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Folic Acid. I. Concentration from Spinach^{1a}

By HERSCHEL K. MITCHELL, ESMOND E. SNELL AND ROGER J. WILLIAMS

In a preliminary note¹ it was announced that we had concentrated a nutritive from spinach that had a high degree of activity in stimulating the growth of certain strains of bacteria. Because of its unusual abundance in numerous leaves, we suggested the name "folic acid" for the substance and defined it as the material responsible for growth stimulation of *Streptococcus lactis* R on a given medium.²

For purposes of convenience in concentration a sample of Wilson liver fraction "B" was established as a standard of "potency = 1." For comparison of folic acid assay values with those of other vitamins, results have been expressed in terms of micrograms of material of "potency 40,000" per gram of tissue.³ Fraction "B" is required in concentrations 40,000 times as great as such material in order to produce the same amount of growth stimulation. One milligram of such a concentrate has been designated by Williams⁴ to contain "one milligram unit," and this unit has been used throughout this paper.

Experimental

The following typical example best demonstrates the application of the concentration procedure.

Preparation of Extract.—One thousand pounds of raw spinach was ground in a hammer mill and transferred to a jacketed pot with sufficient water to bring the volume to about 350 gallons. The suspension was stirred vigorously and the temperature raised to 30–35°. After thirty-six hours standing the mixture was heated at the boiling temperature for fifteen minutes and the pulp strained out by allowing the solution to drain overnight through muslin bags. The resulting solution was adjusted to pH 3.0 to 3.2 with sulfuric acid, followed by addition of 4 gallons of super-cel filter aid. The solution was heated and held at the boiling temperature for about five minutes followed by rapid cooling with cold water in the jacket of the pot. The suspension was filtered in a plate and frame press coated with filter aid to obtain a brilliantly clear filtrate. This material contained 2,700 mg. units; potency of solids = 2.5.

Adsorption on Charcoal 1.—Twelve pounds of charcoal (Darco S-51) was added and the suspension stirred for thirty minutes. Following the addition of 2 gallons of super-cel the suspension was filtered and washed and the filtrate discarded.

Elution from Charcoal 1.—The charcoal containing the adsorbed activity was added to a boiling solution of one gallon of 28% ammonia in 10 gallons of distilled water. The suspension was maintained at boiling temperature for fifteen minutes and filtered while hot. The elution was repeated and the combined filtrates cooled rapidly to 25°. The charcoal was discarded; yield, 1,700 mg. units; potency of solids = 60.

Adsorption on Charcoal 2.—Three pounds of pretreated charcoal^{4a} was added to the cooled ammonia solution and the suspension stirred twenty minutes. After addition of 1 gallon of super-cel the suspension was filtered and washed, discarding the filtrate.

Elution from Charcoal 2.—The charcoal with the adsorbed activity was added to a boiling mixture of 500 g. of aniline in 6 liters of water and the boiling continued for fifteen minutes. The suspension was filtered hot and the elution repeated. The charcoal was discarded. After cooling the solution was extracted twice with 20% of its volume of ether and then adjusted to pH 3.0 to 3.2 with sulfuric acid. A slight precipitate was removed by filtration with filter aid; yield, 1,400 mg. units; potency = 200.

Adsorption of Charcoal 3.—The solution was stirred twenty minutes with 180 g. of pretreated charcoal which was then filtered off and washed.

Elution from Charcoal 3.—The charcoal containing the activity was added to a boiling solution of 75 g. of aniline in 1 liter of water and the boiling continued for fifteen minutes. The elution was repeated and the combined eluates cooled and extracted twice with ether to remove the aniline; yield, 1,270 mg. units; potency = 800.

Lead Precipitation.—A solution of 37 g. of lead acetate trihydrate in 100 ml. of water was added to the aqueous solution from eluate 3 with vigorous stirring. The resulting precipitate was collected and washed by centrifugation. The activity was regenerated by suspension of the precipitate in 3 liters of water containing 150 g. of ammonium sulfate. The solution was boiled twenty minutes, cooled and filtered, and the filtrate retained; yield, 850 mg. units.

Silver Precipitation.—The filtrate from the lead regeneration step was diluted to 6 liters and a solution of 35 g. of silver nitrate in 100 ml. of water was added. The pH was adjusted to 6.5 with ammonium hydroxide with vigorous stirring of the suspension. The precipitate was collected and washed by centrifugation. The folic acid was regenerated from the silver precipitate by five minutes boiling with a solution of 25 g. of ammonium chloride in 150 ml. of water. After cooling the suspension was acidified to pH 6 with hydrochloric acid followed by filtration and washing. The precipitate was discarded; yield, 600 mg. units.

Adsorption on Lloyd Reagent.—The solution from the silver regeneration which contained a high concentration of salts was cooled below 10° and acidified to pH 1.0 with hydrochloric acid. The cold solution was shaken a few minutes with 15 g. of Lloyd reagent. The suspension was filtered and washed and the filtrate discarded.

Elution from Lloyd Reagent.—The Lloyd reagent was shaken with 50-ml. portions of 5% ammonium hydroxide three times. The volume of this combined eluate (150 ml.) was conveniently reduced to a few ml. by addition of 5 volumes of ether and sufficient ethanol to cause the separation of an aqueous layer of 5 to 10 ml. This product was then evaporated rapidly in a vacuum desiccator over sulfuric acid. The dry ammonium salt obtained in this fashion is stable and was stored as such; yield, 380 mg. units; potency = 5,200.

Products obtained from different batches treated as indicated varied in potency from 2,000 to 10,000 depending largely on the amount of activity in the original spinach.

Fractionation on Alumina.—Further purification was effected by fractional elution from alumina and by precipi-

(1a) Nearly all of the large scale concentration work was carried out by Mr. Elmer H. Stuart through the very generous cooperation of the Eli Lilly Co., Indianapolis, Indiana.

(1) Mitchell, Snell and Williams, *THIS JOURNAL*, **68**, 2284 (1941).

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

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

(4) Williams, *J. Am. Med. Assoc.*, **119**, 1 (1942).

(4a) This charcoal (Darco S-51) was pretreated by heating to boiling with 1.5 parts of aniline and 5 parts of water, followed by filtration and washing with hot water.

tation of the acid from a concentrated solution of the ammonium salt. This is best illustrated by the following examples.

A sample (1.86 g.) of potency 3,500 (162 mg. units) was dissolved in 8 ml. of water and 6 ml. of methanol and adsorbed on a column of alumina (Merck according to Brockman) which had been washed with 40% methanol in water. The column was 2 cm. in diameter and 15 cm. long, packed dry. Following adsorption of the sample, the column was washed with 40% methanol. Fourteen 10-ml. fractions were collected followed by a fraction obtained by washing with 50 ml. of 2% ammonia in 40% methanol. Fractions 4-12 were combined giving 330 mg. of potency = 13,000 (143 mg. units). Fractions 1-4 and 13-15 were combined, evaporated and fractionated in an alumina column as before, giving 64.5 mg. of potency 11,000 (17.7 mg. units) and a considerable quantity of low potency material.

The two products, potency 13,000 and potency 11,000, were combined and again subjected to a fractionation on alumina. The sample was dissolved in 40% methanol (5 ml.) and adsorbed on an alumina column 1.5 cm. in diameter by 10 cm. long. Nine fractions of 5 to 10 ml. each were collected followed by one of 30 ml. containing 2% ammonia. Fractions 3-6 were combined giving 148 mg. of potency 25,000. The fractions 1-2 and 7-10 were combined and refractionated giving 36 mg. of potency 25,000 (total activity: 115 mg. units).

Acid Precipitation.—The combined sample of 184 mg. (potency = 25,000) of ammonium folate was triturated with 0.6 ml. of water, centrifuged and the precipitate washed 4 times with 0.1 ml. of water each time. The solution was acidified with 0.06 ml. of concentrated hydrochloric acid and mixed thoroughly. The solution was cooled to 0°, centrifuged and the precipitate washed 3 times with 0.1-ml. portions of ice water. The precipitate was dissolved in dilute ammonia and the solution evaporated in vacuum. The product weighed 88.3 mg., contained 110 mg. units, and had a potency of 46,000.

Fractionation on Alumina.—The sample with potency 46,000 was dissolved in 1 ml. of 40% methanol and adsorbed on a 1.5 × 10 cm. column of alumina as before. The column was washed with 40% methanol containing 1 drop of concentrated ammonium hydroxide per 100 ml. Nine fractions of from 4 to 6 ml. were collected followed by one obtained by using 40 ml. of 2% ammonia in 40% methanol. Fraction number 6 contained 5.8 mg. of solids and had a potency of 105,000. The remaining fractions were recombined and fractionated again under the same conditions. One fraction of 10.9 mg. had a potency of 75,000 (20.5 mg. units) and the remaining high potency fractions contained 28 mg. of potency 48,000 (33.5 mg. units). The total product at this point represented a 42.5% yield of the activity contained in the starting material of 3,500 potency.

Acid Precipitation from Cold Water.—A sample of 9.1 mg. of potency 71,000 (16.1 mg. units) prepared in the same fashion as that described, was dissolved in 0.2 ml. of ice water and 0.1 ml. of concentrated hydrochloric acid added. After a thorough mixing the precipitate was centrifuged and washed twice with 0.05 ml. of ice water. The supernatant liquid and washings contained 6.25 mg. units. The precipitate was triturated with 1 ml. of water at 50°, centrifuged and decanted. The precipitate weighed 1.4 mg. and had a potency of 85,000 (3.0 mg. units). The decanted solution was allowed to cool slowly in the dark to 25° and the resulting amorphous precipitate centrifuged off. It weighed 1.2 mg. and had a potency of 137,000 (4.1 mg. units). The supernatant liquid was cooled to 0° and centrifuged at that temperature. The

product contained 0.6 mg. of potency 100,000 (1.5 mg. units). The remaining solution contained 1.5 mg. of material with 45,000 potency (1.67 mg. units).

Discussion

The illustrations cited represent the best single method of concentration that we have developed in this Laboratory though a great many variations have been tried. The problem was complicated by instability of the active principle to oxidation, reduction, acid, alkali, light and heat, as well as a high degree of reactivity with many organic reagents. One of the most complex processes, that of adsorption and elution from charcoal, involved extended study, and this phase of the work will be reported in a separate paper.

In addition to charcoal, alumina and Lloyd reagent we have utilized many kinds of fuller's earths, amberlites, barium carbonate, bentonites, florisol, floridin and permutite with successful adsorptions and elutions. We have also successfully utilized many precipitants other than lead and silver. Among these were salts of copper, mercury, iron, uranium oxide, phosphotungstic acid, alum, zinc, rhodanilic acid and barium. Recoveries of activity from the precipitates were poor in most cases.

A number of attempts to obtain crystalline products resulted unsatisfactorily. All samples prepared having a potency of 110,000 or greater precipitated in an amorphous state from acid solution and recrystallization attempts from warm water also gave amorphous products. Quinine, brucine, or even ammonium salts gave semicrystalline material on evaporation of the solvent on samples of potency 20,000 and up, but satisfactory recrystallization was not accomplished. Adsorption and fractionation from alumina has been a highly effective means of concentration only after the preliminary steps. This method has been most useful in the potency range of 2,000 to 80,000. Higher potency samples are readily adsorbed by the alumina but are not easily eluted. A preliminary treatment of the alumina with aniline allowed easy elution but no concentration was effected. Other eluants than ammonia such as triethanolamine, triisopropylamine, brucine, aniline, pyridine and quinine were tried with mediocre success.

Summary

A concentration procedure is described whereby folic acid has been concentrated from spinach to such an extent that the product is about 137,000 times as active as a standard material (liver extract).

AUSTIN, TEXAS

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