United States Patent Office 2,741,608 Patented Apr. 10, 1956

2,741,608

5-FORMYL-5,6,7,8-TETRAHYDROPTEROIC ACID AND AMIDES THEREOF AND METHOD OF PREPARING SAME

William Shive, Austin, Tex., assignor to Research Corporation, New York, N. Y., a corporation of New York

No Drawing. Application March 8, 1951, Serial No. 214,625

13 Claims. (Cl. 260—251.5)

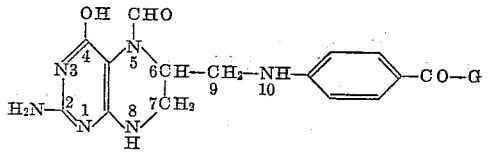
This is a continuation-in-part of U. S. patent application Serial No. 144,868, filed February 17, 1950.

This invention relates to certain pteroic acid compounds, and more particularly to formyltetrahydropteroic acid compounds, and to their preparation.

By this invention there are provided novel growth factors or vitamin-like compounds characterized by hemopoietic activity and additional desirable properties, and methods of preparing those compounds.

The vitamin-like properties of the novel compounds of this invention are readily illustrated by microbiological tests such as inhibition reversal or growth tests. In those tests the compounds exhibit characteristic growth-factor or vitamin-like activity and are more active than folic acid itself. Thus, for example, in an inhibition reversal test employing *Lactobacillus casei* and methylfolic acid as an inhibitor, the compounds are much more effective than folic acid. In an inhibition reversal test employing methylfolic acid and *Streptococcus faecalis*, the compounds are many times as effective as folic acid. More surprisingly, the novel compounds are capable of producing growth effects impossible with folic acid. Thus, it has been found that in a growth deficient synthetic culture medium, the addition of a small amount of one or more of the compounds readily stimulates a maximal growth rate of *Leuconostoc citrovorum* 8081, whereas folic acid is substantially ineffective.

The novel compounds of this invention comprise 5-formyl-5,6,7,8-tetrahydropteroic acid and the glutamyl derivatives thereof, and may be represented by the following formula



wherein G represents a hydroxyl group or a glutamyl residue. By the term "glutamyl residue" as used herein is meant a residue comprising one or more glutamyl groups attached to the formyltetrahydropteroic acid residue by a peptide linkage. A residue containing a plurality of glutamyl groups is attached to the pteroic acid portion of the molecule by a peptide linkage, and the glutamyl groups in the residue are attached to each other in a chain by peptide linkages which can be α or γ linkages. The number of glutamyl groups in the glutamyl residue can be from 1 to about 8.

Also included within the scope of this invention are the cationic salts of the above-represented pteroyl compounds. Illustrative examples of cationic salts are metallic salts such as the alkali and alkaline earth salts, e. g., the sodium, potassium, calcium, magnesium salts, etc.; the heavier metal salts, e. g. the iron, mercury and gold salts; and the metallic-like cationic salts, e. g. the ammonium, and substituted salts. Of the above-mentioned salts, the preferred salts are those of low toxicity

having substantial solubility in water, such as the alkali and alkaline earth metal salts, and simple ammonium salts.

The compounds of this invention are obtained by formylation and hydrogenation of pteroic acid compounds. Formylation is carried out by any of the methods known to the art for introducing formyl groups, and hydrogenation can be effected by chemical, catalytic or electromechanical means. Hydrogenation and formylation can be carried out simultaneously or stepwise. Thus for example, the pteroic acid compound can be hydrogenated and formylated, or alternatively, the pteroic acid compound can first be formylated and then hydrogenated. As will be set forth hereinafter, it is often desirable to subject the product obtained by formylation and hydrogenation to further processing, such as heating or hydrolysis, to obtain increased yields of the 5-formyl hydrogenated pteroyl compounds of this invention.

The pteroic acid compounds used as starting materials can be pteroic acid, or formylpteroic acid, also known as rhizopterin, or a pteroic acid or formylpteroic acid which contains one or more glutamyl groups, for example, folic acid or 10-formylpteroyl di-, tri-, or heptaglutamate. The non-formylated pteroyl compounds are all members of a recognized group of compounds exhibiting a folic acid-like activity. The formylation of the pteroic acid compounds in the 10-position is readily accomplished by the procedure described in J. Am. Chem. Soc. 70, 878 (1948); which comprises heating a pteroic acid compound with a mixture of formic acid and acetic anhydride, and removing any excess reagents and the acetic acid formed in the reaction by evaporation in vacuo. As described in the publication, the heating may be carried out at 100° C., but heating may be dispensed with if desired. Formylation thus carried out produces a 10-formylpteroic acid compound.

Although hydrogenation of a pteroic acid compound formylated in the 10-position produces through a rearrangement an amount of the 5-formyl-5,6,7,8-tetrahydroformylpteroic acid compound, for maximum yields of the compound, the reaction mixture obtained by the formylation and reduction is further treated, as by heating or autoclaving the mixture for a short time, or by allowing the reaction mixture to stand at room or lower temperature for a longer period of time. During the heating or standing step, the formyl group shifts from the 10- to the 5-position. Preferably when catalytic reduction is employed, the hydrogenation catalyst is removed from the mixture prior to this further treatment, but if, as not infrequently happens, the catalyst is peptized during the hydrogenation and so is present in difficultly separable colloidal state, the catalyst is permitted to remain in the mixture during the subsequent treatment, and then is removed. The additional heating or standing step is carried out at a pH of about 5 or above, and preferably at about pH 9. Duration of the heating or standing necessary to produce a maximum yield is dependent upon both temperature and pH. When the mixture is adjusted to alkaline pH, say about pH 9, and the mixture is allowed to stand under anerobic conditions at room temperature, a period of several days is necessary for maximum yield of vitamin-like compound as determined by the increased ability of the product to cause a growth response of *Leuconostoc citrovorum* 8081 in a growth deficient medium. Increasing the pH reduces the length of time necessary to attain a maximum yield. When the mixture is heated or autoclaved subsequent to formylation and reduction, a greatly increased yield of active product is obtained in a relatively short period of time. Thus for example, with the pH of the aqueous solution of reduced formylpteroic acid compound at about pH 5 or above, autoclaving at a temperature of about 120° C.

and a pressure of about 15 pounds for about one half hour gives a greatly augmented yield of new vitamin-like factor.

During the additional heating or standing step, air should be excluded from the vessel containing the aqueous solution of the reduced material since the presence of air (i. e., oxygen) during the heating or standing reduces the yield of active compound. The adverse effect of air is avoided by maintaining the aqueous solution of the reduced compound under an atmosphere of hydrogen, nitrogen or other inert gas, or by having present in the reduced mixture an adjuvant such as a reducing sugar or sugar-like substances. Preferably the adjuvant is incorporated in the mixture prior to the reduction to afford continuous protection to the reduced formylpteroic acid compound. Maintenance of a reducing atmosphere or medium is especially important when the 10-formyl-5,6,7,8-tetrahydropteroic acid compound is present in the reaction mixture in low concentration.

As noted above, hydrogenation of the pteroic acid compound can precede formylation in the production of the 5-formyl-5,6,7,8-tetrahydropteroyl compounds of this invention. It is possible for the hydrogenated pteroyl compound, for example, 5,6,7,8-tetrahydrofolic acid, to be formylated in the 2-, 5- and 10-positions. Mild formylation procedures lead to the formation of the 5-formyl or 5,10-diformyl derivatives or mixtures thereof, and possibly some 10-formyl derivative. Formylation under more drastic conditions yields the 5,10-diformyl derivative and possibly under such conditions an amount of the 2,5,10-triformyl derivative is produced. These di- and triformyl derivatives are converted to the 5-formyltetrahydropteroic acid compound by hydrolysis under mild conditions. The 5-formyltetrahydropteroic acid compounds are quite stable under hydrolytic conditions, whereas the 2- and 10-formyl derivatives are hydrolysed with great facility, the 2-formyl derivative being hydrolysed with exceptional ease.

Although not essential to the attainment of physiologically active compounds, it is usually preferable to obtain the novel compounds of this invention in substantially pure state. Isolation and purification of the compounds are readily accomplished by conventional methods. Chromatography affords an excellent isolation and purification procedure, especially when relatively small amounts of the hydrogenated formylpteroic acid compound are to be separated. A variety of adsorbing agents can be used. For example, chromatography on potato starch can yield a product having a purity ranging upwards of 70 percent. Likewise, alumina and prepared magnesium silicates (e. g. such as those sold under the names "Florisil" and "Magnesol") and other chromatographic agents are useful for effecting purification. An especially suitable procedure for use in those instances in which ascorbic acid has been used as an adjuvant in the reduction or subsequent heating reaction, comprises the adsorption of the active compound on alumina from 50 percent aqueous ethanol solution, elution of the compound with 50 percent aqueous ethanol containing from 2 to 5 percent by volume of concentrated ammonium hydroxide solution, and subsequent chromatography on potato starch with a solvent containing butanol, ethanol, water and acetic acid. An illustrative scheme of purification employing multiple chromatographic procedures is as follows: 120 ml. of a reaction mixture containing about 4 g. of 5-formyltetrahydropteroyl compound as determined by microbiological assay is added to a solvent mixture containing 80 ml. of ethyl alcohol, 200 ml. of n-butanol and 0.8 ml. of glacial acetic acid. After thorough agitation and centrifugation the upper layer is removed by decantation and set aside. To the lower layer are added 100 ml. of water, 80 ml. of ethyl alcohol, 200 ml. of n-butanol and 0.8 ml. of acetic acid and the mixture is centrifuged and the upper layer removed. The extraction is repeated once more with the same amount of solvent mixture, and is repeated three times with double amounts of solvent mixture for a total of six extractions. All of the extracts are combined and are allowed to stand for about 15–20 hours before final centrifugation. The supernatant layer, about 3500 ml. in volume, is further purified by chromatography on a starch column in the following manner:

A starch column is prepared from 4 kilos of dry potato starch slurried in a solvent mixture and packed into a 4-inch glass column. The solvent mixture used for packing the column and for development has the following composition:

	ml.
n-butanol	1000
Water	500
Ethyl alcohol	400
Acetic acid	4.0

After the material is on the column and the column has been developed with about 10 liters of solvent mixture, the column is examined under ultraviolet light. The yellow band at the bottom of the column is found to be essentially inactive, but the first fluorescent band above the yellow band is closely associated with the active fraction. The column is developed until the eluate containing this band is collected. The eluate is made alkaline with ammonium hydroxide and is concentrated in vacuo to about one fourth its volume, whereupon a separation into two layers occurs. The aqueous layer is removed and the butanol layer is washed four times with water. The combined aqueous extracts are concentrated in vacuo to about 75 ml. After addition of an equal volume of ethyl alcohol the material is further purified by chromatography on alumina.

A column of activated alumina is prepared with 50 percent alcohol using 50 grams of alumina per gram of formyltetrahydropteroic acid compound to be purified. The aqueous alcoholic solution of the active material is passed over the column and the column is washed with about 250 ml. of 50 percent alcohol. Elution is carried out with a solvent mixture containing 48 parts of water, 50 parts of ethyl alcohol and 2 parts of concentrated ammonium hydroxide. The eluate is first concentrated to a small volume (25–50 cc.) in vacuo and is then dried from the frozen state to yield a solid preparation of high activity. The activity of the material is such that 0.03–0.04 millimicrogram per ml. of test medium causes a half maximal growth response for the test organisms L. Citrovorum (8081) in the 16 hour growth response assay.

A further increase in purity of this material is achieved by chromatography on "Florisil" from an 0.05 N aqueous calcium chloride solution, followed by fractionation from aqueous alcohol. The resulting product contains substantially pure 5-formyl-5,6,7,8-tetrahydrofolic acid as its calcium salt.

As has been mentioned above, the compounds of this invention are characterized by certain physiological properties as evidenced by microbiological tests. In addition, the compounds can be characterized and differentiated by certain physical properties. Thus, for example, the novel compounds can be characterized by their R_f values. In accordance with the publication by Williams and Kirby in Science 107, 481–3 (1948), R_f values are determined by preparing a paper chromatograph of the substance under test and assigning as the R_f value the quotient obtained by dividing the distance on the paper strip traveled by the compound under test by the distance on the paper strip traveled by the solvent. By employing the same conditions of chromatography, R_f values are readily reproducible. Using a solvent mixture consisting of 25 parts of water, 50 parts of n-butanol and 12 parts of acetic acid, and carrying out the strip chromatography at room temperature, the R_f values determined for certain of the compounds of this invention as as follows: That product obtained by the formylation, hydrogenation and autoclaving of pteroyl-monoglutamate has an R_f value of about 0.56 to about 0.57; that obtained from pteroyl-γ-

glutamylglutamate has an R_f value of about 0.42 to about 0.45; that obtained from pteroyl-di-γ-glutamylglutamate has an R_f value of about 0.33 to about 0.34; that obtained from pteroyl-α-glutamylglutamate has an R_f value of about 0.45 to about 0.51.

The procedures outlined above and the reaction products produced thereby are further illustrated by the following specific examples.

Example 1

To 0.1 ml. of water solution of 200 γ (.0002 g.) of formylfolic acid adjusted to about pH 7.5 by the addition of dilute ammonium hydroxide, was added 0.1 ml. of a water solution of 800 γ (.0008 g.) of ascorbic acid, adjusted to about pH 7.5 with ammonium hydroxide. The mixture was placed in the side arm of a Warburg apparatus. In the main body of the apparatus were placed 1.8 ml. of water and about 1 mg. of platinum oxide catalyst. The apparatus was swept out with hydrogen and brought to a temperature of about 50° C. The apparatus was shaken until the platinum oxide catalyst had been reduced to platinum. The contents of the side arm were then transferred to the main body of the apparatus and hydrogenation continued for about 1½ hours. The hydrogen absorbed during this time approximated one molar equivalent based on the amount of formylfolic acid originally present. The hydrogenated solution was filtered to remove the platinum, and the solution was transferred to a test tube, the tube closed with a cotton plug, and autoclaved for about 40 minutes at a temperature of about 120° C. and 15 pounds pressure.

The mixture thus obtained was tested for growth activity on *Leuconostoc citrovorum* 8081 in a growth-deficient medium in the following manner: An aqueous culture medium was made up containing acid-hydrolysed casein supplemented with tryptophane and cysteine, a complete mixture of synthetic B-vitamins, uracil, guanine, adenine, 0.1 γ of thymidine per ml. of medium and inorganic salts customarily used in such media, and the medium was adjusted to about pH 6.8 with acetate buffer. 10 ml. quantities of the medium were each inoculated with *Leuconostoc citrovorum* 8081. To the separate samples of the medium were added measured, varied amounts of the above aqueous reaction product, the samples were incubated at a temperature of 37.5° C. and the growth of the microorganisms was followed photometrically. 1.5×10^{-5} ml. of the reaction product were found to evoke a maximal growth response.

No appreciable growth of the microorganism could be observed in control culture medium samples to which no reaction product had been added, nor in samples to which folic acid, or a mixture of formylfolic acid and ascorbic acid had been added.

Example 2

The procedure of Example 1 was repeated except that the pH of the aqueous mixture of formylfolic and ascorbic acids was adjusted with sodium carbonate solution instead of ammonium hydroxide.

The reaction product thus produced and tested in the *Leuconostoc citrovorum* test described in Example 1, showed a maximal growth response when 5×10^{-5} ml. of reaction product was added to 10 ml. of growth-deficient medium and half maximal response when 1×10^{-5} ml. were added.

Example 3

To 1 g. of folic acid were added 2 ml. of formic acid and 3 ml. of acetic anhydride, and the mixture was heated at about 40° C. for one hour. The mixture was evaporated in vacuo to remove excess reagents and acetic acid, leaving a solid residue of formylfolic acid.

The residue was suspended in 4 ml. of water, 4 g. of ascorbic acid were added and the mixture was neutralized to pH 7.3 by the addition of solid sodium carbonate. As the neutrality point was approached, all of the solid dissolved. The aqueous solution was reduced in a Parr hydrogenation apparatus with 0.2 g. of platinum oxide and hydrogen at a pressure of about 40 pounds, until about 1 molecular equivalent of hydrogen, based on the amount of formylfolic acid used, was absorbed. The mixture was filtered to remove most of the platinum catalyst and was autoclaved for one hour at 120° C. and 15 pounds pressure. The autoclaved mixture was cooled and refiltered to remove the small amount of catalyst not removed by the first filtration. The filtrate when tested for growth activity according to the procedure described in Example 1 was found to possess growth-factor activity.

Example 4

500 mg. of formylfolic acid was reduced in the presence of 2 g. of ascorbic acid in 50 ml. of water at pH 7 with hydrogen at atmospheric pressure in the presence of Adams' platinum catalyst. After consumption of slightly greater than one molecular equivalent of hydrogen, the mixture was autoclaved for approximately 1 hour. This sample at concentration equivalent to 0.100 γ of the original formylfolic acid per 10 ml. of medium gave a half-maximal response with *Leuconostoc citrovorum* 8081 in the microbiological assay. The sample was diluted with 60 ml. of alcohol (95 percent), and the catalyst was removed by centrifuging the reaction mixture.

Alumina was pretreated with 15 percent sodium cyanide, washed with water, 50 percent acetic acid and again with water, and activated by heating at 180° C. under reduced pressure for four to five hours. A column of this alumina (35 g.) was prepared in 50 percent alcohol, and the alcoholic solution of the reaction mixture was added to the column. The column was washed with 500 ml. of 50 percent alcohol followed by 250 ml. of 80 percent alcohol and 250 ml. of 80 percent alcohol containing 5 percent concentrated ammonium hydroxide. The active principle was eluted by washing the column with 300 ml. of 70 percent alcohol containing 5 percent concentrated ammonium hydroxide and with 100 ml. of 50 percent alcohol containing 5 percent concentrated ammonium hydroxide. The active material (444 mg. of solids) was obtained from the eluate by evaporation under reduced pressure at room temperature.

A potato starch column was prepared in a solvent mixture of the following composition: n-butanol, 250 parts; water, 125 parts; absolute ethanol, 100 parts, and glacial acetic acid, 1 part. The pH of the solvent was approximately 3.9 to 4.0. The sample (444 mg.) was dissolved in 2 ml. of water and 1 ml. of absolute ethanol was added. To this solution, 0.01 ml. of glacial acetic acid and 4 ml. of n-butanol were added. The mixture was centrifuged, and the butanol phase was added to the starch column. The single extract was chromatographed on the column and the column was developed with the same solvent used to dissolve the active material. Approximately 53 ml. of solvent passed through the column before a band observed under ultra-violet light as light yellow began to pass out of the column. The light yellow band passed out of the column in approximately 36 ml. of eluate and was followed after a few ml. of eluate by a band which was deep yellow under ultra-violet light. This band was removed from the column in approximately 21 ml. of eluate. After a few ml. more of solvent passed through the column, a band which was pale yellow was recovered in the eluate in fractions totaling approximately 20 ml. The active principle followed closely after this pale yellow band in a band which was pale blue under ultra-violet light. The active fractions were evaporated under reduced pressure to obtain approximately 12 mg. of solids. Approximately half of this material was freed from starch by adsorption on a small alumina column in a manner analogous to that described above, and was eluted from the alumina to obtain approximately 5.5 mg. of solids free of starch. Solvent fractionation by solution in methanol

and precipitation from methanol by addition of acetone produced substantially pure active principle in an amorphous state.

Example 5

2.5 g. of folic acid was hydrogenated in 50 ml. of formic acid with platinum oxide as a catalyst. The reaction mixture after consumption of two molecular equivalents of hydrogen was evaporated to dryness at room temperature under reduced pressure. The residue was dissolved in 50 ml. of 20 percent ascorbic acid at pH 7, and autoclaved for one hour. The reaction mixture at a concentration equivalent to 0.0007 to 0.001 γ of the original folic acid per 10 ml. of medium stimulated a half-maximal response with *Leuconostoc citrovorum* 8081.

The crude reaction mixture (50 ml.) was diluted with four volumes of ethanol and centrifuged. The alcoholic solution was added to a column of approximately 50 g. of "Florisil" which had been prepared in alcohol solution (80 percent by volume of 95 percent alcohol) buffered to pH 4.5–5.0, and washed well with alcohol. The residue centrifuged from the alcoholic solution was dissolved in a small volume of water and diluted with four volumes of alcohol. The solution was added to the column just as the first 250 ml. of solution prepared above completely entered the column. The residue was again treated in a similar manner, and the column was then developed with the alcohol (80 percent by volume of 95 percent alcohol). The first 200 ml. of solution passing through the column were inactive. The next 200 ml. contained essentially all of the original active principle in total solids amounting to 1.3 g.

The material from the "Florisil" column was dissolved in a small amount of water and an equal weight of calcium chloride was added. The aqueous solution was centrifuged and added to a column of 40–60 g. of "Magnesol." The "Magnesol" column had been prepared by slurrying the "Magnesol" in 200 ml. of buffer solution (sodium acetate-acetic acid at pH 4.5–5.0) containing 10 g. of calcium chloride, pouring the slurry into a column of appropriate diameter, and washing it well with water. The active principle in the aqueous calcium chloride solution was added to the column, and the slight residue obtained upon adding the calcium chloride to the active principle in aqueous solution was washed several times with water, and the washings were added to the column after each preceding solution entered the column. The column was then developed with water. The first 150 ml. which passed through the column usually did not contain any appreciable amounts of the active principle. The next 150 to 200 ml. contained the active principle in good yield. On evaporation of the aqueous solution, approximately 200 mg. of essentially pure calcium salt recrystallizable from water was obtained.

The barium salt is obtained in substantially the same manner by using barium chloride solution instead of calcium chloride solution.

Example 6

A mixture was prepared from 2.5 g. of folic acid, 50 ml. of 98–100 percent formic acid and 50 mg. of platinum oxide catalyst, and the mixture was maintained at a temperature of about 30° C. and stirred under an atmosphere of hydrogen for about 90 minutes, during which time about 235 ml. of hydrogen was absorbed. The mixture was filtered to remove the catalyst and evaporated in vacuo to remove excess formic acid. The residue was dissolved in 100 ml. of boiled water and was neutralized with sodium carbonate. 4 g. of ascorbic acid were added, and the solution was brought to about pH 8 by the addition of sodium carbonate solution. The slightly alkaline solution was autoclaved at 119° C. for one hour. Bioassay of the autoclaved solution showed a pronounced ability of the composition to stimulate the growth rate of *Leuconostoc citrovorum* 8081 in a growth-deficient synthetic culture medium.

Example 7

886 mg. of pteroylglutamic acid are dissolved in 50 ml. of 98 percent formic acid. 100 mg. of platinum oxide catalyst are added and the mixture is hydrogenated at room temperature with hydrogen at a pressure of about 40 pounds until two molecular equivalents of hydrogen are absorbed. 10 ml. of acetic anhydride are added to the mixture and the mixture is allowed to stand at room temperature for about one hour. The catalyst is filtered off and the filtrate is evaporated to dryness from the frozen state. The solid residue which contains a mixture of polyformylated 5,6,7,8-tetrahydrofolic acids is dissolved in 50 ml. of 0.1 N sodium hydroxide solution. The solution is allowed to stand at room temperature for about 24 hours, whereupon 5-formyl-5,6,7,8-tetrahydrofolic acid in the form of its sodium salt is produced. 5-formyl-5,6,7,8-tetrahydrofolic acid is recovered from the reaction mixture, and purified by the procedure described in Example 4.

Example 8

200 γ of formylpteroic acid and 2000 γ of ascorbic acid were dissolved in water, and the solution was neutralized with sodium carbonate solution, and made up to a volume of 2 ml. About 1 mg. of platinum oxide catalyst was added and the mixture was hydrogenated for 2 hours at room temperature at about 30 pounds hydrogen pressure in a modified Parr hydrogenation apparatus. The hydrogenation mixture was autoclaved at a temperature of about 120° C. and a pressure of about 15 pounds for about 1 hour. The reaction mixture thus obtained was tested by the *Leuconostoc citrovorum* test described in Example 1. 5×10^{-5} ml. of the mixture were required for half maximal growth of the organism and 1.5×10^{-2} ml. were required for maximal growth. Isolation of the 5-formyl-5,6,7,8-tetrahydropteroic acid is accomplished by the procedure described in Example 4.

Example 9

The procedure described in Example 8 was followed except that formyl-N-pteroyl-α-glutamylglutamic acid was used in place of formylpteroic acid. Using the test procedure described in Example 1, 2×10^{-3} ml. of the reaction product were required for half maximal growth of the organism and 6×10^{-3} ml. were required for maximal growth. R_f studies showed the product comprised a mixture of the monoglutamyl and di-α-glutamyl 5,6,7,8-tetrahydro-5-formylpteroic acid.

Example 10

The procedure of Example 8 was repeated except that formyl-N-pteroyl di-(γ-glutamyl)-glutamic acid was used in place of formylpteroic acid. The reaction product obtained was tested in accordance with the procedure described in Example 1. 1.5×10^{-3} ml. of reaction product were required for half maximal growth, and 5×10^{-3} ml. were required for maximal growth. The reaction mixture contained 5-formyl-5,6,7,8-tetrahydropteroyl mono-, di- and tri-α-glutamates.

Example 11

0.01 mol of formylfolic acid is added to 200 ml. of water and the mixture is adjusted to about pH 5.3 with anhydrous sodium carbonate. 150 mg. of platinum oxide are added and the mixture is hydrogenated in a hydrogenation apparatus until about 1 molecular equivalent of hydrogen is taken up. The hydrogenation bottle is stoppered and autoclaved for about 2 hours at a temperature of about 120 to 125° C. at a pressure of about 15 pounds.

Six reaction mixtures obtained as described above are combined and are evaporated in vacuo to a volume of about 90 ml. To the concentrated solution are added

60 ml. of ethanol, 150 ml. of n-butanol and 0.6 ml. of glacial acetic acid. The mixture is thoroughly agitated and centrifuged, and the clear supernatant liquid is decanted. To the lower layer consisting largely of water and some semi-solid material, are added 75 ml. of water, 60 ml. of ethanol, 150 ml. of butanol and 0.6 ml. of glacial acetic acid. The mixture is shaken and centrifuged as above, and the supernatant layer removed. The lower layer is extracted six additional times in accordance with the above procedure.

The extracts are combined, and allowed to stand for about 20 hours during which time a small amount of insoluble material separates. The extracts are centrifuged and the supernatant layer is treated by chromatography as follows: 4 kg. of dry potato starch are slurried with 8 liters of a solvent mixture consisting of about 100 parts by volume of n-butanol, 5 parts of water, 40 parts of ethanol and 0.4 part of glacial acetic acid. The slurry is used to prepare a chromatographic column in a 4-inch glass pipe. To the column are added the combined supernatant extracts and the column is developed with about 22 liters of the solvent mixture used to prepare the column. During the development, the flow is adjusted to about 80 to 120 ml. per hour, and a head of about 2 to 3 liters of the solvent is maintained above the starch. The column is developed with about 25 liters of solvent mixture and the eluate discarded. The next two liters of eluate are collected, these fractions possessing the highest activity as determined biologically.

The eluate is neutralized with about 5 ml. of concentrated ammonium hydroxide, and evaporated in vacuo until two layers appear. The lower, predominantly aqueous, layer is separated. The upper, predominantly butanolic, layer is extracted with three 10 ml. portions of water. The aqueous fractions are combined and diluted with 120 ml. of ethanol, and the aqueous ethanol solution containing the active compound is treated in the following manner:

A chromatographic column is prepared with 50 g. of purified activated alumina in a solvent mixture consisting of 50 percent ethanol. The aqueous ethanol solution of the active compound is placed on the column and developed with 50 percent ethanol. A total of about 2350 ml. of developing solvent is used. The column is drained and the alumina extruded, and the top and bottom ½-inch portions of the extruded alumina are removed. The remainder of the column is eluted eight times with 20 ml. portions of an aqueous ethanol solvent mixture containing 5 percent by volume concentrated ammonium hydroxide. The eluate is concentrated to a small volume, is frozen, and is dried from the frozen state. About 10 mg. of the solid residue are dissolved in 1 ml. of water. The solution is cooled and acidified with .25 N hydrochloric acid whereupon a precipitate forms. The precipitate is removed by centrifuging and is washed with 1 ml. of cold water. It is dissolved in 0.3 ml. of dimethylformamide, and water is added to the point of incipient turbidity. The solution is placed in an ice bath, and the sides of the container are scratched with a stirring rod. Upon standing, the solution becomes turbid. The solution is allowed to warm to room temperature gradually, and crystals form along the scratch lines over a period of about 4 hours. The mixture is centrifuged, and the crystals thus isolated are washed with a mixture of 3 ml. of water and 0.5 ml. of dimethylformamide, and are then washed with two 1 ml. portions of ethanol. The crystalline product is dried in vacuo at room temperature.

The product thus obtained was characterized as follows: A sample was dried at about 150° C. in vacuo for a period of 2 hours. The weight loss was determined to be 12.3 percent which corresponds to about 3 molecules of water.

Analysis of the dried sample showed the presence of 50.64 percent of carbon, 5.01 percent of hydrogen and 20.97 percent nitrogen as compared with the values of 50.73 percent carbon, 4.90 percent hydrogen and 20.71 percent nitrogen calculated for 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid ($C_{20}H_{23}N_7O_7$).

Analysis of the dried sample for the weight percentage of formyl groups was found to be 5.5 percent as compared with the calculated value of 6.1 percent.

The crystalline material showed the presence of three groups having a pk a' of about 10.4, 4.8 and 3.1.

The optical rotation of the compound was found to be as follows: $[\alpha]_D^{25}=10.5°$ (c=8.3 in water at pH=8.5).

The ultraviolet absorption spectrum of the crystalline material showed a minimum absorption peak at about 242 mμ and a maximum absorption peak at about 282 mμ.

An infrared examination of the crystalline material mulled in mineral oil, showed distinct absorption maxima at the following wave lengths, expressed as microns: 5.82; 6.1; 6.2; 7.5; 7.8; 8.0; 8.2; 8.4; 9.0; 9.6; 11.9; and 13.1.

X-ray diffraction studies on a sample of the compound crystallized from water gave the following interplanar spacings (D-values) and their relative intensities.

"D" values:	Relative intensities
11.5	.40
10.4	1.00
8.19	.32
7.41	.04
6.97	.04
6.58	.04
6.04	.12
5.79	.02
5.32	.12
4.95	.16
4.76	.12
4.63	.16
4.46	.04
4.37	.16
4.06	.20
3.83	.04
3.76	.04
3.49	.02
3.44	.02
3.29	.60
3.19	.08
2.95	.02
2.84	.02
2.71	.02
2.52	.02
2.45	.02
2.30	.02
2.20	.02
2.04	.02

Example 12

0.1 g. of formylfolic acid and 0.4 g. of ascorbic acid were added to about 8 ml. of water and to the mixture was added sufficient sodium carbonate to effect complete solution of the formylfolic acid. 0.4 g. of sodium hydrosulfite were added to the solution and water was added to bring the total volume of the solution to 10 ml. The solution was adjusted to about pH 3.5 with dilute hydrochloric acid and the adjusted solution was heated for 50 minutes in an autoclave at a temperature of 120° C. and a pressure of 15 pounds. The reaction mixture was tested in accordance with the test procedure described in Example 1. 5×10^{-8} ml. of the mixture gave a half maximal growth response.

Example 13

50 mg. of formylfolic acid and 200 mg. of ascorbic acid were dissolved in 50 ml. of dilute aqueous ammonium hydroxide (5 percent conc. NH_4OH and 95 percent H_2O). The solution was placed in a 100 ml. beaker containing a layer of mercury which, serving as the cath-

ode, was connected to the source of E. M. F. through an insulated wire. The anode which consisted of a platinum plate, was inserted in a porous cup of about 1 inch diameter. The cup was immersed in the solution in the beaker and filled with aqueous ammonium hydroxide to the height of the level of the external catholyte. The electrodes were connected to a battery having an E. M. F. of 8 volts and the electrolysis was carried out at a current intensity of about 25 ma. During the electrolysis which was carried out for about 2 hours, the anolyte decreased in volume and became acidic, and from time to time the volume was restored and the anolyte made basic by the addition of ammonium hydroxide solution. After 2 hours, the catholyte was neutralized with ascorbic acid and was autoclaved for 1 hour at a temperature of 120° C. and a pressure of 15 pounds. The autoclaved solution was tested for growth activity according to the procedure described in Example 1. 1×10^{-5} ml. gave a half maximal growth response.

Example 14

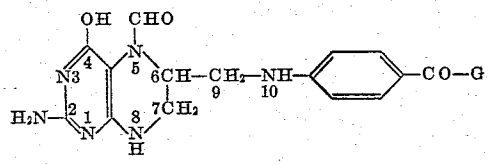
100 mg. of formylfolic acid and 400 mg. of ascorbic acid were suspended in 10 ml. of absolute ethanol. 50 mg. of sodium were added to the suspension and the mixture was heated to 60–80° C. for about 1 hour and was then refluxed for about 20 minutes. An equal volume of water was added to the reaction mixture, the solution was neutralized to about pH 7.2 and autoclaved for 1 hour at a temperature of 120° C. and 15 pounds pressure. The autoclaved solution was tested for growth activity according to the procedure of Example 1. 2×10^{-4} ml. of the autoclaved solution gave a half maximal growth response.

Example 15

100 mg. of formylfolic acid and 400 mg. of ascorbic acid were suspended in water and the suspension was brought to pH 4 by the addition of sodium carbonate whereupon a solution was obtained. To the solution were added 100 mg. of ammonium chloride and 50 mg. of zinc dust, and the mixture was allowed to stand at room temperature for about 2 hours. The reaction mixture was filtered and the filtrate tested for growth activity according to the procedure described in Example 1. 1×10^{-5} ml. of the filtrate gave a half maximal growth response.

I claim:

1. Compounds of the group consisting of compounds represented by the formula



wherein G represents a member of the group consisting of a hydroxyl group and a glutamyl residue; and cationic salts thereof.

2. Cationic salts of 5-formyl-5,6,7,8-tetrahydrofolic acid.

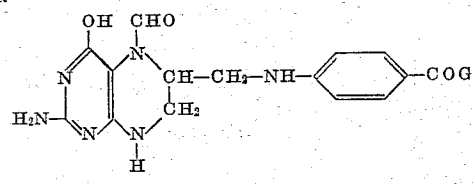
3. 5-formyl-5,6,7,8-tetrahydrofolic acid.

4. 5-formyl-5,6,7,8-tetrahydropteroic acid.

5. 5-formyl-5,6,7,8-tetrahydropteroyl-diglutamate.

6. 5-formyl-5,6,7,8-tetrahydropteroyl-triglutamate.

7. The method of preparing compounds of the formula



wherein G represents a member of the group consisting of a hydroxyl group and a glutamyl residue, which comprises formylating and hydrogenating a pteroic acid compound and subjecting the resulting compound to a pH of at least 5.

8. The method according to claim 7 in which the hydrogenation is effected by hydrogen and a hydrogenation catalyst.

9. The method according to claim 7 in which the hydrogenation is effected by a chemical reducing agent.

10. The method according to claim 7 in which the hydrogenation is effected electrochemically.

11. The method according to claim 7 in which the formylated and hydrogenated pteroic acid compound is further treated by heating at a pH of at least 5.

12. A method according to claim 7 in which the formylated and hydrogenated pteroic acid compound is further treated by hydrolyzing under mild conditions.

13. A method of preparing a novel growth factor which comprises formylating a pteroic acid compound, hydrogenating said compound, and heating the formylated-hydrogenated product at a pH of at least 5, thereby to obtain a 5-formyl-5,6,7,8-tetrahydropteroic acid compound.

No references cited.