

a marked instability of the active principle to oxidation, reduction, acid, alkali, light, dry heat, acylation, esterification, methylation, benzylation, nitrous acid, bromine, hypobromite and certain other specific reagents. There is considerable evidence that some of these reactions involve structural changes that are of a minor nature with respect to the whole complex molecule. For example, partial destruction of physiological activity with alkali takes place very rapidly and with dilute reagents but a long and vigorous treatment is necessary for complete destruction. A similar situation appears to exist in the case of light destruction. With acid destruction physiological activity is lost rapidly but absorption spectra data¹⁰ indicate that only minor modifications of the folic acid molecule are involved. It therefore appears evident that folic acid may be converted by various treatments to other very closely related substances which may have quite different physiological properties than the original.

A summation of analyses of our concentrates combined with information on molecular weight

indicated that the formula of folic acid may be approximated by $C_{15}H_{15}O_8N_5$. Absorption spectra data¹⁷ indicate the presence of a structural unit similar to xanthopterin. Side chains or other rings of an unknown nature but lacking nitrogen or a sugar residue, are indicated according to our evidence. The explanation of the high oxygen content must await further investigation.

Summary

Reactions and stability of folic acid are considered with reference to concentration problems.

The probability is pointed out that a large proportion of the impurities in purified concentrates are of a very similar nature to folic acid. On the basis of analyses of some of these concentrates an approximate empirical formula of $C_{15}H_{15}O_8N_5$ is given.

The absence of a sugar or poly hydroxy group, and the probable presence of a xanthopterin-like structural unit are indicated.

(17) Mitchell, *THIS JOURNAL*, 66, 274 (1944).

AUSTIN, TEXAS

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[CONTRIBUTION FROM THE UNIVERSITY OF TEXAS, BIOCHEMICAL INSTITUTE, AND THE CLAYTON FOUNDATION FOR RESEARCH]

Folic Acid. IV. Absorption Spectra

BY HERSCHEL K. MITCHELL

It became evident from precipitation reactions, in the early stages of concentration, that folic acid was probably related to the pyrimidines, which show a strong absorption of ultraviolet light. There appeared to be a good possibility that such absorption might serve as a basis for quick quantitative determinations of the substance as well as an aid to chemical identification by showing specific absorption bands. It was also hoped that modification of folic acid by various chemical reactions would alter its absorption spectrum in such fashions as to yield information on the structure of the substance.

For comparative purposes (Figs. 1-4) the absorption data for concentrates are given in terms of extinction for a 1-cm. layer of 1% solution. This is represented according to the notation of Morton¹ by $E_{1\text{cm}}^{1\%}$. The data in Figs. 5-11 are plotted in terms of molecular extinction coefficients since most of the substances concerned are pure compounds. In Fig. 11 the molecular weight of the folic acid sample is assumed to be 400.²

All of the absorption work was carried out using a Beckman spectrophotometer and a 1-cm. cell.

(1) Morton, "The Application of Absorption Spectra to the Study of Vitamins, Hormones and Coenzymes," Adam Hilger, Ltd., Camden Road, London, 1942, p. 10.

(2) Mitchell and Williams, *THIS JOURNAL*, 66, 271 (1944).

Experimental

Absorption Curves of Different Concentrates.—The absorption curves for folic acid concentrates covering a wide range of physiological potency are given in Fig. 1. These are illustrative only and represent materials of widely diversified treatment. It is evident that there is little parallelism between absorption and physiological activity. In certain high potency preparations, however, such a parallelism was found. In many low potency preparations, especially among those whose lack of physiological activity was probably due to destruction during fractionation rather than a lack of sufficient fractionation, a high degree of absorption was shown. This is illustrated in Fig. 1 by the two samples of potency 2,400 and 12,000, both of which had undergone more fractionation than the potency 28,000 sample and weight for weight represented much more raw material than either of the high potency samples. It therefore appears probable that concentration processes cause partial inactivation of the folic acid without altering the complex molecule sufficiently to cause a great change in light absorption. This supposition was corroborated by purposely destroying the physiological activity and following the treatment by a determination of the absorption spectra of the products.

Absorption of Inactivated Folic Acid.—Samples of folic acid of potency 33,000 were treated with 1 *N*, 4 *N* and 16 *N* sulfuric acid for one hour at 100°. All of the treatments are sufficient to destroy the folic acid activity. The absorption spectra of these products are compared with the original untreated sample in Fig. 2. It is evident that the molecular structure responsible for the light absorption is not destroyed by these treatments though some changes are produced.

Light absorption curves for products of treatment of folic acid with ultraviolet light in the presence of oxygen

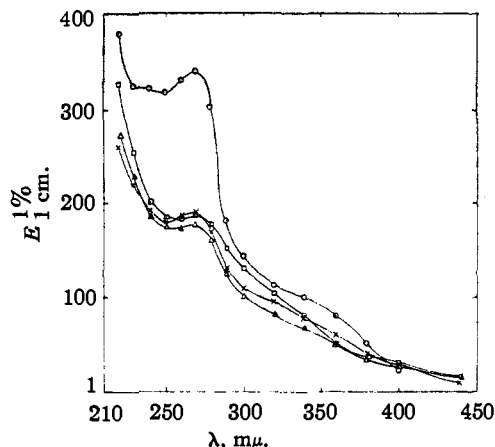


Fig. 1.—○—○—○, potency 75,000; ×—×—×, potency 30,000; △—△—△, potency 12,000; □—□—□, potency 2,400.

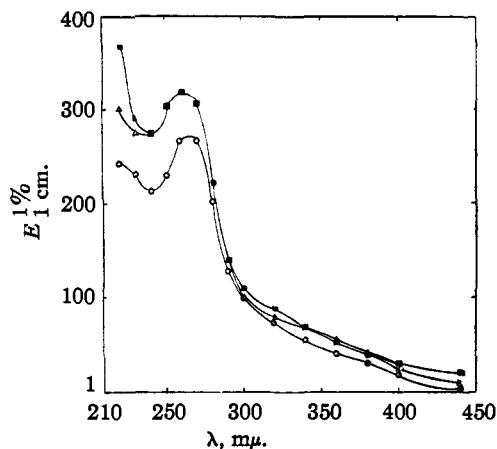


Fig. 2.—○—○—○, untreated sample, potency 33,000; ×—×—×, 1 N H₂SO₄, potency 33,000; □—□—□, 4 N H₂SO₄, potency 33,000; △—△—△, 16 N H₂SO₄, potency 33,000.

and in the absence of oxygen are given in Fig. 3. The physiological activity was destroyed in both cases. A curve in Fig. 3 represents the absorption curve for a benzylated sample of folic acid. This was prepared by heating 10 mg. of silver folate (potency 12,000) for two hours at 100° with 1 ml. of benzyl chloride. The excess benzyl chloride was removed in vacuum. The product was physiologically inactive.

From these representative curves it appears probable that in spite of easy destruction of the physiological activity of folic acid the nucleus of the molecule is quite stable to varied treatments. The complete destruction by ultraviolet light in the presence of oxygen is attributed to the presence of ozone as well as to the light energy itself. Ozone in the dark produces the same effect though much more slowly. The supposition previously set forth that a large portion of the impurity in many folic acid concentrates consists of inactivated folic acid that still maintains its general structure, seems to be supported by these findings on absorption spectra of concentrates in which the folic acid has been purposely destroyed.

Absorption Spectra of Similar Compounds.—Only one compound has been found that has absorption characteristics with a marked similarity to these folic acid concentrates. A comparison of the absorption spectrum of this compound (xanthopterin) with that of a folic acid concen-

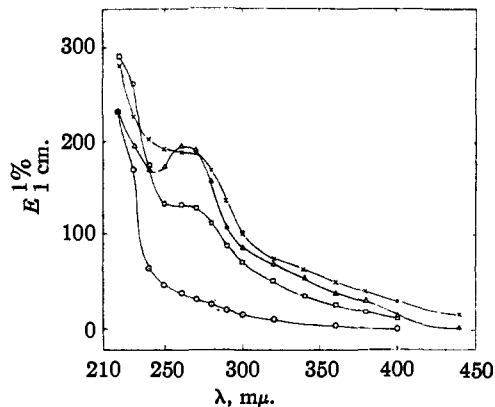


Fig. 3.—△—△—△, untreated folic acid, potency 28,000; ○—○—○, ultraviolet light and oxygen, potency 28,000; ×—×—×, ultraviolet light and nitrogen, potency 28,000; □—□—□, benzylfolic acid, potency 12,000.

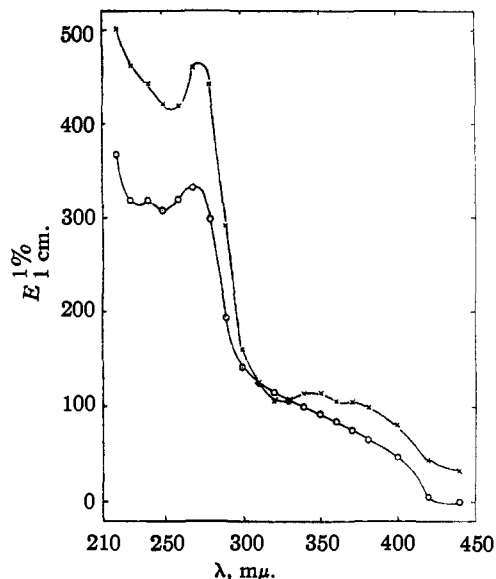


Fig. 4.—○—○—○, folic acid, potency 63,000; ×—×—×, xanthopterin.

trate of potency 63,000 is given in Fig. 4. This indicates a probable similarity in structure.

Effect of pH on Absorption Spectra.—A number of investigators have studied and adequately reviewed recent work on the effects of acid and alkali on the absorption spectra of solutions of several purines and pyrimidines.^{3,4,5} Considerable shifts in absorption bands were observed in compounds containing tautomeric structures which are affected by pH. Evidence has been presented by Stuckey^{6,7} showing that in certain barbituric acids ionization takes place followed at once by a tautomerism with its accompanying change in absorption.

The absorption spectra of xanthopterin and folic acid (potency 63,000) were determined in 0.1 N acid, at neutrality and in 0.1 N alkali. Considerable changes were observed as a result of the pH differences but both substances changed in a very similar fashion. In order to

(3) Loofbourov, Stimson and Hart, *THIS JOURNAL*, **65**, 148 (1943).
 (4) Stimson and Reuter, *ibid.*, **65**, 151 (1943).
 (5) Stimson and Reuter, *ibid.*, **65**, 153 (1943).
 (6) Stuckey, *Quart. J. Pharm. Pharmacol.*, **15**, 370 (1942).
 (7) Stuckey, *ibid.*, **15**, 377 (1942).

obtain more quantitative information from this approach an "absorption titration" was devised in which molecular extinctions at given wave lengths are plotted against pH . It was hoped that such a process would lend information on the number of ionizable groups and on their acidic strengths. Results on 16 compounds^{7a} and a folic acid concentrate are given in Figs. 5-11. The wave lengths were chosen arbitrarily and do not necessarily represent the maximum change in absorption but do adequately represent the pH ranges in which the absorption takes place. The method used was as follows. Solutions of the compounds to be titrated were prepared containing 10 micrograms per ml. of the compound in a mixed buffer 0.1 M each in formic, phosphoric, and boric acids. To a 50 ml. portion, 40% sodium hydroxide was added one drop at a time to bring the solution to the desired pH . The same amount of alkali was added to a 50-ml. portion of buffer at the same time to act as a standard for the absorption determination at each pH interval. Readings were taken at five or six arbitrarily chosen wave lengths in the range of absorption of the compound.

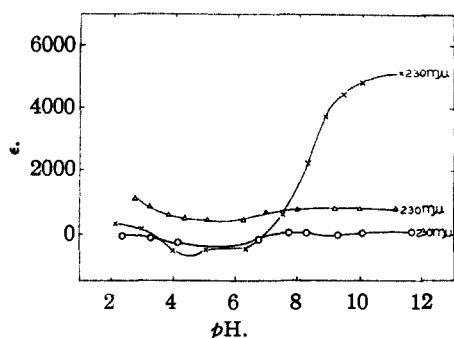
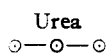
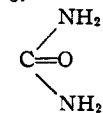
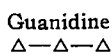
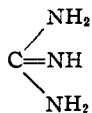


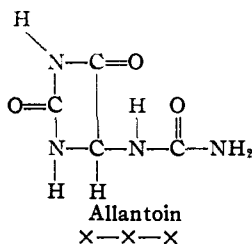
Fig. 5.—



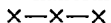
Urea



Guanidine



Allantoin



Discussion

Studies of absorption of ultraviolet light by solutions of folic acid concentrates have indicated that the pure substance has an absorption spectrum similar to that of xanthopterin but it appears to have a much higher molecular extinction coefficient. No significant interpretation of that difference can be made at the present time other than the suggestion that the folic acid may contain more carbon to carbon, or carbon to oxygen double bonds.

(7a) The author is indebted to his colleague, E. E. Snell, for samples of thymine, 5-carboxy-uracil, 4-carboxy-thymine, 4-carboxy-uracil ethyl ester, which had been previously prepared by him in this Laboratory.

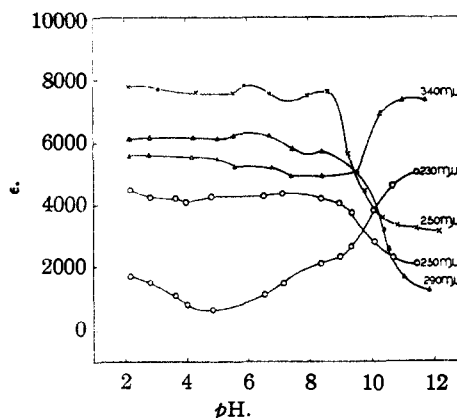
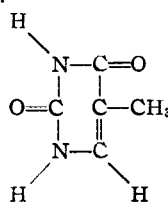
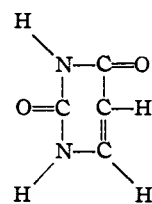


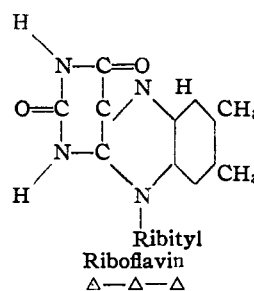
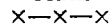
Fig. 6.—



Thymine



Uracil

Riboflavin
Ribityl
 $\Delta \text{---} \Delta \text{---} \Delta$

In order to conserve space, absorption spectra on only a few concentrates are given here and all are on samples that have been highly fractionated by processes already described.⁸ Low potency fractions that have not been subjected to extensive fractionation do not show a high degree of absorption as do the samples of potency 12,000 and 2,400 in Fig. 1 (Curves 3 and 4). It appears probable that these samples consist of folic acid plus a considerable proportion of folic acid with the structure modified to the extent of destroying the physiological activity of the compound. In view of the ease of destruction of this physiological activity² and the stability of the absorbing nucleus, as shown in Figs. 2 and 3, this hypothesis is given considerable support and offers a good explanation for difficulties involved in obtaining fractions of high physiological activity from many low potency concentrates.

The "absorption titrations" given in Figs. 5-11 suggest a strong similarity between the folic acid concentrate and the xanthopterin except for intensity of absorption. Both show two marked inflections in the curves, at about pH 2.5 and at about pH 9.0. Some reasonable interpretations

(8) Mitchell, Snell and Williams, *THIS JOURNAL*, **66**, 267 (1944).

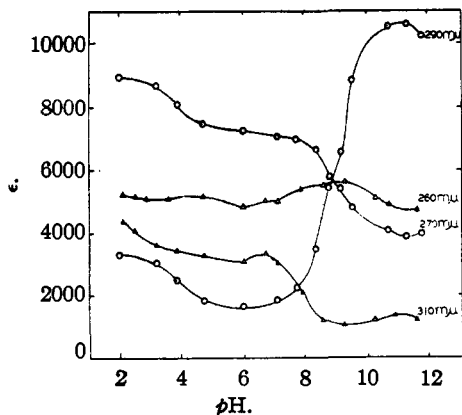
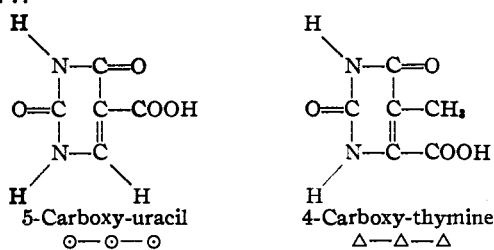


Fig. 7.—



of these data can be made on consideration of the curves obtained on the known compounds. All of the compounds on which determinations were made that had a structure capable of tautomerism in position 6 as induced by pH change, gave major

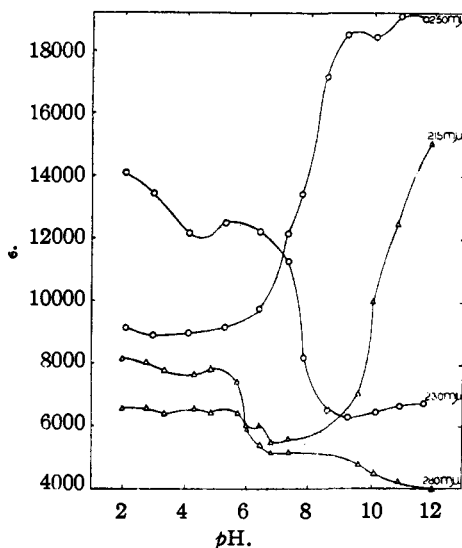


Fig. 8.—

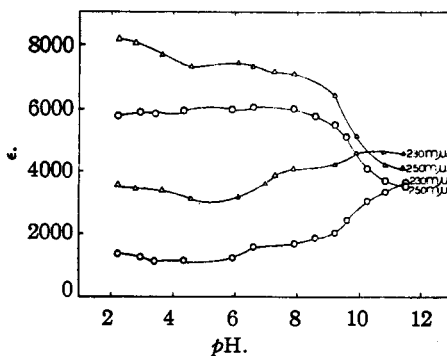
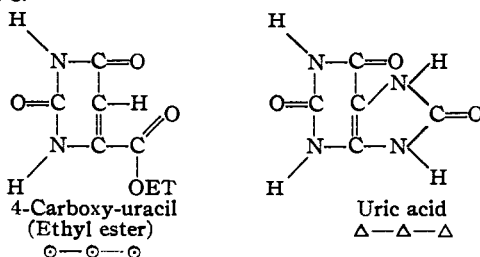
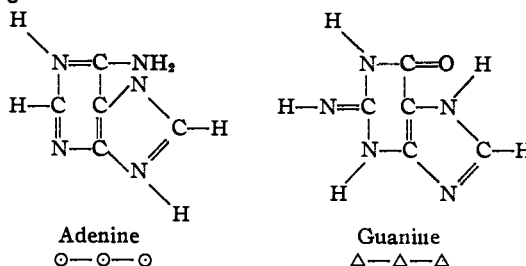


Fig. 9.—



inflections in the curves between pH 7.5 and 10.4. Of these compounds, 4-carboxy-uracil ethyl ester, 4-carboxy-thymine and 5-carboxy-uracil gave inflections at pH 7.5, 7.9 and 8.9, respectively. Six other compounds having similar configurations in position 6 (uracil, thymine, uric acid, guanine, riboflavin and theobromine) showed large inflections in the curves from pH 9.6 to 10.4. It appears evident that the substitution of a carboxyl group in position 5 and especially in position 4 increases the acidity of the ionizable group in position 6. Two other compounds of interest in this connection are theophylline and allantoin. The former has no hydrogen in either the 1 or 5 position to allow enolization, the only available hydrogen being in the 7 position. A three-atom shift could take place with a resulting major change in absorption characteristics. The ionization could be direct at position 7 but according to Stuckey's work on barbituric acids,^{6,7} the ionization alone would not be expected to produce the large absorption changes. Allantoin represents a different series of compounds which appear to have more acidic enolic forms than the pyrimidines. The large inflections in the absorption curves of xanthopterin and folic acid at pH 9 appear to represent enolizable groups at position 6 with acidic strength influenced by both an amino group in position 3 and a relatively strong acidic group at some position closer to 6 (position 8 in xanthopterin).

Inflections in the curves in the acid range are not as easily explained as those on the alkaline side since a neutralization of a weak acid is not involved and the changes in absorption necessitate marked changes in electronic structure but do not necessarily involve ionization. Nearly all of the compounds including urea and guanidine

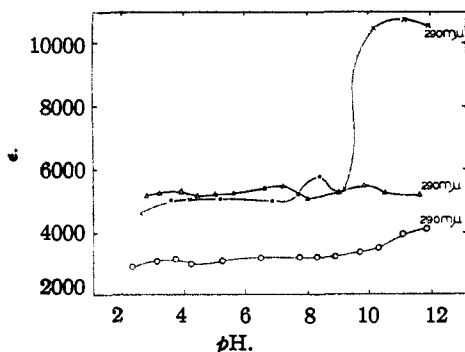
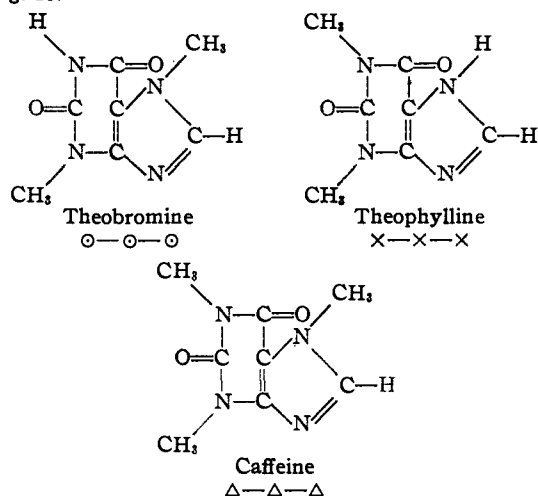


Fig. 10.—



show minor changes in absorption between pH 3.0 and 7.0 at short wave lengths. These changes can therefore be attributed to the urea or guanidine portion of the molecules but are insignificant with the more complex compounds. Five compounds, 5-carboxy-uracil, 4-carboxy-thymine, uric acid, xanthopterin and folic acid, show marked inflections in the curves in the acid pH range at many wave lengths over the entire wave length scale of absorption. In the cases of the two carboxy compounds it appears evident that the absorption changes are due to electronic shifts involving the carboxyl groups themselves since no such inflection was evident with 4-carboxy-uracil ethyl ester. The uric acid similarly must be involved at the strongly acid group in position 8. The changes in absorption of xanthopterin in the acid range must also be due to electronic changes around position 8 though this change is at a much more acidic pH than with uric acid and is in the opposite direction. By inference folic acid also contains an acidic enol in position 8 implying

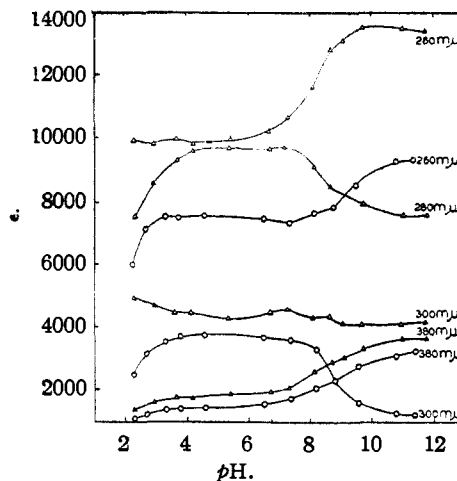
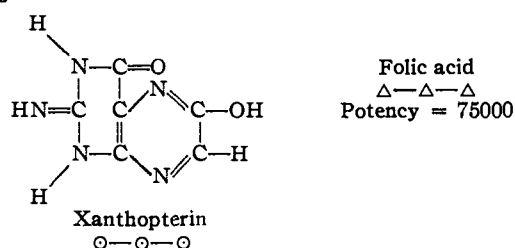


Fig. 11.—



a further similarity in its structure to that of xanthopterin.^{8a} Whether or not these changes in absorption in the acid pH range are related to acidic strength of the groups involved cannot be stated at the present time.

Summary

Evidence has been presented to show that the absorption spectrum of folic acid resembles that of xanthopterin. The light absorbing structure in folic acid is much more stable to light and acid treatment than the physiologically active compound. Evidence is cited indicating that the difficulties in purification of many concentrates are due to the impurities consisting of inactivated folic acid with physical properties only slightly changed.

An "absorption titration" plotting molecular extinction coefficients against pH is described and its significance discussed briefly.

It is concluded that folic acid contains in its structure a unit very similar to xanthopterin.

AUSTIN, TEXAS

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(8a) Wright and Welch⁹ have recently given further evidence of a structural relationship between xanthopterin and folic acid.

(9) Wright and Welch, *Science*, **97**, 426 (1943).