RI bureau of mines
report of investigations 6279

MATERIALS IN COAL INHIBITORY TO THE GROWTH OF MICROORGANISMS

By Martin H. Rogoff and Irving Wender

UNITED STATES DEPARTMENT OF THE INTERIOR

BUREAU OF MINES

1963

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UNITED STATES DEPARTMENT OF THE INTERIOR
Stewart L. Udall, Secretary

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This publication has been cataloged as follows:

Rogoff, Martin H

Materials in coal inhibitory to the growth of microorganisms, by Martin H. Rogoff and Irving Wender. [Washington] U. S. Dept. of the Interior, Bureau of Mines [1963]

13 p. illus., tables. 27 cm. (U. S. Bureau of Mines. Report of investigations 6279)

1. Coal. 2. Micro-organisms. I. Wender, Irving, joint author. II. Title. (Series)

TN23.U7 no. 6279 622.06173

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MATERIALS IN COAL INHIBITORY TO THE GROWTH OF MICROORGANISMS

by

Martin H. Rogoff[1] and Irving Wender[2]

ABSTRACT

The presence of antibiotic materials extractable with polar organic solvents was demonstrated in coals of various ranks. Since these antibiotics occur in low concentrations, 200 pounds of a high-volatile C coal was extracted with acetone at room temperature to obtain sufficient material for detailed study. The biologically active materials in the extract were concentrated by extracting with aqueous sodium hydroxide. The fractions soluble in sodium hydroxide inhibited bacterial cultures when present in concentrations comparable to those at which other antibiotics are active (5 μg/ml). Steam distillation of the extracts did not yield highly active materials, contrary to the finds of other investigators.

Mass spectrometry of the extract indicated, mainly, the presence of 6-ring compounds in the region of mass 324 to 392. The predominance of 3-ring aromatic systems found by ultraviolet analysis suggests compounds such as biphenanthryl or similar structures. The activity of the hydroxide-soluble material decreased markedly when the phenolic hydroxyl groups were transformed into trimethylsilyl ethers. Hydrolysis of the ethers yielded a product having the same activity as the original material, which indicates that phenolic compounds are largely responsible for the antibiotic activity observed.

INTRODUCTION

The presence of biologically active materials (inhibitory to growth of microorganisms) in methanolic extracts of coal was first reported by Evans.[3]

[1] Formerly microbiologist, Pittsburgh Coal Research Center, Bureau of Mines, Pittsburgh, Pa.; now with the Bioferm Corporation, Wasco, Calif.

[2] Project coordinator, Pittsburgh Coal Research Center, Bureau of Mines, Pittsburgh, Pa.

[3] Evans, W. D. Coal-miners' Pneumokoniosis. Colliery Eng., v. 27, 1951, pp. 513-518.

Work on manuscript completed December 1962.

Because the biologically active components came from the vitrain of coal, he designated the fraction that exhibited in vitro inhibition of bacteria as "vitricin." Kosanke[4] showed that acetone and ethanol, as well as methanol, were suitable solvents for the extraction of biologically active material from coals; he demonstrated that bacteriostatic materials were present in lignite, subbituminous and high-volatile bituminous coals. Schenck and Carter[5] reported that these extracts inhibited germination of the spores of a number of saprophytic and plant pathogenic molds. Mills[6] examined the properties of an extract of Tupton coal (about 84 pct C, maf) that proved bacteriostatic to both gram-negative and gram-positive bacteria; the growth of Bacillus subtilis was inhibited by the presence of 34 μg/ml of raw extract (1/33,333 dilution). By steam-distilling a concentrated acetone extract of Tupton coal, he obtained a yellow oil that was strongly inhibitory to gram-positive bacteria and certain molds. Mills attempted to isolate active components from ethanol extracts with aqueous acids, alkalis, and buffered solutions, but little of the biologically active components was transferred to the aqueous phases. Both Mills and Kosanke observed that the highest biological activity resided in subbituminous and high-volatile bituminous coals and that the activity was slight or absent in coals of lower or higher rank.

The presence of material in coal inhibitory to the growth of microorganisms poses some interesting questions on its role in the preservation of coal against microbial attack. Lieske and Hoffman[7] observed that fluorescent pseudomonads, which were found in the present investigation to be resistant to the coal extract materials, were practically the only microbial form found in German brown coal; where these coals were exposed at the surface, the only higher plant that grew was Tussilago furfara. Lieske and Hoffman attributed this restriction of flora to the inhibitory action of the coal on the other forms of plant life.

This paper reports on the investigation of antibiotic activity in a variety of coals, particularly on suitable methods for concentrating and quantitatively estimating the active components, as well as on certain aspects of the nature and composition of the components. This work has been a part of a general study of the microbiology of coal; preliminary studies have already been published. [8] [9]

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[4] Kosanke, R. M. A Bacteriostatic Substance Extracted From the Vitrain Ingredient of Coal. Science, v. 119, 1954, pp. 213-214.

[5] Schenck, N. C., and J. D. Carter. A Fungistatic Substance Extracted From Vitrain. Science, v. 119, 1954, pp. 212-213.

[6] Mills, A. A. Biologically Active Materials in Coal. Nature, v. 184, 1959, pp. 1885-1186.

[7] Lieske, R., and E. Hoffman. Untersuchungen über die Mikrobiologie der Kohlen und ihrer natüralichen Lagerstatten. I. Die Mikroflora der Braunkohlengruben. (Investigations on the Microbiology of Coals and Their Natural Sites. I. The Microflora of Brown-Coal Mines.) Brennstoff-Chem., v. 9, 1928, pp. 174-178.

[8] Rogoff, Martin H., and Irving Wender. Biologically Active Materials in Coal. Nature, v. 192, 1961, pp. 378-379.

[9] Rogoff, Martin H., Irving Wender, and Robert B. Anderson. Microbiology of Coal. BuMines Inf. Circ. 8075, 1962, 85 pp.

MICROBIOLOGICAL METHODS

Test Cultures

Cultures of Bacillus subtilis and Staphylococcus aureus, which are standard test organisms for penicillin assay, were obtained from the Pennsylvania State University culture collection, and then were maintained in nutrient broth.

Turbidimetric Assay for Point of 50 Percent Inhibition

Aliquots of methanol or acetone solutions containing suitable concentrations of biologically active materials were added to 10-ml portions of nutrient broth (Difco)[10] in 17- x 250-mm culture tubes. The tubes were sterilized by autoclaving for 10 minutes at 120° C, and, after cooling, were inoculated with three drops (No. 20 needle) of an 18-hour broth culture of the test organisms. Controls consisted of (1) blank(uninoculated) tubes containing aliquots of extracts to compensate for color and turbidity due to the material in the extracts and (2) a control tube of inoculated broth for determining uninhibited growth. After 18 hours of incubation, all tubes were read against a blank of uninoculated broth at 525 mμ in a Bausch and Lomb Spectronic 20 colorimeter to obtain the optical density (fig. 1). The difference between the optical density of the uninoculated tubes containing aliquots of extracts (1) and the inoculated test set was calculated and plotted as a function of extract concentration (fig. 1). The point of 50-pct inhibition was found by the intersection of the calculated curve with one-half the optical density of the uninhibited growth in the control tube (2).

Paper-Disk Plate Assay

Paper disks, 10 mm in diameter, were saturated with a methanolic solution of known concentration of suspected biologically active materials. The disks absorb 121 μl of solvent; the concentration of the material tested was calculated from this value. After they were dried in air, the disks were placed on the surface of 10 ml of nutrient agar in Petri dishes. This agar had been seeded with either 0.2 ml of an 18-hour culture of S. aureus or 0.1 ml of a 48-hour culture of B. subtilis. Inhibition of the test cultures results in a clear zone on the agar around the paper disk containing an active material (fig. 2); the zone diameter was measured and served as an indication of the comparative potency of the extracts tested.

Broth Assay

With concentrated active (hydroxide-soluble) fractions, the amount of material (contained in a maximum of 0.1 ml of acetone or methanol) completely inhibiting the growth of test cultures did not produce any precipitate or

[10] Reference to commercial materials and models of equipment is made to identify those used; it does not imply endorsement by the Bureau of Mines.

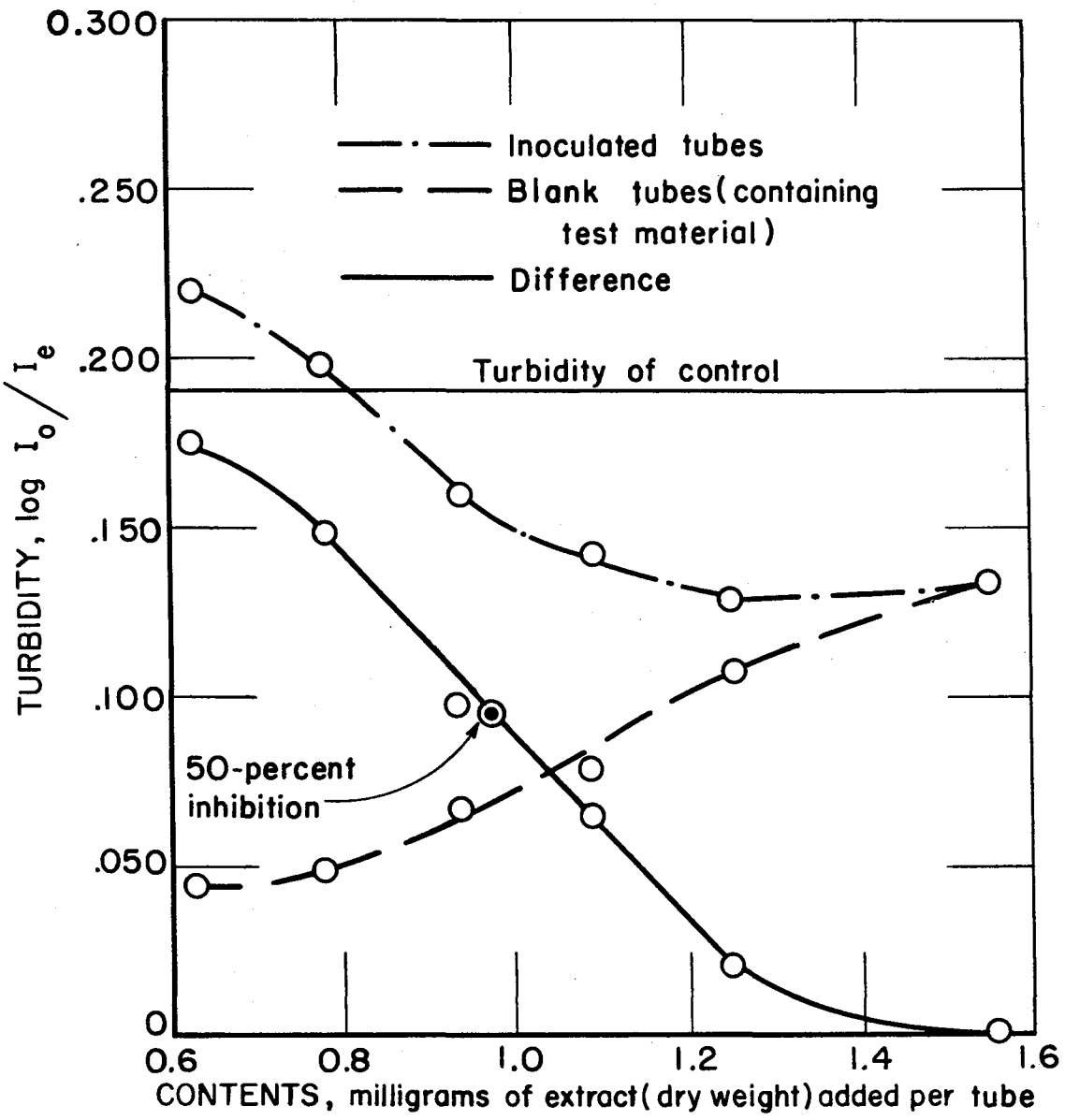

FIGURE 1. - Turbidimetric Assay of a Methanol Extract of North Dakota Lignite. Test organism Staphylococcus aureus.

turbidity when added to tubes containing 10 ml of nutrient broth. Thus determination of growth or inhibition of a test culture could be made visually when compared with a blank tube and an inoculated control tube that received 0.1 ml of pure solvent. Tubes showing no growth or reduced growth were readily apparent after 16 hours incubation at 30° C (fig. 3).

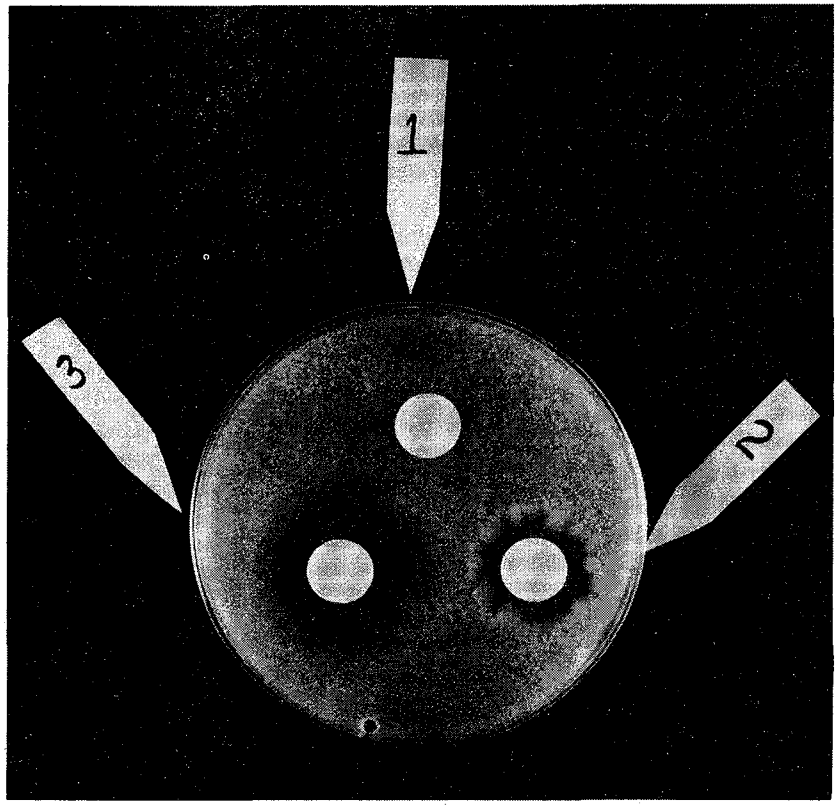

FIGURE 2. - Typical Filter-Paper Disc Plate Assay of Extract of Rock Springs, Wyo., Coal.
No. 1, No extract.
No. 2, 25 μg sodium hydroxide solubles.
No. 3, 100 μg sodium hydroxide solubles.

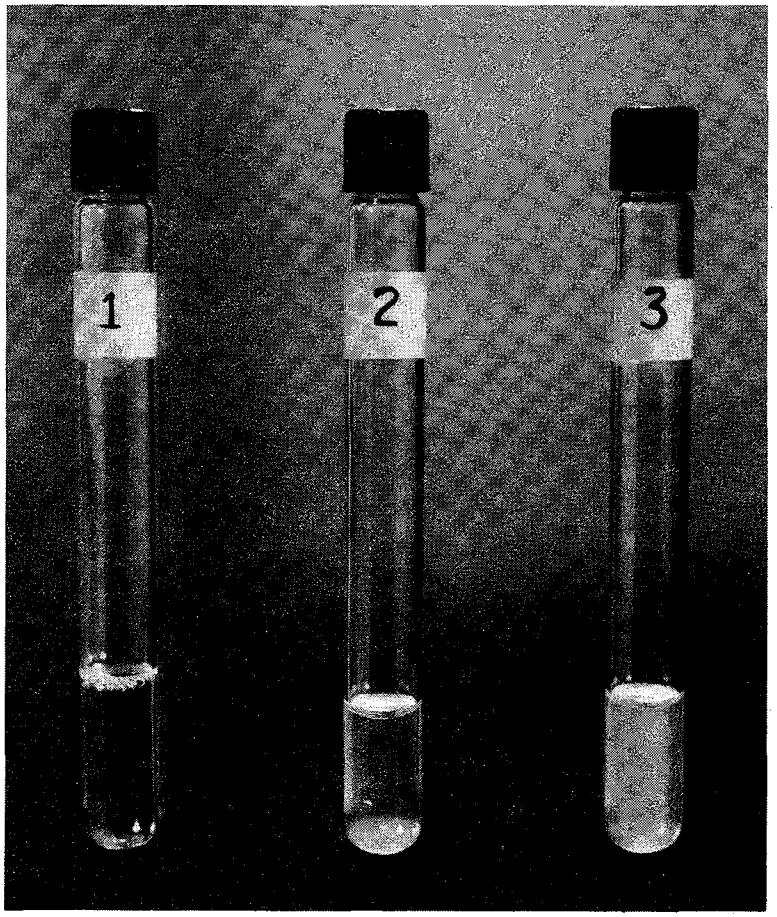

FIGURE 3. - Typical Broth Assay of Extract of Rock Springs, Wyo., Coal.
Tube 1, 20 μg sodium hydroxide solubles.
Tube 2, 5 μg sodium hydroxide solubles.
Tube 3, 1 μg sodium hydroxide solubles.

EXPERIMENTAL PROCEDURES AND RESULTS

Screening of Coals for Biological Activity

The identity and analyses of the coals are given in tables 1 and 2. Samples of peat, lignite, and bituminous coal were ground to pass a 200-mesh screen. The samples were extracted in a Soxhlet apparatus with approximately three times their weight of methanol for 24 hours. The methanol was removed from the extract by warming the extract under reduced pressure; the activities of the dark-brown, friable, solid residues were measured by turbidimetric assay to find the point of 50-pct inhibition. The results, summarized in table 3, confirmed by the findings of Kosanke[11] and Mills[12] that the lower rank bituminous coals are richest in biologically active materials, with a decrease in activity in coals of higher and lower rank. Kosanke found an almost total lack of activity in extracts of anthracite.

TABLE 1. - Identity of coals studied

Item	State	Seam	Mine	County	Rank[1]
1	Minnesota[2]	-	-	-	Peat.
2	North Dakota	Noonan	Baukol-Noonan	Divide	Lignite.
3	Colorado	Laramie	-	Weld	Hvbb.
4	Wyoming	-	Rock Springs	Sweetwater	Hvcb.
5	Pennsylvania	Pittsburgh	Bruceton	Allegheny	Hvab.

[1] Notation for rank:
Hvab, high-volatile A bituminous coal.
Hvbb, high-volatile B bituminous coal.
Hvcb, high-volatile C bituminous coal.
[2] Rice Lake bog.

TABLE 2. - Analyses of coals studied

Item	Analysis, percent					Remarks
	C	H	N	S	O[1]	
1	50.71	5.83	1.26	0.26	-	Air-dried.
2	73.6	5.2	1.2	.6	19.4	Maf.
3	66.91	5.48	1.41	.39	25.8	Maf.
4	79.31	5.41	1.74	.90	12.6	Maf.
5	82.68	5.51	1.61	1.18	9.0	Maf.

[1] Oxygen by difference.

[11] Work cited in footnote 4.
[12] Work cited in footnote 6.

TABLE 3. - Activity of materials extracted from coal with methanol against a culture of Staphylococcus aureus

Source		Solids in extracts, μg/g of dry source material	Range of concentrations tested, μg/10 ml of broth	Concentration producing 50-pct inhibition, μg/10 ml of broth
Rank	Material[1]			
Peat...	1	16,200	500-4,000	1,700
Lignite	2	14,800	80-2,500	990
Hvbb...	3	16,600	330-1,400	490
Hvab...	5	1,900	250-2,000	1,430

[1] Item numbers of materials described in table 1.

Concentration of the Active Components From the Extracts With Aqueous Sodium Hydroxide

An experiment was designed to determine whether the active components were in the acidic and phenolic fractions of the extracts. Ten 2-pound samples of a North Dakota lignite, ground to pass a 200-mesh screen, were extracted in a Soxhlet with methanol. After removal of the solvent from the pooled extracts, 116.5 g of dry material were obtained, which represented 1.28 pct of the whole coal. The dry material was extracted with 2 liters of anhydrous ether and filtered. The filtrate was reduced in volume to 500 ml and was filtered again. The clear filtrate was extracted with three 100-ml portions of 5-pct sodium bicarbonate, and then with three 100-ml portions of 5-pct sodium hydroxide. The two aqueous extracts were acidified by adding hydrochloric acid drop by drop to displace the alkali-soluble materials, which were recovered by first extracting them with ether and then by removing the ether by warming the solution under a stream of nitrogen. During the extraction with aqueous hydroxide, an emulsion formed at the interface. This emulsion and the original material that was insoluble in ether were separated and retained for testing.

Activity of the Concentrates

Measurement of the zones of inhibition of the fractions, which involved use of 1.2 and 0.12 mg of material per filter paper disk (see the section on microbiological methods), showed that the materials soluble in sodium hydroxide were active against the test cultures. The material extracted by aqueous bicarbonate showed little activity; the interface emulsion material showed an activity midway between the two extremes. The material soluble in sodium hydroxide--a hard, dark-brown, resinous mass--was tested quantitatively in the broth assay and was found active against S. aureus at a concentration of 10 μg/ml and against B. subtilis at 5 μg/ml. Because of the greater susceptibility of B. subtilis, it was used in further assays. The activity of these concentrations against test cultures is in the antibiotic range.

Steam Distillation Experiments

Mills[13] reported that steam distillation of a concentrated acetone extract of Tupton coal yielded a yellow oil that was inhibitory to gram-positive bacteria and certain molds. To determine whether similar volatile components might be responsible for the biological activity observed with an extract, steam distillation was conducted on three samples: Colorado whole coal, a montan wax, and active fractions from a North Dakota lignite that were soluble in sodium hydroxide. The distillates were extracted with solvent to obtain the organic matter for testing. These three extracted products are labeled in table 4 as distilled material. With the lignite fraction, the ether solubles in the flask water, and the residue from the distillation also were tested for activity. With the Colorado coal, the residue in the flask was extracted with 1:1 acetone-methanol, and the extract was tested for activity. Finally, an acetone-methanol extract of the original (whole) Colorado coal was also tested. The materials extracted by acetone and methanol apparently were resinous because, after removal of the solvents, the residues were shiny and brittle.

TABLE 4. - Biological activity of fractions obtained by steam distillation of various source materials

Source material	Fraction	Material obtained, μg/g of dry source material	Concentration, μg/ml, showing inhibition
Montan wax.....	Distilled material.....	800	None at 40.
NaOH-solubles from North Dakota lignite.[1]	Flask residue.....	-	10.
	Flask water.....	-	None at 40.
	Distilled material.....	44,000	None at 40.
Colorado (hvb) coal.[2]	Acetone-methanol extract of whole coal.	2,300	20.
	Distilled material.....	100	None at 20.
	Acetone-methanol extract of steam-distilled coal.	2,900	None at 20.

[1] Item 2 of tables 1 and 2.
[2] Item 3 of tables 1 and 2.

Activity of the Distillation Products

The results given in table 4 show that of all the samples only two exhibited biological activity--the acetone-methanol extract of the whole Colorado coal and the residue from steam distillation of the active fraction, soluble in sodium hydroxide, obtained from the lignite extract. The activity of that lignite extract was not decreased by steam distillation. The material obtained from Colorado coal was not typical of the active materials previously tested; it was bacteriostatic rather than bactericidal, and microscopic examination showed abnormal and scanty growth of cells of the test organism.

[13] Work cited in footnote 6.

Biologically Active Materials From Rock Springs Coal

Screening of several coals for biological activity demonstrated that the Rock Springs, Wyo., (high-volatile C) bituminous coal was suitable for obtaining a sample of acetone extract large enough for more detailed study. About 200 pounds of this coal was ground to pass a 200-mesh screen, and one-third of the grind was placed in each of three 55-gallon steel drums fitted with a removable airtight lid. About 30 gallons of acetone was added to each drum, and the resultant slurries were stirred twice daily for a week. After the final stirring, the slurries were allowed to settle for 72 hours. The brown supernatant acetone solution was pumped off, vacuum-filtered through a large Büchner funnel, and collected. Another 19 gallons of acetone then was added to each drum, and the process was repeated. The second acetone extract obtained was combined with the first, and the mixture was then refiltered. The acetone was removed by distillation in batches at 35° C under reduced pressure in a nitrogen atmosphere. When a precipitate appeared in the distillation flask, the contents were transferred to a 4-liter suction flask and the remaining acetone was removed. About 2 pounds of a soft, tarry, dark-brown, viscous material was obtained from the 200 pounds of coal treated. A comparison of analyses of the extracted material and of the original coal is given in table 5.

TABLE 5. - Elemental analysis of Rock Springs, Wyo., coal and its acetone extract

Material	Analysis, percent				
	C	H	N	S	O[1]
Rock Springs coal[2]	79.31	5.41	1.74	0.90	12.64
Acetone extract.....	78.87	9.92	.23	.21	10.77

[1] Oxygen by difference.
[2] Item 4 of table 1.

A 40-gram pilot sample of the extracted material was treated by the process outlined in figure 4. Potency of the various fractions was tested against B. subtilis. As table 6 shows, the active samples were all derived from the fraction of the extract that was soluble in sodium hydroxide.

TABLE 6. - Biological activity of fractions from an acetone extract of Rock Springs, Wyo., coal

Sample[1]	Material obtained, μg/g of dry acetone extract	Concentration of extract showing inhibition, μg/ml
RSE-t.....	-	None at 20.
RSE-o.....	35,000	None at 20.
RSE-2.....	437,500	None at 40.
RSE-3.....	54,000	None at 32.
RSE-4.....	85,500	7.9
RSE-5.....	7,500	[2]8.5
RSE-6.....	5,000	7.2

[1] Samples are identified in figure 4.
[2] Partial inhibition at 4.3 μg/ml was observed with this sample.

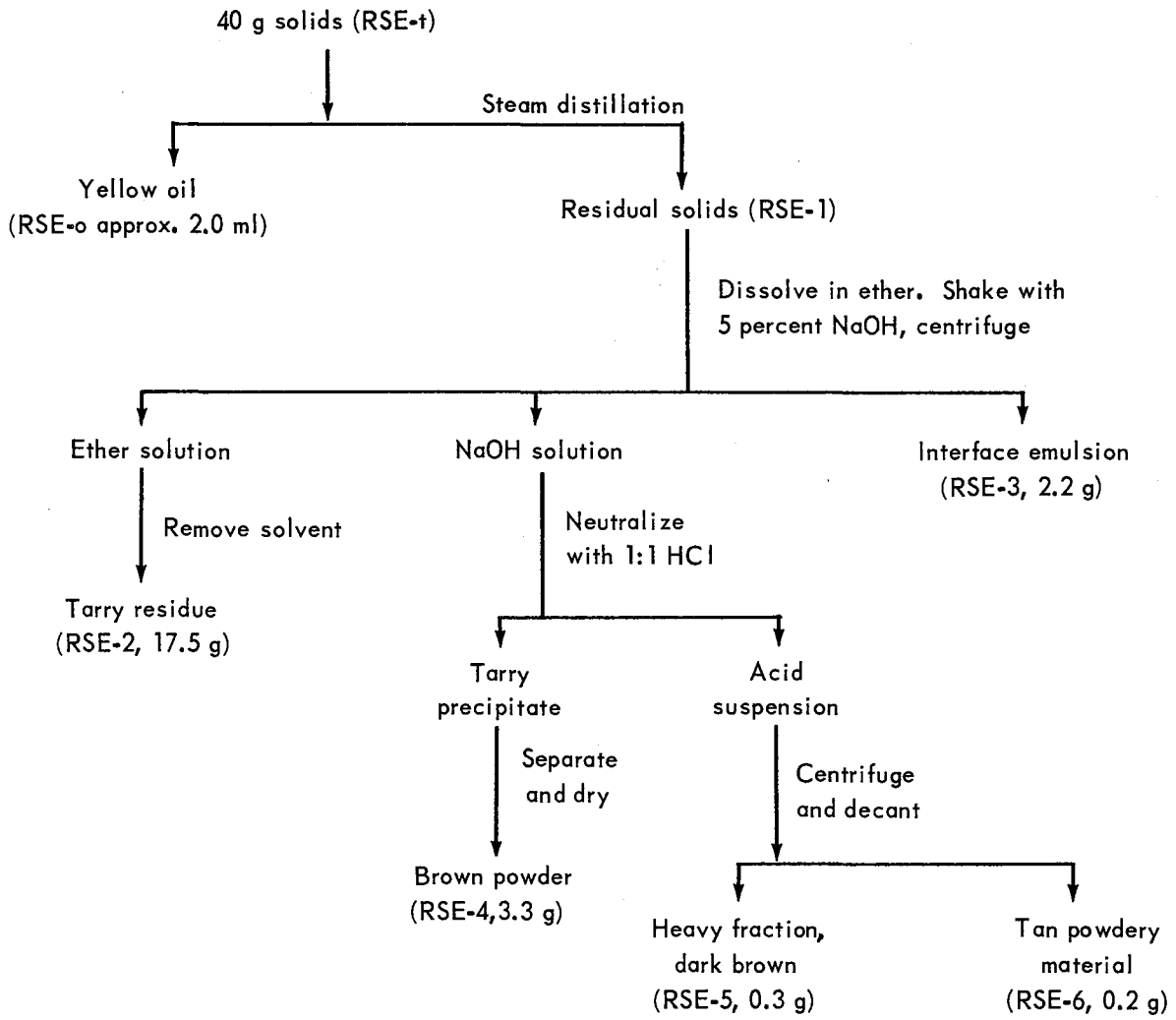

FIGURE 4. - Treatment of Pilot Sample of Acetone Extract of Rock Springs Coal. Designations of sample numbers are enclosed in parentheses.

A 400-gram sample of the extract was then steam-distilled; 14 ml (11.6 g) of a yellow oil was recovered from the distillate by extracting with ether. The oil was separated into its acidic, phenolic, basic, and neutral components by extraction of the ethereal solution with aqueous sodium bicarbonate, sodium hydroxide, and hydrochloric acid, respectively. The only biological activity at low concentration that these extracted materials exhibited was as follows: Partial at 100 μg/ml for the acidic component, and partial at 40 μg/ml for the phenolic component.

A 150-gram sample of the residue from the steam distillation was cooled to -68° C, crushed, and extracted with 10-pct aqueous potassium hydroxide for 5 minutes in a Waring Blendor. Celite was added to the resulting suspension, which was then filtered; more Celite was added to the filtrate and finally removed by filtration. The acidified (1:1 HCl) filtrate was extracted continuously with ether for 16 hours, and the extracted solids were recovered by

removing the solvent. This procedure yielded 6.4 pct of the total solids, a fivefold improvement over extraction of material from ether by aqueous sodium hydroxide. Activity of the extracted fraction against B. subtilis was greatest at 10 μg/ml and with partial inhibition at 5 μg/ml.

A 1.1-gram sample of the material soluble in potassium hydroxide was refluxed for 8 hours with hexamethyldisilazane,[14] $(CH_3)_3SiNHSi(CH_3)_3$, to convert the hydroxyl groups of the active materials to the trimethylsilyl ethers. Residual hexamethyldisilazane was recovered by distillation. The reacted material, when tested against B. subtilis, showed inhibition of the test cultures at a concentration of 40 μg/ml, about one-fourth the activity of the unreacted material. The reacted material was then refluxed for 6 hours with ethyl alcohol to hydrolyze the trimethylsilyl ethers, and heated (54° C) under vacuum to remove hexamethyldisiloxane, alcohol, and water. The recovered solids, when tested against B. subtilis, exhibited their original activity (inhibition at 10 μg/ml). Thus the compounds responsible for the observed biological activity must be mainly those bearing the phenolic hydroxyl group --such compounds would be soluble in potassium hydroxide.

Mass Spectrometric Analysis of the Rock Springs, Wyo., Coal Extract

Mass spectrometry[15] of the total extract from the Rock Springs (hvcb) coal showed peaks at mass units (in order of decreasing intensity) 342, 324, 374, 340, 358, and 392. These results are indicative of compounds containing 6 rings.

To determine whether the compounds obtained by acetone extraction were artifacts, control extractions were conducted in an all-glass apparatus: One on 300 g of coal with 300 ml of acetone as solvent, and another on 300 ml of acetone alone. The extractions were made over a 10-day period; then the solvents were removed from the residual liquids under reduced pressure in a nitrogen atmosphere. Mass spectrometry of the coal extract showed only quantitative differences in the masses found for the larger preparation; qualitatively, the mass distribution was the same as that found previously. No peaks in the 6-membered ring range were found on analysis of the acetone-control residue.

DISCUSSION

The lower rank bituminous coals contain more of the biologically active materials than coals of other ranks. The biological activity of the materials extracted from the various coals was slight; the materials responsible for the activity apparently occur in coal in very small amounts. The active principle(s) must be concentrated before inhibition of bacterial growth can be observed with amounts in the antibiotic range.

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[14] Friedman, S., M. L. Kaufman, W. A. Steiner, and I. Wender. Determination of Hydroxyl Content of Vitrains by Formation of Trimethylsilyl Ethers. Fuel, v. 40, 1961, pp. 33-46.

[15] Mass spectrometric analyses by A. G. Sharkey, Jr., supervisory research physicist, and Janet L. Shultz, mathematician, Pittsburgh Coal Research Center, Bureau of Mines, Pittsburgh, Pa.

No active oils were obtained by steam distillation of the concentrated acetone extracts or of the coals from which they were obtained despite the fact that Mills[16] reported such activity in oil obtained by steam distillation of an extract from Tupton coal. The active principle(s) in the extracts obtained in the present investigation apparently reside in the acidic-phenolic fractions: Concentration by extracting with aqueous sodium hydroxide yielded the most active preparations reported to date. The phenolic nature of the active materials was reflected in the low activity of the preparations when the hydroxyl groups were converted to trimethylsilyl ethers. Hydrolysis of the ethers afforded material with the same activity as the sample before its conversion to the trimethylsilyl ethers.

The acetone extract of Rock Springs, Wyo., coal differs from the parent coal mainly in its high hydrogen content (table 5). Conceivably, acetone could extract the polar compounds from the coal matrix and yield extracted material with an oxygen content higher than the coal, but the ultimate analyses (table 5) show that this is not so.

The mass peaks observed in the low-voltage mass spectrometric analysis represent molecular weights coinciding mainly to 6-ring aromatic systems. Ultraviolet analysis indicated that the extract contained a high percentage of 2- and 3-ring systems, as, for example, biphenanthryl or similarly linked 2- and 3-ring systems. Control experiments demonstrated that the mass peaks observed were not due to compounds derived from polymerization of solvent or those from extraneous sources.

Since such high-molecular-weight compounds should have little solubility in acetone, acetone alone may not be the only solvent involved. On long periods of contact with the coal, certain acetone-soluble materials very likely are extracted that in turn act as solvents for other materials not acetone soluble. Thus extraction proceeds in a continuously changing solvent in contrast to a Soxhlet extraction in which acetone is the lone solvent.

The amounts of biologically active material present in coals are of interest. Calculated on the basis of material soluble in sodium hydroxide, the North Dakota lignite contained 150 μg of active material per gram of coal. The material is inhibitory to test cultures (gram-positive) at a concentration of 5 μg per ml of broth. Evidently, higher concentrations of the biologically active materials occur in coal than are required to inhibit the bacteria under test conditions; thus, these antibiotics may indeed play a role in inhibiting the growth of bacteria in coal at the comparatively high concentrations in which these antibiotics occur, or at least may limit the coal flora to non-susceptible bacterial forms (gram-negative). The same antibiotic substances may well have played an important role in averting the microbial disintegration of the original carbonaceous materials that formed the coal deposits.

The biologically active constituents occur in what are apparently the resinous or waxy ingredients of the coal. Such materials are known to be

[16] Work cited in footnote 6.

resistant to microbial decay. Ferenczy[17] demonstrated that antibacterial substances exist in the seed coats of many plant species; seed coats contribute heavily to the resinous ingredients of coal.

A sample of the antibiotic crude material extracted from Rock Springs coal (see table 5) was analyzed extensively in various paper chromatographic systems by Dr. Werner K. Hausmann at the Lederle Laboratories Division, American Cyanamid Company. In all cases, the active material moved as a single component. It was tentatively concluded that the active substance differs from the presently described antibiotics elaborated by microorganisms. The compound's relationship to antibacterial substances from other natural sources is not known. The concentrate was administered at the maximum tolerated dose to mice infected with S. aureus; the substance had no in vivo activity.

[17] Ferenczy, L. Antibacterial Substances in Seeds. Nature, v. 178, 1956, pp. 634-640.

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