A METHOD FOR THE PREPARATION OF TETRAHYDROFOLIC ACID

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Procedures commonly employed for the preparation of tetrahydrofolic acid involve the catalytic hydrogenation of the pyrazine ring of folic acid (O'Dell et al., 1947) and isolation of the reaction product under conditions which avoid oxidative degradation of the product. This communication describes a rapid and simple method to give a dl-tetrahydrofolic acid preparation suitable for biochemical studies. It involves the use of $Na_2S_2O_4$ (Warburg, Christian and Griese, 1935) for the reduction of folic acid in the presence of ascorbate and purification of the product on DEAE-cellulose (N, N-Diethylaminoethyl cellulose columns (Sober and Peterson, 1954; Usdin and Porath, 1957; Rabinowitz, 1959; Mathews and Huennekens, 1960).

The procedure is essentially that previously developed in this laboratory for the synthesis of dihydrofolic acid (Futterman, 1957) and modified by raising the temperature of the reaction mixture. The synthesis was carried out as follows: 35 mg. of commercial folic acid (90% purity) were suspended in 1 ml. of distilled water and dissolved by the dropwise addition of N-KOH. 3.0 ml. of a solution of potassium ascorbate (pH 6.0, 100 mg./ml.) and 200 mg. of $Na_2S_2O_4$ were then added. The reduction was allowed to proceed at 75° for 90 min. after which the reaction mixture was cooled to 0°. The conversion yield based on microbiological assay was 68% of the theoretical.

To obtain a preparation free of folic and dihydrofolic acids an aliquot was purified by chromatography on DEAE-cellulose. A 50:50 mixture of DEAE-cellulose and Hyflo Super-Cel was packed to a volume of 15 ml. in a conven-

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tional 50 ml. burette and prepared by running through 25 ml. of 0.5 M phosphate buffer, pH 6.0. After the wash of the column was free of phosphate, one-fifth of the chilled reaction mixture was layered carefully at the top of the adsorbant. When the solution had percolated into the column and washed with several ml. of 1% mercaptoethanol the tetrahydrofolate was recovered by gradient elution in which the mixing chamber contained 40 ml. of 1% mercaptoethanol and the reservoir 0.5 M phosphate buffer pH 6 containing 1% mercaptoethanol. The solvents had been chilled to 0° prior to use and the chromatography was carried out at 23°. The flow rate of the eluant under gentle pressure was 1 ml. per minute and 20 five ml. fractions were collected. 75% of the dl-tetrahydrofolic acid was recovered in fractions 11 and 12 and stored at -20° as such. On an analytical column of DEAE-cellulose (characterizing microgram quantities of folate activities) with an ascorbate-phosphate eluant, the product gives a peak at tube 15, as does tetrahydrofolic acid prepared by catalytic hydrogenation or enzymatic reduction. On the same column N^{10}-formyl tetrahydrofolic acid shows a peak at tube 8, N^{5}-formyl tetrahydrofolic acid at tube 12, dihydrofolic acid at tube 26 and folic acid at tube 29.

Absorption spectra of the eluted fractions were determined in 0.1 M phosphate buffer, pH 7.5 containing 1% mercaptoethanol in a Beckman DU spectrophotometer. The active samples showed an absorption maximum at 297 mμ and the absorption ratios 297:320 and 297:340 were 2.12 and 26.2 respectively. In phosphate buffer containing 0.03% mercaptoethanol an absorption minimum is observed at 240-244 mμ.

TABLE I

P. cerevisiae activity and absorption of active fractions

Fraction no.	9	10	11	12	13	14	15
Density Units at 297 mμ per 5 ml. fraction	7.35	21.0	76.5	114.0	24.7	4.35	3.9
Mg. l-tetrahydrofolic acid (microbiological) activity per 5 ml. fraction	.045	.172	.675	.875	.145	.025	.022

The microbiological response for THFA was measured using the aseptic assay for tetrahydrofolic acid with P. cerevisiae as the test organism. (Bakerman, unpublished data). The microbiological activity of the eluted samples paralleled the absorption at 297 mμ (see table).

As is to be expected of tetrahydrofolic acid, on aeration the synthesized product lost its growth activity for P. cerevisiae and on heating at 75° for ½ hour in presence of an excess of formic and ascorbic acids, it gave a product with the microbiological growth response and chromatographic properties of N^{10}-formyl tetrahydrofolic acid.

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