The Identification of Hexa-, Hepta- and Octoglutamates as the Polyglutamyl Forms of Folate Found throughout the Growth Cycle of Yeast

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INTRODUCTION

It appears that in all cells the various intracellular folate cofactors are attached to a number of glutamyl residues (Houlihan & Scott, 1972; Brown, Davidson & Scott, 1973; Brown *et al.*, 1974) and that many cells contain more than a single polyglutamyl chain length. In fact, of the intracellular folates examined to date only yeast (Pfaffner *et al.*, 1946), Clostridia (Curthoys, Scott & Rabinowitz, 1972) and T-even phages (Kozloff & Lute, 1973) have single polyglutamyl forms and in the T-even phages the polyglutamates have a structural rather than a coenzyme role.

To ascertain if yeast synthesizes only the heptaglutamate for the entire growth cycle of the cell, we have re-examined folate polyglutamyl biosynthesis in yeast, using a technique which separates the various polyglutamyl derivatives on previously calibrated ion-exchange columns, after the folates have been cleaved chemically to their corresponding *p*-aminobenzoylpolyglutamyl (*p*-ABGlu) derivatives (Houlihan & Scott, 1972). We found three distinct polyglutamates (the hexa-, hepta- and octo-) throughout the growth cycle; previous reports that only the heptaglutamate is found in yeast appear to be incorrect.

METHODS

Chemicals. Diethylaminoethyl cellulose (Whatman DE52) was purchased from Ward R. Balston Ltd, Maidstone, Kent, Triton X-100 from Koch Light, and the scintillation chemicals 2,5-diphenyloxazole (PPO) and 2,3'-phenylen-bis-(4-methyl-phenyloxazol) (POPOP) from Intertechnique Ltd, Sussex.

Radiochemicals. *p*-Amino-[¹⁴COOH]benzoate (52 mCi mmol⁻¹) was supplied by Schwartz Mann, Orangeburg, New York, U.S.A. 3'5'9-(n)-[³H]pteroylglutamate (15 Ci mmol⁻¹) and pteroylheptaglutamate (PteGlu₇) in which the terminal glutamic acid was labelled U-¹⁴C (1.2 mCi mmol⁻¹) were supplied by the Radiochemical Centre, Amersham, Buckinghamshire.

Radiochemical methods. Radioactivities were determined using a Packard Tri-Carb liquid scintillation spectrometer model 3375. Toluene + Triton X-100 (2:1 w/w) was used with a ratio of 1 part sample to 10 parts scintillation fluid (Turner, 1969).

Organisms. Four *p*-aminobenzoate (*p*-AB)-requiring strains of *Saccharomyces cerevisiae* were supplied from the culture collection of Arthur Guinness and Son, Dublin, and were designated by them as 1167, 1185, 1288 and 1289.

Media. The yeasts were maintained as slopes of sabouraud dextrose agar supplied by Oxoid.

Yeast extract was from Difco. All other constituents were Analar-grade reagents supplied by BDH. Three stock salt solutions, A, B and C, were used. Solution A contained (gl⁻¹): KH₂PO₄, 4.0; K₂HPO₄, 0.5; (NH₄)₂SO₄, 11.5; NaCl, 0.1; MgSO₄.7H₂O, 0.5; CaCl₂.2H₂O,

0.1; ferric citrate, 0.67. Solution B contained (g l^{-1}): $\text{CaSO}_4 \cdot 5\text{H}_2\text{O}$, 4×10^{-4} ; KI, 1.0×10^{-5} ; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 2.0×10^{-5} . Solution C contained (g l^{-1}): calcium pantothenate, 1.0×10^{-5} ; thiamine, 1.0×10^{-5} ; inositol, 2.0×10^{-5} ; pyridoxal-HCl, 1.0×10^{-5} ; nicotinic acid, 5.0×10^{-4} . The final medium consisted of 1 l solution A plus 5 ml solution B and 1 ml solution C; to this was added 1.98 g glucose and 0.25 g yeast extract.

Culture conditions. Inocula were prepared by transfer of a loop of yeast cells from an agar slope to a test tube containing 5 ml of the above medium. After growth at 28°C for 24 h this 5 ml culture was used to inoculate 200 ml medium in a 500 ml Erlenmeyer flask. For the extraction experiments the cells were grown with 25 nCi of $[^{14}\text{C}]p\text{-AB/ml}$, giving a concentration of exogenous $p\text{-AB}$ of 65.9 ng ml^{-1} . Similar concentrations were used in the distribution experiments where $[^{14}\text{C}]p\text{-AB}$ was used for incorporation studies. The cells were grown in shaken culture at 28°C and, unless otherwise stated, for 48 h. After the appropriate time, 10 ml of culture was removed. The yeast was harvested by centrifugation, washed once with 20 ml of distilled water and extracted (see Results). The extract was diluted to 100 ml with distilled water and applied to the columns.

Oxidation of the extracted folates. The extracted folates were oxidized to the corresponding aminobenzoyl- γ -L-glutamates by the addition of 4 ml of 2.0% (w/v) $\text{KMnO}_4/10 \text{ ml}$ extract (Houlihan & Scott, 1972). The mixture was adjusted to pH 8.5 with 1.0 M-tris base. The resulting suspension was kept at room temperature with occasional shaking for 25 min, after which the pH was adjusted to 4.0 with 1 M-HCl. The precipitate was removed by filtration through glass wool and the pH of the filtrate re-adjusted to pH 7.0 with 1.0 M-tris base. The filtrate was diluted to 100 ml with distilled water which always gave a conductivity of less than $3 \text{ m}\Omega^{-1} \text{ cm}^{-1}$.

Chromatography. The oxidized extract was chromatographed on $20 \times 0.7 \text{ cm}$ glass columns containing DEAE-cellulose (Whatman DE52) which had previously been equilibrated with 5 mM-tris-HCl buffer pH 7.0. To each column, 100 ml extract was applied and elution was effected with the non-linear gradient described in Fig. 1. The flow was 10 to 15 ml h^{-1} at 20°C and 6.0 ml fractions were collected. Columns were previously calibrated with p -aminobenzoylpolylglutamates ($p\text{-ABGlu}_{2-7}$) prepared in this laboratory (Houlihan & Scott, 1972).

RESULTS

All four strains of *S. cerevisiae* grew vigorously on a completely defined medium only when supplemented with 0.025% yeast extract. Since no increase in growth was observed upon the addition of $p\text{-AB}$ it can be assumed that the yeast extract has sufficient $p\text{-AB}$ to support optimum growth under the conditions used. However, from the radioactive uptake studies reported in this paper the amount of endogenous $p\text{-AB}$ or other growth factors available in the yeast extract was clearly so small as not to stop accumulation by the organism of the small quantity of radioactive $p\text{-AB}$ added. All four strains, growing in the exponential phase when transferred to fresh medium containing 65.9 ng ml^{-1} of radioactive $[^{14}\text{C}]p\text{-AB}$, removed more than 80% of the label from the medium during 18 h growth. Several methods of extraction of the incorporated folate were examined and it was found that the following procedure usually gave almost total recovery of the incorporated label (70 to 100%). Wet cells (0.25 g) were suspended evenly in 25 ml distilled water and lyophilized overnight. The dry cells were suspended in 10 ml toluene and kept at 40°C for 12 h. To this suspension 100 ml of a 1% (w/v) potassium ascorbate solution pH 7.0 was added. After shaking and standing, the aqueous layer was centrifuged and the supernatant containing the extracted folates was removed. After extraction, the incorporated label must

Fig. 1. DEAE-cellulose chromatography of *p*-amino-[^{14}C]benzoylpoly- γ -L-glutamate derivatives obtained by alkaline KMnO_4 oxidation of native folates from a *p*-AB-requiring strain (1288) of *S. cerevisiae* grown on *p*-amino-[$^{14}\text{COOH}$]benzoate ($\circ$). Standards, either *p*-ABGlu $_7$ ($\bullet$) or a mixture of *p*-ABGlu $_5$ and *p*-ABGlu $_6$ ($\triangle$), were used to calibrate similar columns. Numerals are used to indicate the elution position of these and other standards (i.e. *p*-ABGlu $_x$, where $x = 0, 1 \dots 7$). The non-linear gradient shown was constructed by introducing 235 ml of 1.0 M-KCl in 5 mM-tris buffer pH 7.0 from a cylindrical flask (14×4.5 cm) into a spherical mixing chamber containing 550 ml of 5 mM-tris buffer pH 7.0.

have been in an intact folate polyglutamate, because all of the radioactivity eluted late on Whatman DE52 and, after conjugase treatment, only this area was active for microbiological assay with *Lactobacillus casei*. Permanganate oxidation resulted in earlier elution from the column, primarily as a *p*-aminobenzoylheptaglutamate with one larger and one smaller derivative also present (Fig. 1). The identity of the main derivative was established by re-chromatographing it with authentic [^{14}C]*p*-ABGlu $_7$ chemically prepared from [^{14}C]PteGlu $_7$ by permanganate oxidation. The peak eluting before this was estimated to be *p*-ABGlu $_6$ from the known elution position of non-radioactive standards on this chromatographic system (Brown *et al.*, 1974). The peak eluting immediately after the main heptaglutamate peak was assumed to be the octaglutamate, although no standards were available to determine exactly where this derivative would elute.

This pattern, where folate heptaglutamate constituted 67 to 71 % of the folate present with hexa- (12 to 16 %) and octo- (10 to 13 %) folate polyglutamates representing the remainder, was consistently found throughout the growth cycle of the organism, in samples taken on six occasions from 15½ to 48 h. No other folate polyglutamate was measureable in these experiments or in yeast cells that had entered the stationary phase. This pattern was consistent upon repetition on numerous occasions. Since the presence of a radioactive marker (Fig. 1) makes accurate estimations of the peaks difficult these percentages were calculated for columns calibrated with non-radioactive markers.

DISCUSSION

The strains of yeast used incorporated *p*-AB very well and formed it into long-chain folate polyglutamates of 6, 7 and 8 glutamyl residues (Fig. 1). Small amounts, up to 10 %, of radioactive *p*-AB were sometimes found after extraction. This could be because of a small pool of this precursor existing within the cell, or incomplete removal of the original medium during the washing procedure before extraction. The folate biosynthetic process thus rapidly elongates any newly-incorporated *p*-AB to a long-chain polyglutamate with no intermediate shorter-chain compounds accumulating.

Little definitive information is available on the control of folate polyglutamate biosynthesis or the role of these additional glutamyl residues. On the basis of permeability studies in bacteria (McElwee & Scott, 1973) and human marrow cells (Hoffbrand *et al.*, 1973), it might be suggested that these biosynthetic processes are involved in storage of the vitamin simply by making its exit from the cell impossible. However, the complexity of the forms involved argues against such a role.

Folate or *p*-aminobenzoyl polyglutamates are difficult to synthesize chemically (Krumdieck & Baugh, 1969). Their general lack of availability as markers for the co-chromatography used in the identification of folate polyglutamates from different sources, has limited research in this area to a small number of laboratories. Using the *p*-AB-requiring mutants described in this study, markers ideal for such chromatographic separation are easily available at high specific activity in high yield. The fact that three polyglutamates are isolated is an advantage since this serves to monitor the separation achieved.

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REFERENCES

- BROWN, J. P., DAVIDSON, G. E. & SCOTT, J. M. (1973). The identification of the forms of folate found in the liver, kidney and intestine of the monkey and their biosynthesis from exogenous pteroylglutamate (folic acid). *Biochimica et biophysica acta* **343**, 78–88.
- BROWN, J. P., DOBBS, D., DAVIDSON, G. E. & SCOTT, J. M. (1974). Microbial synthesis of folate polyglutamates from labelled precursors. *Journal of General Microbiology* **84**, 167–172.
- CURTHOYS, N. P., SCOTT, J. M. & RABINOWITZ, J. C. (1972). Folate coenzymes of *Clostridium acidu-urici*. The isolation of (*l*)-5,10-methenyltetrahydropteroyltrimethylglutamate, its conversion to (*l*)-tetrahydropteroyltrimethylglutamate and (*l*)-10-[¹⁴C] formyltetrahydropteroyltrimethylglutamate and the synthesis of (*l*)-10-formyl-[6,7-³H₂]tetrahydropteroyltrimethylglutamate and (*l*)-6,7-[³H₂]tetrahydropteroyltrimethylglutamate. *Journal of Biological Chemistry* **247**, 1959–1964.
- HOFFBRAND, A. V., TRIPP, E., HOULIHAN, C. M. & SCOTT, J. M. (1973). Studies on the uptake of synthetic conjugated folates by human bone marrow cells. *Blood* **42**, 141–146.
- HOULIHAN, C. M. & SCOTT, J. M. (1972). The identification of pteroylpentaglutamate as the major folate derivative in rat liver and the demonstration of its biosynthesis from exogenous [³H]pteroylglutamate. *Biochemical and Biophysical Research Communications* **48**, 1675–1681.
- KOZLOFF, L. M. & LUTE, M. (1973). Bacteriophage tail components. IV. Pteroyl polyglutamate synthesis in T4D-infected *Escherichia coli* B. *Journal of Virology* **11**, 630–636.
- KRUMDIECK, C. L. & BAUGH, C. M. (1969). The solid-phase synthesis of polyglutamates of folic acid. *Biochemistry* **8**, 1568–1572.
- MCELWEE, P. G. & SCOTT, J. M. (1972). Folate metabolism in *Streptococcus faecalis*. *Biochemical Journal* **127**, 901–905.
- PEIFFNER, J. J., CALKINS, D. G., BLOOM, E. S. & O'DELL, B. L. (1946). On the peptide nature of vitamin B₉ conjugate from yeast. *Journal of the American Chemical Society* **68**, 1392.
- TURNER, J. C. (1969). Tritium counting with Triton X-100 scintillant. *International Journal of Applied Radiation and Isotopes* **20**, 499–505.