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Purification of Propionin, an Antiviral Agent from *Propionibacteria.* (31463)

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Extracts of *Propionibacterium freudenreichii* exhibit antiviral activity against Columbia SK virus *in vivo* and *in vitro,* and against vaccinia virus *in vitro*(1,2). The active agent(s) is referred to as propionin. A microcrystalline substance, active primarily against vaccinia virus *in vitro,* has been separated and is called propionin A; it seems to be a minor constituent(3). The purification of the agent(s) active against Col. SK virus is described in this report.

Materials and methods. 1. Source of effluents: The starting materials for these studies were effluents prepared from *Propionibacterium freudenreichii* grown in agitated culture in an enriched medium. The bacteria were separated from the medium by centrifugation and then subjected to moderate heating (80°C) to release their contents.

2. Virus host system in mice: Fractions of propionin were injected subcutaneously 2 hours after intraperitoneal inoculation of LD_{70-90} of Col. SK virus (brain adapted) and continued twice a day for 4 days; observation was continued for an additional 5 days. Ten or 20 mice were treated and 40 mice were controls in each experiment.

3. Purification methods: Precipitation with organic solvents, gel filtration(4), descending paper chromatography(5), dialysis, ion-exchange chromatography(6), and precipitation at different pHs were used.

Experimental and results. Precipitation with organic solvents: A preliminary purification was effected by precipitating the active component by addition of different organic solvents to the source effluent. The activity of the precipitate was correlated with the polarity of the solvent combination, expressed as the dielectric constant. The various precipitates obtained at different dielectric constants were redissolved in water and tested for activity against Col. SK in mice (Fig. 1).

About 40% of the inactive material could be removed at a dielectric constant of 55 (50% methanol) and the subsequent precipitate obtained at a dielectric constant of 28 (one volume of methanol, 2 volumes of n-propanol and 2 volumes of acetone to 1 volume of effluent) contained about 10% of the solids and most of the activity. Precipitates at intermediate dielectric constants exhibited lesser degrees of activity.

Gel filtration on Sephadex G-25: The active precipitate, dissolved in water, was passed through a column of Sephadex G-25, whereon it resolved into 3 different fractions. The first fraction (brown in color) contained most of the activity, while the second fraction (red) and the final fraction (yellow) were toxic and probably inactive (Fig. 2). Similar results were obtained when the crude effluent, without organic solvent treatment was passed through the column. But it is preferable to remove the inactive material with organic solvent to improve the separation and prolong the life of the column. The dry weight of the active fraction is about 7% as against 45 to

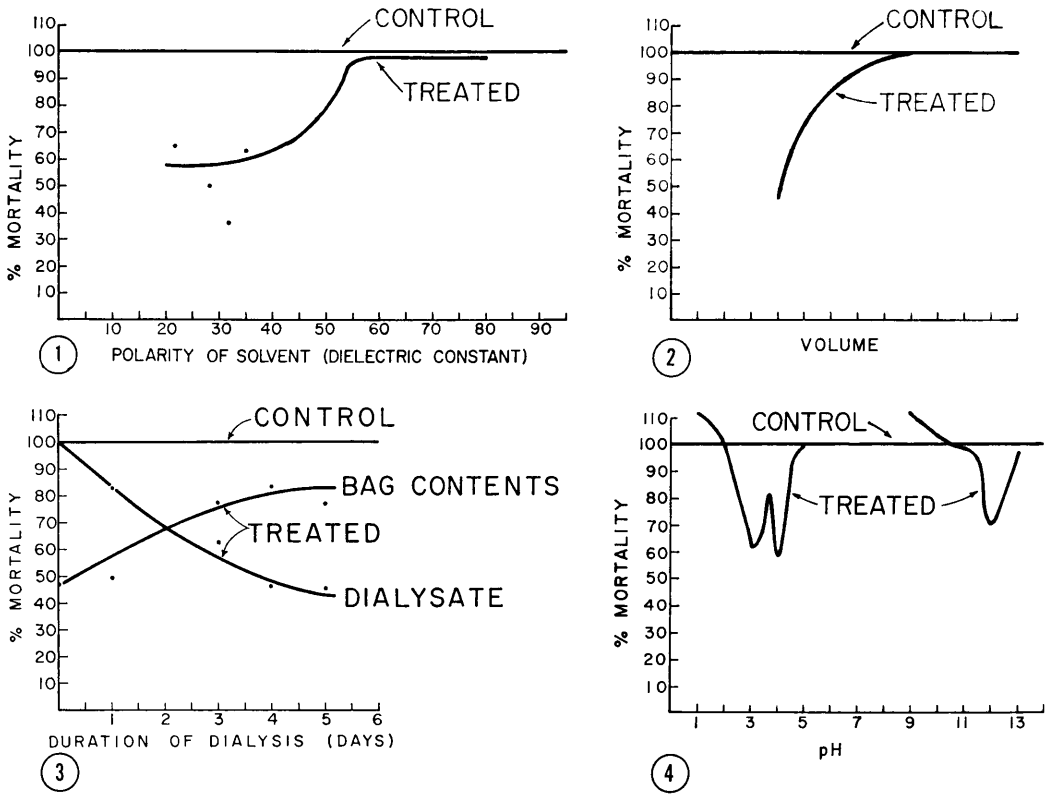

FIG. 1. Activity of solvent precipitates. At a dielectric constant of 25 most of the activity is precipitated; above 55 very little is precipitated. A lowered mortality (Col. SK infections in mice) indicates activity.

FIG. 2. Activity of gel filtration eluates. The first fraction after the void volume is the most active and subsequent fractions become less active. No particular value for the volume is given as columns of different sizes were used. Eluate volume increases to the right.

FIG. 3. Activity of dialysates and bag contents. The dialysate becomes more and more active as the duration of dialysis is increased, while the bag contents become less and less active.

FIG. 4. Activity of pH precipitates. Precipitates at pH 3-4 and 12 show activity.

50% of solids in the source effluent.

Dialysis: The active material was dialyzed against 20 vol. of distilled water at 4°C with constant stirring for a period of 5 days, the water being changed every 24 hours. The dialysates at the end of the 1, 3, 4, and 5 days and the bag contents were tested for activity (Fig. 3). It can be seen that the active material dialyses out gradually, most being out by 4 days. The activity of the bag contents was reduced as dialysis continued.

Paper chromatography: The brown, active fraction from the Sephadex G-25 column was spotted on Whatman No. 1 sheets (46 × 57 cm) 1 ml each (10 mg/ml) and the chromatograms developed (descending) with sec-butanol:formic acid:water (600:60:140 v/v) for 18 to 19 hours. After drying the papers, strips were cut and sprayed with ninhydrin, anthrone, diphenylamine:aniline:phosphoric acid and periodic acid:benzidine. Spots appeared only with ninhydrin, which revealed 8 spots, including the origin. These were cut out, eluted and tested for activity. The results (Table I) indicate that the origin (B_1), and the ninhydrin positive band next to it (B_2), were the most active areas, while the farther moving bands (B_3 to B_8) had little activity. The R_f value of the active fraction was little changed in: methyl ethyl ketone: propionic acid:water (600:200:240 v/v); n-butanol:acetic acid:water (4:1:5 v/v); sec-butanol:ammonium hydroxide 3% (90:40 v/v); and 77% ethanol. No separation was

TABLE I. Inhibitory Effect of Paper Chromatographic Fractions on the Mortality of Columbia SK Virus in Mice.

Fractions	No. dead/ Total No.	Mortality, %
B_1 (chromatograph: origin)	44/100	44
B_2 (chromatograph: next ninhydrin band)	16/40	40
B_3 *Idem*	29/40	72
B_4 "	24/30	80
B_5 "	24/30	80
B_6 "	24/30	80
B_7 "	18/20	90
B_8 (chromatograph: last ninhydrin band)	18/20	90
Control (no drug)	384/510	75

effected by the following solvent systems: sec-butanol:water (85:15 v/v); n-butanol:water (85:15 v/v); and pyridine:water (8:2 v/v).

Ion-exchange chromatography on Dowex-50: The brown fraction (300 mg) was passed through a Dowex-50 (sodium form) column (600 × 45 mm). The column was washed with water and then eluted with 2 N ammonium hydroxide. The water wash was inactive. The ammonium hydroxide eluate contained approximately 30% of the material loaded and most of the activity.

pH precipitation: An attempt was made to precipitate the active substance by changing the pH. The precipitates obtained at different pHs between 1 and 13 were tested for activity (Fig. 4). The precipitates formed at pH 3, 4 and 12 seemed to be the most active.

Statistical analysis: Statistical treatment of the results of the E28 organic solvent precipitate and the brown fraction from Sephadex G-25 showed the data to be highly significant ($P = 0.005$ and 0.001, respectively).

The active band (B_2) on a repeated chromatography revealed 2 different ninhydrin-positive bands. These were cut out, eluted and their homogeneity was established by chromatography. Each was then hydrolyzed with 6 N HCl at 108°C for 24 hours. The hydrolysates on chromatography showed 7, and 4, ninhydrin-positive spots, respectively.

Discussion. Each method used effected a partial purification of propionin and combinations provide further separation. Since the bulk of the activity against Col. SK virus was still present after the separation of propionin A, the latter is probably only a minor constituent, but the effect of the whole effluent on vaccinia *in vitro* is probably due to this component.

The inability of solvents of low dielectric constant (*e.g.*, ether, petroleum ether and chloroform) to extract any active material from the source effluents indicates the probable absence of a lipid moiety in propionin. The ultraviolet absorption spectrum of the active brown fraction showed no characteristic maximum and therefore suggests the absence of any nucleic acids or purines and pyrimidines. Since no characteristic spots are produced by diphenylamine:aniline:phosphoric acid; anthrone; or periodic acid:benzidine, the presence of carbohydrate in propionin is highly doubtful.

The positive reaction with ninhydrin suggests that propionin is a peptide. Further support for this view comes from the observation that propionin on hydrolysis gives rise to different amino acids.

Summary. A potent antiviral agent, propionin, active against Col. SK virus infections in mice, has been isolated from cellular extracts of *Propionibacterium freudenreichii.* Partial purification of propionin by organic solvents, Sephadex and paper chromatography has been achieved. Preliminary identification has been carried out and the available data indicate propionin to be peptide in nature.

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