Production and Isolation of Thermoviridin, an Antibiotic Produced by *Thermoactinomyces viridis* n.sp.[1]

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To our knowledge the only report of an antibiotic produced by a thermophilic actinomycete was published by Schöne (1951). The antibiotic was produced by *Streptomyces thermophilus*, and was named thermomycin. Schöne obtained an antibiotic titer of 256 units per ml in 4 days at 60 C in stationary bottles using a buffered medium which contained 10 per cent blood serum. Extraction of this culture filtrate with ether, followed by evaporation yielded a yellow powder which inhibited *Corynebacterium diphtheriae* at a concentration of 12 μg per ml.

Thermomycin was found to be most active against *C. diphtheriae* and against *Listeria monocytogenes* to a much lesser extent. All other organisms tested were insensitive to thermomycin. Injection of 2 ml of culture filtrate (256 u per ml) into each of 20 mice did not cause a toxic reaction.

The present paper deals with the production, recovery, purification, and microbial spectrum of thermoviridin. The organism which produces this antibiotic is considered to be a new species in the genus *Thermoactinomyces*. The name *Thermoactinomyces viridis* is suggested for the organism.

MATERIALS AND METHODS

In the course of this work, antibiotic activity was measured by the serial dilution, paper disc-agar plate, and agar tube dilution methods. The thermoviridin unit was based on the serial dilution test, and was defined as the minimum amount of thermoviridin in 1 ml which inhibited the growth of a 1 per cent inoculum of *Micrococcus pyogenes* var. *aureus* 209P in broth for 24 hours at 37 C. The medium used for the serial dilution assay contained peptone, 0.6 per cent; trypticase, 0.4 per cent; beef extract, 0.3 per cent; glucose, 0.1 per cent; sodium chloride, 0.5 per cent; and had a pH of 7.0.

Fermentation studies were carried out on a Gump rotary shaker in wide-mouth 500-ml Erlenmeyer flasks. The shaker operated at about 250 rpm and each flask rotated through a two-and-one-quarter-inch diameter circle. The flasks contained 50 ml of medium.

Inoculum for the fermentations was prepared by transferring the growth from a 4- to 10-day-old agar slant culture of the organism into 100 ml of medium in a one-liter Erlenmeyer flask. The flask was shaken for 2 days at 45 C. A second flask inoculated with 2 ml of vegetative growth from the first flask culture was shaken approximately 18 hours. All fermentation flasks received 1 ml of this secondary culture per 50 ml of medium. The medium used for inoculum preparation contained tryptone, 2 per cent; beef extract, 0.5 per cent; and had a pH of 6.9 to 7.1.

Nutrients tested in the development of the fermentation medium were added to a basal medium at levels of 0.5, 1, 2, and 4 per cent. The basal medium contained tryptone, 4 per cent; beef extract, 0.5 per cent; and had a pH of 6.9 to 7.1. Those nutrients which appeared to enhance thermoviridin production were combined and added to the basal medium. The balance of nutrients was then varied.

Thermoviridin was also produced in a 30-liter laboratory fermentor previously described by Olson et al. (1954). The fermentor contained 12 liters of fermentation medium (table 2) and 150 ml of antifoam. The antifoam consisted of mineral oil with 5 per cent octadecanol by weight. The inoculum was 100 ml of a secondary culture obtained by the same procedure as for the preparation of inoculum for shake flask fermentations.

During the fermentation the temperature was maintained at 45 C, the air flow 0.6 volume of air per volume of medium per minute, and the impeller speed 530 rpm.

Recovery of thermoviridin was accomplished by precipitation at pH 3 with phosphoric acid followed by a 2-hour holding period at 0 to 5 C. The precipitate was washed with water and extracted with one-tenth volume of 80 per cent acetone, based on the volume of the culture filtrate. The recovery process is outlined in figure 1.

Two methods were found to be of value in the purification of crude thermoviridin. The first method consisted of fractional precipitation of the active material from a methanol solution using ethyl ether as the precipitating agent.

The second method was the countercurrent distribution method of Craig (1944). This procedure was car-

Whole crude culture

Mycelium (discard) — Culture filtrate

Adjust to pH₃ → Precipitate — Filtrate (discard)

1. H₂O wash (discard)
2. Extract with 80 per cent acetone

Residue (discard) — Acetone extract

Remove acetone and dry from frozen state
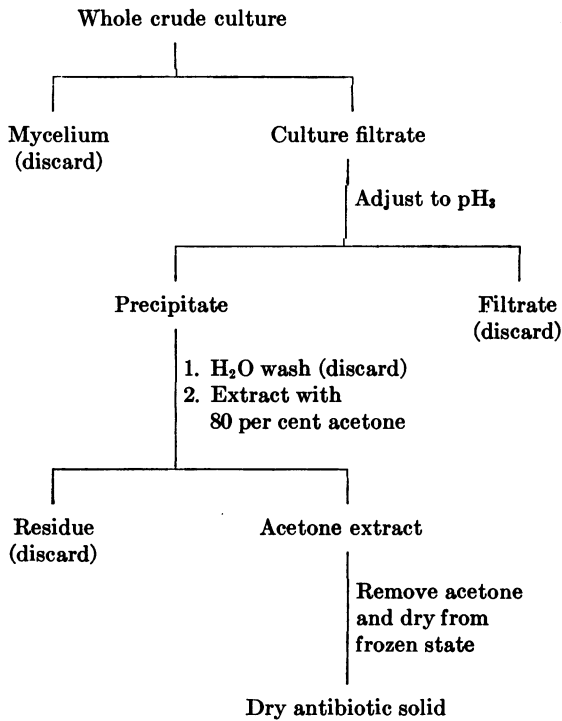
Dry antibiotic solid

FIG. 1. Procedure for the recovery of thermoviridin

ried out in a 30 cell, HO Post, all glass, countercurrent distribution instrument, model 1. This instrument uses 10 ml of each solvent.

RESULTS

Cultural Characteristics

Thermoactinomyces viridis, isolated from a composted manure pile, was found to have an optimum growth temperature of approximately 55 C. At this temperature, growth on agar was fully developed in 3 days. The diameter of the hyphae was approximately 0.5 μ, and oval conidia (about 1.0 x 1.3 μ) were borne singly on short sporophores. *T. viridis* was found to be non-acid fast, and gram negative. The latter reaction is unusual since most actinomycetes are gram positive. However, Lieske (1921) found that certain thermophilic forms were gram negative.

The growth characteristics and biochemical reactions of *T. viridis* on various organic media are presented in table 1. The organism demonstrated amylolytic and proteolytic properties but did not digest cellulose or reduce nitrates.

The glucose-peptone A and B media, glucose-asparagine agar, and starch agar A were prepared according to formulae given by Waksman (1950). The medium for testing cellulose digestion consisted of filter paper strips suspended in a solution of the salts found in Czapek-Dox agar. The nutrient agar referred to in table 1 contained peptone, 1 per cent; beef extract, 0.5 per cent; and had a pH of 6.8 to 7.0.

The organism in many ways resembles *Thermoac-*

TABLE 1. *Cultural characteristics and biochemical reactions of Thermoactinomyces viridis on various organic media*

Medium	Description of Growth or Biochemical Reaction after 14 Days' incubation at 45 C
Nutrient agar	Mycelial growth wrinkled and close to agar surface; blue-green aerial mycelium; emerald-green water-soluble pigment present
Nutrient broth	Flocculent cream-colored submerged growth; surface growth blue-green; green pigment present
Glucose - peptone A agar	Colorless colonies 1–2 mm in diameter; no aerial mycelium
Glucose - peptone A broth	Flocculent cream-colored submerged growth
Glucose - peptone B agar	Wrinkled colorless growth; no aeria. mycelium
Glucose - peptone B broth	Flocculent cream-colored submerged growth
Czapek-Dox agar	No growth
Sabouraud agar	No growth
Glucose - asparagine agar	No growth
Corn meal agar	Flat colorless colonies 1–2 mm in diameter
Calcium malate agar	Colorless colonies 1–2 mm in diameter; few colonies with blue-green aerial mycelium
Potato plug	No growth
Carrot plug	No growth
Blood agar plate	Few large folded colonies; many small flat colonies; no aerial mycelium; hemolysis present
Skim milk	Coagulation followed by digestion
Nitrate broth	Not reduced to nitrites
Cellulose	No digestion
Nutrient gelatin	Liquefaction
Starch agar A	Hydrolysis
Temperature range	Minimum, 37 C; optimum, 55 C; maximum, 60 C
Habitat	Composted manure pile

tinomyces monospora, described by Waksman and Lechevalier (1953). The points of difference, however, are considered significant. *T. monospora* possesses hyphae with a diameter of about 1 μ, has an optimum growth range of 37 to 55 C, and does not coagulate milk.

The hyphae of the organism reported here were approximately 0.5 μ in diameter, the optimum growth temperature was about 55 C, and milk was coagulated and digested. In addition, a green water-soluble pigment was present.

Waksman and Corke (1953) have stated that thermophilic organisms which bear spores singly and produce an aerial mycelium should be classified under the genus *Thermoactinomyces*. For the above reasons, the organism reported here is considered to be a new species for

which the name *Thermoactinomyces viridis* is suggested. The name thermoviridin was chosen for the antibiotic produced by this organism.

Fermentation

The fermentation medium used in the production of thermoviridin is presented in table 2. In this medium, an antibiotic level of 32 units per ml was obtained in shake flask cultures. The addition of buffer to the medium did not increase thermoviridin production. Growth obtained in a 30-liter laboratory fermentor with this medium was good although not so heavy as that in shake flask cultures. The antibiotic titer obtained by this method was only 4 units per ml.

An attempt was made to increase antibiotic potency by supplementing the tryptone-beef extract medium. A list of the nutrients tested is given in table 3. Several nutrients caused a slight increase in antibiotic production, but the increase was not considered significant. Combining these enhancing nutrients in the basal medium did not increase thermoviridin production. The amino acids, tryptophane, and tyrosine were tested because they are stated to be present in substantial amounts in tryptone.

The fermentation medium was also supplemented with several mineral salts. The following metallic ions were added: Ca^{++}, Mg^{++}, K^+, Na^+, Zn^{++}, Fe^{++}, Cu^{++}, Mn^{++}, and Co^{++}. Thermoviridin production was not affected by the presence of these metallic ions at the levels tested.

Antibiotic production at 37 and 45 C has been compared. The relationship of pH changes and thermoviridin production during fermentation at 37 and 45 C may be seen in figure 2. It will be noted that maximum thermoviridin production was reached in 27 hours at 45 C, compared to 48 hours at 37 C. The pH of the fermentation during the period of maximum thermoviridin production was approximately 8.6.

Recovery

The process for recovery of thermoviridin is presented in figure 1. A precipitate formed when the culture filtrate was adjusted to pH 3. There was no apparent loss of antibiotic activity when the acid precipitate was washed with water. The washed precipitate was extracted with 80 per cent acetone. By this process, approximately 80 per cent of the antibiotic activity in the culture filtrate was recovered in the acetone extract. After evaporation of the acetone, the remaining water fraction was dried from the frozen state yielding a solid which possessed up to 64 thermoviridin units per mg.

Purification

The solid material obtained by the recovery procedure was purified by fractional precipitation or by countercurrent distribution. The degree of purification ob-

TABLE 2. *Tryptone-beef extract fermentation medium*

Constituent	Concentration
	g/L
Bacto tryptone	40
Bacto beef extract	5

pH 6.9–7.1.

TABLE 3. *Nutrients tested in the tryptone-beef extract medium for stimulation of antibiotic production*

Glycerol
Glucose
Sucrose
Maltose
Lactose
Corn starch
Corn meal
Corn steep liquor
Whole wheat flour
Wheat germ
L-tryptophane
DL-tyrosine
Casamino acids
Peptone*
Tryptose*
Soy peptone*
Soy bean meal
Milk nutrient GG
NZ amine B*
Brewer's yeast
Yeast extract

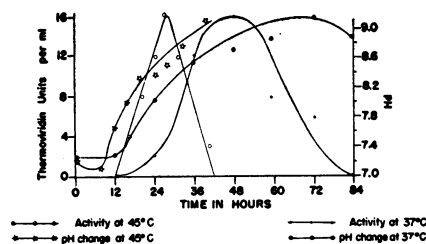* Nutrients showing some stimulation of antibiotic production.

FIG. 2. The relationship of pH changes and thermoviridin production during fermentation at 37 C and 45 C.

tained by the fractional precipitation method may be seen in table 4.

With starting material having 20 units antibiotic activity per mg, a fourfold increase in potency was obtained in the two volume precipitate. The total units recovered in all fractions represented 65.6 per cent of the antibiotic activity in the starting material.

The formation of a precipitate on the addition of one-fourth and one-half volumes of ethyl ether depended upon the potency of the solid material being purified. When the starting material was crude, for example, 5 to 10 units per mg, the one-fourth and one-half volume precipitates were heavy. When the starting material was of higher potency, the precipitate in these fractions was light, or more often absent.

TABLE 4. *Purification of thermoviridin by fractional precipitation*

Fraction	Mg in Fraction	Units per Mg	Units in Fraction	Activity Recovered in Fraction
				%
Starting material*	192.0	20	3840	
Residue	89.9	0	0	0
Precipitated from ½ vol ether	Noppt.			
Precipitated from 1 vol ether	21.8	30	654	17.0
Precipitated from 2 vol ether	16.4	80	1312	34.1
Precipitated from 4 vol ether	18.6	30	558	14.5
Precipitated from 7 vol ether	13.2	0	0	0
Total Recovery	159.9		2524	65.6

* Sufficient methanol added to give an antibiotic concentration of 150 units per ml.

Countercurrent distribution of thermoviridin was carried out in a 30-cell apparatus. The solvent system was butanol saturated with 0.4 M sodium phosphate pH 6.05 buffer and the same buffer saturated with the butanol. Three hundred mg of crude thermoviridin of 8 u per mg was dissolved in 10 ml of buffer for the first cell and the pH adjusted to pH 6.05. The activity was found to be distributed between the water and butanol only in tubes 16, 17, 18, 19 and 20. The butanol fraction of tube 18 was evaporated to dryness and yielded thermoviridin with 360 u per mg.

The single peak of antibiotic activity obtained in the 30 transfer solvent distribution system indicates that thermoviridin is a single entity. Additional support of this statement was obtained by paper chromatography of crude thermoviridin. A solvent system of butanol saturated with 20 per cent sodium phosphate at pH 5.85 was used at 30 C. Ten units of crude thermoviridin (8 u per mg) was added to dry Whatman #1 paper strips saturated with 20 per cent sodium phosphate at pH 5.85. The origin was 12 inches from the bottom of the strip and the strips were developed in a descending manner. In this system, thermoviridin moves as a single component and has an Rf value of 0.1. The location of antibiotic activity was made on two sets of plates, one seeded with *M. pyogenes* var. *aureus* 209P and the other with *Bacillus subtilis* ATCC 6633.

Biological and Chemical Properties

Stability studies with antibiotic beer indicated that thermoviridin was stable for 21 hours in a pH range of 2 to 8 at 37 C. At pH 10, 50 per cent of the activity was lost at 8 hours. The stability of thermoviridin to temperatures higher than 37 C has not been determined.

Several qualitative biochemical tests have been performed using a water solution containing 1 mg per ml of material which had 30 units antibiotic activity per mg. The biuret and ninhydrin tests were negative indicating the absence of proteins and peptides. The xantho-

proteic test was negative suggesting that no phenyl groups were present in the material. The Molisch test for carbohydrates and the lead acetate test for sulfur were also negative.

According to Florey *et al.* (1949), a compound which has preferential solubility in an organic solvent under acid conditions is itself acid in nature. This is true of thermoviridin, which in the presence of both water and butanol favors the butanol phase at acid pH and the water phase at alkaline pH. Thus it appears that thermoviridin is an organic acid which contains no sulfur or phenyl groups.

Thermoviridin was found to be dialyzable and also to be precipitated with saturated ammonium sulfate.

The ultraviolet absorption spectrum of thermoviridin was determined in a Beckman quartz spectrophotometer. Maximum absorption occurred in the range of 268 to 272 mμ. The absorption curves are shown in figure 3. The aqueous solutions A, B, and C were made up from solid preparations which had 30, 80, and 30 thermoviridin units activity per mg respectively. The slight differences in the absorption peaks produced by these preparations were not considered significant. The absorption shown was attributed to thermoviridin and was considered to be characteristic of it.

The microbiological spectrum of thermoviridin is presented in table 5. It will be noted that the activity of the antibiotic was restricted primarily to the gram positive organisms tested.

The sensitivity of *M. pyogenes* var. *aureus* 209P was taken as the standard to which the sensitivity of all other organisms was compared. It will be noted that two of the micrococcus strains were penicillin resistant. Strain 193 was found to require 200 times as much penicillin for inhibition as the 209P strain, whereas the 218 strain required 1600 times as much as the 209P strain. Table 5 shows that a fourfold increase in the concentration of thermoviridin was sufficient for the inhibition of both resistant strains.

The sensitivity of all bacterial cultures except the mycobacteria was tested using the twofold serial dilution method. The agar dilution method was used in testing the sensitivity of the mycobacteria and fungi.

The values given for all bacteria in table 5 except those for the mycobacteria are based on the degree of inhibition after 18 to 24 hours' incubation at 37 C. The values for the mycobacteria are based on the degree of inhibition after 6 weeks' incubation at 37 C, and those for the fungi, on the degree of inhibition after 3 weeks' incubation at room temperature.

The activity of thermoviridin was not impaired by the presence of sheep blood, nor did the antibiotic hemolyze sheep red blood cells.

A preliminary acute toxicity study of thermoviridin was conducted using 20-gram White Swiss mice of the Webster strain from the Michigan Department of

TABLE 5. *The microbiological spectrum of thermoviridin*

Organism	Medium	u/ml Required for Inhibition
Micrococcus pyogenes var. aureus 209P.....	I	1
Micrococcus pyogenes var. aureus 218*.....	I	4
Micrococcus pyogenes var. aureus 193*.....	I	4
Micrococcus pyogenes var. aureus B314.....	I	4
Micrococcus pyogenes var. aureus SM......	I	4
Streptococcus viridans 31 (S. mitis)........	Feltons	16
Streptococcus viridans 143.................	Feltons	16
Streptococcus hemolyticus SFIV...........	Feltons	4
Streptococcus hemolyticus K64C...........	Feltons	4
Streptococcus hemolyticus 316A...........	Feltons	16
Streptococcus hemolyticus 314B............	Feltons	16
Diplococcus pneumoniae type III.........	Feltons	4
Corynebacterium diphtheriae PW8.........	Veal infusion	16
Sarcina lutea PCI-1001W.................	I	0.25
Bacillus subtilis 231......................	I	0.5
Clostridium tetani Pease str..............	I	2
Vibrion (Clostridium) septicum...........	I	0.03
Proteus vulgaris 1414....................	I	64
Escherichia coli FDA....................	I	64
Shigella paradysenteriae 15001............	I	64
Salmonella typhimurium Edward str 9.....	I	64
Mycobacterium tuberculosis var. hominis H37..	II	>26
Mycobacterium phlei 1201.................	II	>26
Trichophyton mentagrophytes..............	III	>26
Histoplasma capsulatum..................	III	>26
Blastomyces dermatiditis..................	III	>26
Nocardia asteroides......................	III	>26
Aspergillus fumigatus.....................	III	>26
Candida albicans.........................	III	>26

* Penicillin resistant.

Medium I: Peptone, 6 g; trypticase, 4 g; yeast extract, 3 g; beef extract, 1.5 g, glucose, 1 g; sodium chloride, 5 g; distilled water to 1000 ml, pH 7.0.

Medium II: Beef extract, 3 g; peptone, 10 g; KH_2PO_4, 2 g; glycerine, 70 g; sodium chloride, 5 g; agar, 20 g; distilled water to 1000 ml, pH 6.9.

Medium III: Medium I with agar, 15 g.

Health colony. Intraperitoneal injection of 32 mg of material which had 25 units per mg antibiotic activity into each of 3 mice showed no toxic effect. The mice were observed for a three-week period and then sacrificed. The gross appearance of the internal organs was normal.

DISCUSSION

Tryptone served as a source of both carbon and nitrogen for *T. viridis*. This is supported by the fact that growth was obtained using only a 4 per cent tryptone solution as the fermentation medium. Beef extract was believed to serve as a source of supplementary growth factors and minerals.

Of the nutrients added to the tryptone-beef extract fermentation medium, whole wheat flour and glycerol stimulated the growth of *T. viridis* to some extent, al-
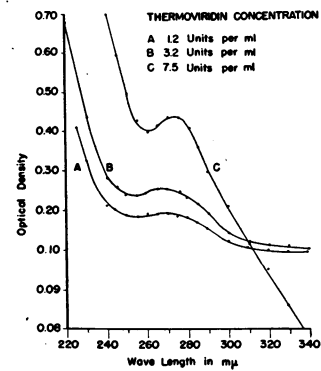

FIG. 3. Ultraviolet absorption spectrum of three lots of thermoviridin in aqueous solution.

though there was no significant enhancement of thermoviridin production. Neither of these constituents was, therefore, included as a permanent constituent of the fermentation medium.

The stability of thermoviridin at temperatures above 37 C was not tested systematically. However, autoclaving 10 ml of culture filtrate for 5 minutes at 121 C caused a reduction in antibiotic activity of approximately 50 per cent.

With the exception of the ultraviolet absorption spectrum, the chemical characteristics of thermoviridin have been determined for the most part by indirect evidence. The negative results of the color tests which were preformed indicate the absence of proteins and carbohydrates in the preparations tested. The solubility characteristics of thermoviridin under acid and alkaline conditions indicate that the antibiotic is an acid. More definite conclusions concerning the chemical nature of this antibiotic cannot be drawn from the accumulated data.

SUMMARY

A thermophilic actinomycete, for which the name *Thermoactinomyces viridis* is suggested, was found to produce an antibiotic which was named thermoviridin. An antibiotic level of 32 units per ml was obtained in shake flask cultures using a medium which contained tryptone, 4 per cent; beef extract, 0.5 per cent; and which had a pH of 6.9 to 7.1. The addition of a number of nutrients did not increase antibiotic potency significantly. Maximum thermoviridin production was reached in 27 hours at 45 C.

Recovery of thermoviridin was accomplished by acid precipitation followed by acetone extraction of the precipitate. Dry material obtained from the acetone extract had up to 64 thermoviridin units per mg.

Fractional precipitation and countercurrent distribution were the methods used in the purification of thermoviridin. A product which had approximately 360 units antibiotic activity per mg. was obtained.

Thermoviridin appeared to be an organic acid which was dialyzable. The antibiotic showed maximum absorption in the range of 268 to 272 mμ.

Thermoviridin was primarily active against the gram positive bacteria tested. A preliminary toxicity test in mice, consisting of a single intraperitoneal injection of 32 mg of material which had 25 thermoviridin units per mg, did not cause death in any case.

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A Laboratory Shaking Machine[1]

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The review of Finn (1954) emphasized the neglect of adequate oxygen supply in common culture methods. Devices for shaking cultures (for aeration) are available in profusion, and it is increasingly common to agitate cultures for recovery of end products and cells. Most of the machines are either very large or designed for small numbers of vessels or for specialized vessels. This is a report of two (similar) shaking machines, assembled so as to accommodate several hundred culture tubes in racks, or four-liter Erlenmeyer flasks. Much of the culture work of this laboratory is conducted at temperatures below 30 C; ambient temperature often exceeds that. These machines were perforce equipped with cooling coils as well as heaters. Their temperatures have been varied with relative ease from 18 to 38 C.

The machines utilize the reciprocating mechanisms from two versions of a small shaker model 75-683 (Eberbach Corp.)[2], which unfortunately so overheated in our uncooled incubator rooms as to render them unsuitable for our culture work. The mechanisms were securely bolted[3] inside refrigerators. The 8½ x 14 inch table of the mechanism is reciprocally shaken through an excursion of 2½ inches by the motion of a connecting rod eccentrically affixed to the shaft of a large pulley. This pulley is beltdriven by a 3½-inch variable-pitch pulley on a 16-inch jackshaft which passes through the back of the cabinet. To the outside end of the jackshaft is attached a large pulley, connected by belting to a small pulley on the ¼ HP AC motor. Grip-link belting is used throughout to facilitate changes in belt length to permit marked alteration in speed required by change in vessel, that is, tubes require more vigorous agitation than is tolerated by fluids in flasks. Without seriously affecting the belting, the speed may be changed from ~85 to 110 cycles per minute.

Machine 1, shown in figure 1, has the following useful inside dimensions: 15 x 26 x 36 inches; is cooled by the coil from a dehumidifier model AFA46,[4] controlled by a Fenwal thermoswitch model 4 732RF[5] activating an appropriate relay; is heated by a 100 Ω wire mesh controlled directly by a Fenwal thermoswitch model 4 732RF. Machine 2, figure 2, has the following useful inside dimensions: 15 x 26 x 26 inches; is cooled by the Crosley sealed unit model N189[6] present in the refrigerator, controlled by a Fenwall thermoswitch model 17501, activiting the relay attached to the refrigeration unit; is heated by a 100 Ω wire mesh controlled by Fenwal thermoswitch model 17500. Sheet metal fastened

[2] Eberbach Corp., Ann Arbor, Mich.

[3] The conversion and installation were made by the All University Laboratory and Research Shop.

[4] Westinghouse Elec. and Mfg. Co., E. Springfield, Mass.

[5] Fenwal, Inc., Ashland, Mass.

[6] Crosley Corp., Cincinnati, O.