[CONTRIBUTION FROM LEDERLE LABORATORIES, AMERICAN CYANAMID CO., PEARL RIVER, N. Y.]

Biosynthesis of the Tetracyclines. III.[1] A New Amino Acid from *Streptomyces aureofaciens*: (+)-*trans*-2,3-Dihydro-3-hydroxyanthranilic Acid[2,3]

BY J. R. D. MCCORMICK, JULES REICHENTHAL, URSULA HIRSCH AND NEWELL O. SJOLANDER

RECEIVED FEBRUARY 3, 1962

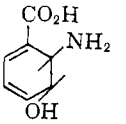
A new amino acid has been isolated from the fermented mash of a *Streptomyces aureofaciens* mutant, S-652. The substance has the empirical formula $C_7H_9NO_3$ and has been characterized as (+)-*trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHAA) (I). DHAA was produced in quantities as high as 10 g./l. in shaker-flask and tank fermentations. C^{14}-Labeled DHAA has been prepared biosynthetically. Using this labeled material we have shown that DHAA probably is not involved as an intermediate in the biosynthesis of 7-chlorotetracycline.

The discovery by Duggar[4] of 7-chlorotetracycline[5] (CTC) as an antibiotic product of *Streptomyces aureofaciens* has led to extensive study of natural and induced mutants of this species in order to obtain strains which would produce greater quantities of the antibiotic. The *S. aureofaciens* mutants isolated in this program have consisted of a wide variety of morphological and physiological types (for example, those described by Backus, Duggar and Campbell[6]). More recently, the fermentation products of several unusual mutants have been studied extensively, and several important compounds related to CTC, such as tetracycline,[7] 7-bromotetracycline,[8] 7-chloro-6-demethyltetracycline,[9] 6-demethyltetracycline[9] and 7-chloro-5a(11a)-dehydrotetracycline[10] have been reported as being formed by various strains of *S. aureofaciens*.

In the continuing study of these mutants of *S. aureofaciens*, frequent occurrence of a substance having characteristic ultraviolet absorption was observed in fermented mashes, particularly from those mutants which elaborated little or none of the tetracyclines. Furthermore, paper chromatographic examination of such fermented mashes indicated that the characteristic ultraviolet absorption could be attributed to a single substance.

Further experience indicated that this substance was also elaborated even by strains which normally produce tetracyclines, especially when these strains were grown under certain conditions that were unfavorable for the production of tetracyclines. This rough reciprocal relationship to the tetracyclines led to early interest in the new substance as a possible intermediate in the biosynthesis of the tetracyclines.

In this paper we present evidence that the new substance probably is not involved as an intermediate in the biosynthesis of the tetracyclines and report the preparation by fermentation and the characterization of the substance as (+)-*trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHAA) (I).



I

DHAA was first observed by the presence of strong absorption at 278 mμ in shaker-flask fermentation mashes of strain S-652, a pale-tan mutant derived by mutation and selection from *S. aureofaciens* NRRL 2209. Strain S-652 produced DHAA in amounts ranging from 7 to 11 g./l. as determined from the optical density of neutral filtrates at 278 mμ. A small quantity of CTC was simultaneously produced. Examination of the neutral filtrates by paper chromatography (butanol–acetic acid[11]) indicated that the ultraviolet-absorbing component was a polar substance having mobility comparable to tyrosine. The characteristic ultraviolet absorption was used as an analytical method in the preparation by fermentation and isolation of DHAA; paper chromatography served to distinguish DHAA from other ultraviolet absorbing components.

A number of other *S. aureofaciens* strains (Table I) also produced DHAA, but in smaller amounts than S-652. An effect of composition of medium on DHAA production also was noted; some *S. aureofaciens* mutants such as S-77-MB-3, B-740, BC-41 and S-609, which produced no detectable DHAA under normal conditions in a corn steep medium, produced significant quantities in a mineral salts–starch–lard oil medium[1] (Table I). Similarly, strain BC-41, a high CTC-yielding strain, produced considerable amounts of DHAA when grown in a corn steep medium at abnormally high temperatures of 32° and 37°, conditions which markedly limited CTC production (Table II). *S. aureofaciens* S-652 fermentations were also successfully carried out in small tanks, with assays for DHAA ranging from 3.2 to 10 g./l.

(1) Previous paper in this series, J. R. D. McCormick, N. O. Sjolander, S. Johnson and A. P. Doerschuk, *J. Bact.*, **77**, 475 (1959).

(2) Presented in part before the Division of Microbial Chemistry and Technology at the Chicago Meeting of the American Chemical Society, September, 1961.

(3) A preliminary report of this work has been published: J. R. D. McCormick, J. Reichenthal, U. Hirsch and N. O. Sjolander, *J. Am. Chem. Soc.*, **83**, 4104 (1961).

(4) B. M. Duggar, *Ann. N. Y. Acad. Sci.*, **51**, 177 (1948).

(5) C. W. Waller, B. L. Hutchings, C. F. Wolf, A. A. Goldman, R. W. Broschard and J. H. Williams, *J. Am. Chem. Soc.*, **74**, 4981 (1952).

(6) E. J. Backus, B. M. Duggar and T. H. Campbell, *Ann. N. Y. Acad. Sci.*, **60**, 86 (1954).

(7) A. P. Doerschuk, J. R. D. McCormick, J. J. Goodman, S. A. Szumski, J. A. Growich, P. A. Miller, B. A. Bitler, E. R. Jensen, M. A. Petty and A. S. Phelps, *J. Am. Chem. Soc.*, **78**, 1508 (1956).

(8) P. Sensi, G. A. De Ferrari, G. G. Gallo and G. Rolland, *Il. Farmaco-Ed. Sc.*, **10**, 337 (1955).

(9) J. R. D. McCormick, N. O. Sjolander, U. Hirsch, E. R. Jensen and A. P. Doerschuk, *J. Am. Chem. Soc.*, **79**, 4561 (1957).

(10) J. R. D. McCormick, P. A. Miller, J. A. Growich, N. O. Sjolander and A. P. Doerschuk, *ibid.*, **80**, 5572 (1958).

(11) R. J. Block, E. L. Durrum and G. Zweig, "A Manual of Paper Chromatography and Paper Electrophoresis," Academic Press, Inc., New York, N. Y., 1955, p. 113.

Table I

Production of DHAA by Various *S. aureofaciens* Strains

Strain	DHAA produced, g./l. Corn steep medium	Mineral salts medium[1]
S-652	10.5	3.2
S-730-14	4.4	
V-11	4.0	
S-730-6	2.9	
S-77-MB-3	<0.8	3.9
B-740	< .8	3.9
BC-41	< .8	3.7
S-609	< .8	1.8

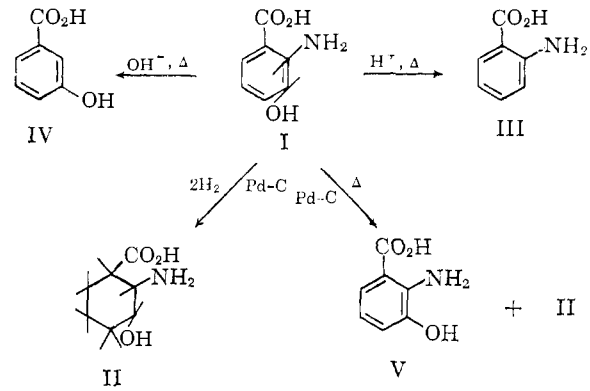
Table II

Effect of Fermentation Temperature on DHAA and CTC Production by *S. aureofaciens* Strain BC-41 in Corn Steep Medium

Temp., °C.	DHAA, g./l.	CTC, g./l.
26.5	<0.8	6.0
32	4.3	1.0
37	5.0	<0.1

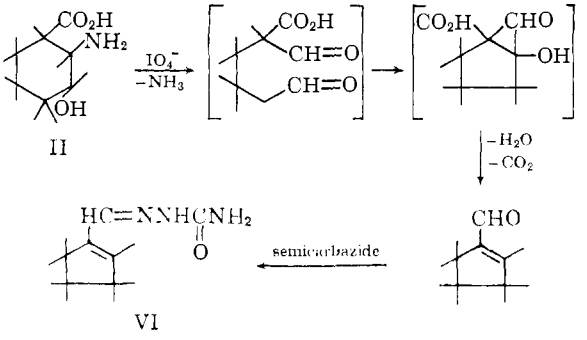
Preliminary investigations of fermentation filtrates showed that DHAA was not extractable from acidic, neutral or alkaline aqueous solutions by such solvents as butanol or methyl isobutyl ketone. DHAA was taken up from solution by both cation and anion exchange resins, and could be eluted from the cation resin by sulfuric acid. After removal of sulfate, the eluates were concentrated and chilled. The crude crystalline DHAA which separated was recrystallized from hot water and from glacial acetic acid. It had no antibiotic activity. The analysis corresponded to the formula $C_7H_9NO_3$. The compound melted with decomposition at 190–191°. It was soluble in water and other hydroxylic solvents but was essentially insoluble in non-polar solvents. The chemical composition of DHAA and its amphoteric nature, as shown by physical properties, behavior with ion exchange resins and the appearance of $-NH_3^+$ (2860 to 3000 cm.$^{-1}$ broad, 2125 cm.$^{-1}$) and $-CO_2^-$ (1590 and 1390 cm.$^{-1}$) absorptions in the infrared spectrum of the crystalline product suggested that DHAA was an amino acid. The ultraviolet absorption of DHAA (λ_{max} 278 mμ, ε 9350 in 0.1 *N* hydrochloric acid) could be accounted for by a chromophore consisting of the carboxyl and two linearly conjugated double bonds.[12] Catalytic hydrogenation of DHAA resulted in the uptake of only two moles of hydrogen to yield another amino acid, II (positive ninhydrin reaction). Thus the original substance, DHAA, was indicated to be a cyclic diene amino acid. Confirmation of this was seen in the ready conversion of DHAA under vigorous acidic conditions to anthranilic acid (III), and under vigorous alkaline conditions to *m*-hydroxybenzoic acid (IV). The presence of acylable amino and hydroxyl groups in DHAA was confirmed by acetylation to an O,N-diacetyl derivative.

Final proof of the constitution of DHAA as a cyclohexadienoic acid and relative placement of the carboxyl, amino and hydroxyl groups was found in the very facile catalytic disproportionation of DHAA to 3-hydroxyanthranilic acid (V) and hexahydro-3-hydroxyanthranilic acid (II). In



addition, since DHAA was optically active, gave no ketone reaction (negative dinitrophenylhydrazine test) and produced no ammonia on mild alkaline hydrolysis, the structure must be one of the four possible stereoisomeric 2,3-dihydro-3-hydroxyanthranilic acids. At this time no conclusive evidence for the configurational relationship of the amino and hydroxyl groups was found, but the vigorous conditions required for dehydration of DHAA by acid to anthranilic acid (Table III) strongly suggested that these groups were *trans*.[13]

The tetrahydro derivative II, produced by catalytic hydrogenation (Pd–C) of DHAA, was isolated and recrystallized from water–acetone. The analysis corresponded to $C_7H_{13}NO_3$. Compound II was a white, crystalline, water-soluble solid which gave a positive ninhydrin reaction, and which reacted smoothly with one equivalent of alkaline periodate. From the periodate oxidation mixture a monosemicarbazone was prepared and isolated. This product was identified as Δ^1-cyclopentenal semicarbazone[14] (VI) as would be expected from the sequence of reactions



At this time no evidence was obtained as to the configuration of the carboxyl-carrying carbon in II relative to the other two asymmetric centers.[13] However, the reduction was shown to be stereoselective by the isolation of the single substance, II, in 72% yield.

(12) E. A. Braude, in "Determination of Organic Structures by Physical Methods," E. A. Braude and F. C. Nachod, Ed., Academic Press, Inc., New York, N. Y., 1955, pp. 131–185.

(13) The complete stereochemistry of II, and therefore of DHAA, is still under investigation.

(14) I. Heilbron, E. R. H. Jones, J. B. Toogood and B. C. L. Weedon, *J. Chem. Soc.*, 1827 (1949).

TABLE III

CONVERSION OF DHAA TO ANTHRANILIC ACID IN CONCENTRATED HYDROCHLORIC ACID AT 60°

Time, hr.	Residual, DHAA, mg.	Anthranilic acid, mg.
0	100	0
1	53	40
2	28	58
3.5	18	68
4.5	10	75
6	5.6	78
6.5	4.6	78

While the work on structure determination was under way, the possibility that DHAA was involved as an intermediate in the biosynthesis of the tetracyclines was investigated by radiotracer techniques.[15] In one experiment, labeled DHAA was prepared by an S-652 fermentation of randomly C^{14}-labeled tobacco starch and was isolated from the mash filtrate by means of preparative paper chromatography. The isolated material was added to a BC-41 fermentation in the active CTC-producing phase. At the end of the BC-41 fermentation period, CTC was isolated by paper chromatographic means and was shown to contain less than 1% of the radioactivity added as labeled DHAA. It therefore was concluded that DHAA is probably not an intermediate in the pathway from carbohydrate to the tetracyclines.[16,17]

Acknowledgment.—The authors wish to express their appreciation to Dr. J. Growich who supplied the original S-652 isolate; to Mr. W. Fulmor and staff for the infrared spectra and interpretations; to Mr. L. Brancone and staff for the microanalyses and titration data; to Mr. J. Goldin and staff for assistance in the large scale steps of the fermentation and isolation; and to Dr. A. P. Doerschuk for his encouragement and advice.

Experimental

Fermentation Procedures.—*S. aureofaciens* spores were transferred to an inoculum medium composed of (g./l.): sucrose, 30; cornsteep liquor, 20; $(NH_4)_2SO_4$, 2; and $CaCO_3$, 7. After incubation for 24 hr. on a reciprocating shaker at 28°, 1 ml. of this inoculum was transferred to a 250-ml. erlenmeyer flask containing 25 ml. of a fermentation medium composed of (g./l.): corn starch, 55; cornsteep liquor, 30; cottonseed meal, 2; $CaCO_3$, 7; $(NH_4)_2SO_4$, 5; NH_4Cl, 1.5; $FeSO_4 \cdot 7H_2O$, 0.04; $MnSO_4 \cdot 4H_2O$, 0.05; $ZnSO_4 \cdot 7H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.005; and lard oil, 20. Fermentations were incubated for 96 to 120 hr. at 26.5° on a rotary shaker operating at about 180 r.p.m. Some shaker-flask fermentations were carried out in a mineral salts–starch–lard oil medium[1] under the conditions described above.

Forty-liter fermentations were run in 60-l. stainless steel tanks at air rates of 0.2 to 0.5 volume of air per volume of medium per min. and agitator speeds of 600 to 650 r.p.m. Corn steep–starch media similar to the fermentation medium above were used.

Isolation of (+)-*trans*-2,3-Dihydro-3-hydroxyanthranilic Acid (DHAA).—Twenty liters of tank-fermented S-652 mash containing 131 g. of DHAA was filtered by suction. The filtrate was adjusted to *p*H 1.5 with concentrated hydrochloric acid. Thirteen liters of the acidified filtrate (containing 85 g. of DHAA) was passed through a 3-in. column containing 3.15 kg. of Amberlite IR-120 cation exchange resin in the hydrogen form. The flow rate used was 2 l./hr. The column was then washed with 20 l. of distilled water. Elution of DHAA was carried out in fractions with 26 l. of 1 *N* sulfuric acid. The DHAA content of the fractions was followed by ultraviolet absorption measurement at 278 mμ. Fifty-one grams (60%) of DHAA was found in the rich fractions. Excess barium hydroxide was added to the acid eluate to bring the *p*H up to about 12. Barium sulfate was removed by filtration. The filtrate was neutralized with 1 *N* sulfuric acid to *p*H 6.8, barium sulfate again being filtered off. At this point the filtrate was concentrated at 35° by vacuum distillation. Crystalline DHAA separated out as the concentrate volume reached approximately 100 ml. The solid was collected by filtration and washed with small portions of cold water. The product was dried for 8 hr. *in vacuo* (40°, 0.05 mm.). Twenty-five grams of material was obtained which assayed 67% pure DHAA by spectrophotometric analysis. Purification was accomplished by repeated crystallization from glacial acetic acid. The recrystallized DHAA (dried at 100° *in vacuo*) decomposed and melted at 190–191°. *Anal.* Calcd. for $C_7H_9NO_3$: C, 54.25; H, 5.80; N, 9.03; mol. wt., 155. Found: C, 53.78; H, 5.95; N, 8.82; neut. equiv., 156; pK_a (water), 8.6; $[\alpha]^{22}D$ (0.5% in water), +445°; ultraviolet absorption, λ_{max} (0.1 *N* hydrochloric acid) 278 mμ, ε 9350; infrared absorption: strong–NH_3^+ absorption at 2860–3000 (broad) and 2125 cm.$^{-1}$, and $-CO_2^-$ absorption at 1590 and 1390 cm.$^{-1}$.

Hydrogenation of DHAA to Hexahydro-3-hydroxyanthranilic Acid (II).—One and a half grams of DHAA (10 mmoles) was dissolved in 50 ml. of water to which was added 200 mg. of 10% palladium-on-carbon catalyst. The reduction was carried out in a modified Parr shaker at 25° and 40 lb. pressure. In 40 min. the reduction was complete with an uptake of 20 mmoles of hydrogen. The reduction mixture was filtered and the filtrate was freeze-dried to give a crystalline solid. The solid was recrystallized from water–acetone to give 1.10 g. (72%) of product II, as a white crystalline solid, soluble in water, insoluble in organic solvents, m.p. 270–276° (dec.). *Anal.* Calcd. for $C_7H_{13}NO_3$: C, 52.85; H, 8.18; N, 8.80. Found: C, 52.68; H, 8.43; N, 8.59; no ultraviolet absorption; positive ninhydrin reaction; $[\alpha]^{25}D$ (0.5% in water), −34.6°; no break in titration curve (water). In a pyridine–water 65:35 paper chromatographic system,[11] II showed only a single ninhydrin-positive spot, R_f 0.62.

N-Benzoylhexahydro-3-hydroxyanthranilic Acid.—Compound II was converted to the N-benzoyl derivative by reaction of the sodium salt in water with one equivalent of benzoyl chloride. The product was recrystallized from methanol–pentane; m.p. 244–245°.

Anal. Calcd. for $C_{14}H_{17}NO_4$: C, 63.85; H, 6.47; N, 5.32; mol. wt., 263. Found: C, 63.46; H, 6.59; N, 5.35; $[\alpha]^{24}D$ (0.5% in methanol), −12.2°; neut. equiv., 267; pK_a (50% ethanol), 6.04.

Acidic Degradation of DHAA to Anthranilic Acid (III).—The rate of conversion of DHAA to anthranilic acid was determined by dissolving 100 mg. of DHAA in 10 ml. of concentrated hydrochloric acid and holding the solution at 60°. At intervals, 0.1-ml. aliquots were removed, diluted to 100 ml. with 0.1 *N* hydrochloric acid, and the absorbancy determined at 230 and 280 mμ. From these results the composition of each aliquot was determined using the known extinction coefficients for DHAA and anthranilic acid at these wave lengths. The results are given in Table III.

(15) P. A. Miller, J. R. D. McCormick and A. P. Doerschuk, *Science*, **123**, 1030 (1956).

(16) Although DHAA does not appear to be involved as an intermediate in the biosynthetic pathway to the tetracyclines, its structure suggests a possible role as an intermediate between shikimic acid and anthranilic acid in the biosynthesis of the essential metabolite tryptophan (B. D. Davis, in "Biochemists' Handbook," Cyril Long, Ed., E. and F. N. Spon, Ltd., London, 1961, pp. 594–596.

(17) It has been suggested by A. Gourevitch and J. Lein (U. S. Patent 2,712,517, July 5, 1955) that shikimic acid might be a precursor in the biosynthesis of the tetracyclines. This possibility was also investigated independently in these laboratories (see ref. 15) with the conclusion that shikimic acid was not incorporated into CTC by *S. aureofaciens*. R. Robinson ("The Structural Relations of Natural Products," Oxford Press, London, 1955, p. 58) and R. B. Woodward [*Angew. Chem.*, **68**, 13 (1956)] have speculated on the probable biogenesis of oxytetracycline through a polyacetyl intermediate. Recent publications by J. F. Snell, A. J. Birch and P. L. Thomson [*J. Am. Chem. Soc.*, **82**, 2402 (1960)] and by S. Gatenbeck [*Biochem. and Biophys. Res. Comm.*, **6**, 422 (1961)], although differing in detail, have offered some experimental confirmation that a polyacetyl biogenesis for the tetracyclines probably is correct.

From a similar experiment in which the reaction solution was held at 60° for 6 hr. without sampling, there was isolated 70 mg. of crude anthranilic acid as the hydrochloride. This was converted to the N-acetyl derivative in the usual manner, m.p. 183–184° (lit.[18] m.p. 182–184°); mixed m.p. with authentic N-acetylanthranilic acid, 183–184°.

Alkaline Degradation of DHAA to *m*-Hydroxybenzoic Acid (IV).—A solution of 250 mg. of DHAA in 25 ml. of 25% sodium hydroxide was kept at 93° in a water-bath for 8 hr. At the end of this time, the reaction mixture was shown to contain 222 mg. (93%) of *m*-hydroxybenzoic acid by its characteristic ultraviolet absorption spectrum in 0.1 *N* sodium hydroxide; 200 mg. of a crystalline solid was recovered from an ether extract of the acidified reaction mixture by evaporation. A small portion of this solid was recrystallized from ether–chloroform yielding a white crystalline product, m.p. 200–201° (lit.[19] 200°). The identity of the product as *m*-hydroxybenzoic acid was further confirmed by the agreement of ultraviolet and infrared spectra with those of an authentic specimen.

Acetylation of DHAA to 2-Acetamido-3-acetoxy-2,3-dihydrobenzoic Acid.—To 110 mg. of DHAA was added 2 ml. of acetic anhydride and 30 mg. of potassium acetate. The mixture was placed on a shaker. At the end of 2 hr., solution was complete. Water was added and the solvent was removed by freeze-drying, leaving 135 mg. of product which was crystallized from methanol; m.p. 177–178°. *Anal.* Calcd. for $C_{11}H_{13}NO_5$: C, 55.25; H, 5.45; N, 5.86; acetyl, 36.0; mol. wt., 239. Found: C, 55.15; H, 5.54; N, 6.11; acetyl, 35.5; neut. equiv., 229; pK_a (50% ethanol), 5.65; λ_{max}; (0.1 *N* hydrochloric acid) 280 mμ, ε 9260. Infrared spectrum confirmed the presence of a secondary amide (1650 and 1540 cm.$^{-1}$), ester (1730 and 1220 cm.$^{-1}$) and carboxyl (1715 and 1250 cm.$^{-1}$) groupings.

Catalytic Disproportionation of DHAA.—To a solution of 1.0 g. of DHAA in 100 ml. of water was added 1 g. of 10% palladium on carbon catalyst. The mixture was refluxed for 2 hr. At the end of this time, the reaction mixture was shown to contain 524 mg. of 3-hydroxyanthranilic acid (V) by its characteristic ultraviolet spectrum in 0.1 hydrochloric acid. The presence of hexahydro-3-hydroxyanthranilic acid (II) in the reaction mixture was detected by the appearance of a chromatographic spot with an identical R_f value and ninhydrin-color response to that of authentic II in the pyridine–water system. Crude crystalline 3-hydroxyanthranilic acid (V) was isolated from the reaction mixture, after removal of the catalyst, by concentrating and cooling. In this manner, a total of 485 mg. of crystalline solid was obtained. This crude solid was decolorized with Darco G-60 activated carbon in hot ethanol solution and recrystallized from methanol–water. The 3-hydroxyanthranilic acid (225 mg.) melted at 255–256° (lit.[20] m.p. 254–255°). The remaining portion of the crude solid was converted to the crystalline hydrochloride by dissolving in hot concentrated hydrochloric acid and cooling. The 3-hydroxyanthranilic acid hydrochloride was dried *in vacuo*, m.p. 227–228° (lit.[20] m.p. 227°).

Periodate Oxidation of II. Δ^1-Cyclopentenal Semicarbazone.—To a solution of 65 mg. of II (0.4 mmole) in 10 ml. of 0.01 *N* sodium hydroxide was added 85 mg. of sodium periodate (0.4 mmole). The reaction solution was allowed to stand at room temperature for 10 min. A saturated solution of barium chloride was added dropwise until no further precipitation of barium iodate occurred. The precipitate was removed by filtration. A solution of 100 mg. of semicarbazide hydrochloride and 150 mg. of sodium acetate in 1 ml. of water was added to the filtrate. The mixture was heated in a water-bath at 70° for 10 min. Crystallization occurred on cooling. The product was recovered by filtration, recrystallized from hot methanol, and shown to be Δ^1-cyclopentenal semicarbazone by the melting point, 208–209°, and by the ultraviolet absorption, λ_{max} (ethanol) 265 mμ, ε 31,200 (Heilbron, *et al.*,[14] reported m.p. 212°, λ_{max} [ethanol] 267 mμ, ε 30,500).

Preparation of C^{14}-Labeled DHAA.—An *S. aureofaciens* S-652 fermentation was begun as described under Fermentation Procedures, except that the medium was modified to contain 90% of the normal amount of starch and none of the cottonseed meal, and the entire medium (except the lard oil) was diluted 4 volumes to 11 volumes with water. After 48 hr. incubation, 1 ml. of the shaker-flask mash was transferred aseptically to a test-tube containing 2.00 mg. of sterile, C^{14}-labeled tobacco starch of specific activity of 10.3 μC./mg. The fermentation was continued for an additional 72 hr. on a rotary shaker.

The neutral filtrate prepared from this fermented mash contained 15.7×10^6 c.p.m., or about 75% of the radioactivity added. (Radioactivity assays were carried out by plating a small aliquot of the solution onto a stainless steel planchet, drying and counting in a windowless gas-flow counter.) The radioactive neutral mash filtrate was applied as a streak to the full width (4 in.) of a paper chromatogram (4 in. × 17 in.) which was developed in a descending solvent system[11] of butanol (40)–acetic acid (15)–water (50). A broad band (R_f 0.44–0.52) corresponding to DHAA was detected by ultraviolet absorption, marked, cut out, and eluted with water. The eluate was found to contain 15.7×10^5 c.p.m., or 10% of the radioactivity present in the neutral mash filtrate. Paper chromatographic examination of a small portion of the eluate showed that it contained only one radioactive component, that component having the R_f of DHAA.

Non-incorporation of DHAA into CTC.—Eluate containing the C^{14}-labeled DHAA (14.9×10^5 c.p.m.) was evaporated to dryness under nitrogen in a test-tube. One ml. of a 48-hr. shaker-flask mash of *S. aureofaciens* BC-41, prepared as described under Fermentation Procedures, was added to the test-tube containing radioactive DHAA and the fermentation was continued for an additional 72 hr. A neutral filtrate was prepared from the BC-41 mash and found to contain 10.7×10^5 c.p.m., or 72% of the DHAA radioactivity added. This filtrate would be expected to contain any unaltered DHAA, but paper-chromatographic examination in the butanol–acetic acid system showed that the major part of the radioactivity was associated with two other components (R_f 0.2 and 0.3), neither being in the expected location for DHAA (R_f 0.44). These two components were not examined further. An acid extract of the neutral mash insolubles, in which the biosynthetic CTC would be found, contained 8.2×10^4 c.p.m., or 5.5% of the DHAA activity added. The CTC present in this acid extract was resolved by paper chromatography in the butanol–acetic acid system as well as in a butanol–*p*H 3 phosphate buffer system.[9] Radioactivity on the strips was measured by means of a scanning device. The recorded radioactivity scans showed no peak in the region corresponding to the CTC spot on each paper strip. The conditions used were such that as little as 1% of the total radioactivity added as DHAA, if incorporated into CTC, would have been detected in the paper chromatograms.

(18) M. T. Bogert and A. H. Gotthelf, *J. Am. Chem. Soc.*, **22**, 522 (1900).

(19) L. Barth, *Ann. Chem., Liebigs*, **154**, 356 (1870).

(20) J. F. Nyc and H. K. Mitchell, *J. Am. Chem. Soc.*, **70**, 1847 (1948).