partial hydrolysis. Sometimes, as with glucosylaniline particularly, a simultaneous or prior reaction (reaction A) took place.

Under some conditions a mutarotation took place without hydrolysis, or the prior occurrence of such a reaction was inferred from the initial rotations. This has been called reaction A and presumably arises from reversible isomerization reactions involving ring or α,β-changes.

All of the glucosylamines when dissolved in glacial acetic acid developed a brown color, and when the rotations could be observed, they became more levorotatory. The nature of this reaction (reaction B) is also unknown, but it may be of importance in the development of colored bodies which are formed readily from glucosylamines. Possibly a reaction like the Amadori-type rearrangement is involved.

Mitts and Hixon[5] have pointed out that with the exception of glucosylamine itself, the hydrolysis of glycosylamines seems to parallel the dissociation constant of the aglycon amine. This relation seems to hold generally for the glucosyl derivatives of simple alkyl and aryl amines and agrees with the known stability of N-acetylglucosylamine and nucleosides. However, not only is glucosylamine an exception, but also glucosylglycine which dissociates readily. Before such a generalization can be made a distinction must be made between the rate and the extent of hydrolysis, and in view of the work of Isbell and Frush,[10] the *p*H at which comparisons are made must be carefully considered. The stability of maltosyldodecylamine also is not consistent with the strong basicity of the parent amine. Since the product is highly surface active, possibly its lack of hydrolysis may arise from its presumed orientation and high concentration in the surface layer. The maltosylhexylamine dissociated readily in water.[11]

Experimental

Preparation of Alkyl Glucosylamines.—Method 1 (based on Weygand[12]). A mixture of 18 g. (0.1 mole) anhydrous glucose, 20.3 g. (0.12 mole) 4-aminobiphenyl and 5.4 g. (0.3 mole) water was heated in a bath of boiling water for 15 minutes. To the clear solution was added 25 ml. of methanol, and the solution was allowed to crystallize in the refrigerator. The crystals were filtered and washed with ether; yield 17.9 g. (66%).

Method 2 (Based on Sorokin).[13]—To a solution of 36 g. (0.5 mole) of *n*-butylamine in 50 ml. of methanol was added 90 g. (0.5 mole) of anhydrous glucose. The stirred solution was heated at 60–65° for 15 to 20 minutes, diluted with 300 ml. of hot ethanol and allowed to cool to room temperature. Crystallization occurred; yield (of two crops) 120 g. (94%). Methanol was found preferable to ethanol as used by Sorokin and others. Higher yields and less discoloration resulted.

The melting points and analyses of the products used are given in Table III.

(11) Several crude maltosylamines and their reduction products have been described previously by J. H. Werntz, U. S. Patent 2,181,929, December 5, 1939.

(12) F. Weygand, *Ber.*, **72**, 1663 (1939).

(13) W. Sorokin, *ibid.*, **20**, 783 (1887).

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

A Synthetic Compound with Folinic Acid Activity

BY EDWIN H. FLYNN, THOMAS J. BOND, THOMAS J. BARDOS AND WILLIAM SHIVE

The preparation and properties of a synthetic compound having biological activities similar to folinic acid, a new B-vitamin related to folic acid, are described. The synthetic factor, folinic acid-SF, is prepared by reduction of formylfolic acid or by reduction of folic acid in the presence of compounds capable of donating a single carbon unit.

In attempts to develop assays for the anti-pernicious anemia principle(s) in refined liver extracts, numerous testing procedures for unknown principles were developed as a result of their particular effects on the toxicities of inhibitory analogs of folic acid and *p*-aminobenzoic acid for certain organisms.[1]

One group of related factors were detected as a result of their enhanced ability, when compared with folic acid, to prevent the toxicity of *x*-methylfolic acid for either *Lactobacillus casei* or *Streptococcus faecalis* R.[2] This group of factors has been termed the folinic acid group, and one of the factors closely related to folic acid has been termed folinic acid.

Purified liver extracts have been reported to contain a factor or factors necessary for the growth of *Leuconostoc citrovorum* 8081 under specified conditions.[3] This growth promoting effect of purified liver extracts has been reported to be the result of a synergistic effect of a combination of thymidine with factors which appear to be identical with the folinic acid group[4]; however, thymidine[5] or folinic acid[4] alone promote growth of this organism at concentrations considerably higher than those required for the two factors combined.

A recent communication has described the preparation from folic acid of a reaction mixture which has the biological activities of folinic acid derived from liver.[6] It is the purpose of this paper to report in detail the preparation in crystalline form and the properties of a synthetic compound, folinic

(1) W. Shive, *Trans. N. Y. Acad. Sci.*, **52**, 1212 (1950); presented before the New York Acad. Science, Feb., 1949.

(2) T. J. Bond, T. J. Bardos, M. Sibley and W. Shive, THIS JOURNAL, **71**, 3852 (1949).

(3) H. E. Sauberlich and C. A. Baumann, *J. Biol. Chem.*, **176**, 165 (1948).

(4) T. J. Bardos, T. J. Bond, J. Humphreys and W. Shive, THIS JOURNAL, **71**, 3852 (1949).

(5) E. E. Snell, E. Kitay and W. S. McNutt, *J. Biol. Chem.*, **175**, 478 (1948).

(6) W. Shive, T. J. Bardos, T. J. Bond and L. L. Rogers, THIS JOURNAL, **72**, 2817 (1950).

acid-SF,[7,8] having a biological activity similar to the folinic acid group.

The steps necessary to produce a reaction mixture with high yields of activity[6] consisted of (1) formylating folic acid, (2) reducing the formylfolic acid with hydrogen and (3) autoclaving the resulting product. The reduction product obtained in step (2) appeared to be unstable to oxygen and had to be protected prior to and during the autoclaving. This was done by carrying out the reduction and heating in the presence of ascorbic acid or by maintaining an inert atmosphere. Omission of any of the steps listed above resulted in decreased activity of the final product. When ascorbic acid was used as a protective agent, it was found that omitting formylation decreased the activity to 3% of that obtained with all steps included. Oxalic acid has been detected in the reaction mixture containing ascorbic acid and presumably served as a source of the single carbon unit in the absence of the formylation step. Similarly, by reduction of folic acid in formic acid, the formylation step could be omitted without appreciable loss in yield if the formic acid was neutralized or removed *in vacuo* prior to the autoclaving step. When autoclaving was omitted the yield dropped to 20% or less of normal. In some cases, hydrogenation of formylfolic acid does not proceed beyond the formation of a dihydro derivative even on long contact with hydrogen. In such cases, autoclaving was essential for production of a reaction mixture with appreciable activity. When folic acid was hydrogenated in the presence of ascorbic acid, *i.e.*, omitting steps (1) and (3), the reduction product had less than 1% of the usual activity. Folic acid reduced in the absence of other organic compounds was essentially inactive.

Isolation of folinic acid-SF was best accomplished by chromatography on potato starch columns. The reaction mixture obtained from folic acid as described above was chromatographed from a solvent mixture of *n*-butanol, ethanol, acetic acid and water. Since the reaction mixture was highly fluorescent in ultraviolet light, it was possible to follow the development of the columns visually. As many as twelve fluorescent zones appeared during the process of development, in addition to several yellow zones visible in natural light. The columns were quite reproducible, only minor variations being observed. After chromatographic behavior of the active substance had been ascertained, microbiological assays were no longer necessary since the folinic acid-SF was associated with one well-defined blue fluorescent zone.

Ultraviolet absorption measurements proved to be useful for following the progress of purification. Concentrates which gave half-maximal growth in the *L. citrovorum* assay[4] at a level of 8×10^{-5} to 4×10^{-5} γ per ml. of medium usually exhibited a minimum between 239 and 242 mμ, and two maxima, one at 280 mμ and the other at 370 mμ. As purification progressed a shift of the minimum to 242 mμ occurred, the maximum at 280 mμ shifted to 282 mμ and that at 370 mμ disappeared. The ratio of the intensity of absorption at 282 mμ to that at 242 mμ was found to be a useful criterion in evaluating purification procedures. This ratio, for the fractions obtained from starch columns, varied from 2.2 to 3.9.

A further purification of the active material from starch columns was readily achieved by using "Florisil"[9] as an adsorbent. Behavior of these columns was also readily reproducible. Chromatography on "Florisil" was carried out with water as the solvent; progress of the separation was followed by ultraviolet absorption measurements on the eluate. The ratio of intensity of absorption at 282 mμ to that at 242 mμ was improved from an initial value of 2.2–3.9 to a maximum value of 4.6 for the eluted activity. The most active fractions did not fluoresce to any appreciable extent in ultraviolet light.

Crystallization of folinic acid-SF was readily effected by adjusting the *p*H of the active fractions to 2.8–3.0 and then concentrating the solutions under reduced pressure. The free acid was only sparingly soluble in water. It was further purified by dissolving in water with the aid of dilute base, followed by acidification to *p*H 2.8–3.0, whereupon the free acid again separated in crystalline form. In the *L. citrovorum* 8081 assay,[4] 1×10^{-5} to 2×10^{-5} γ per ml. of the thrice recrystallized acid gave a half-maximal growth response. Elementary analysis gave values in good agreement for the formula $C_{20}H_{23}N_7O_7$. The weight loss on drying at 150° indicated about 3.5 moles of water of crystallization but varied between 3 and 4 moles depending on the manner in which preliminary drying of the sample was carried out. Electrometric titration showed the presence of three acidic groups with pK_a' values of 3.1, 4.8 and 10.4 ± 0.2. The ultraviolet absorption spectrum of folinic acid-SF is shown in Fig. 1.

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Experimental[10]

Preparation of Folinic Acid-SF from Folic Acid (a).—Folic acid (10.0 g.) was mixed with 20 ml. of 98–100%

(7) Since the relationship of this compound to the factor in liver extracts cannot be conclusively shown until the naturally occurring factor has been isolated in sufficient amounts for detailed comparisons, it is proposed to use the tentative designation folinic acid-SF for the compound prepared from folic acid.

(8) Subsequent to the completion of this work and the preliminary report on a synthetic method,[6] a preliminary communication (J. A. Brockman, Jr., *et al.*, THIS JOURNAL, **72**, 4325 (1950)) has reported the isolation of a crystalline barium salt from reaction mixtures resulting similarly from catalytic hydrogenation of folic acid or formylfolic acid in formic acid solution. This barium salt is effective in promoting the growth of *Leuconostoc citrovorum* 8081 and is the salt of an acid of the same elementary composition as the folinic acid-SF reported in this paper.

(9) "Florisil" is a synthetic adsorbent sold by The Floridin Co., Warren, Pennsylvania.

(10) All ultraviolet absorption measurements were made in 0.05 *N* sodium hydroxide unless otherwise indicated.

formic acid and 3 ml. of acetic anhydride was added. The reaction mixture became warm and suspended solid slowly dissolved. After one hour at 40–50° excess reagents were removed by drying from the frozen state. The formylated folic acid was suspended in 425 ml. of distilled water, and ascorbic acid (40 g.) was added. Solid sodium carbonate was then added until a *p*H of 7.3 was attained and the formylfolic acid was in solution.

Platinum oxide catalyst (2.0 g.) was added, and the solution was hydrogenated at 40–50 lb. pressure until 1.1 mole equivalents of hydrogen had been absorbed (based on a molecular weight of 473 for formylated folic acid). The solution was then autoclaved for one hour at 15 lb. pressure and 120°. An amount of this solution equivalent to 1×10^{-4} γ of the original folic acid per ml. of medium gave a half-maximal growth response with *L. citrovorum* 8081 under the testing conditions.[11]

(b).—Formylated folic acid (4.7 g.) prepared as described above was dissolved in 100 ml. of distilled water by adding sodium carbonate until the final *p*H of the solution was 5.4. Platinum oxide (0.20 g.) was added and 1.1 mole equivalents of hydrogen introduced at 40–50 lb. pressure. After hydrogenation was completed, the bottle was stoppered tightly, care being taken to maintain the hydrogen atmosphere and to exclude air. The stoppered bottle was autoclaved 2 hours at 120° and 15 lb. pressure. A half-maximal growth response of *Leuconostoc citrovorum* was induced by an amount of the reaction product equivalent to 1×10^{-4} of original formylfolic acid per ml. of medium. Similar reaction mixtures gave half-maximal responses at concentrations equivalent to 7×10^{-5} to 1.3×10^{-4} γ of the original formylfolic acid per cc. of medium.

Purification by Chromatography on Potato Starch.—Six batches prepared as described under preparation (b) were combined and the solution concentrated under reduced pressure to a volume of 90 ml. To this was added ethanol (60 ml.), *n*-butanol (150 ml.) and glacial acetic acid (0.6 ml.). The mixture was agitated thoroughly and centrifuged, and the two liquid phases were separated. To the bottom layer was added 75 ml. of distilled water, 60 ml. of ethanol, 150 ml. of *n*-butanol and 0.6 ml. of acetic acid. The mixture was agitated and centrifuged as before. Again, the upper phase was separated, and this extraction process was repeated six additional times on the lower phase in the same manner. The extracts of the lower phase were combined and allowed to stand several hours. Some precipitate formed and was removed by centrifuging. The clear yellow supernatant solution (2475 ml.) gave a half-maximal response at 1×10^{-8} ml. per ml. of assay medium. It was applied to a column prepared as described below.

Dry potato starch (4 kg.) was slurried in 8 liters of a solvent mixture composed of *n*-butanol 100 parts, distilled water 50 parts, ethanol 40 parts and glacial acetic acid 0.4 part. This solvent mixture was also used for developing the column. The starch slurry was poured into a glass column four inches in diameter and allowed to settle several hours; then the excess solvent was drained off.

The 2475 ml. of solution was placed on the column. Development was carried out with the solvent mixture described above. After an initial period during which the adsorbent settled, a flow rate of 80–100 ml. per hour was attained, depending on the solvent head on the column.

About 2 l. of developing solvent passed through before the appearance of the fluorescent zone which later was shown to be associated with the active principle. Seven zones of varying intensity and color in ultraviolet light preceded the active zone. After 5 l. of solvent had passed, removing the zones which could be seen initially, four zones were visible in ultraviolet light. These in ascending order were a yellow-green zone, a blue zone followed by another blue zone which proved to be associated with active material and a yellow-green zone. About 7 l. of developing solvent was passed through the column before activity could be found in the eluate. An additional 4 l. of solvent mixture then removed all the activity. Approximately 90% of the activity applied to the column was recovered in this eluate which consisted of a number of fractions.

The active fractions were processed separately. Concentrated ammonium hydroxide was added in slight excess to neutralize the eluates. Each fraction was then concentrated *in vacuo* until a separation into two layers occurred. The lower aqueous phase was removed and the butanol layer extracted four times with distilled water. The combined aqueous extract was concentrated *in vacuo* to a small volume (10–25 ml.). Ten volumes of ethanol were added and the solid triturated. After centrifuging, the precipitate was redissolved in 10–25 ml. of water and precipitated again with ethanol. The solid was washed once with ethanol and dried *in vacuo*. Typical preparations gave a half-maximal response at 2×10^{-5} to 4×10^{-5} γ per ml. of assay medium.

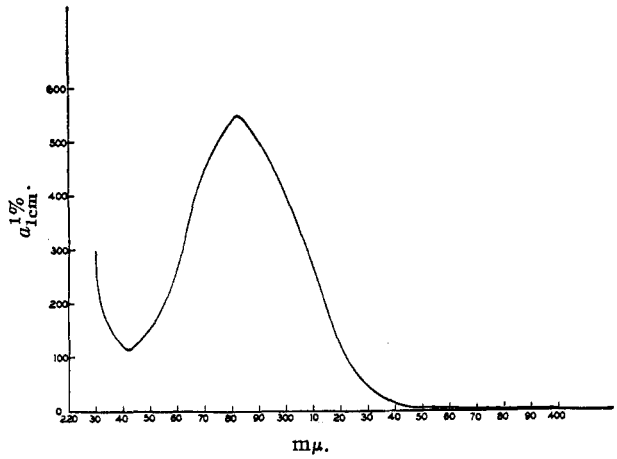

Fig. 1.—Ultraviolet absorption spectrum of folinic acid-SF in 0.1 *N* sodium hydroxide.

Subsequent experiments showed that the starch column described above could satisfactorily accommodate larger amounts of the active reaction mixture. In a typical experiment the reaction mixtures from 0.16 mole (70 g.) of folic acid were combined and extracted as described above using proportionate quantities of solvents in the first three extractions, then doubling these amounts for the last three extractions. The extract, 6,560 ml., which gave a half-maximal response at 1.6×10^{-8} ml. per ml. assay medium was applied to a 4 kg. starch column and developed in the manner previously described. About 35 l. of solvent passed through before active material appeared in the eluate. The active zone was collected in four 2-l. fractions. A quantitative recovery of activity was obtained based on microbiological assay of the solutions. After processing the four fractions individually, the data in Table I were obtained on the solid products. Recovery of activity in the solids was about 75%.

TABLE I

PROPERTIES OF ACTIVE FRACTIONS FROM STARCH COLUMN

Fraction	Weight of solid, g.	Ultraviolet Absorption: λmax.	λmin.	$a^{1\%}_{1cm.}$ (at λmax.)	Ratio[b]	Assay[a]
1	3.76	282	242	454	2.78	8×10^{-5}
2	4.89	282	242	503	3.85	3.3×10^{-5}
3	2.89	280	240	509	2.71	4.2×10^{-5}
4	2.52	280	240	425	2.04	6.6×10^{-5}

[a] γ per ml. of medium required to give a half-maximal response of *Leuconostoc citrovorum* 8081. [b] Ratio of intensity of absorption at the maximum to that at the minimum wave lengths.

Purification by Chromatography on "Florisil."[9]—The "Florisil" (300 g.) column was prepared by making a slurry of the adsorbent in *p*H 5.5 sodium acetate–acetic acid buffer (0.25 *M*) and placing this in a 1.5 inch diameter column. The adsorbent was washed with 5 l. of the buffer solution, then with 1 l. of a solution containing 50 g. of calcium chloride and 50 g. of ascorbic acid adjusted to *p*H 5.5. After a final washing with 3 l. of distilled water the column was ready for use.

An ammonium folinate-SF concentrate (6.58 g.) prepared by starch chromatography as described above was the

(11) A previously described medium (Snell, *et al.*, *J. Biol. Chem.*, **143**, 519 (1942)) supplemented with pyridoxine (3.4 γ per cc.), folic acid (0.01 γ per cc.) and thymidine (0.1 γ per cc.) was employed for this assay.

starting material for this column. This preparation gave a half-maximal response at 5×10^{-5} γ per ml. of assay medium. Ultraviolet absorption measurement showed a maximum at 281 mμ, $a^{1\%}_{1cm.}$ 428. The ratio of absorption density at 281 mμ to that at 241 mμ was 2.2. This concentrate was dissolved in 150 ml. of distilled water, and 6.58 g. of calcium chloride was added. The precipitate which formed was removed by centrifuging and washed with two 25-ml. portions of distilled water. The solution and washings were combined and applied to the column. Development was carried out with distilled water. The column was allowed to flow at the rate of 100–120 ml. per hour and one hundred ml. fractions were collected.

Progress of the column was followed by ultraviolet absorption measurements on the fractions. A highly fluorescent component having an absorption maximum at about 290 mμ appeared first. This material traveled nearly as fast as the solvent front and was completely eluted from the column by the time eleven fractions had been collected. It was closely followed by a zone having a maximum at 282 mμ and minimum at 242 mμ. This material began appearing in fraction 13 and persisted through fraction 29. It was not fluorescent. The ratio of absorption density at 282 mμ to that at 242 mμ of fraction 13 was 4.50 and remained fairly constant through fraction 27, having decreased to 4.1. Fraction 29 had a value of 3.6. At this point, the maximum shifted to lower wave lengths and fluorescence appeared in the eluate. Microbiological assay showed that activity was confined to fractions 12–33.

Crystallization of Folinic Acid.—Fractions 14–27, inclusive, from the above column, were combined and adjusted to *p*H 3.0 with 2.5 *N* hydrochloric acid. The solution was concentrated under reduced pressure to a volume of 400 ml. Crystallization occurred during the concentration. After several hours at room temperature the product was removed, washed with four 5-ml. portions of distilled water, and dried *in vacuo* over phosphoric anhydride. The product weighed 1.63 g., and gave a half-maximal response at 2.1×10^{-5} γ per ml. of assay medium. The $a^{1\%}_{1cm.}$ value at 282 mμ was 615, and at 242 mμ it was 160. The ratio of densities at these wave lengths was thus 3.84.

Properties of Folinic Acid-SF.—A sample (235 mg.) of the compound prepared as described was recrystallized by suspending in 30 ml. distilled water and adding 0.1 *N* sodium hydroxide to *p*H 6.8. The solution was filtered, acidified to *p*H 3.0, then concentrated *in vacuo* until crystallization began. After three recrystallizations in this manner the product was dried *in vacuo* over phosphoric anhydride for one hour. In the ultraviolet, $a^{1\%}_{1cm.}$ at 282 mμ was 545 and the ratio of absorption density at 282 mμ to that at 242 mμ was 4.95. A sample was dried *in vacuo* at 150° for two hours prior to analysis.

Anal. Calcd. for $C_{20}H_{23}O_7N_7$: C, 50.73; H, 4.90; N, 20.71. Found: C, 50.64; H, 5.01; N, 20.97.

The weight loss on drying was 12.3%. A second determination in which the sample was exposed to the air for about two hours gave a value of 14.7%.

The activity of this preparation was such that 1.8×10^{-5} γ per ml. of assay medium gave a half-maximal response. Folinic acid-SF decomposed without melting at 240–250° (uncor.).

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[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE GLIDDEN COMPANY, SOYA PRODUCTS DIVISION]

Sterols. XII. The Partial Synthesis of 4-Pregnene-17α,20,21-triol-3-ones and Reichstein's Substance E[1]

BY PERCY L. JULIAN, EDWIN W. MEYER, WILLIAM J. KARPEL AND WAYNE COLE

Steroids having the 17α,20β,21-triol structure were obtained by the lithium aluminum hydride reduction of either 17α,21-diol-20-ones or 16,17-oxido-21-ol-20-ones, the latter yielding also the 20α-isomer. The ketonic function of 3-keto-4-pregnenes may be conveniently protected from reduction by means of enol ether formation. Thus 4-pregnene-17α,21-diol-3,20-dione 21-acetate was converted into 4-pregnene-17α,20β,21-triol-3-one while this triolone and 4-pregnene-17α,20α,21-triol-3-one were obtained from 16,17-oxido-4-pregnen-21-ol-3,20-dione 21-acetate. Cortisone acetate yielded Reichstein's substance E (4-pregnene-11β,17α,20β,21-tetrol-3-one).

In continuation of our program on the synthesis of steroids occurring in and related to those of the adrenal cortex,[1a] we became interested in certain pregnanes having the 17,20,21-triol structure. Of particular interest were Reichstein's substance E (4-pregnene-11β,17α,20β,21-tetrol-3-one) and 4-pregnene-17α,20β,21-triol-3-one (VII). This latter steroid has been reported by Ungar,[2] in his study of the adaptation syndrome, to possess a rather striking physiological activity. We are now reporting the partial synthesis of these two substances as well as that of the hitherto unknown 4-pregnene-17α,20α,21-triol-3-one (VI).

The first synthesis of 4-pregnene-17α,20β,21-triol-3-one (VII) was reported in the same year from two independent laboratories. Ruzicka and Müller[3] prepared this compound by a series of transformations beginning with $\Delta^{4,20}$-17-isopregnadien-17β-ol-3-one (17-vinyltestosterone). Logemann[4] presented a brief description of what appears to be the identical method, but details are lacking for a more exact comparison with that of the Swiss workers.

We have recently recorded the synthesis of Reichstein's substance S from 16,17-oxido-4-pregnen-21-ol-3,20-dione acetate (I).[5] The availability of this oxido steroid (I) made it an attractive intermediate for the synthesis of the triolone (VII). 16,17-Oxido-4-pregnen-21-ol-3,20-dione acetate (I) reacted smoothly with ethyl orthoformate[6] in the presence of a catalytic quantity of sulfuric acid in dioxane to produce the corresponding 3-enol ether (II) in good yield. Reduction of the enol ether (II) with lithium aluminum hydride in ether–benzene afforded a mixture of triols (III) which was best separated into its components after acid cleavage of the enol ether and acetylation. By crystallization the product was separated into two isomeric diacetates (IV and V); one melting at 189–191° (V) and the other at 251–253° (IV).

(1) Presented in part before the American Chemical Society, Chicago, Ill., Sept. 5, 1950.

(1a) For previous communication in this series, see THIS JOURNAL, **72**, 5145 (1950).

(2) G. Ungar, *Proc. Soc. Endocrinology*, **5**, liii (1947); *J. physiol. et path. gen.*, **39**, 219 (1947).

(3) L. Ruzicka and P. Müller, *Helv. Chim. Acta*, **22**, 755 (1939).

(4) Logemann, *Naturwissenschaften*, **27**, 196 (1939).

(5) Julian, Meyer, Karpel and Waller, THIS JOURNAL, **72**, 5145 (1950).

(6) Schwenk, Fleisher and Whitman, *ibid.*, **60**, 1702 (1938); Serini and Köster, *Ber.*, **71**, 1766 (1938).