ANTIBIOTIC ACTIVITY OF THE FATTY-ACID-LIKE CONSTITUENTS OF WHEAT BRAN

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An appreciable part of the antibiotic activity observed in connection with the culturing of an unidentified microorganism on a medium consisting principally of wheat bran and asparagus juice was found to reside in the medium itself. Investigation disclosed that the wheat bran was the source of this activity. Wheat bran has frequently been used as an ingredient in media assayed for antibiotic activity. Because of the possibility of confusing the antibiotic activity naturally present in wheat bran with the activity produced by microorganisms grown on media containing wheat bran, efforts were made to characterize the active factor involved. A hypothesis is presented that antibiotic activity may be formed by the hydrolysis and saponification (by the action of the microorganisms) of fatty constituents contained in the original substrates.

EXPERIMENTAL PROCEDURE

Preliminary observations indicated that the constituent or constituents of wheat bran possessing antibiotic activity were extractable with 60 or 95 per cent ethanol, petroleum ether, or diethyl ether, but were not appreciably water-soluble. These extracts, particularly those obtained by the use of petroleum ether or diethyl ether, when saponified with KOH, yielded soaps which had even greater activity on an equivalent basis than the original bran. Extraction of the hydrolyzed material with diethyl ether to obtain the neutral and the acid ether-soluble fractions showed that all of the active material was present in the latter.

A quantity of the antibiotically active fraction was obtained by extracting 1,000 grams of wheat bran overnight in a percolator with 2.5 liters of petroleum ether, following which procedure the fraction was drained and washed with an additional 1.5 liters of the ether. These extracts were combined and evaporated almost to dryness on a steam bath. The residue was extracted with aliquots of 95 per cent ethanol, totaling about 300 ml. Three hundred ml of 0.832 N alcoholic KOH were added to the ethanol solution, and the mixture was refluxed for 2 hours. The refluxed solution was concentrated to about 200 ml and then diluted with 1,600 ml distilled water. This solution was then extracted with five 200-ml portions of diethyl ether to remove the "neutral" ether-soluble fraction. The alcohol-water solution was then acidified with HCl to pH 2, and the diethyl ether extraction was repeated. After being washed with water, the combined acid-ether extracts were evaporated, from which about 30 g of a brown, oily residue were obtained. This residue, which contained the active material,

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was then extracted with 95 per cent ethanol. The ethanol-soluble fraction was removed by centrifuging the suspension of the residue in the ethanol, and a potassium salt was prepared from the supernatant by the addition of 90 ml of 0.096 N KOH. This solution, after dilution with about 600 ml of water, was shell-frozen in round-bottomed boiling flasks and then dried under vacuum from the frozen state. The final yield was 27 g.

Tests for antibiotic activity were made on each extract at each step of the separation, and a control containing only the solvent was made on each solvent. It was calculated that the final product contained about 95 per cent of the original activity. It was found that the refluxed alkaline alcohol solution was more active than the original petroleum ether extract, which was probably due to the hydrolysis of some of the fats to free fatty acids. The solvent controls showed no activity at the inhibition levels of the active extracts.

A modified medium II of Schmidt and Moyer (1944) was used for the bioassay. The ingredients of the medium were peptone, 5 g; yeast extract, 1.5 g; beef extract, 1.5 g; "N-Z-amine" type B, 2.0 g; glucose, 3.0 g; NaCl, 3.5 g; 500 ml of KH_2PO_4 (15 g per liter) adjusted to pH 7.0 with NaOH; and distilled water to make 1 liter. The medium was placed in bottles of convenient size, usually in the amount required for any one series of assays. The inoculum was grown by transferring it from an agar slant to 100 ml of the medium in a 250-ml Erlenmeyer flask, and incubating it 18 hours at 37 C. The inoculum was added to the medium at the rate of 20 ml per liter and the serial dilution set up. Two logarithmic series of 10, 100, 1,000, etc., and 5, 50, 500, etc., were set up for the survey assay during the fractionation. When more accuracy was desired, as in the comparison of solutions of various salts of the fatty acids, series of 120, 200, 300, 400, 600, 800, and 1,200, or 10-fold multiples thereof, were set up. Test tubes 18 by 150 mm in size were used. Each series was made in duplicate and inoculated, and sterile control blanks were set up with each series. The tubes were incubated for 4 hours in a water bath at 37 C. At the end of the incubation period the tubes were sterilized, and the turbidity of each tube was measured in a Klett-Summerson colorimeter. The turbidity readings were plotted against the dilutions on a semilogarithmic paper. A sigmoidal inhibition curve was obtained. The point of 50 per cent inhibition as compared with the readings of the antibiotic-free controls was taken as the most accurate measure of degree of inhibition. From the dilution at which this point in the curve occurred the concentration in micrograms per milliliter was calculated. For the comparison of the activity of the wheat bran fraction with some other salts of fatty acids, *Staphylococcus aureus* (Food and Drug Administration strain 209), *Micrococcus conglomeratus* (Merck's N.Y. strain), *Streptococcus faecalis* (ATCC 7080), and *Escherichia coli* (Waksman's strain for testing streptomycin) were used as the assay organisms.

Potassium laurate, sodium oleate, potassium salt of mixed acids of castor oil, potassium salt of the mixed acids of cottonseed oil,[2] and a sample of potassium

[2] Samples of these were supplied by Dr. Ernest Kester of this laboratory.

linoleate made from methyl linoleate[3] were used for the purpose of comparing activities.

RESULTS

Extracts obtained by treating separate 10-g samples of wheat bran with 200-ml portions of water, and with 70 per cent ethanol, produced a 50 per cent inhibition of *S. aureus* at dilutions of 10 and 260, respectively. A comparison with various other salts of fatty acids readily available is given in table 1. The results indicated that the potassium salt obtained from the wheat bran was considerably more active than any of the other salts tested, with the exception of potassium linoleate, which had about the same activity as the salts of the wheat bran extracts.

TABLE 1

Antibiotic activity of the salts of fatty acids from various sources

FATTY ACID SALTS	CONCENTRATION OF SALTS GIVING 50 PER CENT INHIBITION OF		
	S. aureus	*M. conglomeratus*	*S. faecalis*
	μg per ml	μg per ml	μg per ml
Potassium laurate	22	16	27
Sodium oleate	23	18	100
Potassium salts of mixed acids of castor oil*	50	38	45
Potassium salts of mixed acids of cotton seed oil*	50	41	48
Potassium salt of acid ether fraction of wheat bran	4	5.5	10
Potassium linoleate	3.5	4.2	6

* These samples were stored laboratory samples, and it is likely that freshly made samples would have shown higher activity.

Results with *E. coli* indicated no inhibition within the range tested. In fact, with the wheat bran salt, a definite stimulation was noted at 300 micrograms per milliliter. These findings would seem to indicate that these salts would probably be more active against gram-positive organisms than against gram-negative ones.

DISCUSSION

Germicidal and bacteriostatic activity of some fatty acids is well known. Stimulatory action of these and similar materials at certain concentrations also has been reported. Whether the action will be stimulatory, inhibitory, or germicidal apparently depends upon the kind and concentrations of the materials added, on the physical and chemical environment, and on the type of organism employed. In riboflavin assays, an alcoholic extract of fresh liver hydrolyzed with alkali was found to be strongly inhibitory for *Lactobacillus casei* (Feeney and Strong, 1942). Feeney and Strong also found that ether extract of whole blood was stimulatory at low, and inhibitory at higher, concentrations. Kodicek

[3] Supplied by Dr. Gordon Rose of the Enzyme and Phytochemical Research Division of this bureau.

and Worden (1944), also studying factors affecting the riboflavin assay, found that *Lactobacillus helveticus* was inhibited for 24 hours by oleic acid, and for 72 hours or more by linoleic and linolenic acids, when used in concentrations of 160 micrograms per 10 ml of culture, or at 16 parts per million. Avery (1918) was able to suppress the growth of pneumococci and streptococci, while attempting to isolate "*B. influenza*," by adding sodium oleate to a hemoglobin medium. The activity of various fatty acid soaps was tested by Lamar (1911*a*, 1911*b*, 1912). Lysis of pneumococci was obtained at comparatively high dilutions of sodium oleate, potassium linoleate, and potassium linolenate. The latter two salts inhibited growth for 1 hour at dilutions up to 1:4,000 and 1:6,000. It was concluded that the action was directly proportional to the degree of unsaturation of the acid.

Bergström, Theorell, and Davide (1946) found that the presence of fatty acids in the medium interfered with the oxygen uptake of *Mycobacterium tuberculosis*. Di-heptylacetic acid reduced oxygen uptake at a 1:7,000 dilution, whereas the uptake was completely inhibited by oleic acid at 1:10,000, by linoleic acid at 1:15,000, and by linolenic acid at 1:30,000. After extended investigation, Stanley, Coleman, Greer, Sacks, and Adams (1932) concluded that the most active compounds were aliphatic acids which contain from 15 to 18 carbon atoms. They studied the action of chaulmoogra oil and related compounds on *Mycobacterium leprae*, *Mycobacterium tuberculosis*, and other acid-fast bacteria. The most effective acids were good surface tension depressants. This physical property seemed to be more important than the detailed chemical structure. The sodium salts of these acids were found to be effective in dilutions of 1:50,000, or at 20 parts per million.

Barton-Wright (1938) reported that, in the fatty fraction of wheat bran, the total combined acids are 84 per cent unsaturated, with an iodine value of 152.4. It seems safe to assume the presence of a considerable amount of linoleic acid.

The separation from wheat bran of a material with antibiotic properties, which is of a fatty acid nature or is closely associated with the fatty acid, has some rather interesting implications. For instance, when wheat bran, which contains fatty acid constituents, is extracted with 70 per cent ethanol, antibiotic activity is obtained in the extract. Also, the possibility no doubt exists that such fatty constituents may be hydrolyzed by the action of the microorganisms and subsequently saponified. When the culture is subsequently assayed, water-soluble antibiotic activity may be found, and such activity may be attributed to the antibiotic normally produced by the microorganism.

Keeping this hypothesis in mind, it might be well to re-examine the findings reported by Srinivasa (1944), Mohan *et al.* (1946), Moyer and Coghill (1947), and Holtman (1945). For instance, extracts obtained by the extraction of Moyer and Coghill's (1947) wheat bran medium with 70 per cent alcohol at pH 7.5 gave a 50 per cent inhibition of *S. aureus* at 1:200, of *S. faecalis* at 1:108, and of *M. conglomeratus* at 1:60. Hence, if an organism capable of hydrolysis of the fatty constituents of the medium were grown, a water-soluble salt having antibiotic activity might be formed, which, when the medium was assayed, could give a false picture of antibiotic activity produced by the organism. Actual separation

of this fraction from the antibiotic produced by the organism would then be necessary in order to obtain the correct data. Intensive search of the literature might bring to light numbers of instances in which the addition to media of fat- and fatty-acid-containing materials resulted in an increase in antibiotic activity.

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SUMMARY AND CONCLUSIONS

A fraction having antibiotic properties was extracted from wheat bran. This material has the characteristics of a fatty acid and forms a water-soluble potassium salt which has a comparatively high activity against *Staphylococcus aureus*, *Micrococcus conglomeratus*, and *Streptococcus faecalis*. It was inactive against *Escherichia coli*. When materials of plant or animal origin containing fats or fatty acid constituents are used in making microbiological media, the possibility of these constituents having antibiotic activity, which might be confused with activity produced by microorganisms, should be given consideration.

REFERENCES

AVERY, O. T. 1918 A selective medium for *B. influenza*. Oleate hemoglobin agar. J. Am. Med. Assoc., **71**, 2050–2051.

BARTON-WRIGHT, E. C. 1938 Observations of the nature of the lipids of wheat flour, germ, and bran. Cereal Chem., **15**, 723–738.

BERGSTROM, S., THEORELL, H. T., AND DAVIDE, H. 1946 Effect of some fatty acids on the oxygen uptake of *Mycobacterium tuberculosis humanis* in relation to their bactericidal action. Nature, **157**, 306–307.

FEENEY, R. E., AND STRONG, F. M. 1942 The concentration of the substance in blood which is stimulatory for *Lactobacillus casei*. J. Biol. Chem., **142**, 961–962.

HOLTMAN, D. F. 1945 Increasing penicillin yields. J. Bact., **49**, 313–314.

KODICEK, E., AND WORDEN, A. N. 1944 Effect of unsaturated fatty acids upon the growth of *Lactobacillus helveticus* and other gram-positive bacteria. Nature, **154**, 17–18.

LAMAR, R. V. 1911*a* Chemo-immunological studies on localized infections. I. Action on the pneumococcus and its experimental infections of combined sodium oleate and antipneumococcus serum. J. Exptl. Biol. Med. U. S. S. R., **13**, 1–23.

LAMAR, R. V. 1911*b* Chemo-immunological studies on localized infections. II. Lysis of the pneumococcus and hemolysis by certain fatty acids and their alkali soaps. J. Exptl. Biol. Med. U. S. S. R., **13**, 380–386.

LAMAR, R. V. 1912 Chemo-immunological studies on localized infections. III. Some further observations upon the action of certain soaps on the pneumococcus and its experimental infections. J. Exptl. Biol. Med. U. S. S. R., **14**, 256–264.

MOHAN, R. R., RAO, T. N. R., AND SREENIVASAYA, M. 1946 Supplemental value of bran in penicillin production. Current Sci., **15**, 108–109.

MOYER, A. J., AND COGHILL, R. D. 1947 Penicillin. X. The effect of phenylacetic acid on penicillin production. J. Bact., **53**, 329–341.

SCHMIDT, W. H., AND MOYER, A. J. 1944 Penicillin. I. Methods of assay. J. Bact., **47**, 199–208.

SRINIVASA, RAO S. 1944 Production of penicillin. Nature, **154**, 83.

STANLEY, W. M., COLEMAN, G. H., GREER, C. M., SACKS, J., AND ADAMS, R. 1932 Bacteriological action of certain synthetic organic acids and other acid-fast bacteria. J. Pharmacol., **45**, 121–162.