjugase (Fig. 2), the total activities in tubes 1 through 17 (Peaks I, II, and III, Table II) represented not more than 3% of the total folates.

Since the exact degree of conjugation of the yeast folates is unknown, the higher forms (present in tubes 15 to 50) are referred to as polyglutamates. It must be kept in mind that the polyglutamates in these fractions were treated with chicken pancreatic enzyme, which supposedly degraded them to diglutamates (21). Fractions comprising Peaks IV (tubes 18 to 23) and V (tubes 24 to 27), because of their elution order (6) and

TABLE II
Summary of probable predominating folate conjugates occurring in separate chromatographic peaks

<table>
<thead>
<tr>
<th>Chromatographic peak</th>
<th>Conjugated form</th>
<th>Pteroylglutamic acid (folic acid) analogue</th>
<th>Degree of reduction (positions 5-8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Diglutamate</td>
<td>N\textsuperscript{2}-Formylfolate</td>
<td>H\textsubscript{2} and/or H\textsubscript{4} and</td>
</tr>
<tr>
<td>II</td>
<td>Triglutamate</td>
<td>N\textsuperscript{2}-Formylfolate</td>
<td>H\textsubscript{2} and/or H\textsubscript{4} and</td>
</tr>
<tr>
<td>III</td>
<td>Polyglutamate†</td>
<td>Folic acid</td>
<td>H\textsubscript{2} and/or H\textsubscript{4} and</td>
</tr>
<tr>
<td>IV</td>
<td>Polyglutamate†</td>
<td>N\textsuperscript{2}-Formylfolate</td>
<td>H\textsubscript{2} V</td>
</tr>
<tr>
<td>V</td>
<td>Polyglutamate†</td>
<td>N\textsuperscript{2}-Formylfolate</td>
<td>H\textsubscript{2} V</td>
</tr>
<tr>
<td>VI</td>
<td>Polyglutamate†</td>
<td>N\textsuperscript{2}-Formylfolate</td>
<td>H\textsubscript{2} V</td>
</tr>
<tr>
<td>VII</td>
<td>Polyglutamate†</td>
<td>N\textsuperscript{2}-Methylfolate</td>
<td>H\textsubscript{2} and/or H\textsubscript{4} and</td>
</tr>
</tbody>
</table>

* H\textsubscript{2}, H\textsubscript{4}, and H\textsubscript{6} refer to the oxidized compounds folate and dihydrofolate, and to tetrahydrofolate, respectively.
† Only trace amounts of completely reduced forms present.
‡ The conjugated forms predominating in Peaks IV through VII are presumed to be heptaglutamates (20).

TABLE III
Total folate activity of all conjugase-treated and untreated chromatographic fractions

The values listed are derived by comparing growth-promoting activity of the yeast folates with that of graded amounts of leucovorin, and are expressed as micrograms of l-leucovorin per g of dried yeast.

<table>
<thead>
<tr>
<th>Assay organism</th>
<th>Untreated</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. casei*</td>
<td>0.75</td>
<td>30.0</td>
</tr>
<tr>
<td>S. faecalis</td>
<td>0.20</td>
<td>13.7</td>
</tr>
<tr>
<td>P. cerevisiae</td>
<td>0.005</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* The activity for L. casei is the most representative of total folates (see Table I).
their activity for \textit{L. casei} and \textit{S. faecalis}, appear to contain \textit{N}^{10}\text{-formyl}dihydrofolate and \textit{N}^{10}\text{-formyl}folate, respectively (approximately 63% of the total folate activity). The possibility that these two peaks may divide different conjugated forms is not eliminated. More substantial evidence is being sought. Fractions in Peak VI (tubes 28 to 34) contain a completely reduced polyglutamate, most likely the relatively stable \textit{N}^{2}\text{-formyltetrahydrofolate} (approximately 14% of the total activity), as evidenced by the strong \textit{P. corvisiae} response and order of elution. Peak VII (tubes 35 to 42) shows fractions giving exclusively an \textit{L. casei} response characteristic of the \textit{N}^{5}\text{-methyl}tetrahydrofolate (prefolio A) (6) and comprising about 20% of the total folates present. In calculating the percentage of the total folate activity contributed by each peak, only the activity for \textit{L. casei} was considered.

**DISCUSSION**

It is not known if pteroylglutamic acid alone will adequately replace other folic acid-active forms in human nutrition; nor is it known whether man can subsist entirely on methylated or formylated folates as the only dietary form of folic acid. Further observations on the comparative effectiveness of the several folic acid derivatives are necessary.

Herbert (22) has suggested that \textit{L. casei} folate activity found in foods by assay without conjugase treatment is a much closer approximation of the food folate available to man than is \textit{L. casei} activity found by assay following conjugase treatment.

It has been suggested that human may have a specific requirement for pteroylglutamic acid (23), and Sheehy et al. (24) demonstrated the effectiveness of small oral doses of synthetic folic acid in the treatment of tropical sprue. The addition of 25 \mu g of synthetic pteroylglutamic acid to the daily oral intake of certain patients resulted in the correction of the clinical manifestations of the disease. This amount is much less than is measured microbiologically in the diet (25), which alone was not effective. The need for further assessment of the amounts of pteroylglutamic acid available in substituted and unsubstituted forms in foods is indicated by this report.

In the present work, both the qualitative and quantitative results depend on comparison with the biological activity of synthetic leucovorin, a monoglutamate. Variation in microbial responses to the various folate forms may occur, depending in some cases on the concentration and type of the derivative and on the degree of conjugation (7). For example, the formyl derivatives with 2 glutamic acid residues are approximately 50% as active as the corresponding monoglutamates for \textit{S. faecalis} and \textit{P. corvisiae}, while all diglutamyl derivatives are as active as the respective monoglutamates for \textit{L. casei} (3). The methods of choice for the measurement of total folic acid appear to be those which employ \textit{L. casei} as the test organism (26). Concurrent assays with all three organisms, however, supply qualitative information based on known differential responses.

A conjugase preparation from the gas gland of \textit{Physalia physalis} \textit{L.} has been utilized to degrade chicken liver folates completely to monoglutaryl derivatives (3, 7). More uniform analytical results may thus be expected since the reference standard is also a monoglutamate. We were unsuccessful in obtaining such a preparation for use in our investigation owing to the difficulties involved in collecting the glands. The end products formed by digestion with hog kidney conjugase are also reported to be monoglutamates, but this enzyme is reported to be affected by inhibitors, and assays in which it was used have generally given lower results than comparative assays with chicken pancreas conjugase, the end product of which is considered to be the diglutamate (4). However, it was found that the action of the chicken pancreas and hog kidney conjugases on the same substrate produces materials that give identical chromatographic patterns of folate activity (8). The chicken pancreas conjugase preparation appears to be the enzyme of choice at the present time since it has been shown to release the greatest amount of folate activity and its use presents the least problems (26).

In view of the results of the present investigation, any nutritional evaluation of yeast folates must include the effects of the relatively high percentages of polyglutamates occurring as formyl and methyl derivatives, with some of the more labile forms possibly in various stages of oxidation. Workers previously mentioned found that various other foodstuffs contain appreciable amounts of these same derivatives; however, the revelation, in the present report, of significant amounts of methylfolates in yeast gives increased significance to the involvement of this derivative in the nutritional evaluation of yeast. This evaluation, at present, is difficult, because of the lack of knowledge concerning the relative biological potencies of the various folic acid analogues. Former studies have presented evidence indicating that the main members of the folic acid group found in green leaves, bakers' yeast, and beef liver are \textit{N}^{4}\text{-formyltetrahydrofolate}, \textit{N}^{10}\text{-formylfolate}, and another unidentified factor (10). In studies on human blood a similar folate pattern was found (9). Furthermore, it was concluded from studies on ordinary American diets that the major folate derivative present was the \textit{N}^{10}\text{-formyl}folate (8). In earlier reports, pteroylhexaglutamylglutamic acid (20) and \textit{N}^{4}\text{-formyltetrahydrofolate} in conjugated form have been reported as present in yeast (27). The isolation from yeast of \textit{p}-aminobenzoypolyglutamic acid containing 10 to 11 glutamic acid residues suggests the occurrence of even higher conjugates (28). In chicken liver, algae, yeast, bacterial cells, and human blood, the folates appear mainly as polyglutamates (4). In contrast to this, in human livers (29), in blood serum (30), and in leukemic cells of the mouse and mouse liver (6) they occur as monogluitamates.

**SUMMARY**

An aqueous extract of primary dried yeast has been chromatographed on a diethylaminoethyl cellulose column. Seven fractions with folate activity for at least one of three organisms, \textit{Streptococcus faecalis}, \textit{Lactobacillus casei}, or \textit{Pediococcus cervisiae}, were obtained. Treatment of successive eluates with chicken pancreas conjugase was necessary before the microbiological activity of the latter five fractions could be observed. About 97% of the folate activity appears to be due to folic acid derivatives containing more than 2 glutamic acid residues. The cumulative total folate activity (per g of yeast) of conjugase-treated fractions for \textit{L. casei} was equivalent to that of 30 \mu g of the microbiologically active form of leucovorin.

The major compounds in yeast appear to be conjugates of the \textit{N}^{10}\text{-formyl} derivative of either folic acid, dihydrofolate, or both; the \textit{N}^{4}\text{-formyl} derivative of tetrahydrofolate; and the \textit{N}^{4}\text{-methyl} derivative of dihydro- or tetrahydrofolate or both. The elution pattern of the chromatograms suggests that these forms represent about 63, 14, and 20% of the total microbiological activity, respectively. Minor peaks representing about 3% of the total activity indicate the presence of di- and triglutamates of oxidized and reduced formylated forms and possibly of a polyglutamate of pteroylglutamic acid.
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