THE DETERMINATION OF LIVER VITAMIN B₁₂ AND FOLATE ACTIVITY ON A SINGLE EXTRACT

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The microbiological assay of vitamin B₁₂ and folate activity in liver tissue is usually preceded by some form of treatment to make the vitamins available to the assay test organisms. Enzyme treatment and various other procedures usually involving changes in the pH of the preparation have been used to release maximum folate activity.

Romine (1) compared the results obtained by autolysis in the presence of ascorbate buffer and by treatment with ascorbate buffer only. She found that the autolysis did not increase the folate activity for *Lactobacillus casei*.

Bennet *et al.* (2) examined the effect of pH, temperature and incubation time on liver folate levels. They concluded that an alkaline homogenate incubated for 5 hours at 37° in the presence of ascorbic acid gave maximum levels. Noronha and Silverman (3) used autolysed chicken pancreas extraction to detect polyglutamates with good results.

The quantity of material obtained by percutaneous needle biopsy of the liver is usually small. It is therefore desirable with such small specimens to use a single pre-treatment system which will provide material suitable for both assays. Pitney and Onesti (6) used papain at pH 4.6 and as a control used a duplicate series without papain but at pH 6.6. The results showed that higher levels were obtained for both vitamin B₁₂ and folate activity after papain treatment.

This paper reports a series of experiments to determine the merits of papain and chicken pancreas treatment at various pH levels on the vitamin B₁₂ concentration and folate activity of liver.

EXPERIMENTAL

1. Materials and Methods

Liver blocks were taken at autopsy from 30 individuals who died from one of the following causes: coronary occlusion, alcoholism, carcinoma of the stomach or traffic accident.

2. Vitamin Assays

The folate activity was assayed microbiologically using *Lactobacillus casei* as the test organism (4). The vitamin B₁₂ concentration was measured microbiologically using the "Z" strain of *Euglena gracilis* as the test organism (5).

3. Buffers

Acetate buffer, pH 4.6: 10.0 g. of sodium acetate·3H₂O in 950 ml of distilled water. Glacial acetic acid was added to bring the pH to 4.6; the volume was then brought to 1,000 ml with distilled water. *Phosphate buffer*, pH 6.1 (4). *Phosphate buffer*, 0.25 M, pH 7.0. Ascorbic acid was added to each buffer in a concentration of 1.5 mg/ml immediately before use.

4. Enzymes

Papain (Difco) and chicken pancreas (Difco) were used at a concentration of 50 mg/g of liver tissue. Five ml aliquots of a stock solution containing 50 mg/ml in saline were stored at -20° until required.

5. Preliminary Treatment of Liver Tissue

One gram samples of liver tissue were weighed and homogenized with 5.0 ml of the appropriate buffered ascorbic acid. One millilitre of the appropriate enzyme stock solution was added and the volume made up to 20.0 ml with the same buffered ascorbic acid used in homogenization. The samples treated with papain were incubated in a water bath at 50° for 1 hour and then steamed for 20 minutes (6). The samples treated with chicken pancreas were incubated at 37° for 24 hours and then autoclaved for 10 minutes at 10 lb. p.s.i. (7). Enzyme blanks, omitting only the liver tissue, were prepared in the same way. After cooling, the samples were centrifuged at 3,000 r.p.m. for 10 minutes and the supernatants stored at -20° until assayed.

For the assay of either vitamin the samples were thawed and diluted to a stock solution of 1:1,000 with glass distilled water. From this stock further dilutions, where necessary, were made with glass distilled water.

6. Assay of Folate Activity

Samples were set up in duplicate, each tube containing 1.0 ml the appropriate sample dilution; 1.0 ml of fresh buffered ascorbic acid (pH 6.1); 3.0 ml of glass distilled water; and 5.0 ml of double strength assay medium

7. Vitamin B₁₂ Assay

Samples were set up in triplicate, each tube containing 2.0 ml of the appropriate sample dilution; and 2.0 ml of double strength assay medium

RESULTS

All results were corrected for the vitamin activity of the enzyme. Papain: folate activity 27 mμg/50 mg and vitamin B₁₂ nil. Chicken pancreas: folate activity 200 mμg/50 mg and vitamin B₁₂ 0.013 μg/50 mg.

Ten liver samples were assayed for folate activity after treatment with papain at pH levels of 4.6, 6.1 and 7.0 with the following results: pH 4.6, 1.3—6.1 μg/g (mean 3.8 μg/g); pH 6.1, 2.6—5.5 μg/g (mean 4.1 μg/g); and pH 7.0, 2.3—6.8 μg/g (mean 3.9 μg/g) (Table 1).

Duplicate samples of the same livers were assayed for folate activity after treatment with chicken pancreas using the same range of pH levels, with the fol-

TABLE 1
Effect of pH on Conjugate Cleavage Using Papain
 Results are expressed in $\mu\text{g/g}$

No.	pH level		
	4.6	6.1	7.0
1	4.5	4.0	4.0
2	5.7	4.3	4.6
3	2.2	5.0	2.3
4	5.3	3.8	4.7
5	4.6	5.5	4.0
6	6.1	5.4	6.8
7	1.3	2.6	2.3
8	3.3	3.5	3.3
9	3.2	3.6	3.7
10	2.2	3.0	3.7
Mean	3.8	4.1	3.9
SD	1.6	1.0	1.2

The standard error of the difference between the means: Group 1 and 2, 0.58; 2 and 3, 0.49; 1 and 3, 0.63. The differences between the means of the three groups are not significant.

lowing results: pH 4.6, 2.2–5.6 $\mu\text{g/g}$ (mean 3.9 $\mu\text{g/g}$); pH 6.1, 2.8–6.2 $\mu\text{g/g}$ (mean 4.3 $\mu\text{g/g}$); and pH 7.0, 3.6–7.0 $\mu\text{g/g}$ (mean 5.2 $\mu\text{g/g}$) (Table 2).

TABLE 2
Effect of pH on Conjugate Cleavage Using Chicken Pancreas
 Results are expressed in $\mu\text{g/g}$

No.	pH level		
	4.6	6.1	7.0
1	4.4	3.7	5.3
2	4.1	5.1	6.0
3	3.6	3.6	3.6
4	4.9	5.4	6.2
5	5.2	4.9	5.9
6	5.6	6.2	7.0
7	2.5	3.5	4.2
8	3.6	3.7	5.4
9	3.3	3.6	4.6
10	2.2	2.8	3.6
Mean	3.9	4.3	5.2
SD	1.1	1.00	1.1

The standard error of the difference between the means: Group 4 and 5, 0.47; 5 and 6, 0.47; 4 and 6, 0.49. The differences between the means of 4, 5 and 6 are not significant. The difference between the means of 4 and 6 appears to be significant.

Samples from the same liver were assayed for vitamin B₁₂ content after treatment with papain and chicken pancreas at pH levels of 4.6 and 7.0. A control sample omitting only the treatment with the enzyme was included. The results are tabulated in Table 3.

TABLE 3
Vitamin B₁₂ Concentration in Liver
 Results are expressed in $\mu\text{g/g}$

Enzyme used in digestion	pH level	
	4.6	7.0
Nil	1.2	1.3
Papain	1.2	1.3
Chicken pancreas	1.4	1.3

Twenty livers were assayed for both folate activity and vitamin B₁₂ levels after treatment with papain at pH 6.1 and chicken pancreas at pH 7.0. Treatment with papain gave the following results: folate activity, 1.3–7.8 $\mu\text{g/g}$ (mean 3.7 $\mu\text{g/g}$); vitamin B₁₂ concentration, 0.5–1.5 $\mu\text{g/g}$ (mean 0.9 $\mu\text{g/g}$). Treatment with chicken pancreas gave the following results: folate activity, 1.4–8.4 $\mu\text{g/g}$ (mean 3.5 $\mu\text{g/g}$); vitamin B₁₂ concentration, 0.5–1.3 $\mu\text{g/g}$ (mean 0.9 $\mu\text{g/g}$) (Table 4).

TABLE 4
Comparison of Treatment with Papain and Chicken Pancreas on Folate and Vitamin B₁₂ Recovery from Liver

No.	Folate		Vitamin B ₁₂	
	Papain (pH 6.1)	Chicken pancreas (pH 7.0)	Papain (pH 6.1)	Chicken pancreas (pH 7.0)
	$\mu\text{g/g}$		$\mu\text{g/g}$	
1	5.2	4.9	0.5	0.7
2	7.8	8.4	1.2	1.0
3	3.6	2.6	1.4	1.1
4	4.1	5.2	0.8	1.1
5	4.5	3.1	1.1	1.3
6	4.4	4.0	0.7	0.9
7	3.0	3.1	0.8	0.5
8	1.4	1.6	0.7	0.9
9	2.1	1.9	1.4	1.2
10	1.7	2.8	1.0	0.8
11	4.4	4.6	1.1	1.2
12	3.6	3.4	—	0.5
13	6.8	4.3	0.6	0.6
14	1.3	1.4	0.4	0.6
15	2.0	2.8	0.9	1.1
16	2.9	1.7	0.5	0.7
17	6.0	6.0	1.2	1.2
18	2.0	1.8	0.7	0.6
19	2.7	2.2	1.1	0.8
20	5.1	4.7	1.5	1.0
Mean	3.7	3.5	0.9	0.9
SD	1.8	1.7	0.3	0.3

The standard error of the difference between the two means is 0.55.
 Not significant.

The standard error of the difference between the two means is 0.09.
 Not significant.

DISCUSSION

The differences between the means in Table 1 were not significant, indicating that

within the limits of the experiment, pH has no effect on the cleavage of folate conjugates by papain. Treatment with chicken pancreas at pH 7.0 gave the greatest yield of folate activity. However, the differences between the means was statistically significant only between the extremes of the pH range tested.

Pitney and Onesti (6) suggested that treatment with papain increased the yield of vitamin B₁₂, but it is not clear whether the control samples were subjected to the same heating regime. They were of the opinion that while treatment with chicken pancreas increased the yield of folate activity, its vitamin B₁₂ content would make it unsuitable when both vitamins were required to be assayed. The results in Table 3 indicate that pH and enzyme treatment do not greatly affect the yield of vitamin B₁₂, which is dependent on heat for its release from the bound form.

The preliminary experiments (Tables 1 and 2) suggested that chicken pancreas at pH 7.0 would give a greater yield of folate activity. The results in Table 4 do not confirm this finding as the differences between the means are not statistically significant.

Folate activity is heat labile and even when protected by the use of ascorbic acid, prolonged exposure to heat is best avoided. This together with its lower vitamin content indicates papain as the enzyme of choice when both folate activity and vitamin B₁₂ have to be estimated on a single small sample.

SUMMARY

When assaying the vitamin B₁₂ and folate activity of material obtained by needle biopsy of the liver, it is important because of the small size of the sample to be able to choose an extraction method suitable for both estimations.

The release of vitamin B₁₂ from the bound form depends on heat rather than enzyme extraction, whereas the release of folate active material requires preliminary treatment with enzymes such as papain or chicken pancreas. Both materials were tested at various pH levels and both gave comparable results. However, the papain contained no vitamin B₁₂ activity and less folate activity than chicken pancreas and had the added advantage of requiring less heat treatment.

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