oxalacetate. This compound may yield α-ketoglutarate, by way of the tricarboxylic acid cycle, which is then converted to the pyrrolidine ring with glutamic acid as an intermediate. In the 2-hour feeding experiment all of the C^{14} in the pyrrolidine ring was in the 2 and 5 positions, as would be expected if the reactions described had occurred.

This result shows that little or no randomization of the C^{14} occurred during synthesis of the pyrrolidine ring in a 2-hour period. Thus during such a period, synthesis of the pyridine ring also should occur with limited randomization of the isotope. Therefore, if aspartic acid were converted directly to the pyridine ring, more than half of the C^{14} would be expected to reside in position 3. However, in the experiment in which labeled aspartic acid was fed for 2 hours, carbon 3 of the pyridine ring again contained about one-half the C^{14}. If aspartic acid were converted to either fumarate or succinate before incorporation into the pyridine ring the observed labeling could result.

Succinate has been shown to be a precursor of the pyridone ring of ricinine in castor plants (Waller and Henderson, 1961a) and of the pyridine ring of nicotinic acid formed by *E. coli* (Ortega and Brown, 1959).

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Hadacidin, a New Growth-Inhibitory Substance in Human Tumor Systems

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The fermentation broth of *Penicillium frequentans* Westling was found to inhibit the growth of the human adenocarcinoma-1 in the embryonated egg. The active substance in the fermentation broth, which was responsible for the inhibition of tumor growth, was designated hadacidin. It was isolated as a crystalline monosodium salt having the composition $C_3H_4NO_4Na$. The free dibasic acid was converted to a monomethylester. Hadacidin was degraded to formic acid and hydroxyaminoacetic acid. The latter was converted to glycine by hydrogenation, and was identical with a synthetic specimen of hydroxyaminoacetic acid. *N*-Formylation of synthetic hydroxyaminoacetic acid yielded hadacidin, *N*-formylhydroxyaminoacetic acid; the products were compared as monosodium salts. Hadacidin and 6-mercaptopurine show a similar degree of activity against the human adenocarcinoma-1 in the embryonated egg.

Transplantation of human tumors into the conditioned laboratory animal has become a useful laboratory tool, according to Toolan (1958). It is noteworthy that these neoplasms remain human in both their chromosomal (Levan, 1956) and antigenic (Korngold and Lipari, 1955) composition, even though some have been transplanted for several years.

After such human tumors have grown in the conditioned animal, they can be passed serially in the embryonated egg (Dagg *et al.*, 1955; Harris, 1958). The implanted and embryonated egg offers a biological system which serves as a primary screen for new antitumor agents. At this stage, the embryonated egg does not require conditioning by x-irradiation or cortisone.

The use of human tumors offers a new methodology for seeking and evaluating broth filtrates

from microorganisms and products isolated from active broths for antitumor activity. It has seemed (Woolley, 1958) that the use of human tumors enhances the likelihood of finding useful therapeutic agents for neoplasms of man, and further work is of interest.

The growth of the human adenocarcinoma-1 (HAd-1) in the embryonated egg was selected for our study because this tumor represents cancer in the gastrointestinal tract, which is frequently the site of cancer of high mortality. Further, it has been shown (Harris, 1958) that this tumor-egg system has certain reproducibility and can detect antitumor agents.

The results of the initial phase of screening fermentation broths for antitumor activity have been described separately (Gitterman *et al.*, 1962). One of the broths was selected, because of its antitumor activity, for isolation of the active substance, with the tumor-egg system used as an assay. Since the substance is new, it was given the trivial name hadacidin. Its structure was determined, and a synthesis confirmed the structural interpretation.

Results

The lyophilized filtered fermentation broth was extracted with methanol. An aqueous solution of the methanol-soluble material was treated with ethanol, and the sodium salt was crystallized. The crystalline sodium salt exists in the form of a hydrate as well as an anhydrate; each is characterized by its infrared absorption spectrum. The crystalline monosodium salt is of a dibasic acid and was shown to have the empirical formula $C_3H_4NO_4Na$.

Potentiometric titration with 0.1 N hydrochloric acid gave a $pH_{1/2}$ value[1] of 8.9 and an equivalent weight of 138. Hadacidin gives a positive ferric chloride test and an atypical ninhydrin reaction. It is optically inactive, and its ultraviolet absorption spectrum shows only end-absorption.

The monosodium salt was converted to the acid with the cation exchange resin Amberlite IR 120 on the hydrogen cycle. The crystalline acid melts at 119–120° and is unstable in the solid form and in aqueous solution. An aqueous solution of the acid, at room temperature, is slowly hydrolyzed to hydroxyaminoacetic acid and formic acid.

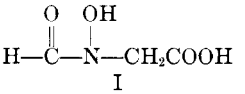
The crystalline form of the acid and ester are also unstable, and after a period of several weeks there is a gradual color change to brown and the solids liquefy.

Potentiometric titration showed two acid groups, $pH^1_{1/2}$ 3.5 and $pH^2_{1/2}$ 9.1; the equivalent weights were estimated as 123 and 111. A disodium salt of hadacidin was obtained by treating an aqueous solution of the monosodium salt or acid with dilute sodium hydroxide; crystallization took place after the addition of ethanol to the aqueous solutions.

Hadacidin gives a monomethyl ester, m.p. 71–72°, with diazomethane in ether solution.

[1] A $pH_{1/2}$ value is defined as the pH at the half neutralization point or midpoint of the titration curve.

Hadacidin was shown to be *N*-formyl hydroxyaminoacetic acid, I, by degradation to formic acid and hydroxyaminoacetic acid.



I

When hadacidin was hydrolyzed with sulfuric acid and the mixture distilled, formic acid was in the distillate. It was isolated as the *p*-bromophenacyl formate. Formic acid also was identified by titration of the distillate of a hydrolysate of hadacidin.

Hydroxyaminoacetic acid was isolated from the aqueous hydrolysate of hadacidin. It was identified by its composition, by its instantaneous reaction with Fehling's solution, and by catalytic reduction to glycine.

Hydroxyaminoacetic acid was prepared from ethylacetoacetate and nitric oxide according to the procedure of Traube (1895). The compositions, melting points, and infrared spectra of hydroxyaminoacetic acid from hadacidin and from synthesis are identical.

Hadacidin was synthesized by formylating hydroxyaminoacetic acid with a mixture of formic acid and acetic anhydride. The product was isolated as the crystalline monosodium salt. The physical, chemical, and biological properties of the isolated and synthetic products are identical.

Table I illustrates some typical results (Gitterman *et al.*, 1962) of tumor growth inhibition by hadacidin and by certain antitumor agents used as standards.

Table I

Growth Retardation of HAd 1 in the Embryonated Egg by Hadacidin and Control Compounds

Compound	Dose (mg/egg)	Embryo Mortality	Growth Retardation (%) Embryo	Tumor
Hadacidin Monosodium salt	6.0	3/24[a]	3.5(0–7)[b]	61(56–65)[b]
Methyl ester	7.5	0/6	14	57
Triethylene melamine	0.025	2/48[c]	10(0–23)[b]	74(48–100)[b]
Azaserine	1.0	2/9	20	62
6-Mercaptopurine	8.0	3/9	6	63

[a] Results represent two different experiments. [b] Numbers in brackets refer to range of values observed. [c] Result represents eight different experiments, six eggs per experiment.

The procedure consists of implanting pieces of tumor tissue on the chlorioallantoic membrane of 9-day-old embryonated eggs and incubating the eggs for 3-4 days. Then, the test agent is injected into the yolk sac of some of those eggs showing a positive "take," the rest being used as controls. After incubation for an additional 7 days, the tumors of the treated and control eggs are removed and weighed. The mean weight of the tumors at the time of injection is subtracted from these

weights; per cent inhibition = 100 − (T/C × 100).

Discussion

In addition to the data of Table I on activity, it has been found (Gitterman *et al.*, 1962) that hadacidin inhibits the growth of Toolan's human epidermoid carcinoma (HEp 3), and the activity data on Skiff's bronchogenic carcinoma (A-42) secured by Harris *et al.* (1962) have been confirmed.

Harris *et al.* confirmed the activity data of hadacidin on HAd 1 and HEp 3 as reported by Gitterman *et al.*, and they extended these studies to include HS 1 and A-42. The latter tumor was found to be the most sensitive to hadacidin.

Harris *et al.* (1962) have demonstrated the inhibitory activity of hadacidin to human tumors in the conditioned rat.

Shigeura and Gordon (1962) have studied the mode of inhibitory action of hadacidin and have reported that the *de novo* biosynthesis of adenylic acid is markedly and specifically inhibited by hadacidin.

It is evident that a human tumor such as HAd 1 in the embryonated egg can be useful for direct screening of fermentation broths for activity and as an assay in the chemical fractionation of broths for isolation of active substances.

It is significant that hadacidin, discovered by this primary screen, exhibits activity against the human tumor in the conditioned animal, such as the rat. The conditioned animal constitutes the secondary and a more advanced biological appraisal.

The discovery of the activity of hadacidin exemplifies the stepwise use of the human tumor-egg system and the human tumor in the conditioned host for the search for new antitumor agents. The retention of the chromosomal and antigenic compositions (Korngold and Lipari, 1955; Leven, 1956; Toolan, 1958) of these human tumors is basic to this method.

Hadacidin is not a potent antitumor agent, but is comparable with 6-mercaptopurine against HAd 1.

Experimental

The Isolation of Hadacidin as a Monosodium Salt.—Three liters of the filtered fermentation broth of *P. frequentans* Westling was lyophilized to yield 245 g of dry solid material. This material was triturated with one 1000-ml and two 700-ml portions of methanol. The combined methanol extract was filtered and evaporated *in vacuo*.

The residue was dissolved in 200 ml of water, and *ca.* 500 ml of ethanol was added until there was no further crystallization. The yield of the ethanol-washed and air-dried salt was 20.8 g. Repeated recrystallization from water by the addition of ethanol gave the pure salt, m.p. 205–210° (decomp.) (microblock).

Anal. Calcd. for $C_3H_4NO_4Na$: C, 25.54; H, 2.86; N, 9.92; Na, 16.3. Found: C, 25.50; H, 2.84; N, 9.91; Na, 16.7.

Potentiometric titration with 0.1 N hydrochloric acid gave $pH_{1/2}$ *ca.* 8.9; equivalent weight 138.

The monosodium salt exists in a hydrous as well as an anhydrous form. The infrared spectra of these two forms in mineral oil suspensions obtained on a Perkin-Elmer Infrared Spectrophotometer Model 137 (Infracord) showed the bands (expressed in μ): 2.8–4.2 (broad), 5.9, 6.3, 6.6, 7.0, 7.6, 8.3, 8.4, 9.8, 10.1, 11.2, and 13.0 (anhydrous form) and 2.8–2.9, 3.7–4.2 (broad) 6.0, 6.3, 6.6, 7.5, 8.2, 10.1, 11.2, and 12.8 (hydrate).

An aqueous solution of the salt exhibits only end-absorption in the ultraviolet spectrum, and is optically inactive.

Conversion of the Monosodium Salt of Hadacidin to the Free Acid.—One gram of the monosodium salt of hadacidin was dissolved in 10 ml of water, and the solution was passed through a column containing 27 ml of the cation exchange resin Amberlite IR 120 on the hydrogen cycle. The combined effluent and water wash of the column (20 ml) was lyophilized, and the resulting residue was triturated with acetone. The acetone solution was evaporated to dryness *in vacuo* to yield 625 mg of residue, which was triturated with ethanol; the ethanol solution was evaporated to dryness. The crystalline residue was purified by repeated recrystallization from acetone by the addition of petroleum ether (30–60°). The crystalline acid melted at 119–120° (microblock). It is soluble in water, methanol, ethanol, acetone, and ether.

Anal. Calcd. for $C_3H_5NO_4$: C, 30.26; H, 4.23; N, 11.76. Found: C, 30.88; H, 4.48; N, 12.21.

Potentiometric titration of an aqueous solution of the acid showed two acidic groups, $pH_{1/2}$ 3.5; eq. wt. 123 and $pH_{1/2}$ 9.1; eq. wt. 111.

Disodium Salt of Hadacidin.—Twelve and one-half milligrams of the acid was dissolved in 0.5 ml of dilute sodium hydroxide. This solution was diluted with ethanol until the crystallization of the disodium salt was complete. The salt was recrystallized from water by the addition of ethanol.

Anal. Calcd. for $C_3H_3NO_4Na_2$: C, 22.10; H, 1.85. Found: C, 21.3; H, 2.3.

The disodium salt was also prepared from the monosodium salt in the same manner. The infrared spectra of the products were identical.

Conversion of the Disodium Salt of Hadacidin to the Monosodium Salt.—One hundred sixty milligrams of the disodium salt of hadacidin was dissolved in 2 ml of water. To this solution was added *ca.* 0.3 ml of glacial acetic acid and then ethanol to a total volume of 8 ml. A small amount of crystalline material which formed was separated by centrifuging, and the solution was diluted with 5 ml of ethanol to give 110 mg of crystalline monosodium salt. The infrared spectrum was identical with that of the salt isolated from the fermentation broth.

Methyl Ester of Hadacidin.—To an ether solution of 2.5 g of hadacidin, an ether solution of 1 g of

diazomethane was added dropwise with constant stirring. When the addition was completed, the solution was evaporated to dryness *in vacuo*. The residue was dissolved in acetone and the solution was diluted with petroleum ether (30–60°). After a short time, the ester began to crystallize. The crystalline ester melted at 71–72° (microblock).

Anal. Calcd. for $C_4H_7NO_4$: C, 36.09; H, 5.30; N, 10.52. Found: C, 36.18; H, 5.36; N, 10.74.

Hydrolysis of N-Formyl-Hydroxyaminoacetic Acid (Hadacidin) to Hydroxyaminoacetic Acid.—One and three-tenths grams of the monosodium salt of hadacidin was dissolved in 15 ml of water, and the solution was passed through a column containing *ca.* 30 ml of the cation-exchange resin Amberlite IR 120 on the hydrogen cycle. The combined effluent and water wash of the column (40 ml) was allowed to remain at *ca.* 25° for 5 days. The solution was then evaporated to dryness *in vacuo*, and the residue was washed with 10 ml of water. The remaining 280 mg of solid was dissolved in 10 ml of hot water and, after the solution was cooled, 215 mg of crystalline hydroxyaminoacetic acid, m.p. 145–150° (decomp.) (microblock), was obtained.

Anal. Calcd. for $C_2H_5NO_3$: C, 26.37; H, 5.53; N, 15.38. Found: C, 26.38; H, 5.00; N, 15.40.

Hydroxyaminoacetic acid was synthesized according to the method of Traube (1895) from ethyl acetoacetate and nitric oxide.

The infrared spectra of the synthetic product and the hydroxyaminoacetic acid from hadacidin were identical.

Hydrogenolysis of the Hydroxyaminoacetic Acid Obtained from Hadacidin.—Sixty-three milligrams of hydroxyaminoacetic acid, obtained from degradation of hadacidin, was dissolved in 25 ml of water; the hydrogenolysis was conducted for 2 hours with a platinum catalyst used under slightly more than atmospheric pressure. During this time, 98% of one equivalent of hydrogen was consumed. The solution was separated from the catalyst and evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml of water and, after the addition of ethanol to the solution, glycine crystallized from the solution; yield 35 mg. This glycine was identified by its melting point behavior (245° decomp., microblock), infrared absorption spectrum, and the analytical data.

Anal. Calcd. for $C_2H_5NO_2$: C, 32.00, H, 6.71; N, 18.67; Found: C, 32.46; H, 7.02; N, 18.25.

Hydrolysis of N-Formyl-Hydroxyaminoacetic Acid (Hadacidin) to Formic Acid.—One gram of the monosodium salt of hadacidin was dissolved in 7 ml of water, and 1 ml of concentrated sulfuric acid was added. Five milliliters of the volume of this solution was distilled into a dilute sodium hydroxide solution. The alkaline solution was acidified with hydrochloric acid and then refluxed with an alcoholic solution of *p*-bromophenacyl bromide for 1 hour. After the solution was cooled, the *p*-bromophenacyl ester of formic acid crystallized. The ester was separated, dried, and then recrystallized from ethanol. The crystalline product melted at 137–139°. A sample of authentic *p*-bromophenacyl formate melted at 137–139°. There was no change in the melting point of a mixture of the two samples.

The hydrolysis product was further identified as formic acid by hydrolysis of hadacidin with sulfuric acid and potentiometric titration of the distillate. The *pKa* found, 4.15, is in agreement with the *pKa*, 4.17, found by the titration of a dilute solution of formic acid.

Synthesis of Hadacidin.—Three hundred and fifty-five milligrams of synthetic hydroxyaminoacetic acid was dissolved in a mixture of 5 ml of 98–100% formic acid and 1 ml of acetic anhydride. The solution immediately gave a positive ferric chloride test and became slightly warm. After 15 minutes, the solution was heated at 50–55° for 10 minutes, and then evaporated *in vacuo*. The residue, which contained a small amount of acetic acid, was dissolved in 2 ml of water, and dilute sodium hydroxide was added until the *p*H was 6–7. This solution (10 ml) was concentrated *in vacuo* to a volume of 3 ml, and 27 ml of ethanol was added. The salt which crystallized was separated, washed with ethanol, and dried; it was recrystallized from 4 ml of water by the addition of 16 ml of ethanol. The yield was 357 mg.

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