Further evidence of the dual nature of the adsorption was obtained from adsorption isotherms (Figs. 1, 2 and 3). The fact that the change in slope of these curves occurs at very low concentrations may indicate that this behavior is related to the change in energy of adsorption which occurs when the adsorbing surface is covered with a single layer of molecules, and further adsorption involves the formation of secondary layers. Further studies relating the amount of material adsorbed to the surface area of adsorbent exposed might shed light upon the exact mechanism involved.

The slow rate of equilibrium of adsorption is rather surprising, requiring at least forty-two hours in the case of riboflavin. From the shapes of the adsorption curves after different lengths of time (Figs. 2 and 3) it appears that the primary irreversible adsorption is the slow step since this step becomes more sharply defined with increasing time. Data not shown indicated that the equilibrium was nearly reached in forty-two hours with riboflavin and in twenty-two hours with thiochrome.

From a practical standpoint the value of these observations is evident. In dealing with concentration of substances which adsorb and do not readily elute from the adsorbent it appears highly advantageous to pretreat the adsorbent, for a considerable period of time, with a solution of one or more compounds that will block the irreversible adsorption. A dilute solution of a crude tissue extract often may be effective.


## Summary

Quantitative studies of charcoal adsorption of folic acid from crude preparations and from concentrates with subsequent elutions have been described. Elution of folic acid after adsorption from crude preparations is much easier than from relatively pure solutions. It has been found that this behavior is apparently due to the presence of interfering substances which affect the manner of adsorption. A working hypothesis of the mechanism of adsorption is proposed.

A study of the adsorption isotherms of folic acid, riboflavin, and thiochrome upon charcoal at low concentrations is reported. There is evidence that the adsorption process is of a dual nature, since the slopes of the isotherms change markedly in the range covered.

The application of these observations to concentration procedures using charcoal is pointed out.


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# Folic Acid. III. Chemical and Physiological Properties

BY HERSCHEL K. MITCHELL AND ROGER J. WILLIAMS

In order to better evaluate methods of concentration, various studies on stability, reactions and physical properties of folic acid were carried out. For most of this work the alteration of physiological activity of concentrates by various treatments was used as a criterion of reaction. Obviously a lack of destruction of activity by a given reagent does not prove the absence of a reaction since the product might be physiologically active. However, the destruction of activity does indicate a reaction.

## Experimental

**Esterification.**—Two 1-mg. samples of ammonium folate, potency = 11,000, were each suspended in 1 ml. of methanol containing 0.01 ml. of fuming sulfuric acid. After ten minutes at 30° one sample was diluted with ice water and neutralized. After thirty minutes the other sample was treated in a similar fashion and both assayed for folic acid content using *S. lactis R.* The destruction of activity was 90 and 96%. In spite of considerable experimentation on means of hydrolysis of the ester, no consistently satisfactory method has been devised. Treatment with 1 *N* alcoholic potassium hydroxide at 30° for ten minutes usually gave a 60 to 80% recovery of activity.

Fractional adsorption and solubility experiments indicated the properties of the compound were not changed to any great extent by formation of the methyl ester. That he methyl ester was still acidic in nature was demontrated by the fact that it could be more readily extracted from acid solution with butanol than from neutral or basic solution.

**Acylation.**—Acetylations using acetic anhydride and sodium acetate or acetic anhydride and pyridine at 100° for twenty minutes resulted in complete destruction of activity. Acetyl chloride as well as ketene (thirty minutes treatment at room temperature) also caused complete destruction of physiological activity. No method was found for regeneration of activity from any of these reaction products. Benzoyl chloride and β-naphthyl-sulfonyl chloride caused similar irreversible destruction of activity.

Solubility characteristics of acylation products were not markedly different than those of the original starting material.

**Methylation.**—Attempts to esterify folic acid using methyl iodide plus silver folate resulted in a product with no physiological activity. No activity could be recovered by methods used for hydrolysis of esters produced by acid–alcohol esterification. Similar results were obtained using benzyl chloride instead of methyl iodide, but the products in both cases did not show any great difference in solubility as compared to the starting material.

**Solubility.**—The solubility of free folic acid is very limited. In water it was found to be in the order of 2 mg. per ml. at 30° and about 1 mg. per ml. at 0°. The acid is slightly soluble in glacial acetic acid and liquid ammonia but essentially insoluble in dry methanol, ethanol, butanol, acetone, ether, dioxane, benzene, petroleum ether and chloroform. The ammonium salt is quite soluble in aqueous alcohols and is very soluble in water.

**Stability to Various Treatments.**—Frequent losses of activity during concentration processes necessitated studies of the stability of the concentrates at various

stages of purification. It was observed repeatedly that the activity of higher potency fractions disappeared under milder conditions than that from crude extracts. Numerous tests for stability were performed. Treatments which involved about 50% destruction of activity included: material of potency 75,000, treated with 1 *N* sulfuric acid one-half hour at 100°, or with 1 *N* sodium hydroxide for one and one-half hours at 100°, or with 1 *N* sodium hydroxide for twenty-four hours at 28°. More or less vigorous treatment yielded results about as would be expected on the basis of the above results.

The material in the form of the ammonium salt (potency 17,000) was relatively stable in the neutral condition (even when exposed to ultraviolet light) but was destroyed slightly (17% in twenty minutes) at the boiling temperature in the presence of oxygen gas. Material of even higher potency (45,000) was stable at room temperature when kept at a *p*H of 3, but was destroyed to a considerable extent when exposed at this *p*H to ultraviolet light either in the presence of nitrogen or oxygen. At *p*H of 1, folic acid (potency 2,000–3,000) was readily destroyed by contact with an atmosphere containing ozone. By standing at room temperature for an hour in the presence of 1% hydrogen peroxide, it was about 90% destroyed.

Bubbling hydrogen sulfide through a solution either in 0.1 *N* ammonium hydroxide or at *p*H 4 resulted in 30–40% destruction in a half hour. Nitrous acid under Van Slyke conditions for thirty minutes caused about 90% destruction. Dry heat (98°) caused moderate destruction and heating in the neutral condition in the presence of zinc dust for fifteen minutes caused 50% destruction even in the case of low potency material. In 50% acetic acid at 100° for fifteen minutes, 100% destruction resulted from adding zinc dust, whereas in the presence of the acetic acid alone no destruction took place. Folic acid activity was completely destroyed by bromine, hypobromite and hydroxylamine in the presence of sodium acetate.

**Analyses.**—Qualitative elementary analyses were carried out at several stages of purification indicating the presence of C, H, O and N, and the absence of halogens, P and S. Following the work on absorption spectra of various fractions some C, H and N determinations were made on highly fractionated material having different physiological activities. It was considered probable that such fractions were made up principally of folic acid and inactivated folic acid and it was therefore expected that analyses of materials of different activities would give similar results.

| Potency | C, % | H, % | N, % |
|---|---|---|---|
| 80,000 | 45 | 3.6 | 19.2 |
| 28,000 | 46 | 5.0 | .. |
| 3,000 | 45 | 5.1 | .. |

Micro-Kjeldahl determinations on the potency 80,000 and 28,000 samples gave N values of 10 to 12%. It was found that micro-Kjeldahl determination on xanthopterin under the same digestion conditions accounted for only about three-fifths of the theoretical amount of nitrogen present.

**Determination of Glycols.**—The apparent high oxygen content of the folic acid concentrates analyzed suggested the possibility of a sugar group being present. Five-micromole samples of erythritol, mannitol, dextrose, riboflavin, xanthopterin, folic acid of potency 80,000 (assuming a molecular weight of 400) and folic acid of potency 28,000 were placed in small test-tubes with 0.5 ml. of solution containing 5 mg. of potassium periodate. One-half ml. of 0.4 *N* sulfuric acid was added and the mixture allowed to stand for twenty-four hours. The formaldehyde produced was distilled into 1 ml. of Schiff reagent. No formaldehyde was obtained from the xanthopterin and the folic acid samples, but strong positive tests were obtained with the rest of the compounds. The absence of a glycol was thus indicated.

**Quantitative Acetylation.**—As a check on the absence of polyhydroxy groups in folic acid, quantitative acetylations were carried out on 5-micromole samples of folic acid concentrate of potency 28,000 (assuming an average molecular weight of 400 for its ingredients), xanthopterin, mannitol, and riboflavin. The samples were weighed into 0.3 × 5 cm. Pyrex tubes followed by 0.039 ml. of 20% acetic anhydride in pyridine. The reagent was measured with a calibrated capillary pipet and the rinsings from each tube placed in a labeled erlenmeyer flask. The tubes were sealed, heated for one hour at 100°, placed in the proper flasks and the tubes crushed with the end of a glass rod. All samples except the xanthopterin had dissolved in the hot reaction mixture. Five ml. of water was added and the samples titrated with 0.01 *N* sodium hydroxide after standing thirty minutes. The number of moles of acetic acid used per mole of compound is given as follows: mannitol, 5.95; riboflavin, 4.05; folic acid, 1.15; xanthopterin, 0.5.

Since hydroxyl groups were found not to be abundant and since analysis indicated that a concentrate of this potency contained as high a percentage oxygen as a concentrate of potency 80,000, it appears probable that the high oxygen content of folic acid is due to structural features other than polyhydroxy groups.

**Molecular Weight.**—The determination of molecular weight by diffusion has been previously described and applied.[1,2] An application of this method to low potency fractions of folic acid gave the molecular weight as about 500. Results of more recent studies are described as follows: A sample of ammonium folate, potency = 65,000, was diluted to 20 micrograms per ml. with 0.1 *N* potassium chloride and the solution placed in one compartment of each of three calibrated diffusion cells. The other compartment of the cells contained 0.1 *N* potassium chloride. After twenty hours of diffusion time at 35°, samples were removed from both sides of the cells and the concentration of folic acid determined by biological test. Concentration of material in the solutions was also determined by fluorescence in a Pfaltz and Bauer fluorophotometer. The results are summarized below in terms of diffusion coefficients calculated in sq. cm. per day.

| Cell | Physiological test *D* (sq. cm./day) | Fluorometric *D* (sq. cm./day) |
|---|---|---|
| E | 0.453 | 0.495 |
| F | .445 | .449 |
| J | .396 | .462 |

The molecular weight was determined by comparison of these diffusion coefficients with those obtained from studies of a number of compounds of known molecular weight.[2a] The molecular weight appears to be 400 ± 50.

In order to test further the hypothesis that highly fractionated concentrates are made up of molecules of the same type as folic acid (folic acid plus products of its inactivation) a diffusion experiment was carried out on a sample of potency 10,000. This sample had been subjected to extensive chromatographic and solvent fractionation without appreciable increase in potency of products. Concentrations of the material in the solutions after diffusion were determined in

(1) Moquin and Cathcart, THIS JOURNAL, **57**, 1571 (1935).

(2) Williams, Lyman, Goodyear, Truesdail and Holaday, *ibid.*, **55**, 2912 (1933).

(2a) The experimental work involved in these diffusion experiments was carried out by E. H. Frieden and will be published later.

parallel by (1) physiological test, (2) fluorescence and (3) ultraviolet light absorption at various wave lengths. Results are given as follows

| | *D* (sq. cm./day) |
|---|---|
| Physiological test | 0.48 |
| Fluorescence | .47 |
| Light absorption | .49 |

It appears from these results that the concentrate either was composed of molecules of very nearly the same molecular weight (about 380) or else the impurities involved showed no fluorescence or light absorption. Since 9 micrograms of this sample (potency 10,000) was found to be equivalent to 7 micrograms of potency 63,000 in ultraviolet light absorption the second possibility is ruled out.

**Physiological Properties.**—Folic acid was originally defined[3] as the active principle required for the growth of *S. lactis R* under specified conditions.[4] A considerable number of known compounds have been tested for activity under these conditions. One compound, thymine, replaces folic acid if present in sufficient quantity (1 $\gamma$ per ml. of culture medium). This activity is not sufficient to cause interference with the test for folic acid. A considerable number of other compounds show a slight activity at very high concentrations (10 $\gamma$ per ml. of culture medium). Among these are hypoxanthine, alloxazine, alloxantine, guanidine, theobromine, xanthopterin, alloxan, allantoin and uric acid. Compounds tested that were completely inactive were: nicotinic acid, pyridoxin, pantothenic acid, ascorbic acid, taurine, asparagin, biotin, riboflavin, *p*-aminobenzoic acid, creatine, inositol, phytin, 4-carboxy - uracil, 4 - carboxy - thymine, 5 - carboxy-uracil, uracil, adenylic acid, indolbutyric acid, cozymase, cystine, theophylline and caffeine.

Until pure folic acid becomes available, specific physiological properties of the compound must necessarily remain uncertain. However, a number of indications as to these physiological activities have been obtained using highly purified concentrates.

In this Laboratory activity of concentrates (potency 400) on four strains of yeast was demonstrated. In the very early stages of concentration "Old Process" yeast was used as a test organism but it was discarded in favor of the more specific and otherwise superior test using *S. lactis R*. Concentrates were found essential for *L. casei*, *L. delbruckii*, and *Clostridium tetanii*,[5] and stimulated the growth of *L. arabinosus*.[3] The "eluate factor" of Snell and Peterson[6] has been identified with folic acid on the basis of a test using *L. casei* but the exact relationship between the two factors is not yet clear. In view of the recent findings of Pfiffner, *et al.*,[7] and of Stokstad,[8] it appears certain that pure compounds promote the growth of both *S. lactis R* and *L. casei*, but the relative activity of preparations from different sources differs for the two organisms. Keresztesy, *et al.*,[9] have reported isolation of a material of extremely high activity for *S. lactis R* which is almost without activity for *L. casei*. We have found concentrates from spinach acting in a somewhat similar fashion to those from liver described by Keresztesy, *et al.*,[9] *e. g.*, a sample of concentrate of potency 30,000, by *S. lactis R* test had only a potency of 15,000 by *L. casei* test, both being referred to the same standard. These findings support the conclusion drawn here and in a subsequent paper[10] that very minor changes in structure may produce great changes in activity for various organisms.

Antianemia properties of liver concentrates for chicks were reported by Hogan,[11] and this active principle was recently identified with folic acid though some uncertainty still exists.[7]

Early experiments on rats in this Laboratory indicated a higher erythrocyte count with animals receiving a folic acid concentrate than with controls. The difference was about 10% but an insufficient number of animals was used. Consequently, through the very generous coöperation of Dr. Earl R. Norris of the University of Washington, a sample of potency 75,000 folic acid concentrate was tested for antianemia activity on trout. The material was found to be one-fifth as active as xanthopterin for this purpose, since 10 micrograms per g. of fish was required to produce a good response in erythrocyte regeneration.

Concentrates high in folic acid were demonstrated to stimulate growth of chicks[12] and indications were obtained of stimulation of the growth of rats.[3] In both cases the concentrates used were of too low a potency to be certain of the biological specificity of the preparations. That intestinal bacteria can serve as a source of folic acid for the rat was demonstrated in this Laboratory[13] while several investigators have been able to produce a deficiency[14,15,16] by impairment of intestinal production with succinylsulfathiazole. In one case[14] this deficiency was cured by administration of a highly purified concentrate of folic acid obtained from this Laboratory.

## Discussion

Investigation of stability of folic acid to a number of reagents and conditions demonstrated

(3) Mitchell, Snell and Williams, THIS JOURNAL, **63**, 2284 (1941).

(4) Snell and Mitchell, *Proc. Natl. Acad. Sci.*, **27**, 1 (1941).

(5) Mueller and Miller, *Proc. Soc. Exptl. Biol. Med.*, **49**, 211 (1942).

(6) Snell and Peterson, *J. Bact.*, **39**, 273 (1940).

(7) Pfiffner, *et al.*, *Science*, **97**, 404 (1943).

(8) Stokstad, *J. Biol. Chem.*, **149**, 573 (1943).

(9) Keresztesy, Rickes and Stokes, *Science*, **97**, 465 (1943).

(10) Frieden, Mitchell and Williams, THIS JOURNAL, **66**, 269 (1944).

(11) Hogan and Parrott, *J. Biol. Chem.*, **132**, 507 (1940).

(12) Hutchings, Bohonos, Hegsted, Elvehjem and Peterson, *ibid.*, **140**, 681 (1941).

(13) Mitchell and Isbell, *Univ. Texas Pub.*, **4237**, 125 (1942).

(14) Nielson and Elvehjem, *J. Biol. Chem.*, **145**, 713 (1942).

(15) Martin, *Proc. Soc. Exptl. Biol. Med.*, **51**, 353 (1942).

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

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 data[10] 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 data[17] 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).

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# 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 Morton[1] by $E^{1\%}_{1\,cm.}$. 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