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CHROMATOGRAPHY OF FOLATES ON SEPHADEX G-10

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SUMMARY

Folates have been separated on columns of Sephadex G-10 using spectrophotometric and microbiological detection with *Lactobacillus casei*. The method is suitable especially for the separation of folic acid from other folates, and of dihydrofolic acid derivatives from their tetrahydrofolic acid forms. Distribution constants of seven standard folates have been determined. The method has been applied to the determination of folates in cow's milk. Chromatographic separation of conjugase treated and untreated samples of cow's milk has made it possible to distinguish between free and bound forms of folates.

INTRODUCTION

Many types of folates, which have properties similar to the vitamin folic acid, occur in foodstuffs. Besides the free forms of folates, there are also bound forms, polyglutamates, from which the free forms can be released by enzymatic hydrolysis. The vitamin activity of the individual types of folates is different and therefore many workers have tried to separate the different forms. Thus column chromatography on several kinds of ion-exchangers, *e.g.*, DEAE- and TEAE-cellulose[1–5], QAE-Sephadex A-25[6,7] and Dowex 1[8], gel chromatography on Sephadex G-15 and G-25[6,7,9] and affinity chromatography have been applied[10]. Different buffers in the pH range 6.0–9.5 have been used for elution[1,5,7]. Since several folates are easily oxidized by air or oxygen, some inhibitors of oxidation are usually added to the eluents. The substances most often employed for this purpose are 2-mercaptoethanol[11,12] and L-ascorbic acid[13,14].

Folates in the effluent can be detected in various ways. Spectrophotometric detection is limited by the relatively low sensitivity and many substances can cause interference. Microbiological tests are very sensitive (*ca.* 0.2 ng/ml) and are not affected by ascorbic acid used as anti-oxidant. Microorganisms having different specific sensitivities to various types of folates have also been applied. Radioisotopic

determination of folates labelled with ^{3}H or ^{14}C radionuclides is also a very sensitive method[15]. However, the microbiological test with *Lactobacillus casei* combined with spectrophotometric detection, is preferable for the determination of the distribution of various folates in model systems as well as in foodstuffs. The major advantage of this test is its sensitivity to all of the folates which exhibit vitamin activity.

This separation procedure has been applied to the determination of folates in cow's milk. Its application to other food materials is the subject of a further study.

EXPERIMENTAL

Materials

The folic acid standard was obtained from Calbiochem, Los Angeles, Calif., U.S.A. The folate derivatives were prepared in the Laboratory of Protein Metabolism, Charles University, Prague. Sephadex G-10 was obtained from Pharmacia, Uppsala, Sweden. Media for the microbiological assay, Bacto-Lactobacillus agar and Bacto-folic acid casei medium, were obtained from Difco, Detroit, Mich., U.S.A. Other chemicals used were supplied by Lachema, Brno, Czechoslovakia.

Preparation of hog-kidney conjugase

γ-Glutamyl-carboxypeptidase (EC 3.4.12.10) (conjugase) gradually splits off the terminal glutamic acids from polyglutamates to monoglutamates. Fresh hog kidney was homogenized with acetone which had been chilled to —10° and was quickly washed in a büchner funnel with a 20-fold excess of acetone[16]. The slightly pink powder which was obtained was then ground for 15 min with 10 ml/g of chilled McIlvain buffer (pH 4.6). Activated carbon was then added to remove the folates present (1.5 g per g of conjugase preparation). After filtration, the extract was frozen until required for use.

Preparation of samples

Synthetic folates (1–5 mg) were dissolved in 1 ml of 0.1 *M* sodium bicarbonate. Barium salts of some folates were dissolved in ammonium sulphate, and the precipitated barium sulphate was removed by centrifugation.

Two samples of pasteurized whole milk (4 ml) were hydrolyzed with 10 ml of McIlvain buffer (pH 4.6), containing 50 mg of ascorbic acid in 100 ml, in an autoclave at 121° for 10 min. After cooling, 0.2 ml of hog-kidney conjugase was added to one sample and the enzymatic hydrolysis of polyglutamates was carried out for 2 h at 37°. The conjugase was then inactivated by placing the tube containing the sample in a bath of boiling water for 5 min. The second sample was treated under identical conditions but without enzyme addition. Both samples were then adjusted to 20 ml with water, filtered and kept in a refrigerator until the chromatographic separation. Samples which were not chromatographed were diluted with McIlvain buffer (pH 7.0) in the ratio $1:10^2$ and assayed directly.

Chromatography of folates on Sephadex G-10

Samples (1 ml) of synthetic folates or extracts (0.1 ml) of cow's milk were applied to a Pharmacia K15/90 column (85 × 1.5 cm) of Sephadex G-10 which was equilibrated with 0.1 *M* phosphate buffer (KH_2PO_4 and Na_2HPO_4, pH 6.8) containing

10 mg of L-ascorbic acid in 100 ml. When stable synthetic folates were used L-ascorbic acid was omitted. The same buffer was used for elution at a flow-rate of *ca.* 24 ml/h. During the separations the column was protected against light with aluminium foil. Folic acid derivatives in the effluent were determined by spectral analysis[17,18], and simultaneously by microbiological assay with *Lactobacillus casei* after dilution of the individual fractions in the ratio $1:5 \cdot 10^5$ and $1:5 \cdot 10^6$.

In the case of the separation of folates in cow's milk it was only possible to use the microbiological assay procedure because of the low content of folates in the sample.

Microbiological assay of folates

The assay procedure was carried out according to ref. 19 with *Lactobacillus casei*, strain ATCC 7469, using the appropriate Difco medium. The inoculation medium was prepared from the test medium by adding folic acid to give a final concentration of 0.2 ng/ml. For the evaluation of the effluents, 0.5-ml portions from each fraction were added to 5 ml of the inoculation medium in a test-tube. The intensity of the Lactobacillus growth after incubation for 18–20 h at 37° was measured turbidimetrically at 540 nm using a Pulfrich photometer with Elpho objective adaptation (Carl Zeiss Jena, Jena, G.D.R.). In order to estimate the amount of folates in milk samples which were not chromatographed, the method of standard addition, as modified in our laboratory[20], as well as the usual method of a calibration graph, were used.

RESULTS

Separation of synthetic folates

Synthetic folates were chromatographed separately on Sephadex G-10, because the preparations were not of pure standard compounds, due to the instability of most of the compounds. Pure fractions of folates obtained in this way were used for spectral analysis as well as for the determination of the vitamin effect by microbiological tests.

TABLE I

SEPARATION OF FOLATES ON SEPHADEX G-10

Experimental conditions are as given in the text. The void volume (V_0) was 50 ml. All of the values of V_R are arithmetic means of two or three determinations.

Folate	*Molecular weight*	*Elution volume V_R (ml)*	*Distribution constant, K*	*Stability**
N^5-Methyltetrahydrofolic acid	459.5	65	0.15	u
N^5-Formyltetrahydrofolic acid	473.4	67	0.17	s
Tetrahydrofolic acid	459.5	77	0.27	u
N^{10}-Formylfolic acid	469.4	90	0.40	s
Dihydrofolic acid	443.4	95	0.45	s
N^{10}-Formyldihydrofolic acid	471.4	102	0.57	s
Folic acid	441.4	155	1.05	s

* s = Stable, addition of protecting agent (L-ascorbic acid) being unnecessary; u = unstable, addition of protecting agent being necessary.

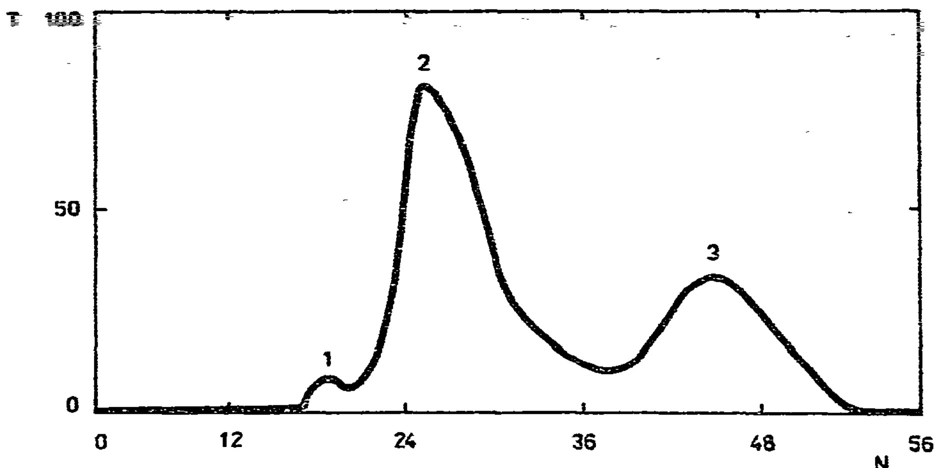

Fig. 1. Separation of synthetic folates on Sephadex G-10 recorded on a UV analyzer at 253.7 nm. N = Number of fractions each of 3.6 ml. Peaks: 1 = N^5-formyltetrahydrofolic acid; 2 = N^{10}-formylfolic acid; 3 = folic acid. The experimental conditions were as described in the text. In this case, phosphate buffer was used without L-ascorbic acid.

On the basis of these experiments, the elution volumes (V_R) and distribution constants (K) of seven folate derivatives were determined (Table I). The data demonstrate that Sephadex G-10 is suitable mainly for the separation of folic acid from all of its derivatives and for the separation of the reduced forms, especially dihydrofolic acid derivatives, from tetrahydrofolic acids. The resolution was not as good when the individual tetrahydrofolic acid derivatives were separated. This disadvantage could be compensated by separating these compounds on Sephadex G-15 or G-25 (ref. 9). On the other hand, the derivatives of folic and dihydrofolic acids could not be well separated on Sephadex G-10. Therefore the separation of folates on Sephadex G-10 could be used to supplement the present separation techniques. Examples of

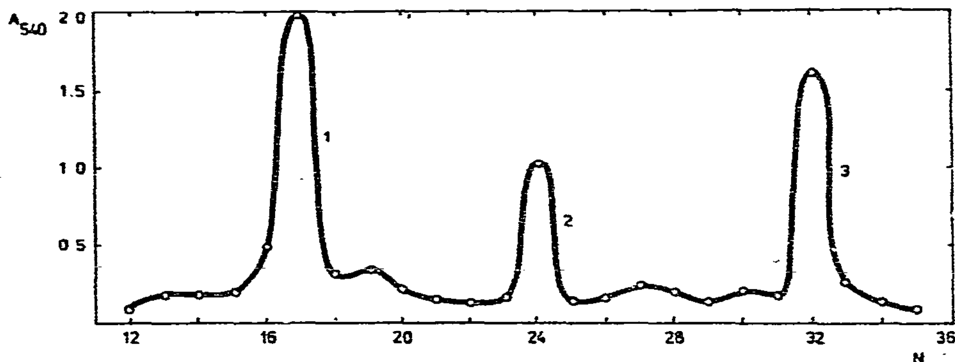

Fig. 2. Separation of synthetic folates on Sephadex G-10 as evaluated by the microbiological test with *L. casei*. N = Number of fractions each of 3.2 ml. Peaks: 1 = N^{10}-formyltetrahydrofolic acid; 2 = tetrahydrofolic acid; 3 = formyldihydrofolic acid. The experimental conditions were as described in the text. In this case, 10 mg of L-ascorbic acid were added to the phosphate buffer used for elution.

the separation of synthetic folates, detected by spectrophotometric or microbiological methods, are shown in Figs. 1 and 2, respectively.

Determination of folates in cow's milk

Samples of cow's milk were treated as described and then analyzed in the same way as the standard solutions. Because of the low content of folates in cow's milk, it was only possible to use a microbiological test for their detection. We were interested in determining whether polyglutamates, the vitamin effect of which differs from that of monoglutamates[12], are present in milk. Although the bound forms of folates are present in almost all kinds of plant and animal material, their occurrence in milk has not yet been proved satisfactorily. While Karlin[21] found a remarkable increase in folates in cow's milk after its treatment with chicken-pancreas conjugase, we did not find any increase in the vitamin effect when applying the hog-kidney conjugase preparation to fresh or powdered milk (Table II). This is why we applied the separation on Sephadex G-10 to the samples of milk extracts which were either treated or untreated with the conjugase preparation. Both experiments are compared in Fig. 3. The upper elution diagram shows the different folate derivatives detected by *L. casei* before enzymatic hydrolysis, the lower elution diagram shows the derivatives detected after enzymatic hydrolysis. Both elution diagrams represent a satisfactory separation of the folate derivatives in a number of fractions.

TABLE II

CONTENTS (μg/100 g) OF FOLATES IN MILK

Sample	*"Free" before hydrolysis*		*"Total" after hydrolysis*	
	Standard addition	*Calibration graph*	*Standard addition*	*Calibration graph*
Fresh milk (pasteurized)	2.5	1.8	2.5	2.0
Powdered milk	20.0	23.0	20.0	23.0
Fresh milk (pasteurized) after Karlin[21]	—	3.7	—	5.9

The intensity of peaks 2, 4, 5 and 6 in Fig. 3 is much lower after hydrolysis than before the hydrolysis. It seems likely that these peaks are due to various polyglutamates (containing most probably two or three glutamic acids). Their identification will require further investigations. On the other hand, the amount of some free forms of folates increased after enzymatic hydrolysis, *e.g.*, peak 1 due to free N^5-methyltetrahydrofolic acid. This observation is in agreement with those of Ford *et al.*[22] who found that the content of this folate in cow's milk is the highest in comparison with its analogues. Peak 3 most likely represents N^{10}-formyldihydrofolic acid and again increased in intensity after enzymatic hydrolysis. The intensity of peak 7, corresponding to free folic acid, also increased after hydrolysis. Undoubtedly, the increased concentration of these three free forms of folate derivatives must be due to their release from polyglutamates. The analysis of conjugase treated and untreated milk extracts on Sephadex G-10 therefore also indicates the possibility of the occur-

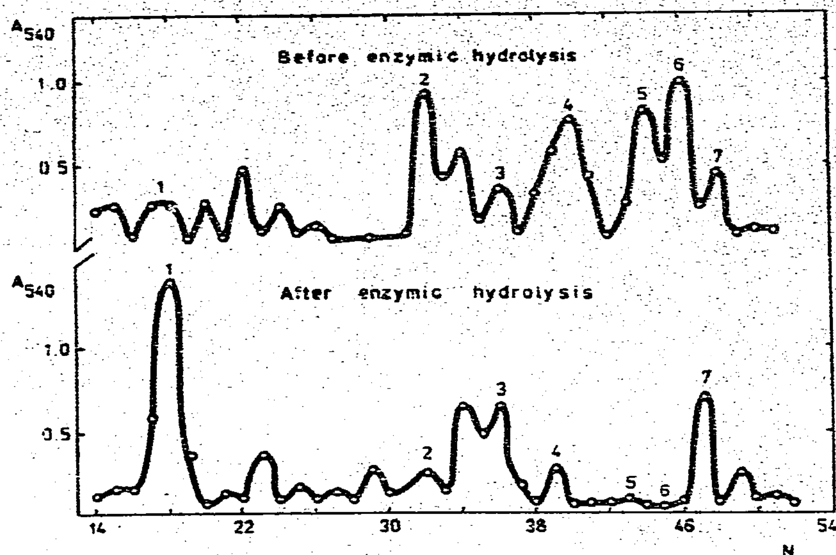

Fig. 3. Separation of folates from pasteurized whole milk on Sephadex G-10 as evaluated by the microbiological test with *L. casei*. N = Number of fractions each of 3.3 ml. Peaks: 1 = N^5-methyltetrahydrofolic acid; 2, 4, 5 and 6 = unidentified polyglutamates; 3 = N^{10}-formyldihydrofolic acid; 7 = folic acid. The experimental conditions were the same as in Fig. 2.

rence of polyglutamates in milk. Provided that bound forms of folates are present in milk, one may assume that their vitamin effect is the same as that of the free folates released from polyglutamates by enzymatic hydrolysis (Table II).

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