SOME CHARACTERISTICS OF A GROWTH STIMULANT IN CORN STEEP LIQUOR FOR LACTOBACILLUS CASEI

A. H. HEIMBUCH, L. W. AURAND, AND M. L. SPECK

Department of Animal Industry, North Carolina State College, Raleigh

Received for publication April 13, 1956

In a previous report by Kennedy and Speck (1955), it was shown that the addition of corn steep liquor to milk stimulated the growth of several species of lactic acid bacteria. Additional studies (Kennedy et al., 1955) have shown the stimulatory factor to be different from other stimulatory factors reported for the lactic acid organisms. These investigators also observed that corn steep appeared to shorten the lag growth phase of the lactic acid organisms. It has been reported by Malmgren and Heden (1947) that in microorganisms moderate amounts of nucleic acids have been formed by the end of the lag phase in growth, and in the logarithmic phase the nucleic acid content is at its maximum. Hoffman and Pavcek (1952) observed that uridine, a compound found in corn steep, stimulated the early growth of Streptococcus faecalis.

Since corn steep contains nucleic acids, the possibility exists that the stimulatory factor(s) is a nucleic acid derivative.

By the use of paper chromatography, bioautography and qualitative analysis, a stimulatory substance has been isolated from corn steep which gave characteristics of a nucleoside. The results of these studies are reported in this paper.

EXPERIMENTAL METHODS

The organism used for the bioassay was Lactobacillus casei strain ATCC 7469. The methods used to estimate the potency of the corn steep factor(s) were those which have been developed by Kennedy et al. (1955). The assay media used were skim milk (8.5 per cent solids) and two semi-synthetic media; one was reported by Roberts and Snell (1946) and the other by Rabinowitz et al. (1948) with modifications by Kennedy et al. (1955). The bioautograph technique used to determine the location of the stimulant on the paper chromatogram was that described by Kennedy et al. (1955).

Paper chromatography was used in the characterization studies of the corn steep factor(s). Fractions which had been subjected to various physical and/or chemical treatments were applied to strips of Whatman No. 1 chromatography paper, air dried, placed in chromatography jars and equilibrated with the developing solvent for a period of 8 hr. Descending solvent flow, at room temperature, was used in developing the chromatogram. Ethanol, benzene and water (6:1:2) was used to develop chromatograms for routine checking by the bioautograph. Other solvent systems used were 1-butanol, acetic acid and water (4:1:5) and 2-propanol plus 0.6 per cent aqueous ammonium bborate (1:1).

Qualitative reagents used on the paper chromatograms were as follows: (a) alkaline permanganate was used as a spray reagent to detect carbohydrates (Pascu et al., 1949); (b) a solution of 0.25 per cent ninhydrin in anhydrous acetone was used as a dipping reagent for amino acids (Toennies and Kolb, 1951); (c) the presence of sulfhydryl compounds was tested for by the use of a spray reagent containing sodium nitroprusside (Toennies and Kolb, 1951); (d) lactones and esters of sugars were tested for by spraying with alkaline hydroxylamine followed with ferric chloride (Abdel-Akher and Smith, 1951); (e) a reagent described by Feigl (1954) for spot plate tests of secondary aliphatic amines was used as a spray reagent. A solution of 1 per cent sodium nitroprusside in 10 per cent (by volume) acetaldehyde was sprayed on the chromatogram, followed by a spray solution of 2 per cent sodium carbonate.

The eluate of the active spots (determined by the bioautograph) was tested for the presence of a pentose by the use of orcinol and ferric chloride.

1 Published with the approval of the Director of Research, North Carolina Agricultural Experiment Station, Raleigh, as Paper no. 730 of the Journal Series.

2 These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and North Carolina State College (NR 135-111).
in hydrochloric acid (Meijbaum, 1939) and for phosphorus by the method of Fiske and SubbaRow (1925). An ultraviolet lamp (254 mμ) was used on chromatograms to detect the presence of compounds which would absorb or fluoresce. A Beckman Model DU spectrophotometer was used to examine the eluates from the biologically active spots present on the paper chromatograms.

RESULTS

The results of the experiments reported in this paper are based on bioassays as determined by the method of Kennedy et al. (1955) unless otherwise stated.

Extraction studies. Various organic solvents were tested as extracting agents in an attempt to effect purification and concentration of the corn steep factor(s). None of the solvents tested compared favorably with phenol (Kennedy et al., 1955) for extraction of the corn steep factor.

The ionic strength of corn steep solutions was increased by saturation with sodium chloride in order to observe its effect upon extraction with organic solvents. Acetone, 1-butanol, and ethyl acetate were used to extract saturated salt solutions of corn steep at pH 1.5, 3.3, and 8.7. Only a trace of activity was extracted by acetone and 1-butanol, both at pH 1.5. No activity was extracted by ethyl acetate.

According to Vischer and Chargaff (1948), treatment of yeast ribonucleic acid with anhydrous methanol-hydrogen chloride yields a precipitate which contains the purine hydrochlorides; the pyrimidine nucleotides are not hydrolyzed. It was thought that a similar study using corn steep dialyzate might be of value in identifying the corn steep factor(s). Therefore, a lyophilized corn steep dialyzate was extracted with anhydrous methanol. The methanol extract was cooled in an ice bath while dry hydrogen chloride was passed into the solution for 2 hr. A white, granular precipitate formed which was removed from the solution by filtration. This precipitate dissolved in water and possessed no activity. The hydrogen chloride was removed from the methanol solution by repeated evaporations to dryness under reduced pressure. Approximately 50 per cent of the activity was recovered from the methanol soluble phase. The methanol soluble factor gave an Rf value of 0.50, when bioautographed, compared to 0.48 of the control dialyzate chromatogram.

Chromatographic studies. Chromatographic studies, coupled with the bioautograph, showed the biologically active spot to have an Rf value of 0.51 ± 0.02 at 30°C when ethanol, benzene and water (6:1:2) was used as the developing solvent. It was noted that a lower temperature caused a decrease in the Rf value; at 20°C the Rf value was 0.44 ± 0.02. Therefore, the Rf value of a test fraction was compared to that of a control (starting material) rather than an absolute value.

Characterization studies. Qualitative tests were made to determine some of the general characteristics of chromatograms developed in ethanol, benzene and water (6:1:2). The results of the qualitative tests are presented in figure 1, and can be summarized as follows: (1) negative tests were obtained for carbohydrates, sulfhydryl compounds, lactones and esters; (2) absence of a clear fluorescent area coincident with the activity; (3) when the chromatogram was sprayed with ninhydrin, there were generally 5 or 6 ninhydrin positive areas, one area overlapped about one-half the area of the active spot, but there was no definite band coincident with the center of the active spot; (4) a positive, blue color for secondary aliphatic amines was present in the area of the active spot.

Representative absorption spectra of the

Figure 1. Paper chromatograms of corn steep dialyzate which have been subjected to analytical treatment. (1) 1% KMnO4 in 2% Na2CO3 solution sprayed on the chromatograms gave yellow-green colored spots. (2) Fluorescent to ultraviolet light. (B) Biologically active spot on bioautograph. (2) Positive response to the ninhydrin test. (4) Positive response to secondary aliphatic amine test (Feigl).
eluates from the active areas of the chromatograms are presented in figure 2. An absorption spectrum maximum at 265 μμ and a minimum at 245 μμ was obtained at pH 2. The absorption peak disappeared in dilute potassium hydroxide (pH 12.0); it was present, however, in neutral and dilute ammonium hydroxide solutions (pH 12.8).

In an effort to determine whether the absorption spectrum and the positive secondary amine test were properties of the same compound, successive developments of the chromatogram were carried out with three different developing solvents. The developing solvents used were ethanol, benzene and water (6:1:2); 1-butanol, acetic acid and water (4:1:5); 2-propanol and 0.6 per cent aqueous ammonium borate (1:1) respectively. The dialyzate containing the active factor was placed on a paper strip and developed; after ascertaining its Rf value by the bioautograph, the active area was eluted with water and spotted on a paper strip; the chromatogram was then developed with the second solvent system. The same procedure was repeated using the third solvent system. It was found that an absorption spectrum maximum (at pH 2) of 265 μμ and a positive secondary aliphatic amine test were characteristic of the biologically active spots. Another interesting fact was noted when the chromatogram developed with butanol, acetic acid and water (4:1:5) was treated with ninhydrin. A distinct yellow band was observed which coincided with the front one-third of the active spot. A yellow color with ninhydrin is characteristic of proline, although Knight (1951) reported a number of peptides which do not contain proline but give a yellow color with ninhydrin.

Since the eluate of the active areas from the chromatograms gave an absorption spectrum characteristic of nucleic acid derivatives, some attention was given to the study of the biological activity of known purine and pyrimidine compounds. Various nucleic acid derivatives were obtained and assayed for activity in the same manner as the corn steep factor. Thymidylic acid, uridylic acid (2' and 3'), cytidylic acid, xanthosine, yeast nucleic acid and calf thymus nucleoprotein elicited slight responses.

**DISCUSSION**

The moderate stability of the active factor(s) toward mineral acid at elevated temperatures would suggest the possibility of a low molecular weight compound. The observations that the factor(s) was readily dialyzable and not precipitated at any pH (excluding heavy metals) would also tend to support this view. Furthermore, strepenogen is not involved because of the moderate stability of the corn steep factor toward mineral acid.

The results of heavy metal precipitation were not too conclusive. Little or no activity was recovered when ammoniacal silver nitrate, basic lead acetate, and ethanolic barium chloride were used as precipitating agents. In the case of ammoniacal silver nitrate, activity was completely lost, but it is felt that loss of activity may have been due to oxidation. The results of the ethanolic barium chloride would indicate the factor is not an organic phosphate compound.

Most organic solvents were ineffectual as extracting agents of aqueous solutions of crude corn steep. Methanol, ethanol and pyridine extracted over fifty per cent of the activity; however, these solvents are water miscible and as a result little purification of the factor was ob-
Phenol extracted most of the activity from an aqueous solution of corn steep.

In general, the percentage activity extracted increased as the polarity of the solvent increased. For example, acetone extracted only a small amount of the active factor, but by increasing the concentration of hydrogen chloride in the acetone solution, at least fifty per cent of the activity was extracted.

A purine or pyrimidine derivative is indicated by the fact that eluates of the active spot from chromatograms of dialyzate gave an absorption spectrum maximum at about 265 mμ and also gave a positive pentose reaction. It was interesting to note that the absorption peak of the eluate disappeared in dilute potassium hydroxide (pH 12.0) but persisted in N ammonium hydroxide (pH 12.8). Although there was no ninhydrin positive band coincident with the center of the active spot, there was ninhydrin positive material within the active area. Part of the ninhydrin positive material gave a yellow color reaction which is indicative of proline. Likewise, it was found that a positive test for secondary aliphatic amines was obtained on a chromatogram in the area of the active spot.

The accumulated evidence for a nucleic acid derivative would suggest that either a pyrimidine nucleotide or nucleoside is involved. The corn steep factor is moderately stable to mineral acid which is true of a pyrimidine nucleotide; a purine nucleotide is much more labile (Hunter and Hlynka, 1937). No activity was recovered from the precipitate when lyophilized dialyzate was treated with dry hydrogen chloride in anhydrous methanol. Vischer and Chargaff (1948) reported that the purine nucleotides of yeast nucleic acid are hydrolyzed by this method, whereas, pyrimidine nucleotides are unaffected and remain in solution.

Since the eluate of the active spot from the paper chromatogram gave a characteristic absorption spectrum for a nucleic acid derivative, and since uridine was reported to be present in corn steep (Hoffman and Pavcek, 1952), nucleic acid derivatives of known composition were tested for biological activity. Of the compounds tested, only thymidyllic acid, uridylic acid (2' and 3'), cytidylic acid, xanthosine, yeast nucleic acid and thymus nucleic acid showed slight activity. Neither uridine nor uridine-5'-monophosphate showed any activity.

Kennedy et al. (1955) reported that the factor was presumably a new one since its properties were not in agreement with those of previously reported factors. Recently new and unidentified growth factors for lactobacilli have been reported. Shorb and Veltre (1954) describe a growth factor in fermentation products for avian Lactobacillus bifidus which was soluble in water, phenol, methanol and tertiary butanol. It was stable to autoclaving at pH 9 or 10 for 30 min at 15 pounds pressure, but the activity was completely destroyed by refluxing for 1 hr with 6 N hydrochloric acid. The L. bifidus factor is different from the corn steep factor in that it was insoluble in pyridine and the activity was lost when the factor was rechromatographed on paper strips. A growth factor in the nutrition of L. casei strain ATCC 7469 was reported by Rege and Sreenivasan (1954) to be found in APF supplement, liver fraction "L," yeast extract and peptone. It appears to be different from the corn steep factor in that it is more soluble in 1-butanol than in water. Furthermore, liver fraction "L" showed high potency in their assay medium; this material was inactive in the semi-synthetic medium used in the present study.

**SUMMARY**

Studies on the chemical and physical properties of a growth factor(s) contained in corn steep liquor have been made.

The results of stability tests, precipitation with heavy metals and solubility studies are presented.

Paper chromatography was used to resolve the active factor present in the corn steep. Location of the activity on the paper chromatogram was determined by bioautography. Qualitative tests of the paper chromatogram gave positive tests in the area of the active factor for (a) pentose, (b) secondary aliphatic amine and a positive ninhydrin area which was not coincident with the center of the active spot. Eluates of the active spot absorbed in the ultraviolet region, having a maximum at 265 mμ and a minimum at 245 mμ.

Corn steep dialyze was developed and eluted sequentially on paper strips using neutral, acidic and basic solvents, respectively. Efforts to
separate the secondary aliphatic amine component from the substance which absorbed in the ultraviolet region were, however, unsuccessful.

In view of its stability toward mineral acid, its absorption of ultraviolet light and the results of chromatographic studies, the corn steep factor appears to be a nucleoside.

REFERENCES


Mejbaum, W. 1939 On the determination of small amounts of pentose, especially in derivatives of adenylic acid. Z. Physiol. Chem., 258, 117-120.


