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Folic Acid. II. Studies on Adsorption

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The procedure for the concentration of folic acid described in a previous paper[1] is based to a considerable extent upon successive adsorptions upon and elutions from activated charcoal. During the early stages of the work on this problem it was observed that while the active principle from crude extracts adsorbed upon either norite or Darco could readily be eluted by treatment with hot ammonia, the principle from purified preparations, similarly adsorbed, was eluted to a negligible extent with this reagent. This rather peculiar behavior has been subjected to a more extended investigation in order to understand better the phenomenon and make use of it in concentration processes.

Experimental

Elution Experiments.—For preliminary quantitative experiments two extracts of spinach were prepared, one by hot water (110°) extraction of fresh spinach macerate for one-half hour and one by autolysis under toluene at 37° for two days. After the preliminary treatment, the samples were autoclaved for fifteen minutes at 15 lb. pressure. Both samples were then adjusted to *p*H 3.0 and filtered to remove precipitated protein material. Finally, each sample was adjusted to *p*H 6.5–7.0, sterilized, and stored. A concentrate of potency 280 was used as indicated.

The following experimental procedure was used. Aliquots of solution containing known amounts of folic acid were adjusted to *p*H 3.0 and shaken with weighed amounts of Darco S-51 (1 mg. Darco per mg. unit). The shaking was repeated several times during the course of one hour. The suspensions were then centrifuged, and the supernatant liquid removed. The residue was eluted with 5% ammonia (equal in volume to the supernatant liquid) at 90° for ten minutes, filtered while hot, and both filtrate and the supernatant solution assayed for folic acid. Folic acid assays were performed essentially according to the method of Mitchell and Snell,[2] and in all cases adsorption, as measured by the folic acid content of the supernatant solution, was found to be nearly complete (92–100%).

The results of the elution experiments are indicated in Table I.

Table I shows that at comparable concentrations the elution of folic acid from crude preparations is considerably more complete than when purified samples are used. It appeared, therefore, that the extent of elution with ammonia was dependent upon the concentration of substances present that interfered in the adsorption. This conclusion was verified by an experiment. A sample of the water extract referred to in Table I was exposed to ultraviolet light at *p*H 1 in order to destroy the folic acid present. This folic acid-free solution was then used as the solvent in the preparation of a solution of concentrate, and the adsorption-elution procedure repeated as described. The results are indicated in Table II.

TABLE I

ELUTION OF FOLIC ACID FROM DARCO S-51 BY 5% AMMONIA

Source of sample	Folic acid concentration, mg. units/ml. ($\times 10^{-4}$)	Eluted, %
Spinach autolysate	8.7	54
Spinach autolysate	3.35	69
Spinach autolysate	0.87	92
Spinach autolysate	.32	91
Spinach water extract	6.7	74
Spinach water extract	3.35	70
Spinach water extract	0.67	89
Spinach water extract	.67	92
Concentrate potency = 280	6.9	4.0
Concentrate potency = 280	3.4	5.4
Concentrate potency = 280	1.7	6.8
Concentrate potency = 280	0.69	25.6

TABLE II

EFFECT OF THE ADDITION OF FOLIC ACID FREE EXTRACT UPON THE EXTENT OF ELUTION BY AMMONIA

Source of sample	Folic acid concentration, mgu./ml. ($\times 10^{-4}$)	Eluted, %
Concentrate potency = 280	9.6	41
Concentrate potency = 280	4.4	65
Concentrate potency = 280	2.4	100
Concentrate potency = 280	0.96	100

Comparison of the data of Table II with those of Table I indicates the considerably increased elution yields due to the presence of added material. Results like those of Table II were also obtained when the irradiated material. instead of being added to folic acid solutions, was used to pretreat samples of charcoal. When this charcoal was used for adsorption and subsequent elution of purified folic acid, increased yields again resulted. A ten-fold dilution of irradiated extract was as effective in facilitating elution as was the undiluted material.

It seemed reasonable to suppose that the action of irradiated extract in facilitating elution of folic acid could be duplicated by known compounds. This hypothesis was tested by experiment. Samples of norite were shaken with 10 parts of 10% solutions of aniline, hippuric acid, allantoin, and triethanolamine at boiling temperature. The suspensions were filtered, washed with a small volume of water, and air dried overnight. The treated charcoals so

TABLE III

EFFECT OF PRETREATMENT OF NORITE UPON THE ELUTION OF FOLIC ACID

Description	Folic acid concentration, mgu./ml. ($\times 10^{-4}$)	Eluted, %
Norite, untreated	1.15	13
Norite, untreated (added irradiated extract)	1.15	88
Aniline-treated norite	1.15	100
Hippuric acid-treated norite	1.15	71
Allantoin-treated norite	1.15	55
Triethanolamine-treated norite	1.15	44

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

(2) Mitchell and Snell, *Univ. Texas Pub.*, 4137, 36 (1941).

obtained were tested for adsorption and subsequent elution of concentrate as before. The results are indicated in Table III.

This increase in elution yields by pretreatment of adsorbent with aniline has been found useful in large-scale concentration processes.

Adsorption Isotherms.—From the quantitative adsorption and elution experiments it appeared probable that two different mechanisms of adsorption of folic acid were involved. Since this difference should be evident from adsorption isotherms, the problem was considered from that standpoint. Only two studies of adsorption isotherms at extremely low concentrations were found in the literature. In 1914, Trumpler[3] studied the adsorption of fluorescein upon activated charcoal at equilibrium concentrations ranging upward from 10^{-7} g. per liter. He found that adsorption was essentially complete in one and a half hours, but that slow adsorption continued over a period of thirty hours. For a "reaction time" of one and a half hours, the isotherm was found to be linear from log x/m = -1.6 to log x/m = 0.5, where the symbols have their usual significance.[4] More recently, Cheldelin and Williams[4] reported adsorption studies for a large number of ampholytes upon Darco G-60. Of the substances studied only calcium pantothenate, biotin, pyridoxin hydrochloride and thiamin hydrochloride were investigated at very low concentrations. In all cases, linear log x/m − log C relations were found. No dependence of adsorption on time was noted.

Experiments were carried out on a folic acid concentrate of potency 10,000 using norite charcoal as the adsorbent. The concentration of folic acid was varied from 0 to 0.05 mgu. per ml. and the charcoal concentration was 0.2 mg. per ml. The adsorptions were carried out at pH 3. The suspensions were centrifuged, and the supernatant solution assayed for folic acid. The data are given in Fig. 1, plotted according to the Freundlich equation. Quantities of folic acid are expressed in milligram units.

Despite the scattering noted, the curve obtained does appear to exhibit the dual character predicted.

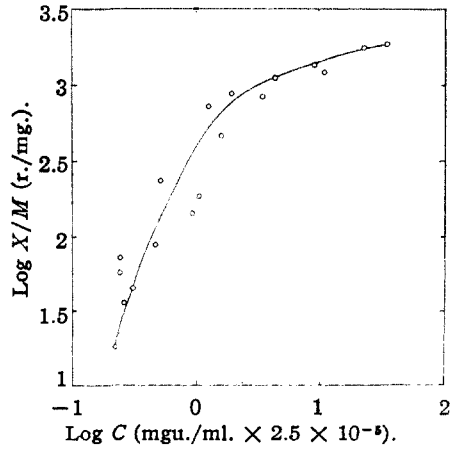

Fig. 1.—Adsorption isotherms of folic acid on norite at 25°.

Since a considerable part of the uncertainty probably lies in the use of a partially purified substance as well as the inherent limitations of microbiological assay methods, the studies were continued using riboflavin and thiochrome, analyses being made fluorometrically. Merck riboflavin was used, and the same Company very generously supplied sufficient pure thiochrome. Fluorescence was determined with a Pfaltz and Bauer fluorophotometer, equipped with a very sensitive triple mirror galvanometer. Equipped with suitable filters, the instrument is sensitive to 3×10^{-4} γ/ml. of riboflavin, and to 2×10^{-4} γ/ml. of thiochrome per galvanometer division.

(3) Trumpler, *Kolloid-Z.*, **15**, 10 (1914).

(4) Cheldelin and Williams, THIS JOURNAL, **64**, 1513 (1942).

Preliminary experiments followed the procedure used for folic acid. Erratic results were obtained, and were traced to a definite dependence of adsorption on time. Accordingly, the following procedure was adopted. Mixtures of norite and the compound being studied were prepared, and placed in an automatic shaking machine. At stated intervals, aliquots were withdrawn, centrifuged, and the supernatant fluid analyzed. The data obtained are shown in graphical form in Figs. 2 and 3.

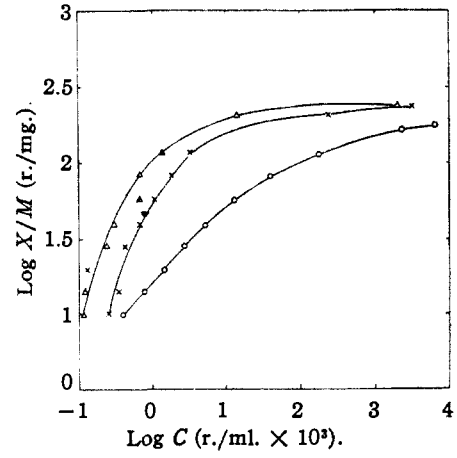

Fig. 2.—Adsorption isotherms for riboflavin at 25°: ⊙—⊙—⊙, after 1.1 hours; ×—×—×, after 17 hours; Δ—Δ—Δ, after 42 hours.

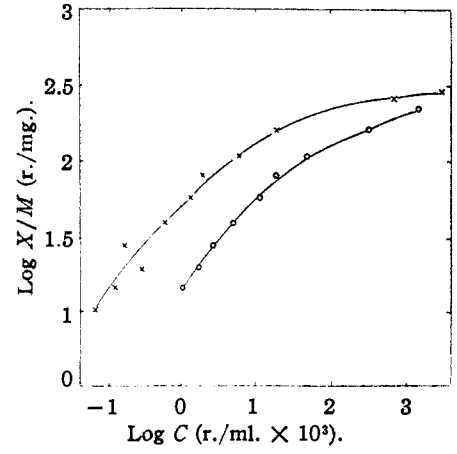

Fig. 3.—Adsorption isotherms for thiochrome on norite at 25°: O—O—O, after 1.5 hours; ×—×—×, after 22 hours.

Discussion

Quantitative elution and adsorption experiments suggest that adsorption of folic acid and presumably some other ampholytes occurs in two steps. From relatively pure solutions of folic acid the active principle is adsorbed in such a way that the resulting union is a very firm one and not readily reversible. If part or all of the charcoal surface is blocked by pretreatment, or by preferential adsorption of another compound, another type of binding manifests itself. In contrast to the initial "primary" adsorption, this "secondary" adsorption appears less tenacious, and its reversibility is more readily effected.

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

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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