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SYNTHESIS AND ISOLATION OF FOLINIC ACID

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The first reference to folinic acid (I) was in 1948 [1], when it was shown that a substance isolated from sheep liver and also present in rice bran and yeast extracts was a growth factor for *Leuconostoc citrovorum* 8081. It was observed almost simultaneously [2] that natural folinic acid removed the inhibitory effect of aminopterin on *L. citrovorum*.

Folinic acid (5-formyl-5,6,7,8-tetrahydrofolic acid, leucovorin, citrovorum factor (CF), N-{4-[(2-amino-5-formyl-1,4,5,6,7,8-hexahydro-4-oxo-6-pteridinyl)methyl]amino}-benzoyl-L-glutamic acid) is one of the coenzyme forms of folic acid (II). Reduced forms of folic acid, which are cofactors for a series of enzyme systems responsible for the transfer of one-carbon groups (formyl, hydroxy-methyl, formiminyl, methyl) to the respective acceptors, participate in metabolic processes in the biosynthesis of purines, pyrimidines, and amino acids. Folinic acid is the immediate cofactor of glutamate formyltransferase (EC 2.1.2.6), which is responsible for the reversible formulation of L-glutamic acid [3], of 5,10-methenyltetrahydrofolate synthetase (EC 6.3.3.2), which participates in the formation of 5,10-methenyltetrahydrofolic acid [4], and also of formyltetrahydrofolate synthetase (EC 6.3.4.3), which catalyzes the synthesis of 10-formyltetrahydrofolic acid [5], and subsequently inosinic acid. The latter two enzymatic reactions proceed with the participation of ATP.

The folinic acid molecule has two centers of asymmetry: one at the C(6) of the pteridine ring, the other at the α-carbon atom of the L-glutamyl residue. Natural folinic acid is the (−)-L-diastereomer in which C(6) has an S configuration [6]; synthetic folinic acid (±)-L-folinic acid, is a mixture of the diastereomers with R and S configurations. The biological activity of natural folinic acid is twice that of the synthetic acid.

Lately the interest in folinic acid has arisen significantly in relation to its application in medicine as an antidote in the treatment of malignancies with large methotrexate doses [7-9].

The first folinic acid syntheses were described in 1951-1952 [10-11]. In these syntheses folic acid was formylated to N(10)-formylfolic acid, which was then reduced at the pyrazine ring to give N(5), N(10)-methenyltetrahydrofolic acid (III). Subsequent hydrolysis of compound III in the presence of sodium hydroxide gave a mixture of substances containing 20–25% folinic acid. Pure folinic acid was obtained by a multiple chromatographic purification (on starch, florisil). The yield of folinic acid in its calcium salt form did not exceed 10%. Another synthesis is described [12], proceeding from the labile 5,6,7,8-tetrahydrofolic acid (IV). Compound IV is formylated by formic acid to N(5), N(10)-methenyltetrahydrofolic

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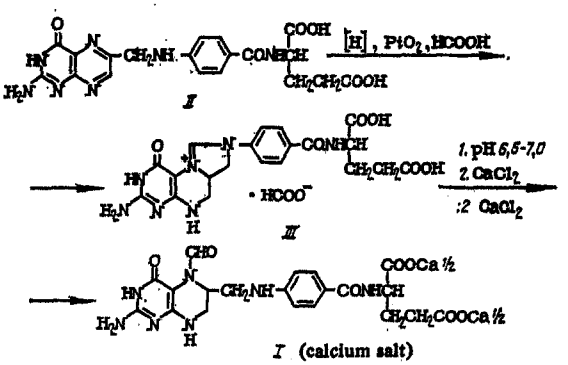
acid, which in then hydrolyzed in an alkaline medium, with purification of the folinic acid formed by repeated chromatography on DEAE-cellulose. Recently it was established [13] that formylation of 5,6,7,8-tetrahydrofolic acid with methyl formate in a mixture of DMSO with pyridine (5:1) led directly to folinic acid which requires, however, chromatographic purification. A folinic acid synthesis from folic acid was published in 1979 [14] which proceeds with a 45% yield and an approximate 78% purity of the resulting calcium salt of folinic acid. Folic acid was reduced with a 12-fold excess of sodium borohydride followed by formylation of the resulting 5,6,7,8-tetrahydrofolic acid by formic acid in the presence of trifluoroacetic acid (approximately 2%). Hydrolysis of the N(5),N(10)-methenyltetrahydrofolic acid obtained was conducted in a neutral or weakly alkaline medium (pH 6.2-7.0), in contrast to earlier works [10-12], which resulted in a decreased amount of impurities in the folinic acid. There is evidence [15] that opening of the imidazoline ring of N(5),N(10)-methenyl-tetrahydrofolic acid also proceeds with good yield when organic amines (methylamine, triethylamine, morpholine, etc.) are used.

We carried out the synthesis of folinic acid (I) in the following manner. Folic acid was converted to N(5),N(10)-methenyltetrahydrofolic acid (III) in one stage by catalytic hydrogenation over the Adams catalyst in formic acid at room temperature and atmospheric pressure with subsequent heating of the reaction mixture in a nitrogen atmosphere. Compound III was purified by chromatography on cellulose in formic acid in the presence of mercaptoethanol. The overall yield of compound III was approximately 45% with a purity of 80-85%. We carried out its hydrolysis, for imidazoline ring opening and folinic acid formation, at various pH values. Data indicating that optimal results are obtained by hydrolysis at pH 6.0-7.0 [14] fully corroborate our investigations. The best yields (more than 30%) of the purified calcium salt of folinic acid (I) were obtained via hydrolysis of compound III by boiling its aqueous solution at pH 6.5-7.0. Hydrolysis of compound III at pH 7.5-8.0 resulted in a low folinic acid yield (about 25%), and at pH 12.0 for 1 h at 90-95°C, the yield of compound I was less than 15%.



The folinic acid isolated from the reaction mixture after hydrolysis of compound III contains small amounts of 10-formylfolic acid, 7,8-dihydro-10-formylfolic acid, *p*-aminobenzoyl-L-glutamic acid, and 6-hydroxymethylpterin and insignificant unidentified impurities.

Folinic acid was purified by a chromatographic method as well as by other means [14]. Chromatography was conducted on a cellulose column in 0.1 M phosphate buffer, pH 7.0, and in borate buffer, pH 7.8. The folinic acid fractions obtained during chromatography in phosphate buffer were adsorbed on carbon and desorbed with a mixture of ethanol, ammonium hydroxide, and water (4:1:3). Folinic acid was isolated as the calcium salt from a concentrated extract by alcohol precipitation. As a result of chromatographic purification, the calcium salt of folinic acid was obtained in an amount greater than 92% (on the basis of anhydrous substance). Compound I was determined by comparison with a standard in paper chromatography in 0.1 M phosphate buffer, pH 7.0, with subsequent elution of the corresponding spots and spectrophotometric analysis.

As established previously [14], the calcium salt of folinic acid can be isolated from the reaction mixture after hydrolysis of compound III in amounts of 75-79% by dilution with alcohol. We attempted to further purify this product, not by a chromatographic method, but by recrystallization, reprecipitation, or complexation with nitric acid [16]. Reprecipitation

of the product with alcohol from a 5% aqueous solution previously treated with activated carbon led to a calcium salt yield of about 60% with a content of more than 90%, on the basis of anhydrous substance.

Thus, as a result of our investigations, we have developed a one-step method of obtaining N(5),N(10)-methenyltetrahydrofolic acid via catalytic reduction of folic acid in formic acid followed by heating in a nitrogen atmosphere and a method for hydrolysis of N(5),N(10)-methenyltetrahydrofolic acid to folinic acid. Methods are proposed for folinic acid purification by chromatography on a cellulose column in phosphate and borate buffers and for reprecipitation of the calcium salt of folinic acid by alcohol from a 5% aqueous solution treated with activated carbon. The calcium salt obtained showed an activity analogous to that of a known preparation in studies of its effect on the antitumor activity of methotrexate and its reduction of the toxic effects of the latter.

EXPERIMENTAL

(+)-L-N(5),N(10)-Methenyl-5,6,7,8-tetrahydrofolic acid, formate (III).

To 2 g of 98% (in anhydrous substance) folic acid (II) mixed with 80 ml of 98% formic acid was added 0.5 g of platinum oxide, and reduction was carried out at room temperature and normal pressure until hydrogen consumption ceased (depending on the degree of hydrogenation, the reddish-brown reaction mixture acquires a yellow color). When hydrogenation was complete, the mixture was heated to 90-92°C in a nitrogen atmosphere over a 40 min period, then cooled to room temperature and the catalyst filtered off. The filtrate was concentrated to a volume of about 10 ml by evaporation *in vacuo* at a temperature no higher than 40°C and then mixed with 150 ml acetone. The resulting precipitate was separated by filtration, washed with acetone, and dried, giving 2 g of compound III with a content of about 60% (for the pure substance $\varepsilon = 26.5 \times 10^3\ M^{-1}\ cm^{-1}$ at λ_{max} 348 nm in 0.1 N HCl [17]). The technical product was purified by column chromatography (60 × 350 mm) in 0.1 M formic acid with 2-mercaptoethanol (0.01 M), with a solvent passage rate of 1.2-1.5 ml/min. Fraction recovery began with the appearance of the first colored drops of eluate shortly before elution of the first rapidly moving yellow band. Fractions were monitored by paper chromatography in 1 M formic acid with 0.01 M 2-mercaptoethanol and UV spectroscopy. Those fractions for which $D_{355}/D_{268-275} > 1.6$ were combined and lyophilized, and the resulting amorphous powder was washed with small amounts of alcohol and dessicated *in vacuo* over P_2O_5. We obtained 1.2 g of compound III with a content of about 80%, yield 45.4%; R_f 0.4-0.5, spot with white fluorescence in UV light in descending paper chromatography (Whatman No. 1) in 1 M formic acid with 0.01 M 2-mercaptoethanol.

Calcium salt of (+)-L-5,6,7,8-tetrahydrofolic acid (I).

Compound III, 3 g with a content of about 80%, was hydrolyzed in 75 ml water at pH 6.5-7.0 at 100°C over an 11 h period according to the method in [14]. Isolation and purification of the folinic acid formed proceeded by three methods.

A. The reaction mixture was concentrated to a volume of 20 ml *in vacuo* and subjected to chromatography on a cellulose column (50 × 300 mm) in 0.1 M phosphate buffer, pH 7.0, containing 0.02 M 2-mercaptoethanol. Fractions were monitored by UV spectroscopy. Fractions with λ_{max} 282-284 nm and λ_{min} 242 min for which $D_{282-284}/D_{242} > 2.0$ were combined, acidified to pH 4.0 with 10% HCl, and mixed for 30 min at 20°C after the addition of 12 g of activated carbon. The carbon was removed by filtration, washed with distilled water, thoroughly pressed out, and extracted in several stages with 200 ml of a hot ethanol—ammonium hydroxide—water mixture, 4:1:3. The extract was concentrated to a volume of 40 ml, the pH was brought up to 7.0, and a solution of 1 g $CaCl_2$ in 3 ml water was added. After mixing for 10-15 min, the pH was adjusted to 12.5 with 5 N NaOH, and the resulting precipitate was separated by filtration. The filtrate was acidified to pH 7.0-7.2 with 10% HCl. When a precipitate formed during this procedure, it was removed. To the clear, bright yellow filtrate (about 50 ml) were added 5 ml ethanol, the precipitate formed after cooling was separated by filtration, and 150 ml alcohol were added to the filtrate. The resulting suspension was kept at 3-5°C for 2 h. The precipitate was separated by filtration and dried *in vacuo* over P_2O_5, giving 0.9 g of the calcium salt of folinic acid with a content of 83%, yield 30.5%, moisture content 10.5%, $D_{282}/D_{242} > 4.5$, R_f 0.75-0.85 in paper chromatography

in 0.1 M phosphate buffer, pH 7.0, containing 0.5% 2-mercaptoethanol. Insignificant amounts of 10-formylfolic acid (R_f 0.85-0.9), 7,8-dihydro-10-formylfolic acid (R_f 0.6-0.7), *p*-aminobenzoyl-L-glutamic acid (R_f 0.94-0.98), 6-hydroxymethylpterin (R_f 0.4-0.45), and insignificant unidentified substances (R_f 0.2-0.25, yellow fluorescence, and R_f 0.03-0.05) were present as impurities in the obtained calcium salt of folinic acid (according to UV spectra and paper chromatography in 0.1 M phosphate buffer, pH 7.0, together with reference compounds).

B. After hydrolysis the reaction mixture was concentrated to 3 ml and subjected to chromatography on a cellulose column (40 × 300 mm) in 0.2 M borate buffer, pH 7.8, collecting fractions of 15-20 ml. The fractions for which D_{282}/D_{242} in the UV spectrum (in 0.1 N NaOH) was equal to or greater than 3.5 were combined (approximate 200 ml total volume), and a solution of 0.8 g $CaCl_2$ in 6 ml water was added followed by 20 ml of ethanol. The mixture was cooled at 2-5°C for 2 h and the yellow precipitate filtered off and discarded. To the filtrate were added 620 ml of ethanol, and the mixture was cooled for 3 h at 0-5°C. The resulting precipitate was separated by filtration, washed in the filter with alcohol, and dried *in vacuo*, giving 1.87 g of a substance which, when reprecipitated with alcohol from a 5% aqueous solution, gave 0.97 g of the calcium salt of folinic acid with a content of 86.0%, moisture content 10.3%. The yield was 32.8% (from III). D_{282}/D_{242} = 4.8.

C. (According to the method in [14]). The solution was kept in a stream of nitrogen for 8 h at 20°C, followed by addition of 3 ml of a solution containing 1.3 g $CaCl_2$ at pH 7.6-7.8. After 10-15 min, 0.1 volume of alcohol was added to the reaction mixture (pH about 7.5), and the suspension was cooled for 2-3 h at 0-5°C. The yellowish-brown precipitate was removed by filtration and 260 ml alcohol were added to the clear filtrate. The precipitate resulting after cooling for 12 h at 0-5°C was separated by filtration, washed with alcohol, and dried *in vacuo* over P_2O_5, giving 2.2 g of the calcium salt of folinic acid with a constant of 78.5%, yield 70.4%. The calcium salt obtained was further purified. A 5% aqueous solution was prepared, 0.9 g activated carbon added, and after mixing at room temperature for 20-30 min, the carbon was removed by filtration. To the filtrate was added 0.1 volume of alcohol, the mixture was cooled for 2-3 h at 0-5°C, the precipitate was filtered off, and 3 volumes of alcohol were added to this filtrate followed by cooling at 0-5°C for several hours. The precipitate was separated by filtration, washed in the filter with alcohol, and dried *in vacuo* over P_2O_5, giving 1.26 g calcium salt with a content of 82.4%, moisture content 10.1%. The yield after reprecipitation was 60.1%, the yield calculated from III, 42.3%. D_{282}/D_{242} = 4.3.

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