low the CMC throughout at least region B. On the other hand since micelles are certainly present in this region at the time of preparation of the solutions it is not possible to rule out on this basis the possibility that micelles cause the denaturation step. In view of the fact that we have shown that micelles do not form in the dialyzate of detergent solutions, however, the results of Fig. 2 clearly show that the all-or-none step proceeds in the absence of micelles, although slowly. This result has also been obtained with O. The much faster reaction when detergent and protein are mixed directly may mean that the reaction proceeds more rapidly through a mechanism involving micelles although this cannot be definitely concluded as yet.

On the Mechanism of the Combination.—It thus appears that a conclusive study of binding of detergent ions by proteins will have to await the availability of a really pure detergent preparation, much more homogeneous than any yet available for such purposes. In the meantime it seems possible to draw some conclusions about the probable mechanism. It is suggested that there is a series of reactions of the following sort

$$\left.\begin{aligned} A + I &\rightleftharpoons AI \\ AI + I &\rightleftharpoons AI_2 \\ &\vdots \\ AI_{m-1} + I &\rightleftharpoons AI_m \end{aligned}\right\} \quad \text{I}$$

$$AI_m + I \longrightarrow A^*I_{m+1} \quad \text{II}$$

$$\left.\begin{aligned} A^*I_{m+1} + I &\rightleftharpoons A^*I_{m+2} \\ &\vdots \\ A^*I_{n-1} + I &\rightleftharpoons A^*I_n \end{aligned}\right\} \quad \text{III}$$

$$\left.\begin{aligned} A^*I_n + I &\rightleftharpoons A^*I_{n+1} \\ &\vdots \end{aligned}\right\} \quad \text{IV}$$

The first set of equations (I) is essentially statistical in character. When a critical number m of ions is bound the protein is opened up or denatured as indicated (II). Whether or not this step is reversible cannot be concluded but it seems probable that it is not.[11] Once the protein is opened up, however, a series of equilibria (III) is made available, the binding constants being so great that the formation of AI_n is essentially complete before further combination with AI_m takes place. Finally there are the further equilibria IV which are not important until formation of AI_n is substantially complete.

This mechanism would predict that the free detergent concentration should be substantially constant throughout region B, *i.e.*, the curves in Figs. 3 and 4 should be nearly vertical. Clearly the curves are approaching such behavior more closely with increasing purification and it seems probable that the slope is largely due to the heterogeneity. This is also the explanation, almost certainly, for the failure to find any breaks in the binding curves corresponding to the changes in binding mechanism indicated by the electrophoretic behavior.

There are other minor inconsistencies, notably the slight increase in the mobility of both components in region B in the cases of both proteins. It seems probable that these effects can be explained on the basis of the binding, to a small degree, of lower homologs but it does not seem profitable to discuss these points further at this time.

Acknowledgments.—We are indebted to Armour and Company for generous samples of the bovine plasma albumin used, and to Mr. J. L. Kucera who performed most of the electrophoretic analyses.

(11) That the all-or-none component is not observed in the electrophoretic pattern when heat denatured protein is used has been previously shown for O.[4] The authors have verified this and have shown also that this is true in the case of A.

AMES, IOWA

[CONTRIBUTION FROM THE U. S. PUBLIC HEALTH SERVICE, TUBERCULOSIS RESEARCH LABORATORY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Aromatic Biosynthesis. IX. The Isolation of a Precursor of Shikimic Acid[1]

BY IVAN I. SALAMON[2] AND BERNARD D. DAVIS

RECEIVED JULY 22, 1953

A precursor of shikimic acid in the biosynthesis of five aromatic metabolites has been isolated from culture filtrates of a mutant strain of *Escherichia coli*. It has been characterized as a previously unknown unsaturated dihydroxy-monoketocarboxylic acid containing seven carbon atoms. Its formulation as 5-dehydro-shikimic acid is discussed.

Mutant strains of *Escherichia coli* and other bacterial species have been described which require for growth five aromatic metabolites: tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid and *p*-hydroxybenzoic acid.[3,4] The genetic change in each of these strains has resulted in inability to carry out one of the reactions in the synthesis of a sequence of common precursors of these metabolites. Analysis of this sequence is facilitated by the fact that each mutant accumulates in its culture filtrates the substrate of the blocked reaction, and can utilize later members of the sequence to replace the required aromatic metabolites.

Strains blocked in a relatively late reaction in this biosynthetic sequence (Fig. 1) accumulate considerable amounts (up to 600 γ/ml.) of shikimic acid ($3\beta,4\alpha,5\alpha$-trihydroxy-$\Delta^{1:6}$-cyclohexene-1-carboxylic acid[5]) in their culture filtrates,[3b,6] while

(1) (a) Paper VIII of this series, B. D. Davis and U. Weiss, *Arch. exp. Path. Pharmakol.*, **219**, 549 (1953). (b) Presented in part before the 119th Meeting Am. Chem. Soc., Boston, 1951, Abstracts of Papers, p. 21C. (c) Aided by a grant from the Squibb Institute for Medical Research.

(2) National Institutes of Health Research Fellow, 1950–1951.

(3) B. D. Davis, (a) *Experientia*, **6**, 41 (1950); (b) *J. Biol. Chem.*, **191**, 315 (1951); (c) *Nature*, **166**, 1120 (1950); (d) *J. Bact.*, **64**, 729 (1952).

(4) The requirement for *p*-hydroxybenzoic acid is in most of the mutants a relative one, and is dependent on the carbon source. A sixth, unknown aromatic factor is also required when the *p*H of the culture is raised to 7.5. *Cf.* refs. 3c and 3d.

(5) H. O. L. Fischer and G. Dangschat, *Helv. Chim. Acta*, **20**, 705 (1937); G. Dangschat and H. O. L. Fischer, *Biochim. Biophys. Acta*, **4**, 199 (1950).

(6) H. Shigeura and D. B. Sprinson, *Federation Proc.*, **11**, 286 (1952).

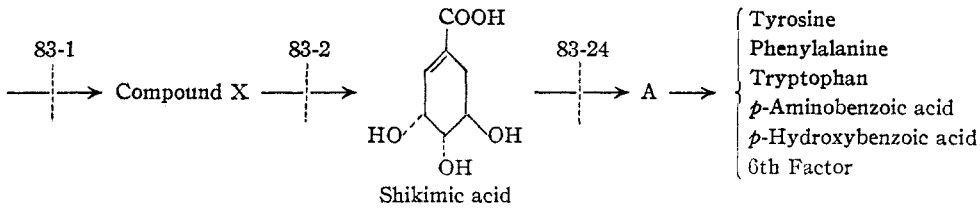

Fig. 1.—Partial scheme of aromatic biosynthesis.

strains blocked in earlier reactions can utilize this compound as a growth factor. It has therefore been concluded that shikimic acid is a precursor of the five aromatic metabolites.[3b] In turn, certain of these strains that are blocked in earlier reactions accumulate, and the others can utilize, a precursor of shikimic acid which has been provisionally designated as compound X.[3b] It could be differentiated from shikimic acid by its inactivity as a growth factor for strain 83-2,[3b] its different chromatographic mobility on paper,[3b] and its extreme sensitivity to alkali. It is moderately stable in mildly acidic solutions at temperatures up to 65°. All steps in its isolation were controlled by bioassay with *E. coli* mutant 83-1, which responds well to either compound X or shikimic acid as a growth factor.

The starting material was prepared by incubating mutant strain 83-2 of *Escherichia coli* with aeration in minimal medium supplemented with the required aromatic metabolites. After two to three days the culture was acidified and the bacteria were removed; the filtrate contained 100–200 γ of compound X per ml. The filtrate was concentrated *in vacuo*, the concentrate was freed from the bulk of inorganic salts by addition of ethanol, the ethanol was evaporated, and the aqueous concentrate was purified by extraction with ether and *n*-pentanol-1. The active material remained in the aqueous phase, from which it was isolated in crystalline form after chromatography on purified charcoal (yield about 60%, in terms of biological activity). It was later possible to prepare compound X by a simpler procedure, in 25% yield, using crystals for seeding impure concentrates.

As a growth factor for the assay organism the crystalline material was about 75% as active as shikimic acid, and the activity remained constant when further purification was attempted. Aside from being somewhat hygroscopic, the crystals were stable under ordinary conditions. They were very soluble in water; less so in methanol, ethanol or acetone; only slightly soluble in ethyl acetate, acetic acid, or *n*-butanol-1; and insoluble in ether, chloroform or hexane. Aqueous solutions of the pure compound were moderately stable if neutral or weakly acidic, but quite unstable if strongly acidic. In alkaline solutions biological activity was destroyed within a few seconds, and at the same time extensive changes in light absorption occurred.

Compound X is a monobasic acid (pK 3.2) of empirical formula $C_7H_8O_5 \cdot H_2O$. It is levorotatory ($[\alpha]_D$ −57° in ethanol) and shows selective absorption in the ultraviolet (ϵ_{234} 12,100 in ethanol; *cf.* Fig. 2). On heating it loses its water of crystallization at 85–90° and melts at 150–152° with slight decomposition. If heated above 152° the melt resolidifies and shows a second m.p. at 202° accompanied by sublimation; there is complete loss of biological activity. The sublimate, of m.p. 204°, was identified as protocatechuic acid, which could also be formed by heating the compound briefly with concentrated hydrochloric acid.

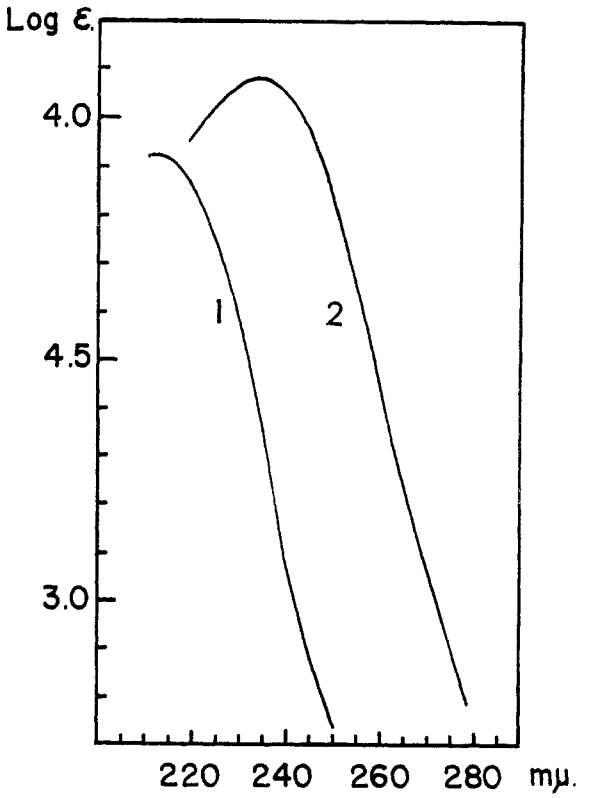

Fig. 2.—Absorption spectra of shikimic acid (1) and compound X (5-dehydroshikimic acid) (2) in ethanol.

Compound X reduces Fehling solution and Tollens reagent very rapidly but does not form chromogens in the Molisch test for carbohydrates, with tetranitromethane, or with ferric chloride. It forms a phenylosazone and a very insoluble semicarbazone. From the latter it can be recovered in about 50% yield on acid hydrolysis. The semicarbazone probably also hydrolyzes slowly under the conditions of the bioassay since it shows weak biological activity.

Compound X decolorizes diazomethane at −40° instantly, yielding a crystalline methyl ester, $C_8H_{10}O_5$, of m.p. 126°, $[\alpha]_D$ −47°, and ϵ_{234} 11,600. This ester is stable to high vacuum sublimation at 100°. It shows biological activity for strain 83–1 corresponding to about 50% that of its parent compound (on an equimolar basis), but with a slower response of the microörganism. It forms a

2,4-dinitrophenylosazone, and it can be acetylated with acetic acid in the presence of acetic anhydride and a catalytic amount of perchloric acid[7] to yield a sirupy methyl ester diacetate, $C_{12}H_{14}O_7$, $[\alpha]_D$ $-18°$, ϵ_{234} 11,600, which is apparently also stable to high-vacuum distillation. The complete lack of biological activity of the methyl ester diacetate may be due to its insolubility in water.

A well characterized diacetate of the free acid could not be readily prepared. The product obtained with acetic anhydride and pyridine had some biological activity, but also contained an inhibitor of bacterial growth. Following high-vacuum sublimation at 100° the activity was lost; the main fraction of m.p. 205°, λ_{max} at 252 and 284 mμ, gave protocatechuic acid on hydrolysis, and was probably identical with 3-monoacetylprotocatechuic acid[8] of a similar m.p. Acetylation with acetic acid, using perchloric acid as catalyst, resulted in an even less stable sirupy product which reacted similarly if heated in a high vacuum. All the above esters of compound X decomposed on standing for a few days in ethanolic solution at room temperature.

The reactions described above show that compound X is a monocarboxylic acid containing a carbonyl group and two acylable hydroxyl functions; one of the latter probably participates in an α-ketol group. The ready aromatization of this compound and its esters, which is reminiscent of the behavior of inososes, suggests that it has a cyclic structure. The empirical formula, which differs from that of shikimic acid by two hydrogen atoms, implies that such a structure would also contain a double bond; and this is confirmed by the ultraviolet absorption spectrum, which indicates the presence of an α,β-unsaturated carbonyl group. These facts, and the ready conversion *in vivo* to shikimic acid, are consistent with the formulation of compound X as 5-dehydroshikimic acid. Proof of this structure and configuration will be presented in a forthcoming publication.

Methods. Organisms and Culture Media.—The organisms employed were mutants obtained by ultraviolet irradiation from the W strain of *Escherichia coli* (ATCC 9637). Their isolation by the penicillin method[9] and their nutritional responses[3b,3d] have been described. Minimal medium A[10] was used throughout; it contains glucose, citrate and NH_4^+ as sole carbon and nitrogen sources, and has a *p*H of 7.0. Solid media were prepared by the addition of 1.5% agar. Stock cultures of the organisms in liquid medium A, enriched with 0.2% casein digest (N-Z Case, Sheffield) and 0.2% yeast extract (Difco), were adequately stable for several months if stored at 3°.

Bioassay.—Assays of compound X were carried out with strain 83-1 either turbidimetrically in liquid media or by means of filter paper disks on heavily seeded solid media. The turbidimetric procedure was similar to that previously described for a proline precursor.[11] In the disk assay the diameter of the zone of visible growth was measured after incubation for 18 to 24 hours at 35°. The disk method, though less precise, was more frequently used because of its convenience: the seeded plates were stable for several weeks at 3°, and sterilization of the test solutions and elimination of volatile organic solvents were unnecessary. In both methods shikimic acid served as a standard for comparison with the unknown samples, and the assay medium was supplemented with 20 γ of L-tyrosine and 40 γ of DL-phenylalanine per ml. in order to make the assay more sensitive and to insure rapid response of the organisms.[12] For the turbidimetric assays compound X was sterilized by filtration or by heating at 65° (at *p*H 4).

Experimental[14,15]

Accumulation of Compound X.—An 18-hour culture of strain 83-2,[16] in medium A enriched with 0.2% casein hydrolyzate and 0.2% yeast extract, was centrifuged and resuspended in the same volume of water. One ml. of this suspension was inoculated into cotton-plugged 1-liter erlenmeyer flasks, each containing 700 ml. of sterile medium A (0.5% glucose) supplemented with 10 mg. of L-tyrosine, 20 mg. of DL-phenylalanine, 5 mg. of L-tryptophan and 0.01 mg. of *p*-aminobenzoic acid per liter. The cultures, aerated by shaking, were incubated for 2 to 3 days at room temperature (25–32°). The bacteria were then removed in a Sharples centrifuge and the effluent was acidified to *p*H 3 with HCl and clarified with "Hyflo Super-cel." The resulting culture filtrates contained compound X in a concentration equivalent to 100 to 200 mg./l. of shikimic acid. They were stored at 3° under toluene until used.

Stability of Compound X in Filtrates.—Samples (2.5 ml.) of a culture filtrate of strain 83-2 were adjusted to various *p*H values and heated. They were then neutralized, brought to 5-ml. volume, and assayed turbidimetrically. The results are summarized in Table I.

TABLE I

STABILITY OF COMPOUND X

*p*H	35° 18 hr.	60° 2 hr.	80° 2 hr.	100° 45 min.	120° 15 min.
1.0	90[a]	70	35	50	25
3.5	100	100	60	80	70
7.1	100	60	35	0	0
9.0	0	0	..	..	..

[a] Activity expressed as γ shikimic acid/ml. original volume. Original activity: 100 γ shikimic acid/ml.

Purified Aqueous Concentrate.—This was prepared in several ways; a satisfactory procedure was the following: 8.4 l. of culture filtrate (activity 180 γ/ml. shikimic acid, or 1.5 g. total) was adjusted to *p*H 4.3 and evaporated under N_2 *in vacuo* at 30–35° (bath) to a volume of about 100 ml. During the evaporation toluene was added in small portions to prevent mold growth. The concentrate was diluted with 1.5 l. of ethanol and the resulting deposit of mineral salts was filtered off and washed with ethanol. The combined filtrates (2 l.) were assayed turbidimetrically; no activity had been lost. To this filtrate 65 ml. of water was added and the ethanol was removed at 30° *in vacuo* until the volume

(7) *Cf.* J. B. Conant and G. M. Bramann, THIS JOURNAL, **50**, 2305 (1928); B. Whitman and E. Schwenk, *ibid.*, **68**, 1865 (1946).

(8) G. Ciamician and P. Silber, *Ber.*, **25**, 1476 (1892); E. Fischer, U. Bergmann and W. Lipschütz, *ibid.*, **51**, 74 (1918); R. Lesser and G. Gad, *ibid.*, **59**, 233 (1926). *Cf.* also K. Ono and M. Imoto, *J. Chem. Soc. Japan*, **55**, 517 (1934); *Bull. Chem. Soc. Japan*, **10**, 323 (1935).

(9) J. Lederberg and N. Zinder, THIS JOURNAL, **70**, 4267 (1948); B. D. Davis, *ibid.*, **70**, 4267 (1948); B. D. Davis, *Proc. Natl. Acad. Sci.*, **35**, 1 (1949).

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

(11) H. J. Vogel and B. D. Davis, THIS JOURNAL, **74**, 109 (1952).

(12) The assay organism utilizes shikimic acid or compound X more slowly to replace tyrosine and phenylalanine than to replace the other three required aromatic metabolites. This fact, and the related demonstration that compound X is a competitive inhibitor of the utilization of its own biosynthetic product, shikimic acid, are discussed in detail elsewhere.[13]

(13) B. D. Davis, *J. Bact.*, **64**, 749 (1952).

(14) Analyses by Dr. William C. Alford (National Institutes of Health, Bethesda, Maryland) and Dr. Joseph F. Alicino (Microanalytical Laboratory, E. R. Squibb and Sons, New Brunswick, New Jersey). The samples were dried to constant weight in high vacuum over P_2O_5 at 50°.

(15) Melting points were taken on a Kofler micro-stage and are correct to ±2°.

(16) All available mutants blocked between compound X and shikimic acid accumulate X; strain 83-2 was used because it yielded nearly colorless filtrates. This strain requires only three of the metabolites listed above (tyrosine, phenylalanine and tryptophan). However, this property does not alter its position in the sequence of Fig. 1, since the decreased requirement of this strain has been shown elsewhere[3d] to be due to the incompleteness of its genetic block.

was about 50 ml. (At this stage evaporation to a sirup resulted in complete loss of activity.) The solution was diluted with water to 500 ml. and its *p*H was adjusted to 2.5 with HCl. It was then extracted with 3 portions of 750 ml. of ether followed by 3 portions of 400 ml. of *n*-pentanol-1 saturated with water. The organic layers were washed with 4 portions of 250 ml. 0.001 *N* HCl; the aqueous phases were combined, freed from pentanol by azeotropic distillation at 30° *in vacuo*, and brought to 2 l. with water. The total activity of this solution was 80% that of the original filtrate. The ether phases left on evaporation a partly crystalline, biologically inactive residue. The pentanol phases yielded a dark, sirupy residue which contained about 10% of the original activity, but was discarded.

Chromatography and Crystallization of Compound X.—Originally, samples of the aqueous concentrate were chromatographed on animal charcoal ("Merck"). This adsorbent gave the best results, but had to be purified extensively for this purpose. In consequence, for work on a preparative scale activated charcoal ("Darco G-60") was used as follows: 1.5 l. of the purified aqueous concentrate (*v. supra*) (total activity, 900 mg. shikimic acid) was filtered through a column (50 × 130 mm.) prepared from a mixture of 70 g. of Darco G-60[17] and 30 g. of Celite. The column was washed with 2 l. of 0.001 *N* HCl followed by 250-ml. portions of the solvents indicated in Table II. The aqueous effluents

TABLE II

CHROMATOGRAPHY OF COMPOUND X

Fraction no.	Solvent	Solids, mg.	Assay,[a] activity per fraction
4- 6	Water–ethanol (10–30% E)	280	−
7- 8	Water–ethanol (40–50% E)	317	+
9–15	Water–ethanol (75–100% E)	1099	+++
16–18	Ethanol	128	++
19–20	Ethanol–benzene (2–5% B)	35	++
21–22	Ethanol–benzene (10–20% B)	32	+
23–25	Ethanol–benzene (50–100% B)	50	−

[a] 0.01 ml. of each 250-ml. fraction was assayed by the disk technique. The signs denote: −, no activity; +, diameter of growth area less than 2.5 cm.; ++, diameter 2.5–4 cm.; +++, diameter over 4 cm.

showed no activity. Fractions 9 to 19 (crude weight 1.232 g.) crystallized from ethanol on addition of a small amount of water; the crystals were washed with ethanol–ethyl acetate (1:5). The mother liquors darkened on standing and gave an amorphous precipitate which was inactive. On dilution with ethyl acetate a few colorless needles of m.p. 198–203° (dec.) separated; they were biologically inactive, and different from protocatechuic acid. After removal of the ethanol, the remaining mother liquor was distributed between water and ethyl acetate, and another crop of crystalline compound X was isolated from the aqueous phase. In the ethyl acetate layer, which contained most of the colored impurities, a significant amount of activity was also found. This material was purified by filtration of its ethanolic solution over Darco G-60[17] and crystallization. Altogether 723 mg. of crystalline compound X was obtained from this chromatogram. The residual mother liquors, combined with fractions 7 to 8 and 20 to 22, were treated with semicarbazide (*v. infra*). They gave 257 mg. of semicarbazone, corresponding to 213 mg. of compound X, the total yield of which thus represented 61% of the activity in the original filtrate.

Simplified Isolation Procedure.—16.8 liters of culture filtrate (activity: 200 γ/ml. shikimic acid, or 3.3 g. total) was adjusted to *p*H 3, stirred for 30 minutes with 400 g. of Darco G-60 (untreated), and filtered. The charcoal was washed with 20 l. of 0.001 *N* HCl and with 2 l. of 15% ethanol–water. It was then extracted with 20 l. of ethanol and the extract was evaporated *in vacuo*. The sirupy residue was dissolved in 100 ml. of ethanol, filtered from a slimy precipitate, and, after removal of the ethanol, dissolved in 200 ml. of water. This solution was filtered from amorphous matter and washed with 4 portions of 200 ml. of chloroform. The aqueous phase was concentrated *in vacuo* to a sirup (11 g.) which, after addition of 5 ml. of ethanol and inoculation with crystalline compound X, crystallized on standing for 4 days at 3°. Recrystallization from ethanol with a trace of water gave 1.15 g. (25% of the original activity) of pure compound X. The mother liquor was treated with semicarbazide (5 g. in 50 ml. of methanol containing 3 ml. of acetic acid); 1.26 g. of crude semicarbazone was obtained.

Crystalline Compound X.—Monoclinic prisms, P 2_1, β-angle 111°, $a:b:c$ = 5.87:8.56:8.92 kX,[18] with 1 mole of H_2O. The water is lost at 85–90°, m.p. 150–152° with slight decomposition (on slow heating, m.p. 141–152°). The melt resolidifies at 161–168° and melts again at 201–202° (subl.); $[\alpha]^{28}D$ −57.5 ± 1° (*c* 1.065 in ethanol);[19] ultraviolet absorption ϵ_{234} 12,100 (ethanol); ϵ_{235} 11,600 (0.1 *N* HCl); ϵ_{234} 11,900 (Na salt at *p*H 7.5 in water).

Anal. Calcd. for $C_7H_8O_5 \cdot H_2O$: C, 48.84; H, 4.68; H_2O, 9.48; neut. equiv., 190; mol. wt. (anhydrous), 172. Found: C, 48.83; H, 4.85; H_2O, 9.29; neut. equiv. in water, 197 (pK_a 3.2 ± 0.2); mol. wt. (micro Rast), 162 (decomposes).

Assay.—Microbiological activity (turbidimetric assay) was 80% (18-hours incubation), 80.5% (24 hours), 75.2% (48 hours) that of an equimolecular amount of shikimic acid.[20] Samples melted at 152° and resolidified showed biological activity; those heated to 202° showed none.

Compound X reduces Fehling solution at room temperature, and ammoniacal silver oxide solution on slight warming, in a few seconds. It is stable as an anion in aqueous solution at *p*H 7.5–8 for 6 hours at room temperature. At *p*H 10–10.5 50% of the activity is lost in 15 minutes and none can be detected after 1 hour. On addition of 0.05 *N* NaOH a strong chromogen ($E^{1\%}_{1cm}$ 989 at 233 mμ and 340 at 292 mμ) is formed which then decays slowly. The originally red solution turns yellow in 3 minutes.

Protocatechuic Acid from Compound X. (A).—Two mg. of compound X was heated on a hot stage. Almost quantitative sublimation occurred between 165 and 210°. The sublimate (fine prisms) was biologically inactive, m.p. 203–204°, mixed m.p. with authentic protocatechuic acid[21,22] 202–204°. The ultraviolet absorption (maxima at 219, 259 and 295 mμ in ethanol) and the color with aqueous $FeCl_3$ solution (blue-green, maxima at 385, 455 and 684 mμ in water) were the same as those of protocatechuic acid.

(B).—One mg. of compound X was boiled for 20 seconds with 0.1 ml. of 38% HCl. The protocatechuic acid formed was identified by its absorption spectrum and by the spectrum of its ferric complex. The peak at 234 mμ, characteristic of compound X, could no longer be detected.

Compound X Semicarbazone.—Eighty mg. of compound X was dissolved in 2 ml. of methanol and a solution of 200 mg. of semicarbazide in 0.1 ml. of acetic acid and 0.8 ml. of methanol was added. The semicarbazone crystallized within a few seconds. It was filtered, washed with methanol, and dried. It was insoluble in all the usual solvents and could not be recrystallized, m.p. 189–197° (dec.).

Anal. Calcd. for $C_8H_{11}O_5N_3$: C, 41.92; H, 4.84; N, 18.33. Found: C, 41.02; H, 5.03; N, 19.08.

Assay.—Weak activity (paper disk technique); on incubation for several days the response increased progressively, probably due to slow hydrolysis of the semicarbazone under the conditions of the assay.

Hydrolysis.—1.26 g. of crude semicarbazone (from mother liquors of compound X, *v. supra*) was shaken with 120 ml. of 2 *N* HCl at 37° until dissolved (1 hour). The solution was diluted with 380 ml. of water, shaken for 10 minutes with 30 g. of purified Darco G-60,[17] and filtered. The charcoal

(17) Purified by successive extractions with hot concd. HCl, water, ethanol, benzene and acetic acid; dried at 300° *in vacuo*. Impurities removable by extraction with ethanol–benzene (1:1): 0.01%.

(18) We are indebted to Dr. I. Fankuchen of Brooklyn Polytechnic Institute, Brooklyn, New York, for the X-ray measurements.

(19) This figure is computed for the anhydrous compound. The specific rotation of an air-dried sample of the monohydrate was $[\alpha]^{28}D$ −52.1 ± 1° (*c* 1.176 in ethanol).

(20) The somewhat low activity of compound X may well be due to decomposition during the assay, which involves prolonged incubation at *p*H 7.0 and 37°.

(21) Kindly furnished by Dr. Karl Pfister III, Merck and Co., Inc., Rahway, New Jersey.

(22) M.p. 203–204° on our stage. Hlasiwetz and Barth, *Ann.*, **130**, 348 (1864); D. S. Pratt and G. A. Perkins, THIS JOURNAL, **40**, 224 (1918); G. Lock, *Ber.*, **62**, 1186 (1929), give m.p. 199–200°; E. Späth and H. Quietensky, *ibid.*, **60**, 1882 (1927), obtained m.p. 204–205° after sublimation.

was washed with 700 ml. of 0.001 *N* HCl and extracted with 700 ml. of ethanol. On evaporation the extract gave 560 mg. of crude compound X; recrystallization yielded 340 mg. of m.p. 150–152°. The semicarbazone was not hydrolyzed by 1 *N* HCl (aqueous or ethanolic) after 18 hours at 37°. Cleavage with benzaldehyde resulted in complete loss of activity.

Compound X Phenylosazone.—To 50 mg. of compound X in 0.5 ml. of water, 0.35 ml. of freshly distilled phenylhydrazine in 1.2 ml. of 25% acetic acid was added. The deep orange osazone crystallized after 6 minutes at 100°. Leaflets (from dioxane–ether–hexane), m.p. (194) 197–199°, or needles (from ether), m.p. (220) 225–227° (dec.); biologically inactive.

Anal. Calcd. for $C_{19}H_{18}O_3N_4$: N, 15.99. Found: N, 15.84.

Compound X Methyl Ester.—A solution of 202 mg. of compound X in 6 ml. of ethanol was cooled to −40°, and a cold solution of diazomethane in ether was added until the yellow color remained stable. After 2 minutes the excess diazomethane and the solvents were removed *in vacuo*, and the residue was crystallized from ethanol–ether; fine needles (135 mg.), m.p. 124–126°, $[\alpha]^{24}D$ −47.1 ± 3° (*c* 0.237 in ethanol), ϵ_{234} 11,600 ± 500 (ethanol). The compound sublimed unchanged at 90–110° and 10^{-4} mm.

Anal. Calcd. for $C_8H_{10}O_5$: C, 51.61; H, 5.55. Found: C, 51.86; H, 5.61.

Assay.—Microbiological activity (turbidimetric) was 32.5% (18-hours incubation), 38% (24 hours) and 39.5% (48 hours) that of an equimolecular amount of shikimic acid (*i.e.*, about 50% that of compound X, with slow response of the assay organism). Shikimic acid methyl ester[23] was similarly tested and found to give an even slower response.

2,4-Dinitrophenylosazone.—To 35 mg. of compound X methyl ester in 1 ml. of ethanol 100 mg. of 2,4-dinitrophenylhydrazine in 2.5 ml. of ethanol and 0.2 ml. of 38% HCl was added. The osazone crystallized after boiling for 3.5 minutes; red prisms (69 mg.) from pyridine–methanol–pentane, m.p. 250–256° (dec.), biologically inactive.

Anal. Calcd. for $C_{20}H_{16}O_{11}N_8$: N, 20.58. Found: N, 20.35.

Compound X Methyl Ester Diacetate.—To an ice-cooled solution of 44 mg. of compound X methyl ester in 1 ml. of acetic acid and 0.4 ml. of acetic anhydride, 1 ml. of acetic acid containing 0.001 ml. of 72% perchloric acid was added with stirring. The mixture was kept at 10° for 15 minutes. It was then poured into 20 ml. of cold, saturated sodium hydrogen carbonate solution. This was extracted with ether and the extract was washed with sodium hydrogen carbonate solution and water, dried and evaporated. It left 51 mg. of a light yellow oil which was distilled at 95° and 10^{-4} mm.; colorless oil (47 mg.), $[\alpha]^{24}D$ −18.0 ± 1° (*c* 0.996 in ethanol), ϵ_{234} 11,600 ± 500 (ethanol), biologically inactive.

Anal. Calcd. for $C_{12}H_{14}O_7$: C, 53.33; H, 5.22. Found: C, 53.28; H, 5.31.

(23) H. O. L. Fischer and G. Dangschat, *Helv. Chim. Acta*, **17**, 1200 (1934).

Acetylation of Compound X. (A).—A solution of 50 mg. of compound X in 0.6 ml. of pyridine and 0.15 ml. of acetic anhydride was kept for 48 hours at 3° and, after addition of 1 ml. of methanol, evaporated to dryness at 20° *in vacuo*. The residue was dissolved in ethyl acetate and the solution was washed with 1 *N* HCl and water, dried and evaporated. The material showed on disk assay a zone of inhibition surrounded by a zone of growth. From ether–pentane it gave impure crystals of m.p. 95–125°, $E^{1\%}_{1cm}$ 449 at 234 mμ in ethanol (inflections at 283 and 297 mμ, indication of strong absorption beyond 210 mμ with minimum at 223 mμ). The mother liquor distilled slowly at 95–105° and 10^{-4} mm., and gave two fractions: (1) rosettes from aqueous methanol or ether–pentane, m.p. 157–159°, ultraviolet absorption similar to that of the crystals of m.p. 95–125°,[24] and (2) fine needles from methanol–ether–pentane, m.p. 203–205° (subl.), λ_{max} 252 and 284 mμ (ethanol), showing no color reaction with $FeCl_3$.

Anal. Calcd. for $C_9H_8O_5$: C, 55.11; H, 4.18. Found: C, 55.59; H, 4.49.

Hydrolysis.—Four mg. of the needles of m.p. 205° was boiled under reflux for 30 minutes with 1 ml. of methanol and 1 ml. of 0.1 *N* HCl. The methanol was evaporated and the aqueous solution was extracted with methylene chloride. The extract was dried and evaporated and the residue was sublimed at 12 mm.; m.p. of the sublimate 200–204°. Mixed m.p., ultraviolet absorption, and absorption of its ferric complex showed its identity with protocatechuic acid.

(B).—To a suspension of 51 mg. of compound X in 2 ml. of acetic acid and 0.5 ml. of acetic anhydride, 1 ml. of acetic acid containing 0.001 ml. of 72% perchloric acid was added at 10°, with shaking. After 30 minutes at 10°, 5 ml. of ethanol was added to the clear solution. This, after a few minutes, was buffered with 1 ml. of 10% aqueous potassium acetate solution and evaporated to dryness at 30° *in vacuo*. The residue was extracted with ethyl acetate and the extract was washed with water, dried and evaporated. It left 61 mg. of a light yellow oil, which did not crystallize and showed weak biological activity, ϵ_{235} 11,000[25] in ethanol (single peak). When heated in a vacuum of 10^{-4} mm. to 95–105° the oil behaved like the product prepared according to method (A).

Acknowledgment.—We are indebted to the late Dr. Konrad Dobriner of the Sloan-Kettering Institute for Cancer Research, New York, N. Y., for use of a recording ultraviolet spectrophotometer and a precision polarimeter. The skillful assistance of Marlies M. Salamon and Elizabeth S. Mingioli is gratefully acknowledged.

NEW YORK 21, N. Y.

(24) This material is possibly diacetylprotocatechuic acid (m.p. 159°, *cf.* ref. 8); however, no authentic sample was on hand for comparison.

(25) Calcd. for $C_{11}H_{12}O_7$.