THE EXTINCTION COEFFICIENT OF PURIFIED TETRAHYDROFOLIC ACID

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Received January 4, 1965

Earlier studies (O'Dell et al., 1947; Hatefi et al., 1960; Rabinowitz, 1960) concerning the determination of the extinction coefficient of tetrahydrofolic acid (THFA) have used, for the most part, material which was not highly purified. Moreover, even the concentration of purified THFA has not been estimated by any direct means because, due to its extreme lability, THFA cannot be weighed. Its concentration has been based on the assumption that all of the weighed starting material (i.e., folic acid) is reduced to THFA. Certain investigators (Kalckar et al., 1950) have used the spontaneous breakdown of THFA to *p*-aminobenzoylglutamic acid (PABG) and pteridine as a means for determining the concentration of THFA. However, at least one worker (Zakrzewski, 1960) has maintained that THFA does not oxidize quantitatively to PABG and pteridine. This note is concerned with a series of determinations of the extinction coefficient of highly purified THFA. Since methods had to be used for promoting complete conversion of THFA to PABG, the concentration of THFA in the samples has been determined directly by using the method

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of Hutchings et al. (1947). This method was originally proposed by them as a means for estimating the concentration of folic acid. It was felt that the amount of amine formed after zinc reduction of THFA would also give a true representation of the amount of this factor present - provided the samples contained THFA alone.

The method of preparation and purification of THFA was essentially that of Silverman and Noronha (1961) with certain modifications to increase the flow rate through the chromatographic column and to avoid oxidation of THFA. DEAE cellulose resin was charged with 0.5M, pH 6.0 $KHPO_4$ buffer. Then it was washed with distilled water until the washings were completely free of phosphate as tested by the Fiske and SubbaRow method (1925). The treated resin was then packed to a height of 15 cm in a 1 cm x 35 cm chromatography tube fitted with a fritted glass disc. The closed gradient elution system was first flushed with argon for 20 minutes before the reaction mixture was introduced. Elution of the fractions was accomplished with gentle argon pressure.

Certain precautions were taken to retard the oxidation of THFA. Folic acid was reduced under an atmosphere of flowing argon gas. The reaction mixture containing THFA was removed through a hypodermic syringe without disturbing the argon atmosphere in the reaction vessel. The mixture was then injected directly into the preflushed system described above. One percent 2-mercaptoethanol was included in both the preparation and development systems.

The absorption spectrum of aliquots of the eluted fractions was determined in 0.1M, pH 7.0 Sorensen's phosphate buffer containing 0.5

percent 2-mercaptoethanol. Samples containing the purified THFA displayed a typical absorption curve (Rabinowitz, 1960) with a maximum at 297 mμ. The presence of THFA in the fractions which showed an absorption maximum at this wavelength was authenticated further by assaying microbiologically with Pediococcus cerevisiae ATCC 8081 using the technique of Bakerman (1961).

The concentration of THFA in micromoles per ml (C) in the above samples was calculated from the following equation, which is derived from the information given by Hutchings et al., (1947) for the estimation of folic acid:

$$\text{Eq. 1.} \quad C = A_t \times \left(\frac{MW_{THFA}}{MW_{PABA}} \right)$$

A_t = Micromoles diazotizable amine produced per ml following reduction with zinc (total amine)[1/]

MW_{THFA} = Molecular weight of THFA (445.4)

MW_{PABA} = Molecular weight of p-aminobenzoic acid (used as standard) (137)

The extinction coefficient (ϵ) could then be calculated:

$$\text{Eq. 2} \quad \epsilon = \frac{E}{C} \times 1000$$

E = Absorbance of purified THFA at 297 mμ

C = Concentration of THFA as determined in equation 1.

The results of a series of separate analyses of purified THFA are given in Table 1. There was no consistent value obtained for the extinc-

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1/ The amount of amine produced was measured colorimetrically by the Bratton-Marshall (10) procedure using the Klett-Summerson photoelectric colorimeter with filter #54. Control tests of reagents without THFA revealed that there was no interference with the color reaction. All determinations were carried out in duplicate.

tion coefficient of THFA; it ranged between 25,600 and 39,000. These values are considerably higher than those previously reported for THFA; they ranged between 19,000 and 25,000 (O'Dell et al., 1947; Hatefi et al., 1960; Rabinowitz, 1960). An average of our results as given in Table 1 places the extinction coefficient for THFA at approximately 32,000.

Table 1

EXTINCTION COEFFICIENTS FOR TETRAHYDROFOLIC ACID

Analysis Number	Extinction Coefficient *
1	32,050
2	30,510
3	31,500
4	35,866
5	39,000
6	33,950
7	25,600

REFERENCES

Bakerman, H.A., Anal. Biochem. 2, 588 (1961).
Bratton, A.C., and Marshall, E.K., Jr., J. Biol. Chem. 128, 537 (1939).
Fiske, C.H., and SubbaRow, Y., J. Biol. Chem. 66, 375 (1925).
Hatefi, Y., Talbert, P.T., Osborn, M.J., and Huennekens, F.M., Biochem. Prep. 7, 89 (1960).
Hutchings, B.L., Stokstad, E.L.R., Boothe, J.H., Mowat, J.H., Waller, C.W., Angier, R.B., Semb, J. and SubbaRow, Y., J. Biol. Chem. 168, 705 (1947).
Kalckar, H.M., Kjeldgaard, N.O., and Klenow, H. Biochim. Biophys. Acta 5, 575 (1950).

*Each figure in the Table is an average value obtained for two to four separately prepared samples of THFA analyzed on the same day.

O'Dell, B.L., Vanderbilt, J.M., Bloom, E.S., Pfiffner, J.J., J. Am. Chem. Soc. 69, 250 (1947).

Rabinowitz, J. in "The Enzymes" (Boyer et al. Eds.) Vol. 2, 2nd Ed., 185, Academic Press, New York, 1960.

Silverman, M., and Noronha, J.M., Biochem. Biophys. Res. Comm. 4, 180 (1961).

Zakrzewski, S.F., J. Biol. Chem. 235, 1776 (1960).