THE USE OF CONJUGASE PREPARATIONS IN THE MICROBIOLOGICAL ASSAY OF FOLIC ACID*

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(Received for publication, August 16, 1948)

Taka-diastase (1) and a preparation of hog kidney enzyme (2) have been used routinely to liberate folic acid from its conjugates in the microbiological determination of the vitamin (3–6). However, Olson *et al.* (7) reported recently that taka-diastase and certain proteolytic enzymes are of doubtful value in releasing the vitamin from plant tissues. Hog kidney conjugase also does not completely liberate folic acid in every case with fresh plant materials (8) or plant extracts (2, 9, 10). It has been demonstrated that, with homogenates of rat liver, autolysis at pH 7.0 results in a rapid increase in the folic acid content (11–13), whereas at pH 4.5 neither autolysis of the liver nor digestion of heated samples with hog kidney conjugase causes release of the vitamin (13). Apparently there are bound forms of folic acid not hydrolyzable by the conjugase preparations now available. According to Luckey *et al.* (3) no one method could be prescribed to attain maximum folic acid values in all types of materials. Charkey *et al.* (14) also suggest that there may be more than one form of the conjugate present in yeast.

In spite of the wide-spread occurrence in tissues and organs of enzymes capable of converting the conjugated pteroylglutamic acid to the free acid (15), there is little information as to whether conjugases differ in respect to their mechanism of action. In this communication are reported the results of certain preliminary observations which suggest that conjugases may vary in their ability to liberate folic acid or folic acid-active substances from natural sources.

EXPERIMENTAL

Methods—The hog kidney enzyme used in these studies was a clarified water extract of hog kidney prepared and stored frozen as described by

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from Swift and Company, Chicago, Illinois.

We wish to thank the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for synthetic pteroylglutamic acid and pteroyltriglutamic acid and Parke, Davis and Company, Detroit, Michigan, for crystalline vitamin B_c conjugate used in these studies.

Bird *et al.* (2). The chicken pancreas conjugase (15, 16) was prepared from fresh, frozen chicken pancreas. The pancreas was homogenized with 0.1 M phosphate buffer at pH 7.0 (2 ml. of buffer per gm. of pancreas) in a Waring blendor. The homogenate was allowed to autolyze 24 hours at 37° and was then centrifuged to remove fat. The extract was transferred to tubes and frozen before storage. Homogenates of rat liver were prepared by the procedure outlined by Olson *et al.* (13) and were used at a dilution of 1:5. Folic acid activity was determined by using *Streptococcus faecalis* and the turbidimetric method of Luckey *et al.* (17) (the medium being modified by the addition of Salts B (18)). Synthetic pteroylglutamic acid (Lederle) was used for the standard.

Liberation of Folic Acid from Yeast Samples with Chick Pancreas and Hog Kidney Enzymes—For hydrolysis with hog kidney enzyme, 5 ml. of a

TABLE I

Folic Acid Content of Yeast Samples

Enzyme used	Sample I. Difco yeast extract	Sample II. Brewers' yeast, dried, non-debittered	Sample III. Brewers' yeast, dried, debittered	Sample IV. Dried yeast
	γ per gm.	*γ per gm.*	*γ per gm.*	*γ per gm.*
None	3.8	2.2	1.3	2.0
Hog kidney enzyme	30.0	13.2	13.2	15.3
Chick pancreas enzyme	45.0	9.0	9.0	15.0
" " " followed by hog kidney enzyme	125.0	19.0	22.0	16.0

solution or fine suspension, equivalent to 1 to 10 mg. of the air-dry product, were added to 5 ml. of McIlvaine's disodium phosphate-citric acid buffer at pH 4.5 containing 2 ml. of the enzyme preparation. The mixture was incubated under toluene at 37° overnight (18 to 20 hours), heated in a boiling water bath for 2 minutes, cooled, neutralized to pH 6.8 to 7.0, made to a convenient volume, and filtered. Aliquots of the solution were used for microbiological assay. The procedure for hydrolysis with the chick pancreas conjugase was similar. In this case, however, the sample was incubated with 1 ml. of the enzyme preparation in buffer at pH 7.0 for 6 to 8 hours. When the two enzymes were used successively, the mixture, after incubation with the first enzyme, was adjusted to the pH optimum for the second enzyme before its addition. Blanks for the enzyme preparation were subtracted from the values reported. Table I gives a typical set of results obtained.

It may be observed that, with the exception of Sample IV, successive hydrolyses with the two enzymes gave much greater folic acid activity

than the employment of either enzyme alone. The same results were obtained regardless of which enzyme was used first. It appeared as if the two enzymes acted specifically on different types of substrates, although it was quite possible that there was some overlapping in the action of the two enzymes.

With Difco yeast extract as substrate and the two enzyme preparations as above, the values obtained for folic acid by means of *Lactobacillus casei* and the titrimetric method of Teply and Elvehjem (18) were as follows: free folic acid, 3.5 γ per gm.; hydrolyzable by hog kidney enzyme, 20.0 γ per gm.; hydrolyzable by chick pancreas enzyme, 35.0 γ per gm.; and folic acid hydrolyzable by both enzymes, 93.0 γ per gm. While the general nature of the results obtained was the same as with *Streptococcus faecalis*, the values were somewhat lower than those obtained with the latter organism (Table I). Higher values with *S. faecalis* have also been reported by Fager *et al.* (8) in various vegetable and plant extracts. Differences in the response of the two microorganisms in the folic acid assay have been recognized (7) and may arise as a result of variability in their utilization of the different forms of folic acid, pteroic acid, or the SLR[1] factor (19). Further studies were confined only to the use of *S. faecalis* as the test organism for microbiological assay.

Liberation of Folic Acid from Yeast by Rat Liver Enzymes—Since previous workers indicated the existence of two enzyme systems in rat liver, acting at pH 4.5 and 7.0 respectively (13), trials were carried out with 1 ml. lots of rat liver homogenate as the enzyme source and Difco yeast extract (5 ml. of solution containing 1 mg. of yeast extract) as substrate. The incubation time was 4 hours in every case; this period was sufficient for maximum release of folic acid. In a typical experiment, the results obtained with the liver enzymes at pH 4.5, pH 7.0, and at the two pH values successively were 40 γ, 50 γ and 100 γ of folic acid respectively per gm. of yeast extract after allowing for the blanks obtained by incubating the liver preparations alone. The enzyme systems in rat liver presumably correspond to both the hog kidney and chick pancreas enzymes.

Liberation of Folic Acid from Rat Liver Homogenate by Hog Kidney and Chick Pancreas Enzymes—In the following experiments, 1.0 ml. of heated rat liver homogenate, containing 0.2 gm. of liver, was used as substrate. The values obtained by action of the rat liver enzymes are included for comparison (Table II).

The observations of Olson *et al.* (13) that maximum liberation of folic acid from liver takes place only at pH 7.0 are borne out by these data (Table II); at this pH, chick pancreas enzyme releases nearly as much folic acid as the liver enzyme itself. Further, the inability of hog kidney

[1] *Streptococcus lactis* R. This organism is also known as *Streptococcus faecalis* R.

enzyme to free the vitamin from its combination in the liver corresponds to that of the liver enzyme at pH 4.5. The values (Sample I) obtained after autolysis at pH 4.5 or treatment with hog kidney enzyme at this pH were somewhat higher and less consistent than when the samples were collected under more rigid conditions of refrigeration (Sample II). Apparently the liver enzymes release folic acid rapidly in the intact liver at room temperature.

TABLE II

Release of Folic Acid from Rat Liver by Conjugases

Treatment	Folic acid values, γ per gm. liver	
	Sample I	Sample II
Heated liver, unincubated	1.0	0.46
" " + hog kidney enzyme, pH 4.5, overnight	1.3	0.43
" " + chick pancreas enzyme, pH 7.0, 8 hrs.	2.3	
" " + fresh liver, pH 7.0, 4 hrs.	2.5	
Fresh liver, autolyzed at pH 7.0, 4 hrs.	2.5	2.5
" " " " " 4.5, 4 "	1.6	0.50
" " " " " 4.5 for 4 hrs. and at pH 7.0 for 4 hrs.	2.3	

TABLE III

Release of Folic Acid on Incubation of Enzyme Mixtures

	Folic acid values					
	Sample I	Sample II	Sample III	Sample IV	Sample V	Sample VI
	γ	γ	γ	γ	γ	γ
2 ml. hog kidney enzyme, pH 4.5, overnight	0.02	0.01	0.01	0.03	0.01	0.01
1 ml. chick pancreas enzyme, pH 7.0, 8 hrs.	0.48	0.65	0.35	0.50	0.45	0.14
2 ml. hog kidney enzyme + 1 ml. chick pancreas enzyme, pH 4.5, overnight	0.65	0.92	0.40	0.61	0.50	0.22

Since the liver conjugate is largely hydrolyzable at pH 7.0, it would probably appear that this is the form in which folic acid reserve is held in the liver. With yeast, evidently there is more than one form of substrate.

Liberation of Folic Acid from Hog Kidney and Chick Pancreas Enzyme Mixtures—In the course of the foregoing experiments with mixtures of hog kidney and chick pancreas enzymes, it was noticed that, in the blanks obtained for the enzymes alone, the folic acid amounted frequently to much more than the sum of the values for the individual enzymes. Some of the

results obtained with different lots of the two enzyme preparations are given in Table III.

In these experiments, the mixture of enzymes was in each case incubated overnight at pH 4.5 before microbiological assay, since this was the procedure followed whenever the enzymes were used successively with the various natural sources of folic acid compounds studied. Chick pancreas enzyme, in addition to giving a high blank for folic acid, obviously contains a folic acid compound hydrolyzable by hog kidney enzyme at pH 4.5, thus accounting for the increased folic acid in the mixture of enzymes. It was ascertained in one instance (Sample I, Table III) that, when the mixture of enzymes was incubated at pH 7.0 for 8 hours so as to correspond to the addition of enzymes for successive hydrolyses by hog kidney enzyme

TABLE IV

Folic Acid Content of Some Natural Materials

	Untreated	With chick pancreas enzyme	With hog kidney enzyme	With both enzymes
	γ per gm.	*γ per gm.*	*γ per gm.*	*γ per gm.*
Dry liver preparation, unknown origin	0.7	1.5	1.4	3.0
Condensed fish solubles, 50% total solids	0.04	0.20	0.12	0.30
Dried egg yolk	0.11	0.52	0.47	0.55
Whole " I, fresh basis		0.020	0.033	0.030
" " II, " "		0.063	0.065	0.060
Spinach, dried	2.42	6.15	7.05	9.25
Mustard greens, dried	0.97	4.55	4.10	4.75
Soya flour	0.46	2.55	2.80	3.35

followed by chick pancreas enzyme, the folic acid content at the end of treatment was only slightly higher than the sum of the values for the individual enzymes (actually, 0.5 γ). Hog kidney enzyme preparations, in addition to giving a low blank, do not presumably contain other forms of folic acid hydrolyzable at pH 7.0.

Enzymatic Liberation of Folic Acid from Other Natural Sources—The values obtained, by the procedure outlined above, for a few other materials are listed in Table IV. In the case of dried liver and condensed fish solubles, higher values for folic acid were secured by successive hydrolyses. The results with egg samples and with dried greens would suggest that in these cases folic acid, or at any rate a large part of it, is present in a form which is mostly released by hog kidney enzyme alone and perhaps also by chick pancreas enzyme.

Liberation of Folic Acid from Pteroyltriglutamic Acid and Pteroylhepta-

glutamic Acid—With a view to gaining an insight into the mechanism of action of the two enzymes, crystalline pteroylheptaglutamic acid (Parke, Davis) and synthetic pteroyltriglutamic acid (Lederle) were used as substrates for hydrolysis. With the use of 5 ml. lots of solutions of the two conjugates (1 ml. ≡ 0.1 γ of folic acid) the percentage recoveries of folic acid are given in Table V.

While pteroyltriglutamic acid is completely hydrolyzed by both chick pancreas and hog kidney enzymes, it may be seen that the heptaglutamic acid is not fully hydrolyzed by either enzyme, the hog kidney enzyme being, however, more powerful of the two for this conjugate. The only available supply of pteroylheptaglutamic acid was a 10 γ per ml. solution and it is possible that this solution had undergone some change or degradation into other forms not hydrolyzable by hog kidney enzyme. From a qualitative point of view, however, these results again emphasize the nature of the

TABLE V

Release of Folic Acid from Pteroyltriglutamic Acid and Pteroylheptaglutamic Acid

	Per cent folic acid recovered			
	Without hydrolysis	With chick pancreas enzyme	With hog kidney enzyme	With both enzymes
Pteroyl triglutamate	4.7	94	100	100
Pteroyl heptaglutamate 1	6.0	20	76	105
" " 2	6.7	36	78	92

differences in the mode of action of the two enzyme preparations. The hydrolytic action of chick pancreas enzyme was extended for 24 hours, but there was no significant difference in the extent of release of available folic acid, thus showing definitely that folic acid is not fully liberated from heptaglutamic acid by this enzyme.

DISCUSSION

Luckey *et al.* (3) had observed that, when various hydrolytic methods, enzymatic as well as non-enzymatic, were compared, certain treatments gave higher values than others for some samples, while the reverse was true for others. The values obtained for yeast extract (Difco) by various treatments were so variable that they concluded it was difficult to determine its folic acid content. Bird *et al.* (2) obtained low results by microbiological assay for certain plant extracts as compared to chick assay and suggested that conjugase inhibitors might account for this observation. However, the fact that different treatments are required to give maximum values

for different materials would indicate that the compounds in the folic acid group are bound in natural materials by different chemical unions.

On the basis of the results presented here, it is not possible to outline a definite procedure for the use of conjugase preparations which would result in the maximum liberation of folic acid in all cases. However, it seems definite that conjugases differ in regard to their specificity of hydrolytic action on folic acid-active materials, although a certain amount of overlapping may occur. The suggestion of Olson *et al.* (7) that certain folic acid complexes are first degraded to pteroylheptaglutamic acid and subsequently to pteroylglutamic acid could account for the observation reported here that successive hydrolytic action by more than one enzyme does not necessarily result in additive values for folic acid. Only when the chemical nature of the conjugates or other folic acid complexes is known will it be possible to ascertain whether conjugases, so widely distributed in nature, vary in their mechanism of action. Meanwhile it should be possible to standardize the conditions of time, temperature, and pH in the acidic hydrolysis of materials, first reported by Briggs *et al.* (20, 21) and later by others (22, 3) for the liberation of free folic acid from its bound forms.

SUMMARY

1. The use of hog kidney and chick pancreas enzymes for the release of folic acid from yeast samples resulted in different values for the vitamin as assayed microbiologically, with use of either *Streptococcus faecalis* and the turbidimetric method or *Lactobacillus casei* and the acidimetric method. Successive hydrolyses by the two enzymes gave much higher values for folic acid than when they were used singly.

2. Similar differences were observed in the enzymatic hydrolysis of yeast extract by the rat liver enzymes at pH 4.5 and 7.0, maximum liberation of folic acid being obtained by the use of the two enzymes successively.

3. Rat liver homogenate increased in folic acid content on autolysis at pH 7.0 or when hydrolyzed by chick pancreas enzyme, but not on autolysis at pH 4.5 or when hydrolyzed by hog kidney enzymes.

4. It is shown, from data on the enzymatic treatment of a few other natural materials and of pteroyltri- and pteroylheptaglutamic acids, that conjugases may vary in their ability to hydrolyze different forms of folic acid that may occur in plant materials and that therefore their use does not necessarily result in maximum folic acid values.

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