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Analytical Methods Section

Investigation of the Conjugase Treatment Procedure in the Microbiological Assay of Folate

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Microbiological assays have been conducted to determine the growth responses of *Lactobacillus casei* to pteroyl di-, tri-, tetra-, hexa- and hepta- glutamates relative to the monoglutamate (folic acid). Deconjugation using human blood plasma conjugase at acidic pH (4·5) results in a substantial degradation of folates while between pH 6·0 and 7·0, optimum results could be obtained using a slight excess of plasma. The end products of conjugase treatment with either plasma or chicken pancreas extract as the source of conjugase depend on, besides the conjugase source, the incubation pH and the inhibitory components present in the incubation mixture.

INTRODUCTION

Microbiological assay is by far the most commonly used method for the quantification of folate in foods. The natural folates exist as a mixture of monoglutamates and polyglutamates of pteroic acid and its five derivatives in the reduced form. The organism used for the assay, *Lactobacillus casei*, does not respond equally to all these forms of folate. Tamura *et al.* (1972) have reported that *L. casei* responds equally to folic acid (pteroyl monoglutamate), di- and triglutamates, and much more slowly to the higher glutamates. The higher glutamates are hence converted to mono- or diglutamates using an enzyme, γ-carboxy peptidase (EC 3.4.22.12) (also referred to as conjugase), prior to the actual microbiological assay. Chicken pancreas and hog kidney are the most commonly used sources of the conjugase while human and rat plasma, rat pancreas and rat liver have been used to a lesser extent (Keagy, 1985). The end product with the chicken pancreas conjugase is reported to be a diglutamate, while plasma, liver and kidney conjugases result in a monoglutamate end product (Lakshmaiah & Ramasastri, 1975a; Silink *et al.*, 1975; Leichter *et al.*, 1977; Wilson & Horne, 1984; Day & Gregory, 1985). Because of inconsistencies in the results obtained from hog kidney conjugase treatment and chicken pancreas conjugase treatment. several authors have studied these two procedures in detail in order to optimize the conditions to yield consistent results (Phillips & Wright, 1983; Kirsch & Chen, 1984; Pedersen, 1988; Engelhardt & Gregory, 1990). Lakshmaiah and Ramasastri (1975*b*) have however reported comparable results for a few food items using conjugases from human blood plasma, chicken pancreas and hog kidney. Relatively very few studies have been reported on the plasma conjugase (Lakshmaiah & Ramasastri, 1975*a, b, c,* 1980). A detailed study of the plasma conjugase treatment procedure is needed at this time to evaluate its merits and demerits in the folate assay.

Many investigators have reported unequal *L. casei* growth responses among some of the monoglutamate derivatives present in foods and biological materials (Ruddick *et al.*, 1978; Phillips & Wright, 1982; Reingold & Picciano, 1982). Recently, the authors have studied the relative responses of all these monoglutamate derivatives by a new method of growth determination using a CO_2 analyzer. Significant differences were observed among the different forms, i.e. with respect to folic acid, as low as 45% response was observed for the dihydrofolic acid while values in the 80–115% range were obtained for the tetrahydro derivatives (Goli & Vanderslice, 1989). As an extension of this work, the authors have investigated the conjugase treatment procedure, with synthetic pteroyl polygluta-

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mates, for its efficacy in the assay procedure. Plasma conjugase treatment is studied in greater detail while chicken pancreas conjugase treatment is also briefly studied. The authors' interest in the plasma conjugase is two-fold. First of all, human blood plasma is easily available and a very small quantity (0·2 ml) is required for the assay. Because of the very low folate content of plasma (Lakshmaiah & Ramasastri, 1975*a*), it can be used without any purification. Secondly, since the end product of plasma conjugase treatment is reported to be a monoglutamate (Lakshmaiah & Ramasastri. 1975*a*), plasma could be used for deconjugation for the HPLC analysis for total folates.

EXPERIMENTAL

Materials

Folic acid (pteroyl glutamic acid) was purchased from Sigma Chemical Co. (St Louis, MO, USA). Pteroyl di-, tri-, tetra-, penta- and hexaglutamates were purchased from Schirck's Laboratories (Jona, Switzerland). Pteroyl heptaglutamate was prepared by the method of Krumdieck and Baugh (1980) and was purified by chromatography on a DEAE cellulose column with a gradient of ammonium acetate. The lyophilized sample of the hepta-glutamate contained approximately 10% of the hexaglutamate (by HPLC). Pteroyl mono- through hexaglutamates were found to be of high purity by HPLC. All the samples were stored at 0°C in the freezer. Folic acid casei medium, Bactolactobacilli agar and desiccated chicken pancreas were obtained from Difco Labs (Detroit, MI). Fresh human blood plasma was obtained from the Red Cross in a 250 ml bag. The contents of the bag were stored at –30°C in aliquots of 25 ml in sealed vials. The microorganism, *Lactobacillus casei* (*L. casei*, ATCC 7469), was obtained from ATCC (Rockville, MD).

Microbiological assays

All the microbiological assays were carried out in sealed vials under anaerobic conditions at a pH of 6·1 and analyzed on a Bactec CO_2 analyzer. Details of the procedure are presented in an earlier report (Goli & Vanderslice, 1989). Stock solutions of approximately 42 μM concentration of pteroyl mono through heptaglutamates (equivalent to 20 mg liter^{-1} folic acid) were prepared in 0·05M tris buffer (pH 7·5) containing 0·05M 2-mercaptoethanol. These solutions were stored at –30°C for further use.

For the study of relative growth responses of *L. casei* to pteroyl mono-, di-. tri-, tetra-, hexa- and heptaglutamates, assays were conducted without the conjugase treatment. Stock solutions were diluted in 0·05M phosphate buffer (pH 6·1) containing 1·5 g liter^{-1} ascorbic acid to give 2·1 nM solutions. *L. casei* assays were conducted in the 0–0·21 nM concentration range of these folates, which is the usual range for the standard curve of folic acid (0–0·1 ng ml^{-1}).

Preliminary experiments on the conjugase treatments were carried out with both pteroyl hexa- and heptaglutamates. Results were identical and the further work was done mostly with the hexaglutamate. Along with each conjugase treatment (plasma or chicken pancreas) of the hexaglutamate, an equimolar solution of folic acid was treated identically in order to check for degradation, if any, of the folates under the conditions of conjugase treatment. Plasma conjugase treatment at a pH of 4·5 was carried out by the procedure of Lakshmaiah and Ramasastri (1975*b*) as described by Keagy (1985). The folate solutions for conjugase treatment (2·1 μM) were prepared by dilution of the stock solutions in 0·10 M phosphate buffer (pH 4·5) containing 10 g liter^{-1} ascorbic acid. An aliquot of diluted folate solution (5·0 ml) was incubated for 3 h or 20 h with 100 mM 2-mercaptoethanol (0·6 ml), 0·2M sodium acetate buffer (pH 4·5) (1·0 ml), plasma (0·2 ml) and 2–3 drops of toluene. For plasma conjugase treatments at pH 6·0, 6·5 and 7·2, 0·10 M phosphate buffers of respective pH containing 10 g liter^{-1} ascorbic acid were used for the dilution of stock solutions. A plasma aliquot (0·3 ml) was used while the sodium acetate buffer was omitted from the incubation mixture.

The conjugase treatment using chicken pancreas extract was carried out following the standard procedure described by Keagy (1985). Folate solutions (2·1 μM) were prepared by dilution of stock solutions in 0·1M phosphate buffer (pH 7·2) containing 10 g liter^{-1} ascorbic acid. A portion of desiccated chicken pancreas (100 mg) was dissolved in the same buffer (20 ml) and stirred at ambient temperature for 1 h. The solution was centrifuged and the supernatant was used for the assay. An aliquot of folate solution (5·0 ml) was incubated overnight with the chicken pancreas extract (10·0 ml) and 2–3 drops of toluene.

After incubation, the conjugase treated (plasma or chicken pancreas) samples were autoclaved at 15 psi for 5 min, cooled and the volumes were made up to 50 ml with 0·05M phosphate buffer (pH 6·1) containing 1·5 g liter^{-1} ascorbic acid. These solutions were further diluted (1:100) in the same buffer to give 2·1 nM folate solutions. A fresh 2·1 nM folic acid solution (standard) was prepared by dilution of the stock solution also in the same buffer. Comparative *L. casei* assays were carried out with the three folate sources namely, standard folic acid solution, conjugase treated folic acid solution and the conjugase treated hexaglutamate solution.

Chromatographic analysis

The high performance liquid chromatographic separation was carried out on a Bio-Gel weak anion exchange

column (TSK-DEAE-5PW: 75 × 7·5 mm). A Perkin-Elmer Series 4 Solvent Delivery system was used for isocratic and gradient solvent delivery. A Hitachi model 100-10 spectrophotometer set at 281 nm was used in conjunction with Shimadzu C-R1A Chromatopac for data acquisition. Separation of the pteroyl mono- through hexaglutamates was effected by a salt gradient using 0·05M ammonium acetate, pH 6·5 (Solution A) and 0·05M ammonium acetate (pH 6·5) + 1·0M NaCl (Solution B). The column was equilibrated with a 85:15 (A:B) mixture and the elution was carried out with a 15 min linear gradient to a 80:20 (A:B) solvent composition followed by a 10 min linear gradient to 75:25 (A:B) and a 10 min isocratic elution with 75:25 (A:B) solvent mixture.

Solutions of approximately 100 μM pteroyl tetra- and hexaglutamates were separately prepared in 0·05M tris buffer of required pH (4·5, 6·0 or 7·0) containing 0·1M 2-mercaptoethanol. The absorptions at 281 nm (on Beckman model 25 spectrophotometer) were used to determine the exact concentrations. An aliquot (2·0 ml) of equimolar (approximately 50 μM) mixture of the pteroyl tetra- and hexaglutamates was used for each of the conjugase treatments unless otherwise specified. For plasma conjugase treatment an aliquot of thawed human blood plasma (0·5 ml) was added to the folate mixture (2·0 ml) in a 8 ml screw cap vial and the mixture was incubated for 3 h or 20 h, as desired. After incubation at 37°C, the samples were incubated in a boiling water bath for 10 min, cooled and the supernatant was filtered through a 0·45 μ filter before analysis of end products by HPLC. Desiccated chicken pancreas (20 mg) was dissolved in water (10 ml), stirred for 60 min, centrifuged and the extract was used for conjugase treatment. An aliquot of the chicken pancreas extract (0·5 ml) was used for the pteroyl tetra- and hexaglutamate (50 μM each) mixture (2·0 ml) and incubated for 3 h or 20 h. Further processing of the samples was identical to the processing of plasma conjugase treated samples. In the study of inhibitory action of citrate and other salts (tartarate, oxalate, chloride, acetate), 0·2 mM of the respective sodium salts were added to the folate mixture (100 mM effective concentration (2·0 ml)) before the addition of the conjugase. Inhibitory action of the citrate was also studied at the 10 mM citrate concentration.

RESULTS AND DISCUSSION

The pteroyl mono- through hexaglutamates had identical spectra with maxima at 281 nm and 354 nm with molar extinction coefficients of $2{\cdot}51 \times 10^4$ and $6{\cdot}60 \times 10^3$ respectively in 0·05M tris buffer of pH 7·5 containing 0·05M 2-mercaptoethanol. These extinction coefficients, although slightly different from the reported values ($2{\cdot}76 \times 10^4$ and $7{\cdot}3 \times 10^3$ respectively at pH 7·0) (Blakley, 1969), appear to be more accurate since the literature values were estimated from a spectrum published earlier (Rabinowitz, 1960).

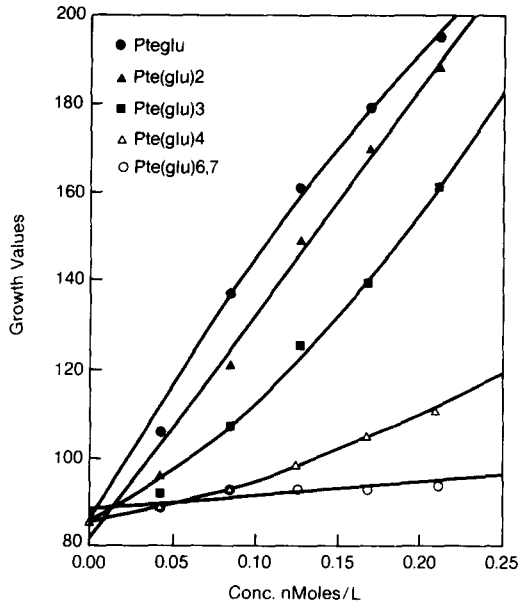

Fig. 1. *L. casei* growth responses of pteroyl oligoglutamates.

Microbiological assay results

The relative responses of *L. casei* growth to pteroyl mono-, di-, tri-, tetra-, hexa- and heptaglutamates are presented in Fig. 1. The *L. casei* growth responses to the di- and triglutamate were 88% (±2%) and 59% (±5%) respectively of the response of the monoglutamate in the upper half of the concentration range. These numbers represent mean relative responses for assays performed at three different times. (Conventional turbidimetric assays also gave similar results). Tamura *et al.* (1972) have reported that *L. casei* responds equally to the mono-, di- and triglutamates and more slowly to the higher glutamates. The negligible

Table 1. *L. casei* Growth Responses of Plasma Conjugase Treated Folic Acid and Pteroyl Hexaglutamate as Percentages of the Response of the Folic Acid Standard

pH	Time of incubation (h)	% Response[a]	
		FA	$Pte(glu)_6$
4·5	20	68	75
4·5	3	81	74
6·0	20	95	91
6·0	3	93	94
6·5	20	96	90
7·2	20	100	86

[a] The numbers are averages from at least two separate assays. The standard deviation is ±2%.

responses of the tetra-, hexa- and heptaglutamates (Fig. 1) are in agreement with their results while the results of the present study for the diglutamate and especially the triglutamate do not corroborate their findings.

The results of microbiological assays following the plasma conjugase treatment at different pH are summarized in Table 1. The tabulated values represent the percentage responses of identically conjugase treated folic acid and the hexaglutamate with respect to the untreated (standard) folic acid solution. Comparison of the results for folic acid and conjugase treated folic acid should indicate its degradation, if any, under the conditions of the conjugase treatment procedure, while comparison of results for conjugase treated folic acid and the hexaglutamate should enable us to estimate the efficiency of the enzyme in the deconjugation process. The plasma conjugase treatment procedure recommended by Lakshmaiah and Ramasastri (1975*b*) (pH 4·5, 20 h incubation) resulted in a substantial (32%) degradation of folic acid while the hexaglutamate resulted in a 75% response. Reducing the incubation time to 3 h gave similar results for the hexaglutamate while the degradation of folic acid itself was lower. It appears that the major proportion of degradation occurs during the autoclaving step which follows the incubation with the conjugase. At the incubation pH of 6·0, the deconjugation seems to be complete in 3 h with a slight excess of plasma (0·3 ml instead of 0·2 ml). Degradation seems to be negligible since the conjugase treated samples of folic acid as well as of the hexa-glutamate consistently resulted in a 90–95% response of the standard sample. Increasing the incubation pH to 6·5 resulted in similar results while incubation pH of 7·2 resulted in a slightly lower response for the hexaglutamate (85%), whereas incubated folic itself showed a 100% response. Because of the high instability of folates under acidic pH (Keagy, 1985), it is suggested that the plasma conjugase treatment be carried out at a near neutral pH (6·0–6·5) with a slight excess of the plasma.

The chicken pancreas conjugase treatment following the standard procedure (Keagy, 1985) resulted in a 95% response for the incubated folic acid and approximately 90% response for the hexaglutamate. Enzyme blanks, however, were higher than those for plasma conjugase. No further investigation was conducted on the chicken pancreas conjugase treatment by microbiological assay procedure.

Chromatographic results

Figure 2 shows the elution pattern of the pteroyl mono- through hexaglutamates under chromatographic conditions. The peak at 4·09 min is due to the 2-mercaptoethanol present in the solution while the pteroyl mono-, di-, tri-, tetra-, penta- and hexaglutamates elute at 15·85, 18·75, 24·69, 31·69, 37·82 and 42·89 min respectively. The elution times for the mono- and diglutamates changed slightly (± 0·5 min) in the presence of the plasma conjugase and the chicken pancreas conjugase, in which case the peak identities were confirmed by addition of the standard to the test sample.

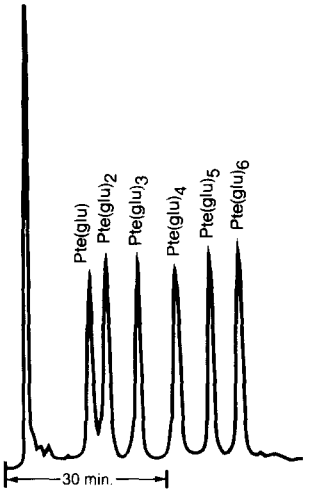

Fig. 2. Chromatogram of pteroyl mono- through hexaglutamates obtained on a Bio-Gel TSK-DEAE-5PW (75 × 7·5 mm) column by elution at pH 6·5 with a salt (NaCl) gradient (see experimental section for details). Approximately 40 μm concentration of each in 0·05M tris buffer (pH 7·0) with 0·1M 2-mercaptoethanol, UV detection at 281 nM.

Based on the enzyme activity determined for plasma (Lakshmaiah & Ramasastri, 1975*a*), 0·5 ml of plasma would contain 0·05 enzyme activity units at pH 6·0 while 0·5 ml of the extract of desiccated chicken pancreas (20 mg) in 10 ml would contain approximately 0·07 enzyme activity units at pH of 6·1 (Chen *et al.*, 1983). One enzyme activity unit is defined as the amount that would release 1 nM of folate (mono- or diglutamate) in 1 min (Chen *et al.*, 1983; Leichter *et al.*, 1977). Theoretically, 0·5 ml of plasma and 0·5 ml of the above chicken pancreas extract should be able to deconjugate only 9 nM and 11 nM of the polyglutamates, respectively, in 3 h. The tetra- and hexaglutamate mixture used in the present study contained a total of 200 nM of the polyglutamates to be deconjugated. However, the above amounts of the conjugases were found to be sufficient for complete deconjugation (trial and error) at pH 6·0 in 3 h and hence were used in further studies. A mixture of the tetra- and hexaglutamates was used instead of a single glutamate to examine if any differences could be observed between the deconjugation rates of these two pteroyl glutamates.

Starting with an equimolar mixture of pteroyl tetra- and hexaglutamates, the end products of the plasma conjugase treatments are presented in Table 2 and Fig. 3. When incubated for 20 h, plasma conjugase resulted in a monoglutamate end product with incubation pH values of 4·5 and 6·0 whereas a negligible amount of diglutamate was formed at pH 7·0. However, at pH 4·5, the chromatogram showed the presence of a new

Table 2. Composition of the End Product after Plasma Conjugase Treatment of Equimolar Mixture of Pte(glu)$_4$ and Pte(glu)$_6$

pH	Time of incubation (h)	Citrate (mM)	Percentage (molar) composition[a]					
			1	2	3	4	5	6
4·5	3	0·0	100[b]	—	—	—	—	—
4·5	3	100	17·0	17·5	2·9	33·7	3·2	25·7
4·5	20	0·0	100[b]	—	—	—	—	—
4·5	20	100	58·8[b]	30·4	1·9	2·6	2·0	2·1
6·0	3	0·0	78·8	21·2	—	—	—	—
6·0	3	10	61·8	38·2	—	—	—	—
6·0	3	100	9·4	16·8	3·7	37·1	3·0	30·5
6·0	20	0·0	100	—	—	—	—	—
6·0	20	10	95·0	5·0	—	—	—	—
6·0	20	100	56·6	27·5	3·9	6·2	2·1	3·8
7·0	3	0·0	51·5	45·1	—	2·6	—	0·8
7·0	3	10	33·8	55·8	5·5	4·9	—	—
7·0	3	100	5·9	9·8	1·4	42·3	2·3	38·3
7·0	20	0·0	~97·0	~3·0	—	—	—	—
7·0	20	10	83·5	13·5	—	—	—	—
7·0	20	100	39·4	40·8	3·4	8·6	2·0	5·8

[a] Numbers 1, 2, 3, 4, 5 and 6 represent pteroyl mono-, di-, tri-, tetra-, penta- and hexaglutamate respectively.
[b] Chromatogram contains an additional peak of degradation product of folic acid.

compound as a shoulder to the monoglutamate peak. This was attributed to the *L. casei* inactive degradation product (*p*-aminobenzoyl glutamic acid) in light of the results of microbiological assay at this pH. Independent experiments with folic acid and chromatographic analysis on an ODS column proved this hypothesis. At both pH 6·0 and 7·0, the proportion of the diglutamate end product was higher with 3 h incubation than with 20 h incubation. The presence of citrate, especially at 100 mM concentration, in the incubation mixture resulted in a higher proportion of diglutamate and higher glutamate (3–6) end products. Severe inhibition to the deconjugation was observed in 3 h incubation samples. In all the cases, the inhibitory effect was clearly higher at higher pH values. As expected, the inhibitory effect of citrate (100 mM) was lower when higher amounts of plasma were used (data not presented in the table). With five times excess plasma, the higher glutamates (3–6) were present at less than 2% of the total. In the presence of citrate, conversion to the monoglutamate was however not complete even with ten times excess plasma conjugase (pH 6·0, 20 h incubation), i.e. the end product was a mixture of 63% monoglutamate and 37% diglutamate.

The results of chicken pancreas treatment are presented in Table 3 and Fig. 4. With incubation at pH 7·0 (usual assay pH is 7·2) as well as 6·0, the end product was mostly the diglutamate when incubated for only 3 h while a substantial amount of the monogluta-

Table 3. Composition of the End Product after Chicken Pancreas Conjugase Treatment of Equimolar Mixture of Pte(glu)$_4$ and Pte(glu)$_6$

pH	Time of incubation (h)	Citrate (mm)	Percentage (molar) composition[a]					
			1	2	3	4	5	6
6·0	3	0·0	4·6	95·4	—	—	—	—
6·0	3	10	1·3	89·0	—	5·4	—	4·3
6·0	3	100	—	36·1	—	34·9	—	29·0
6·0	20	0·0	27·7	72·3	—	—	—	—
6·0	20	10	4·8	95·2	—	—	—	—
6·0	20	100	—	95·1	—	1·8	—	2·1
7·0	3	0·0	5·0	95·0	—	—	—	—
7·0	3	10	—	99·0	—	0·5	—	0·5
7·0	3	100	—	52·6	—	25·3	—	22·1
7·0	20	0·0	17·2	82·8	—	—	—	—
7·0	20	10	5·0	95·0	—	—	—	—
7·0	20	100	—	98·0	—	1·0	—	1·0

[a] Numbers 1, 2, 3, 4, 5 and 6 represent pteroyl mono-, di-, tri-, tetra-, penta- and hexaglutamate respectively.

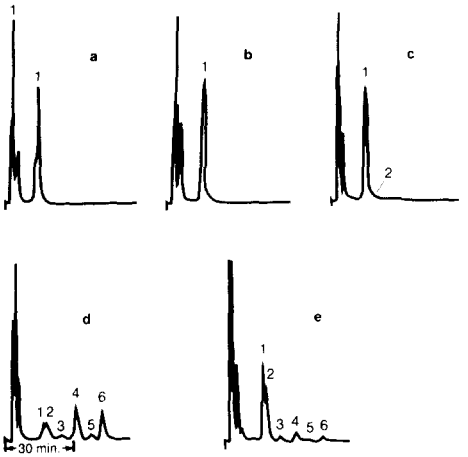

Fig. 3. Chromatograms illustrating the results of plasma conjugase treatment on a equimolar (50 μM) mixture of pteroyl tetra- and hexaglutamate. (a) pH 4·5, 20 h incubation; (b) pH 6·0, 20 h incubation; (c) pH 7·0 20 h incubation; (d) pH 6·0, 3 h incubation with 100 mM citrate; (e) pH 6·0, 20 h incubation with 100 mM citrate. Numbers 1, 2, 3, 4, 5 and 6 represent pteroyl mono-, di-, tri-, tetra-, penta- and hexaglutamate respectively.

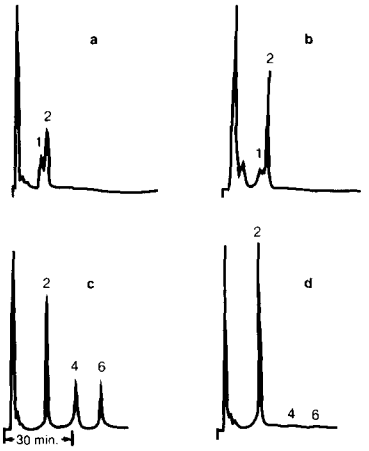

Fig. 4. Chromatograms illustrating the results of chicken pancreas conjugase treatment on an equimolar (50 μM) mixture of pteroyl tetra- and hexaglutamate. (a) pH 6·0, 20 h incubation; (b) pH 7·0, 20 h incubation; (c) pH 7·0, 3 h incubation with 100 mM citrate; (d) pH 7·0, 20 h incubation with 100 mM citrate. Numbers 1, 2, 3, 4, 5 and 6 represent pteroyl mono-, di-, tri-, tetra-, penta- and hexaglutamate respectively.

mate was formed when incubated for 20 h. An excess of the conjugase resulted in a higher proportion of the monoglutamate with as much as 20% (3 h, pH 7·0), 53% (20 h, pH 7·0), 24% (3 h, pH 6·0) and 91 % (20 h, pH 6·0) of the monoglutamate with five times excess of the chicken pancreas extract (data not presented in the table). Citrate, as in the case of plasma conjugase, inhibited the deconjugation process. The end product was a mixture of di-, tetra- and hexaglutamates (Fig. 4c). With 100 mM citrate, as much as three times excess conjugase was required to complete the deconjugation in 3 h. The presence of as low as 10 mM citrate dramatically affected the proportion of monoglutamate end product, for example, a 91:9 mixture of mono:diglutamate (pH 6·0, 20 h, five times excess conjugase) changed to 14:86 mixture in the presence of 10 mM citrate.

No significant difference was found between the extent of deconjugation of the tetraglutamate and the hexaglutamate by both plasma and the chicken pancreas conjugases. Besides the relative amounts of the mono and diglutamate end products, another significant difference between the two conjugase treatments is that in the presence of citrate, with plasma conjugase, all the glutamates, mono through hexa, were present in the end product while with chicken pancreas conjugase, only di-, tetra and hexaglutamates were present in the end product mixture. Plasma conjugase appears to truncate the polyglutamyl chain successively by a single glutamate (exopeptidase). Reisenauer and Halsted (1981) reported similar behavior of folate conjugase from human jejunal brush border folate conjugase. With chicken pancreas conjugase however, it is possible that the polyglutamyl chain could be successively truncated in the units of two glutamates or that the polyglutamyl chain is cleaved as a whole at the diglutamate-triglutamate junction. Independent experiments with pteroyl pentaglutamate resulted in a mixture of di- and pentaglutamate end product (in the presence of citrate) which implies that the latter supposition is true. The inhibitory effect of citrate to the deconjugation of the polyglutamyl chain seems to be less in the case of chicken pancreas conjugase than in the case of plasma conjugase. However, inhibition of the conversion of the diglutamate to the monoglutamate seems to be more in the case of chicken pancreas conjugase than in the case of plasma conjugase. It appears that the chicken pancreas conjugase consists of two enzymes: a predominant fraction of the one which converts the pteroyl polyglutamates to the diglutamate (endopeptidase) and a lesser amount of the other enzyme (similar to plasma conjugase) which converts the pteroyl diglutamate to the monoglutamate. This supposition is in agreement with the results of Leichter *et al.* (1977) who have reported the presence of two enzymes in the chicken pancreas conjugase though the deconjugation properties of individual enzymes were not identified. Rosenberg and Neuman (1973) on the other hand, working with chicken intestine extract, reported the presence of three enzymes; two similar to the ones mentioned above and a third one which catalyzes the hydrolysis of poly-γ-glutamates.

Other salts tried for their inhibitory action (oxalate, tartarate, chloride and acetate) showed a much lower inhibitory effect, almost equal to that of 10 mM citrate concentration at 100 mM concentration of these salts. It

is interesting to notice that salts like chloride and acetate inhibit the deconjugation process.

Lakshmaiah and Ramasastri (1975*b*) have reported plasma conjugase to have an acidic pH optimum (4·5) with a monoglutamate end product while Leichter *et al.* (1977) have reported near neutral pH optimum (8·5) and a diglutamate end product for chicken pancreas conjugase. From the above results, it is apparent that a working pH different from the optimum value and the presence of inhibitory components yields a variety of results. Citrate is supposed to be an inhibitor of conjugases from most of the sources. Engelhardt and Gregory (1990) have reported that citrate as well as most of the food extracts inhibit the conversion of the pteroyl triglutamate to monoglutamate with hog kidney conjugase. Kirsch and Chen (1984) have reported considerably higher inhibition by citrate of the chicken pancreas conjugase than of the hog kidney conjugase. Pedersen (1988) while reporting higher inhibition of the chicken pancreas conjugase than the hog kidney conjugase by the food extracts, has also reported that deconjugation using these sources could be maximized using excess amounts of the enzymes. Pedersen has also noted that on the basis of enzyme activity, a large excess (at least 10^3 times the theoretical amount) of chicken pancreas is being used in the standard assays. Although no systematic study of inhibitory effects of food components on the plasma conjugase has been done, Lakshmaiah and Ramasastri (1975*b*) have reported comparable results with standard conjugase treatments with hog kidney, chicken pancreas and the plasma for some common food items. Lakshmaiah and Ramasastri (1975*b*) have not mentioned the use of any excess amounts of the plasma conjugase. It is apparent that plasma conjugase, hog kidney conjugase and the chicken pancreas conjugase could be inhibited by several components of food extracts. Enzyme treatment at near neutral pH using a large excess of conjugase could alleviate the problem though not eliminate it. Preliminary experiments and recovery studies would be needed on an individual basis to optimize the amounts of conjugase needed.

Use of plasma conjugase at pH 6·0 or higher for deconjugation prior to chromatographic analysis of total folates will be complicated because of the mixed end products. Although a lower incubation pH (4·5) results in mostly monoglutamate, degradation at the acidic pH will offset this advantage.

CONCLUSIONS

Contrary to the general belief (Keagy, 1985) that *L. casei* responds equally to pteroyl mono-, di- and triglutamates, the authors have found that the relative responses of the di- and triglutamates are approximately 90% and 60% respectively of the response of the monoglutamate.

Plasma conjugase treatment following the standard procedure at pH 4·5 results in a substantial degradation of the folate, while a near neutral pH (6·0–7·0) with a slight excess of the plasma results in improvement of results. Plasma conjugase treatment gives a monoglutamate end product at pH of 4·5 and 6·0 while at pH 7·0, a small amount of the diglutamate end product is formed. Chicken pancreas conjugase on the other hand, gives a mixture of mono- and diglutamate end products at both pH 6·0 and 7·0. Plasma conjugase seems to contain single enzyme which truncates the pteroyl polyglutamyl chain successively by a single glutamate while the chicken pancreas conjugase seems to contain two enzymes: a major fraction of the one that cleaves the polyglutamyl chain at the diglutamate-triglutamate junction and a small fraction of the enzyme that converts the diglutamate to the monoglutamate.

Polyglutamate deconjugation using hog kidney, plasma or chicken pancreas conjugase is inhibited by citrate and other food extracts (present work; Phillips & Wright, 1983; Kirsch & Chen, 1984; Pedersen, 1988; Engelhardt & Gregory, 1990). Use of excess conjugase could alleviate the problem. However, since the different components of food extracts can have different inhibitory effects on these conjugases, generalization on the amount of conjugase needed is very difficult. Because of the instability of folates at acidic pH, the hog kidney conjugase treatment, which is generally carried out at pH 4·9 (Gregory *et al.*, 1984; Keagy, 1985; Engelhardt & Gregory, 1990) may result in a substantial degradation of the folates. If the properties of the hog kidney conjugase and the plasma conjugase are similar, the conjugase treatments using these two sources may suffer similar disadvantages, namely, degradation at acidic pH and mixed mono- and diglutamate end products at near neutral pH.

It can be said that each one of these (plasma conjugase, hog kidney conjugase and chicken pancreas conjugase) treatments has its own inherent problems and that no entirely satisfactory procedure can be developed for the conjugase treatment of folylpolyglutamates either for the analysis by microbiological assay or for the deconjugation for HPLC analysis of total folates.

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