Enzymatic Hydrolysis of Pteroylpolyglutamates in Cabbage[1] (36924)

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Folates occur in nature mainly as polygammaglutamyl derivatives of folic acid.

Since these polyglutamates have reduced activities for the organisms employed in routine microbiological assays, they have to be hydrolyzed enzymatically before assay. Conjugases (pteroylpolygammaglutamylcarboxypeptidases) which accomplish this are present in a wide variety of biological materials (1–7), which also contain the substrates for this reaction, namely, the pteroylpolyglutamates. In the identification of the polyglutamates occurring in biological materials, it is important that the procedures employed are such that the polyglutamates are not altered in any way by the conjugase(s) present intrinsically within materials during extraction. These precautions have been emphasized by Kondo, Iwai and Yoshida (8). Baugh and Krumdieck (9) have also stressed the importance of the inactivation of conjugase(s) in assay materials.

We have observed that "free folate" values (*L. casei* activity prior to conjugase treatment) in fresh cabbage were significantly higher than those in cooked cabbage, although there were no differences in "total folate" (*L. casei* activity after conjugase treatment) values in both fresh and cooked cabbage. It was reasoned that these apparent high free folate values were the result of the presence of an active conjugase in this tissue which functioned from the time the tisue was homogenized until the sample was autoclaved.

In this paper we present evidence for the existence of conjugase(s) in cabbage and describe some of its properties.

Materials and Methods. L. casei (ATCC 7469) and *S. faecalis* (ATCC 8043) were employed as the assay organisms. The microbiological assay procedure using *L. casei* has been previously described (10). For *S. faecalis*, 5 ml of inoculum broth containing 10 ng of PteGlu[2] were used for daily transfers and 0.1 *M* sodium phosphate buffer (pH 6.7) containing 1.0 mg/ml of ascorbic acid was used to keep the initial pH of the medium at 6.7. It has been established in preliminary studies that the response of *S. faecalis* is more sensitive and reproducible at this pH than at lower values. Incubation of *S. faecalis* was carried out for 24–30 hr at 37°. Treatment with hog kidney conjugase was made according to the method of Bird, McGlohon and Vaitkus (11). The method for folate extraction was based on that of Hurdle, Barton and Searles (12). Fresh cabbage was obtained from ordinary commercial sources. A 10 g portion of cabbage was homogenized in a blender with 100 ml of 0.1 *M* sodium phosphate buffer (pH 6.0) containing 1% (1 g/100 ml) ascorbic acid. A portion of this homogenate was centrifuged (1000*g*) for 10 min and the supernatant was used as the enzyme source. The other portion of the homogenate was autoclaved at 15 lb pressure for 15 min, centrifuged and the supernatant was used for folate determination. For inactivation of conjugase, small portions of cabbage were placed in boiling phosphate ascorbate buffer for 3 min and then homogenized. This insured inactivation of conjugase. Pteroylpolyglutamates were synthesized by the methods described by Baugh and his coworkers (13, 14). Radioisotopic assay of con-

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[2] Abbreviations: PteGlu, pteroylmonoglutamic acid. For $PteGlu_n$, the number of glutamic acid residues in pteroylpolyglutamates is specified by subscript.

TABLE I. Effect of Heat Treatment on "Free" and "Total" Folate Activities in Cabbage Homogenates.

Expt.	Free folate[a] L. casei activity without hog kidney conjugase treatment (μg/g of cabbage[b])	Total folate L. casei activity with hog kidney conjugase treatment (μg/g of cabbage)	(Free folate/total folate) $\times$ 100 (%)
Heated after homogenization[c]	0.54 ± 0.19 (6)[d]	0.67 ± 0.29 (6)	81
Heated before homogenization[e]	0.09 ± 0.09 (6)	0.64 ± 0.27 (6)	14

[a] Free folate values were calculated at half-maximum point of *L. casei* response curve (10).
[b] g of cabbage wet weight.
[c] Cabbage was not heated before homogenate preparation.
[d] Mean $\pm$ SD, values in parentheses represent number of experiments.
[e] Cabbage was heated before homogenate preparation.

jugase activity was carried out according to Baugh, Stevens and Krumdieck (13).

Results and Discussion. As shown in Table I, about 80% of total folate was active to *L. casei* without hog kidney conjugase treatment (*i.e.*, cabbage homogenate was autoclaved within 15 min after preparation of homogenate), but once cabbage was heated for 3 min in the boiling phosphate ascorbate buffer prior to homogenization, only 14% of total folate became active to *L. casei* as free folate. These data indicate the presence in cabbage of an endogenous enzyme system for the hydrolysis of folate polyglutamates. The results further suggest that the activity of such an enzyme system is destroyed by prior heating of the tissue, although values of the total folate activity measured after hog kidney conjugase treatment are not affected by boiling.

The presence of such a conjugase was directly established by incubating cabbage homogenates with PteGlu$_3$-U-^{14}C-Glu-Glu as substrate in a series of buffers [sodium acetate, sodium phosphate and tris(hydroxymethyl)aminomethane (Tris)] over the pH range of 3.0–9.0. The results are shown in Fig. 1. Cabbage conjugase(s) showed 2 optimum pH values around pH 5.0 in acetate buffer and pH 8.0 in Tris buffer. However, when PteGlu-U-^{14}C-Glu-Glu was employed as substrate, only 1 optimum pH at around pH 5.0 in acetate buffer was evident as shown in Fig. 2. These data indicate that cabbage conjugase(s) can hydrolyze polyglutamates to monoglutamates at low pH and at high pH only to PteGlu$_3$ or PteGlu$_2$. As shown in Fig. 3, microbiological assay with *L. casei* and *S. faecalis* was carried out using PteGlu$_7$ as substrate for cabbage conjugase. These data gave the same optimum pH values and indicated that the final product at

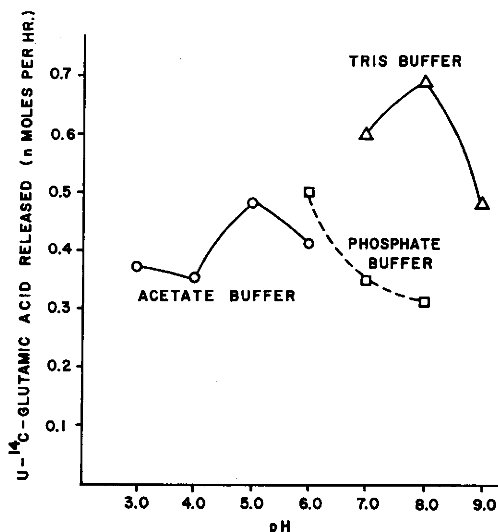

FIG. 1. Effect of pH on conjugase activity using PteGlu$_5$ as substrate. 2.25 nmoles (sp act 0.4 μCi/μmoles) of PteGlu$_3$-U-^{14}C-Glu-Glu were incubated with cabbage conjugase (equivalent to 60 mg of cabbage, wet wt) for 1 hr at 37° in the buffer indicated in a final volume of 10 ml. The final buffer molarity in all cases was 0.07 *M*. Cabbage conjugase was prepared by homogenizing 10 g of tissue with 100 ml of water and centrifuging as described in Materials and Methods.

high pH is $PteGlu_3$, since *S. faecalis* is fully active to both PteGlu and $PteGlu_2$ (13).

The results presented in this communication clearly established the presence in cabbage of an active enzyme system for the splitting of gamma-linkages in polyglutamates. It is shown that the hydrolysis of $PteGlu_5$ proceeds optimally at pH 5.0 and 8.0 in acetate and Tris buffers, respectively, while the hydrolysis of $PteGlu_3$ is maximum at pH 5.0.

The possibility that cabbage contains either 2 enzymes or 1 enzyme with 2 different pH optima remains open.

Two reports have previously appeared which suggest the existence of 2 conjugase activities in hog kidney and human liver (9, 15), although details have not been reported. The present investigation emphasizes that inactivation of endogenous conjugases is essential in studying the nature of folates in biological materials unless it is proved that such conjugases are not active under the assay conditions

Summary. The presence of folate conjugase(s) was established in cabbage. The conjugase(s) was found to have its pH optimum at 5.0 and 8.0 in acetate buffer and Tris buffer, respectively. At pH 8.0 this conjugase was able to split down to third gamma-linkage yielding pteroyltriglutamate while the polyglutamates were split to monoglutamate at pH 5.0.

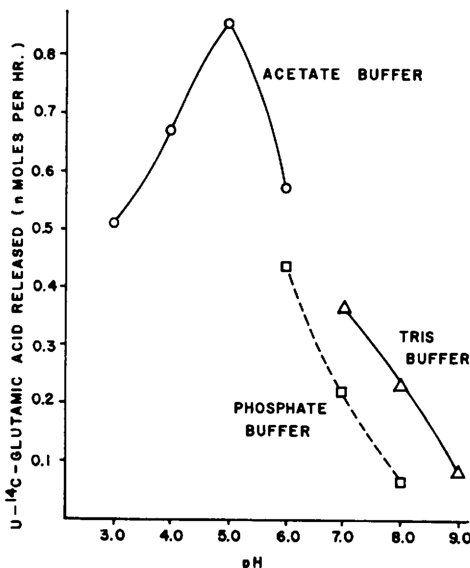

FIG. 2. Effect of pH on conjugase activity using $PteGlu_3$ as substrate. 2.5 nmoles (sp act 0.3 μCi/μmoles) of PteGlu-U-^{14}C-Glu-Glu were incubated with cabbage conjugase. Incubations were carried out in the same manner as described in Fig. 1.

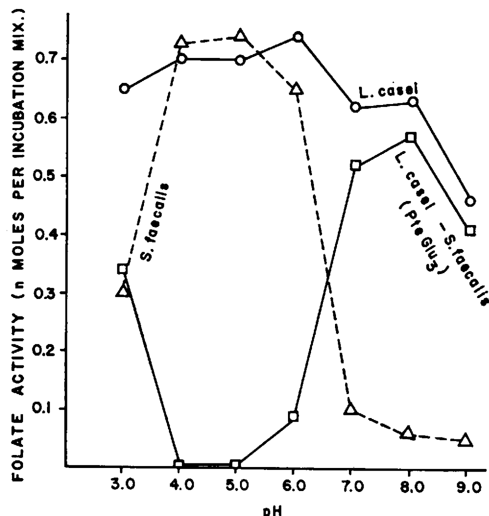

FIG. 3. Effect of pH on conjugase activity. 0.75 nmoles of $PteGlu_7$ were incubated with cabbage homogenate (equivalent to 100 mg of cabbage, wet wt) in a final volume of 10 ml of buffer (0.1 *M* sodium acetate pH 3.0–5.0 and sodium phosphate 6.0–9.0) containing 1% ascorbate for 6 hr at 37°. Endogenous folate values were subtracted at each point. The final product of conjugase treatment at high pH is active for *L. casei*, but not for *S. faecalis*. This indicates that the end product of conjugase treatment at high pH is $PteGlu_3$.

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1. Bird, O. D., Robbins, M., Vandenbelt, J. M., and Pfiffner, J. J., J. Biol. Chem. **163**, 649 (1946).

2. Mims, V., and Laskowski, M., J. Biol. Chem. **160**, 493 (1945).

3. Wolff, R., Drouet, L., and Karlow, R., Science **109**, 612 (1949).

4. Bernstein, L. H., Gustein, S., and Weiner, S. V., Amer. J. Clin. Nutr. **23**, 919 (1970).

5. Krumdieck, C. L., and Baugh, C. M., Anal. Biochem. **35**, 123 (1970).

6. Nielsen, N., and Holstrom, B., Acta Chim. Scand. **11**, 101 (1957).

7. Iwai, K., Mem. Res. Inst. Food Sci., Kyoto Univ. **13**, 1 (1957).

8. Kondo, K., Iwai, K., and Yoshida, T., Bull. Res. Inst. Food Sci. **13**, 67 (1954).
9. Baugh, C. M., and Krumdieck, C. L., Ann. N.Y. Acad. Sci. **186**, 7 (1971).
10. Tamura, T., Shin, Y. S., Williams, M. A., and Stokstad, E. L. R., Anal. Biochem., in press.
11. Bird, O. D., McGlohon, V. M., and Vaitkus, J. W., Anal. Biochem. **12**, 18 (1965).
12. Hurdle, A. D. F., Barton, D., and Searles, I. H., Amer. J. Clin. Nutr. **21**, 1202 (1968).
13. Baugh, C. M., Stevens, J. C., and Krumdieck, C. L., Biochim. Biophys. Acta **212**, 116 (1970).
14. Krumdieck, C. L., and Baugh, C. M., Biochemistry **8**, 1568 (1969).
15. Laskowski, M., Ann. Rev. Biochem. **19**, 21 (1950).

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