[CONTRIBUTION FROM THE U. S. PUBLIC HEALTH SERVICE TUBERCULOSIS RESEARCH LABORATORY, CORNELL UNIVERSITY MEDICAL COLLEGE, AND THE DEPARTMENT OF PHARMACOLOGY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

Aromatic Biosynthesis. XV.[1] The Isolation and Identification of Shikimic Acid 5-Phosphate[2a,2b]

BY ULRICH WEISS[3] AND ELIZABETH S. MINGIOLI

RECEIVED DECEMBER 8, 1955

Certain bacterial mutants blocked in aromatic biosynthesis accumulate in their culture filtrates shikimic acid (I) together with a conjugate of I, compound Z2 (II). II has been isolated in pure form and shown to be shikimic acid 5-phosphate. This identification rests upon the fact that II is oxidized by periodic acid (which excludes shikimic acid 4-phosphate (IV)) and that it is different from authentic shikimic acid 3-phosphate (III), which was prepared from I. The observed differences between II and III are those to be expected from the presence in III, but not in II, of a *cis*-1,2-glycol grouping.

The pathway of biosynthesis of certain aromatic metabolites (phenylalanine, tyrosine, tryptophan, *p*-aminobenzoic and *p*-hydroxybenzoic acid) has been studied in this Laboratory through the use of bacterial mutants blocked in the synthesis of these compounds.[4] This pathway has been found to involve a series of common intermediates, proceeding from 5-dehydroquinic acid[5] *via* 5-dehydroshikimic acid[6] to shikimic acid.[7,8] These compounds are accumulated in the culture filtrates of mutants that are blocked after them, and can replace the above mixture of aromatic compounds as growth factors for mutants with earlier blocks. Furthermore, culture filtrates that contained shikimic acid (I) were also observed to contain regularly a conjugate of I, provisionally named compound Z2 (II).[9] This conjugate, in contrast to I itself, is nutritionally inactive (presumably because of impermeability) for mutants blocked in early reactions in this sequence, but it can be recognized readily through liberation of I on heating with acid. II has a low chromatographic mobility in butanol–formic acid–water, indicating that it is a highly polar compound.

The present paper reports the isolation of II in pure form (as a salt) and its identification as shikimic acid 5-phosphate. Its metabolic significance is not certain; as has been pointed out,[4] it may well be a metabolic side-product of I rather than an obligatory intermediate, since no mutants have been found to be blocked between I and II.[9a]

II is accumulated in the filtrates of certain mutants (*e.g.*, *Aerobacter aerogenes* strain A170-40) in amounts of 500–1000 mg. per liter.[10] From such filtrates, acidified to *p*H 3, II was adsorbed on a charcoal column. After washing with water, 5% ethanol, and 10% ethanol, all acidified to about *p*H 2.5 with HCl, II was eluted with non-acidified 5% ethanol.[11]

The eluate fractions containing the compound yielded an apparently microcrystalline brucine salt. Removal of the brucine as the insoluble picrate, removal of excess picric acid by ether extraction, and evaporation gave purified II as a colorless sirup. Attempts to crystallize the free acid were not successful, but a solid potassium salt of II was obtained from methanolic solution.

Elementary analysis of several preparations of this salt gave values[12] in close agreement with those calculated for a monohydrate of the mono-potassium salt of a monophosphate of I. Some less pure preparations (about 80%) were further purified by addition of barium acetate to an aqueous solution followed by precipitation of the barium salt of II with ethanol.

The purity of the K and Ba salts was further demonstrated by assay of the I and the H_3PO_4 liberated in stoichiometric amounts by intestinal or potato phosphatase. The I obtained on hydrolysis, previously recognized by bioassay and paper chromatography,[9] was unequivocally identified by isolation from such enzymatic digests.

The position of attachment of the phosphate group in II had now to be determined. This position cannot be the carboxyl of I since such a mixed anhydride would be much less stable than II and would be insensitive to the action of phosphatase. II must therefore be phosphorylated on the 3-, 4-, or 5-position of I (compounds III, IV or V, respec-

(1) Paper XIV: H. Yaniv and C. Gilvarg, *J. Biol. Chem.*, **213**, 787 (1955).

(2) (a) Aided by a grant from the Squibb Institute for Medical Research and by U. S. Public Health Service Grant G-4235; (b) in previous papers[4,9] this compound has been referred to as phosphoshikimic acid.

(3) New York Botanical Garden, New York 58, N. Y.

(4) For a review of this work see B. D. Davis, in "Amino Acid Metabolism," W. D. McElroy and B. Glass, Editors, Johns Hopkins Press, Baltimore, Md., 1955, p. 799.

(5) U. Weiss, B. D. Davis and E. S. Mingioli, THIS JOURNAL, **75**, 5572 (1953).

(6) I. I. Salamon and B. D. Davis, *ibid.*, **75**, 5567 (1953).

(7) B. D. Davis, *J. Biol. Chem.*, **191**, 315 (1951).

(8) In an earlier paper[5] the formulas of these compounds were presented with the dotted and the straight lines inadvertently interchanged.

(9) B. D. Davis and E. S. Mingioli, *J. Bact.*, **66**, 129 (1953).

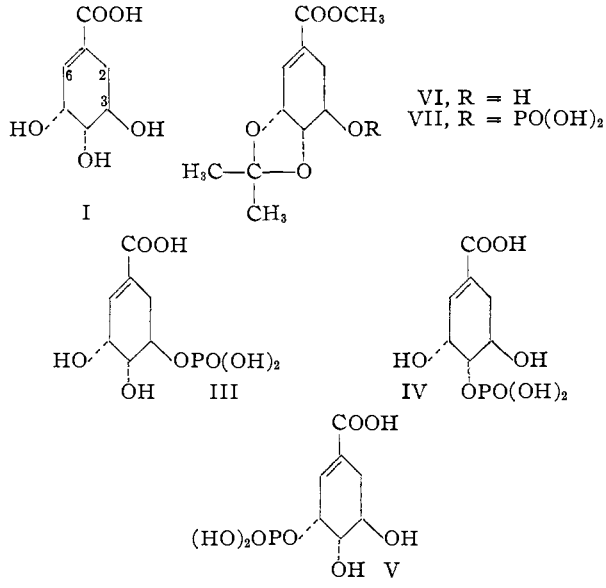
(9a) ADDED IN PROOF.—The view that II is not on the path of aromatic biosynthesis now appears unlikely on the basis of recent findings. Compound Z1 (see ref. 9) is a conjugate of I and pyruvic acid (C. Gilvarg, quoted in B. D. Davis, *Federation Proc.*, **14**, 691 (1955)), that appears to be an intermediate in aromatic biosynthesis. Dr. E. B. Kalan of the Department of Pharmacology, New York University College of Medicine, has found that extracts of a mutant blocked after Compound Z1 can synthesize this substance, in the presence of phosphoenolpyruvate, from II but not from I. II therefore appears to be an intermediate between I and Compound Z1, despite the absence of a mutant blocked between I and II. The authors are much indebted to Dr. Kalan for permission to quote these results.

(10) This figure is based on microbiological assay of the I obtained by enzymatic cleavage with phosphatase (see Experimental Part), a procedure worked out after II had been recognized to be a phosphate ester. Earlier, however, the purification steps were followed by assay after HCl hydrolysis.[9] This method proved adequate for the purpose, although it was found, when pure II became available, to yield only a fraction of the I contained in II (see footnote 21).

(11) If the solutions are not acidified, II comes off the column even with water. This peculiar behavior is not exhibited by shikimic acid 3-phosphate (see below).

(12) Analyses by Dr. W. C. Alford, National Institutes of Health, Bethesda, Md., and by Schwarzkopf Microanalytical Laboratory, Woodside, N. Y. The authors are much indebted to Dr. Alford for his great interest in this work.

tively). Of these, III or V should consume one mole of periodate, while IV should be resistant. Since II readily took up one mole it cannot be IV.



The two remaining possibilities could be distinguished on the basis of the fact that III, but not V, should undergo various reactions based on the presence of a pair of adjacent *cis*-hydroxyl groups. The results of such tests with II were consistent with its formulation as V. However, to reach a decisive conclusion it seemed desirable to prepare an authentic sample of III or V for comparison.

No convenient method for synthesizing V seemed available. However, unequivocal synthesis of III was made possible by the fact that acetonide formation covers the hydroxyl groups on positions 4 and 5 of I, leaving that on position 3 free. The synthesis was accomplished by phosphorylating the known[13] methyl ester of 4,5-isopropylidene-I (VI) with $POCl_3$ in pyridine to yield VII (not isolated), hydrolyzing this compound with NaOH and HCl, and isolating the resulting III as the Ba salt.

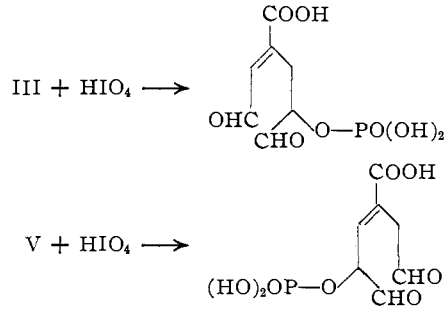
The Ba salts of II and III were found to differ in several respects: acetonide formation, isomerization by acid, formation of a copper complex, rate of oxidation by periodate and the ultraviolet absorption spectrum of the dialdehyde formed by periodate. II must therefore be formulated, by exclusion, as V. It should be further noted that in each of these comparisons between II and III the direction of the observed difference gave evidence for the expected presence of a pair of adjacent *cis*-hydroxyls in III and its absence in II.

Acetonide formation was tested because it is known that under the usual conditions of this reaction *cis*- but not *trans*-1,2-glycols of the cyclohexane series are reactive. II gave no evidence for acetonide formation, while under the same conditions III reacted readily. The acetonide of III was not isolated, but its presence was established by treatment with phosphatase to yield the acetonide of I, which was recognized by paper chromatography and by the demonstration that hydrolysis with acid yielded free I, detected by its growth factor activity for appropriate bacterial mutants.

Isomerization by acid was investigated on the basis of the fact that in V, but not in III, the phosphate group is adjacent to a hydroxyl in *cis* orientation and so should be able to form a cyclic phosphate. This structure is known to be a necessary intermediate[14] in the acid-catalyzed isomerization of phosphates of the general formula $R\text{–}CHOPO_3H_2\text{–}CHOH\text{–}R'$. Hence treatment with acid should convert V, but not III, to an equilibrium mixture with IV, which can be recognized readily through its resistance to periodate. Such isomerization was found to occur on refluxing II for 10 minutes with 80% acetic acid[15]: about 20% of the material became periodate-resistant, as shown by the decreased uptake of periodate and by the fact that subsequent destruction of residual periodate and treatment with phosphatase yielded some I, detected microbiologically.[16] (Non-isomerized II gave no residual I after similar treatment with periodate and phosphatase.) In contrast to II, synthetic III, as expected, yielded no periodate-resistant material on identical treatment with acid.

The copper complex test was based on the fact that many 1,2-glycols form intensely blue water-soluble complexes.[17] It seemed likely that the formation of such complexes would be stereospecific.[18] In fact, the addition of sodium hydroxide and cupric acetate gave a clear blue solution with III, I, or quinic acid, which each possess a pair of *ortho*-hydroxyl groups in *cis*-orientation. In contrast, II gave a greenish-blue precipitate in a colorless solution.

Additional evidence on the structure of II was based on the reaction with periodate. The dialdehyde resulting from III should have a CHO group conjugated with the double bond, while that from V should not.



On treatment of II and III with periodate it was indeed found that the resulting solutions had markedly different absorption spectra (Fig. 1). Furthermore, the nature of the difference furnished posi-

(13) H. O. L. Fischer and G. Dangschat, *Helv. Chim. Acta*, **18**, 1206 (1935). The authors are much indebted to Prof. H. O. L. Fischer for advice concerning the synthesis.

(14) D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952); D. M. Brown, D. I. Magrath and A. R. Todd, *ibid.*, 2708 (1952), and literature quoted.

(15) Conditions used by D. M. Brown and A. R. Todd, *ibid.*, 44 (1952), for analogous equilibrations.

(16) The isolation of IV from the reaction mixture was not attempted but should be feasible on the basis of its periodate resistance.

(17) R. Klimek and J. K. Parnas, *Biochem. Z.*, **252**, 392 (1932); J. K. Parnas and R. Klimek, *Z. physiol. Chem.*, **217**, 75 (1933).

(18) *Cf.* the work of R. E. Reeves (THIS JOURNAL, **71**, 209 (1949), and subsequent papers) on stereospecificity of formation of copper complexes in the carbohydrate series.

tive support for the formulation of II as V: periodate-treated III had a broad absorption maximum at 230–240 mμ (*cf.* maximum at 234 mμ for a compound with a similar conjugated system, 5-dehydroshikimic acid[6]), while periodate-treated II exhibited weaker absorption in the short-wave ultraviolet, with no maximum (Fig. 1). In addition, the reaction of periodate was considerably more rapid with III than with II, which is consistent[18a] with the interpretation that the pair of *ortho*-hydroxyl groups has the *cis* configuration in III and the *trans* configuration in II.[19]

The existence of these several differences between II and III, as well as their direction, thus established the formulation of II as V. It remained to show whether the compound isolated from culture filtrates was the same as that originally excreted by the bacteria. It has been shown above that IV and V are equilibrated in acidic medium, V apparently being strongly favored. Since the isolation procedure involved prolonged exposure to acid, the II (=V) obtained might conceivably be an artifact formed from IV originally present. Accordingly, a fresh bacterial culture filtrate containing II was treated with neutral periodate and then with phosphatase. This procedure would have liberated I from IV but not from V. Since no I was found under these conditions, no significant amount of the periodate-resistant isomer IV was present in the filtrate. The original bacterial product must therefore, like the isolated II, be V.

Experimental

Accumulation.—Culture filtrates were obtained by a procedure previously described,[5] except that the organism used was *Aerobacter aerogenes* mutant A170-40,[9] and citrate was omitted from the medium. (This omission greatly increased the accumulation of II at the expense of I.[9]) The filtrates were found by bioassay to contain 500–1000 γ/ml. of II, estimated in terms of I as a standard. Variable small amounts of free I were also present.

Assays.—*Escherichia coli* mutant 83-1, which has a quintuple aromatic requirement and responds to I[7] (but not to II[9]), was used as assay organism.

(a) **Bioassay of II. HCl Hydrolysis.**—0.1–0.5 ml. portions of the material to be assayed, yielding 2–20 γ of I, were placed in capped colorimeter tubes, and equal volumes of 0.2 *N* HCl were added. After autoclaving at 120° for 30 min., each tube was assayed microbiologically for its content of I by a procedure previously described,[9] using medium A[20] containing 0.5% glucose. Differential assay for free I in the filtrate was performed by an identical procedure, using 0.2 *M* Na_2CO_3 instead of 0.2 *N* HCl.

This HCl hydrolysis released only 25–30% of the I present in II, but was adequate for following the isolation of II.[21]

(b) **Bioassay of II. Enzymatic Hydrolysis.**—A mixture of about 2 μmoles of II, 0.1 mmole of sodium acetate buffer (*p*H 5.3), 0.01 mmole of $MgSO_4$, and 0.2 ml. of potato phosphatase solution (16 mg. protein/ml.),[22] in a total volume of 1 ml., was incubated at 37° for 2 hours. An equal volume of 12% trichloroacetic acid was then added and the mixture was heated for 10 minutes in a boiling water-bath and centrifuged. The supernatant was assayed for I as described in the preceding section. Subsequent references to the content of II in various materials are based on this assay.

Tests with larger amounts of enzyme showed that this procedure gave complete hydrolysis.

(c) **Determination of II by Assay of P Liberated.**—Samples of II sufficiently purified to be free of inorganic phosphate were also assayed by determining the inorganic phosphate[23] released by the enzymatic hydrolysis described in (b). It was found that this hydrolysis released equimolar quantities of I and phosphate.

Isolation of II.—Five liters of culture filtrate, acidified to *p*H 3 and containing II equivalent to about 2.5 g. of I, was passed through a column of 170 g. of charcoal (Darco G 60) under slight pressure of air or nitrogen. The column was washed next with the following solvents acidified to about *p*H 2.5: 3 × 500 ml. of H_2O, 3 × 500 ml. each of 1.5, 5 and 10% ethanol. The effluents and washings were free of II. II was eluted with 500-ml. portions of unacidified 5% ethanol, until the concentration of II in the eluates dropped well below the maximum observed; about 10 portions were necessary. On subsequent washing with unacidified 10 and 25% ethanol, appreciable further quantities of II were obtained. The total recovery in the eluates was 80–90%. The 5, 10 and 25% ethanol eluates were worked up separately and gave products of similar purity.

The 5% ethanol eluates, containing II equivalent to about 1.2 g. of I, were combined and evaporated *in vacuo* at 40° to a few ml. of thick sirup weighing about 3 g., which was dissolved in 20 ml. of ethanol and, if necessary, decolorized with charcoal. A solution of 10 g. of brucine in 30 ml. of ethanol was added. Some flocculent precipitate formed immediately, which tended to redissolve. Precipitation was improved by adding an equal volume of ethyl acetate and keeping in a freezer for 1–2 days. The precipitate was then filtered with suction, washed with ice-cold ethanol: ethyl acetate 1:10, and dried *in vacuo*. The dried brucine salt weighed 11 g., yield 80–90% of the II originally present in the 5% ethanol eluates. Attempts to recrystallize the salt were unsuccessful.

The brucine salt was dissolved in 80 ml. of water, and the solution was decolorized with charcoal and brought to a volume of 120 ml. with H_2O. The brucine was precipitated by adding about 300 ml. of saturated aqueous picric acid and chilling for a few hours. The brucine picrate was filtered off and washed with cold water. The aqueous filtrate was continuously extracted with ether for 8–10 hours to remove excess picric acid (and possibly some ether-soluble impurities). Evaporation of the aqueous phase *in vacuo* yielded a colorless glassy residue which contained practically all of the II originally present in the brucine salt. Attempts to induce crystallization of this free acid were unsuccessful.

Preparation of the Potassium Salt.—The free acid was taken up in 10 ml. of methanol, a small amount of insoluble material was filtered off, and the filtrate was brought to a volume of 40 ml. with methanol. The K salt was precipitated by slow addition of about 8 ml. of 1 *N* KOH in 95% methanol to a *p*H of approximately 7; the salt would be redissolved by an excess of alkali. After storage in the refrigerator for a day the precipitate was separated by centrifugation, washed by resuspending twice in methanol, and dried *in vacuo*. The resulting white powder weighed about 1.2 g. and contained 50–60% of the II originally present in the brucine salt. In different preparations the purity of the product, calculated for the monohydrate of a monopotassium salt, varied between 70 and 100%. A second crop of less pure material was obtained by adding an equal volume of 100% methanol to the mother liquors. Attempts to recrystallize the K salt were unsuccessful.

Anal. Calcd. for $C_7H_{10}O_8PK \cdot H_2O$ (mol. wt. 310.3): C, 27.10; H, 3.90; P, 9.99; K, 12.60. Found for a sample dried *in vacuo* over P_2O_5 at room temperature: C, 27.35; H, 3.61; P, 10.05; K (by flame photometry), 13.18; for a

(18a) Criegee, *Sitzber Ges. Beförder. ges. Naturw. Marburg*, **69**, 25 (1934); C. C. Price and M. Knell, THIS JOURNAL, **64**, 552 (1942).

(19) The authors are indebted to Dr. Charles Gilvarg and Dr. Bernard Davis for these experiments with periodate. Dr. Gilvarg also provided much helpful assistance in the synthesis of III.

(20) B. D. Davis and E. S. Mingioli, *J. Bact.*, **60**, 17 (1950).

(21) Later experiments on the acid stability of pure II showed that autoclaving at 120° in 0.1*N* HCl yielded 41% of its content of I in 1 hour, 64% in 4 hours, and 70% in 6 hours. Further heating, up to a total of 11 hours, yielded no additional I. In contrast, inorganic phosphate was released at a greater rate and reached 94% in 11 hours. Since control determinations on I treated in the same way showed only 10% destruction in 11 hours, it is clear that heating with HCl causes a significant fraction of II to be destroyed without release of I.

(22) Potato phospbatase was prepared by the method of A. Kornberg (personal communication), carrying the purification as far as the first precipitation with ammonium sulfate followed by dialysis. Intestinal phosphatase, kindly furnished by Dr. G. Schmidt, was also satisfactory for this hydrolysis.

(23) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **66**, 375 (1925).

sample dried *in vacuo* at 80°: C, 27.00; H, 3.82; P, 9.71. The water of crystallization was not expelled *in vacuo* at 140°. Presence of very firmly bound water is not unprecedented among compounds of this type, especially salts of inositol phosphoric acids,[24] $[\alpha]^{29}D$ −107.6° (*c* 1.02 in water).[25]

Preparation of the Barium Salt.—1.2 g. of K salt of II (about 70% pure) was dissolved in 15 ml. of H_2O, yielding a cloudy, light brown solution, to which was added 5 ml. of 1 *M* barium acetate. A very light precipitate containing most of the color was filtered off after addition of charcoal, and 45 ml. of 95% ethanol was slowly added, with stirring, to the clear solution. A heavy white precipitate formed. The suspension was chilled for 24 hours and the salt was isolated by centrifuging, resuspending in 50% and then 95% ethanol, and finally drying *in vacuo* at room temperature. 1.83 g. of white amorphous material was recovered; assay following enzymatic hydrolysis revealed an equivalent weight of 704 (per mole of phosphate and I released) and a yield from the K salt of 98% of the II present. This barium salt was reprecipitated by making a saturated solution in about 30 ml. of H_2O, filtering off a small amount of insoluble material, and adding 3 ml. of ethanol dropwise to the chilled solution over a period of 2 days. Assay of the supernatant fluid showed that the precipitation was about 60% complete. The precipitate was isolated by centrifugation, washed 3 times with cold ethanol:water 1:10 and twice with cold 95% ethanol. After drying *in vacuo* at room temperature the Ba salt weighed 750 mg., had an equivalent weight (per mole of phosphate) of 504, and represented a yield of 57% of the starting product. On further attempts at purification the equivalent weight remained at 500–510. This product was shown, by drying *in vacuo* at 100° over P_2O_5, to contain about 2.5 molecules of water per molecule of phosphate or I. (The formula $Ba_3II_2 \cdot 5H_2O$ would have an equivalent weight of 502 per mole of II.) However, during this dehydration some decomposition took place, indicated by release of about 5% of the phosphate as inorganic phosphate.[26]

Isolation of Shikimic Acid from Enzymatic Hydrolysis of II.—104 mg. of pure K salt of II was mixed with 2 ml. of potato phosphatase solution (as in Assay Method (b)), 3 ml. of 1 *M* sodium acetate buffer (*p*H 5.3), 3 ml. of 0.1 *M* $MgSO_4$, 22 ml. of H_2O, and a few drops of toluene. After incubation at 37° for 40 hours the solution was heated in a boiling water-bath for 15 minutes and the precipitated protein was removed by centrifugation. Bioassay indicated 91% conversion of II to I. The clear supernatant was passed through a column of 10 g. of charcoal (Darco G 60), which was then washed with 50-ml. portions of the following solutions: 4 × H_2O, 4 × 2.5% ethanol, and 4 × 5% ethanol. After this, I was eluted with 15 × 50 ml. of 10% ethanol. The pooled eluates, containing about 41 mg. of I (78% of that present in the original starting material), were evaporated to dryness *in vacuo*. The residual colorless sirup was covered with a few drops of ethyl acetate and allowed to crystallize in the cold. The crystallization was improved by the addition of 0.2 ml. of glacial acetic acid. The crystals were isolated by centrifugation and washing with 0.5 ml. of cold glacial acetic acid (much of the material redissolves), and dried *in vacuo;* yield 14 mg. Melting points: I obtained from II sint. 185°, m. 186–188°; authentic I sint. 186°, m. 189°; mixture sint. 185°, m. 187–189°. The material was identical with I in its activity as a growth factor for several mutants blocked before I, and its inactivity for mutants blocked after I.

Uptake of Periodate by II.—50 μmoles of II (pure K salt) plus 85 μmoles of $NaIO_4$ in 25 ml. of water was placed in a refrigerator. At intervals portions were titrated for residual periodate.[27] The results showed that 10.0 μmoles of II took up 8.3 μmoles of $NaIO_4$ in 1 hr., 10.1 in 2 hr., and 10.1 in 3 hr.

Synthesis of III.—Five mmoles (1.07 g.) of the acetonide[13] of I was dissolved in 9 ml. of methanol in a flask connected to a dropping-funnel. Ethereal diazomethane, obtained from 4.12 g. of N-nitrosomethylurea,[28] was added in small portions to the solution cooled in ice. The resulting yellow liquid was at once evaporated to dryness *in vacuo* in the same flask and stored under vacuum until use. It was then dissolved *in situ* in 8 ml. of pyridine which had been freshly distilled from BaO in a rigorously dried apparatus. The mixture was frozen by immersion in Dry Ice–acetone. A 40% excess[29] of freshly distilled $POCl_3$ (0.64 ml.) was placed in the dropping funnel, diluted with 8 ml. of redistilled pyridine, and added in portions of 2–3 drops. The frozen mixture was allowed to melt immediately before each addition, mixed rapidly after the addition, and immediately refrozen. The residual liquid in the dropping funnel was rinsed into the flask with a few ml. of pyridine and the mixture was kept frozen overnight.

After melting, an insoluble phase (crystals of pyridine HCl) was eliminated by filtration with suction. The solution (28 ml.) was diluted with an equal volume of water and concentrated under vacuum to less than half its volume, yielding a yellowish liquid, *p*H about 6, containing some oily droplets. The solution was acidified with 2.2 ml. of 6 *N* HCl (strong effervescence), heated in a boiling water-bath for 20 minutes, cooled, neutralized with NaOH, and refrigerated for several days. The solution then contained a large amount of colorless crystals (presumably mostly NaCl) and some resinous material. The solid was separated from the mother liquor by decantation. Enzymatic assay showed that this solid contained less than 4% of the expected yield of III; it was discarded. The mother liquor was brought to *p*H 11 with 2.5 ml. of 10 *N* NaOH, causing it to separate into two phases. After heating in a boiling water-bath for 20 minutes the mixture was cooled and the phases were separated. Assay showed that the aqueous lower phase contained 70% of the expected bound phosphate and bound I, while the oily upper phase contained only 2%.

The aqueous phase was acidified to about *p*H 2 and put on a column of 40 g. of Darco G 60. The column was washed with 100-ml. portions of the following solvents: 3 × H_2O, *p*H 2; 6 × 5% ethanol, *p*H 2; and 10 × 5% ethanol, unacidified.[11] Ten portions of 10% ethanol then eluted a mixture of I and III and 15 portions of 50% ethanol contained the remaining III, with little I. The yield from the column was 0.5 mmole of free I and 2.2 mmole of bound I and phosphate. The eluates containing III were evaporated to small volumes.

Isolation of the Barium Salt of III.—A pool of some of the concentrated eluates, containing 1.65 mmoles of III and 0.4 mmole of I, was brought to 15 ml. with H_2O. Addition of barium acetate and ethanol, as in the isolation of the Ba salt of II, precipitated a Ba salt of III which was reprecipitated from ethanol–H_2O and was dried over P_2O_5 at room temperature. The product contained no I. Assay after enzymatic hydrolysis revealed an equivalent weight of 485 (per mole of phosphate and I released); the yield was 60% of the III present in the pooled eluates.

Anal. Calcd. for $C_{14}H_{22}O_{19}P_2Ba_3$: ($Ba_3III_2 \cdot 3H_2O$; mol. wt. 968.4): C, 17.36; H, 2.29; P, 6.40; Ba, 42.55. Found: C, 17.22; H, 2.79; P, 6.55; Ba, 41.96. On heating at 100° *in vacuo* over P_2O_5 the material lost weight, but this dehydration, like that of BaII, was accompanied by a significant release of inorganic phosphate.[26]

Comparison of II and III. Acetonide Formation.—Ten mg. (32.3 μmoles) of pure K salt of II and 10 mg. (20.6 μmoles) of pure Ba salt of III were each shaken for 2 days at 37° with 10 ml. of acetone and 0.25 g. of acetone-washed acidic resin[30] (Amberlite IR-120) in a tightly stoppered flask. An acetone-soluble and an acetone-insoluble fraction of each was obtained by the following procedure. The resin and some insoluble material were filtered off and washed with several 1–2 ml. portions of acetone. The filtrate and washings were combined and evaporated to dryness *in vacuo;* the resulting sirup was taken up in 10 ml. of water and neutralized with solid $NaHCO_3$. The acetone-insoluble fraction was recovered by washing the material on the filter with several 1–2 ml. portions of water, bringing the volume of this filtrate to 10 ml., and neutralizing with

(24) S. Posternak, *Helv. Chim. Acta*, **4**, 150 (1921), esp. p. 164, footnote 1.

(25) Rotation kindly determined by Dr. I. I. Salamon of the Sloan-Kettering Institute.

(26) X-Ray crystallography of the Ba salts of II and III (kindly performed by Dr. I. Fankuchen) revealed no diffraction pattern, indicating that these materials were not crystalline despite the sharp edges seen under the microscope.

(27) E. L. Jackson, "Organic Reactions," Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 341.

(28) F. Arndt, "Organic Syntheses," Coll. Vol. II, John Wiley and Sons, Inc., New York, N. Y., 1943, p. 165.

(29) F. Lynen and H. Bayer, *Chem. Ber.*, **85**, 905 (1952).

(30) Y. E. Cadotte, F. Smith and D. Spriestersbach, THIS JOURNAL, **74**, 1501 (1952).

solid $NaHCO_3$. Both fractions were found to be free of inorganic phosphate and I, indicating that there had been no appreciable hydrolysis of II or III during exposure to the acidic resin.

Since II and III are inactive in the bioassay, the fractions were treated with potato phosphatase as in Assay Procedure (b), except that no trichloroacetic acid was added at the completion of the reaction. (It was shown that subsequent heating removed the protein adequately, and that the overall procedure did not split authentic acetonide of I.) Since the acetonide of I is inactive[6] in the bioassay for I, the phosphatase-treated solutions were assayed for free I in the usual manner, and for the sum of I and the acetonide of I by first acidifying the solutions to *p*H 1 and heating at 100° for 20 min., conditions shown to split the acetonide of I. The results are summarized in Table I; they indicate that a considerable fraction of III, but no detectable fraction of II, had been converted to an acetonide.

TABLE I

MICROBIOLOGICAL ASSAYS FOR I AND ITS ACETONIDE

	Product	I, μmoles	I plus acetonide of I, μmoles	(Calcd.) μmoles acetonide of I
II	Acetone-soluble	10.1	10.1	0
	Acetone-insoluble	12.6	12.6	0
III	Acetone-soluble	1.4	6.3	4.9
	Acetone-insoluble	6.8	6.8	0

Further evidence for acetonide formation was obtained by paper chromatography, developing with *n*-butanol–formic acid–water and detecting the spots with Aerobacter mutant A170-143S1 in the presence of 20 γ/ml. L-tyrosine and 40 γ/ml. DL-phenylalanine.[9] The phosphatase-treated acetone-soluble fraction obtained from III yielded a spot which was identical with authentic acetonide of I in its R_f value (0.84), and also in requiring for its detection that the paper, before bioautography, be sprayed with dilute HCl and heated. (The R_f value of I was 0.36; this compound was not detected in the small sample of the fraction tested, which had been shown by bioassay to consist predominantly of the acetonide.)

Isomerization by Treatment with Acetic Acid.—12.4 mg. of pure K salt of II was refluxed for 10 minutes with 2 ml. of 80% glacial acetic acid. Most of the acetic acid was then removed by repeatedly adding a few ml. of H_2O and evaporating *in vacuo* nearly to dryness. The sirup was taken up in 1 ml. of H_2O (*p*H of the solution 4.5), neutralized with NaOH, and brought to 10 ml. with H_2O. 8 ml. of this solution (T) and 8 ml. of an equimolar (4 m*M*) solution of untreated II (U) were each mixed with a stoichiometric quantity of $NaIO_4$,[31] brought to 25 ml. with water, and placed in a refrigerator. After 2 and 3 hours aliquots were titrated for residual periodate.[27] The results showed that all of the periodate originally present was taken up by U but only 78% by T, indicating that in T approximately 22% of the II originally present had been isomerized and thus had become resistant to periodate. This conclusion was verified by destroying any possible excess of periodate in both solutions with a few crystals of inositol, treating with potato phosphatase, and assaying for inorganic phosphate and I. In both solutions all the phosphate was liberated; but U yielded no I (< 0.01 μmole/ml.) while T yielded 0.27 μmole/ml. (about 20% of the amount originally present in the form of II).

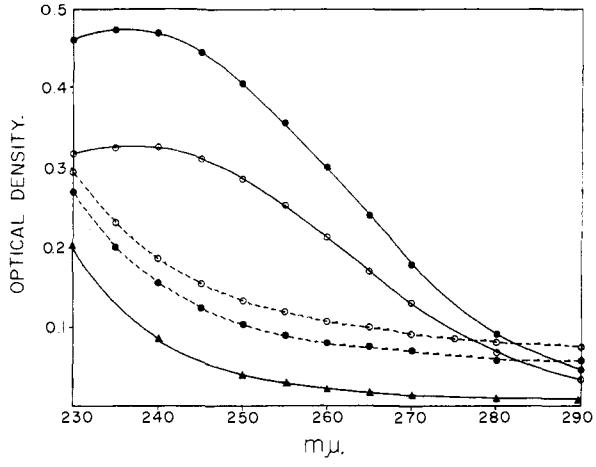


Fig. 1.—Ultraviolet absorption spectra of periodate-treated II and III: ▲, 0 time, either compound; ●, 15 minutes incubation with periodate; dotted line, II; full line, III; O, same plus 30 minutes incubation after destruction of periodate.

Similar results were obtained with the Ba salt of II. In contrast, identical treatment of the Ba salt of III produced no detectable isomerization.

Dialdehydes from II and III.—0.2 ml. of 1.5 m*M* Ba salt of II and III were each mixed with 0.2 ml. of 2.1 m*M* HIO_4 and 0.1 ml. of 1 *M* phosphate buffer at *p*H 7.0 In a control pair of tubes (0 time) the periodate was destroyed immediately by adding 0.2 ml. of 10 m*M* Na_2SO_3 and, 2 minutes later, water to bring the volume of each to 3 ml. The experimental pair of tubes were incubated at room temperature for 15 minutes before destroying the periodate. The absorption at 230–290 mμ was measured immediately, and again 30 minutes later, in a Beckman spectrophotometer against a reagent blank lacking II or III. As shown in Fig. 1, the periodate-treated III showed a marked increase in absorption, with a plateau at 230–240 mμ. The absorption fell considerably on standing, presumably owing to instability of the dialdehyde. In contrast, periodate-treated II showed much less increase in absorption and no plateau. In addition, it exhibited a slight further increase in absorption on standing.

In similar experiments with varying duration of exposure to HIO_4 III yielded maximal absorption by 5 minutes, while that obtained from II was still increasing at 60 minutes, with a slight peak at 260–270 mμ. In view of the instability of the dialdehydes the absolute values observed have little significance, but it is clear that III reacts with periodate more rapidly than II and that the products have quite different ultraviolet absorption spectra.

Formation of Copper Complex.—About 1 mg. of each sample was dissolved in 3 drops of water. Two small drops of 10% NaOH were added, followed by 3 small drops of 2% cupric acetate and 3 more drops of NaOH. II gave a bluish-green precipitate in a colorless solution, while III, I, and quinic acid each gave a clear blue solution, indicating formation of a copper complex by III but not by II.

Effect of Periodate on II in Culture Filtrates.—50 ml. of culture filtrate, containing about 150 μmole of II, was concentrated to 5 ml., about 550 μmole $NaIO_4$ was added, and the volume was brought to 10 ml. with H_2O. The mixture was allowed to stand 24 hours at room temperature. A qualitative test with potassium iodide–starch then revealed residual periodate, which was destroyed by addition of a few crystals of inositol. Because the high salt concentration of this solution interfered with enzymatic hydrolysis, aliquots were assayed after hydrolysis by autoclaving for 30 minutes with 2 *N* HCl. Under these conditions, no growth factor activity was detected in 0.05, 0.1 and 0.2 ml. of the concentrated mixture, aliquots inhibited the growth response of the assay organism to 5 γ of I. The sensitivity of this method would have allowed detection of 2 γ/ml. IV in the original filtrate (equivalent to 0.2% of the II present).

(31) It has been noted above that II reduces exactly one mole of periodate.

NEW YORK 16, N. Y.