ENVIRONMENTAL LETTERS, 5(4), 237-248 (1973)

ISOLATION OF METAL-BINDING AGENTS FROM COAL DUST AND THEIR EFFECTS ON MITOCHONDRIAL FUNCTION

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ABSTRACT

Bituminous coal samples from mines in Utah and Pennsylvania were extracted with a series of organic solvents, used in order of increasing polarity. The extracted materials, amounting to 58-70 mg/g of Utah coal and 12-15 mg/g of Pennsylvania coal, were each fractionated on a carboxymethyl cellulose column in the Cu (II) form. The copper-complexing fractions for Utah and Pennsylvania were 30% and 22%, respectively, of the total extract. Copper-binding capacity was 400 ug copper/g Utah coal and 40 ug/g for Pennsylvania. The copper-binding ligands from both coals uncoupled oxidative phosphorylation of isolated rat liver mitochondria, a system containing copper and iron metalloenzymes. The inhibitory activity per gram of extracted material was higher for Pennsylvania than Utah. These results suggest

that the extraction and absorption of metal-binding agents from coal may adversely alter vital cellular energetics.

Coal dust and its effects on human health is of concern because of the prevalence of respiratory disorders incurred by coal mine workers[1]. In epidemiological studies it has been found that 10% of working miners showed definite radiological evidence of pneumoconiosis[2,3]. Another survey revealed that nearly 50% of working miners suffered from some degree of dyspnea[3].

Several investigators are studying the effects of coal as a particle. However, in the present study coal dust is being investigated as a carrier of chemicals. Coal dust has been shown to contain significant amounts of heavy metals such as Fe, Ni, Al, Cd, Pb, and many others[4,5]. In this paper we are concerned with the nature of the organic chemicals in coal, particularly in the presence of metal-binding agents. It is our contention that metal-binding ligands in coal (if extractable and absorbed) may interfere with trace metal metabolism by interacting with essential trace metals. In this manner, they may act as metalloenzyme inhibitors and thus may play an important role in health effects such as pulmonary, cardiovascular, neoplastic, and collagen diseases in which alterations of trace metal metabolism have been implicated.

Metal-binding agents have been isolated from tobacco

smoke condensate[6] and from the smoke of other burned vegetable matter[7]. These constituents were found to inhibit several metalloenzymes, i.e., galactose oxidase and catalase[8] as well as to inhibit the in vitro function of copper-and iron-requiring biological systems such as respiratory activity of rat liver slices and uncoupling of rat liver mitochondria (V. Elia, unpublished results).

Therefore, the goal of this study was a) to devise a method for extraction of coal dust; b) to establish whether the extracts contain metal-binding agents; and c) to obtain evidence that these agents may interfere with trace metal metabolism or act as inhibitors of metalloenzyme-requiring biological systems.

Two types of bituminous coal, one obtained from a mine in Pennsylvania and the other from a mine in Utah were examined. The Pennsylvania coal is a harder variety, contains more heavy metals, and causes higher incidence of coal miner's pneumoconiosis in comparison to the Utah coal[2].

EXPERIMENTAL

Materials

Acetone (MCB, reagent) and 4-methyl-2-pentanone (MIBK) Eastman, b.p. 114-116°) were used as received. Carboxymethyl cellulose (Cellex CM) was obtained in the sodium form from Bio-Rad Laboratories. The coal samples were

supplied by the National Institute of Occupational Safety and Health (NIOSH).

Extraction of Coal Dust

The method of extraction selected was an organic solvent procedure beginning with solvent of low polarity and then increasing solvent polarity. The Pennsylvania or Utah coal dust (25g) was placed into a 250 ml centrifuge bottle and 200 ml of the appropriate solvent was added. The extraction was carried out by stirring the mixture with a magnetic stirrer for 24 hours at room temperature. The coal was extracted three times successively with MIBK; MIBK: acetone (1:1); acetone; MIBK: acetone: 95% ethanol (1:2:1); MIBK: acetone: 80% ethanol (1:2:1); and finally with 5% ethanol in water to act as a wetting agent. The organic solvent extracts were combined and the final water extracts were considered separately. The metals were removed from the water extracts by adjusting the pH to 7.0, evaporating the supernatant to near dryness, redissolving in 10% ammoniacal ethanol and passing over a CMC-H^+ column.

Fractionation on Carboxymethyl Cellulose in the Cu (II) Form

The method used was that previously described for fractionation of tobacco smoke condensate and the smoke condensate from lettuce cigarettes [6,7].

Assay for Uncoupling Oxidative Phosphorylation by Isolated Rat Liver Mitochondria

Sprague-Dawley male rats weighing 150-200g were sacrificed by decapitation and the livers were immediately removed, placed in 50 ml of ice-cold isolating medium (0.01M Tris pH7.4, 2mM EDTA, 0.21M mannitol and 0.07M sucrose) and rinsed once with isolating medium. The mitochondria were isolated according to the method of Ozawa et al.[9]. The mitochondrial pellet from half of the liver was carefully suspended in 2.0 ml of respiratory medium (0.3M mannitol, 0.01M KCl, 0.01M Na H_2PO_4, 0.0025M $MgCl_2$ and 0.0002M EDTA) at 0°C.

Oxygen uptake was measured by a Clark type polarographic electrode attached to a YSI Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co.). The effects of the coal fractions on uncoupling were determined by adding 0.2 ml of mitochondrial suspension to 2.6 ml of respiratory medium which was preincubated and saturated with air at 25°C. Then 0.1 ml of 0.11M succinate was added followed by the addition of 5 ul of 0.005M ADP. After respiration returned to the succinate rate, 0.1 ml of a coal fraction was added followed by addition of 5 ul of ADP. The respiratory control index and ADP:O ratios were calculated by the method of Chance and Williams[10].

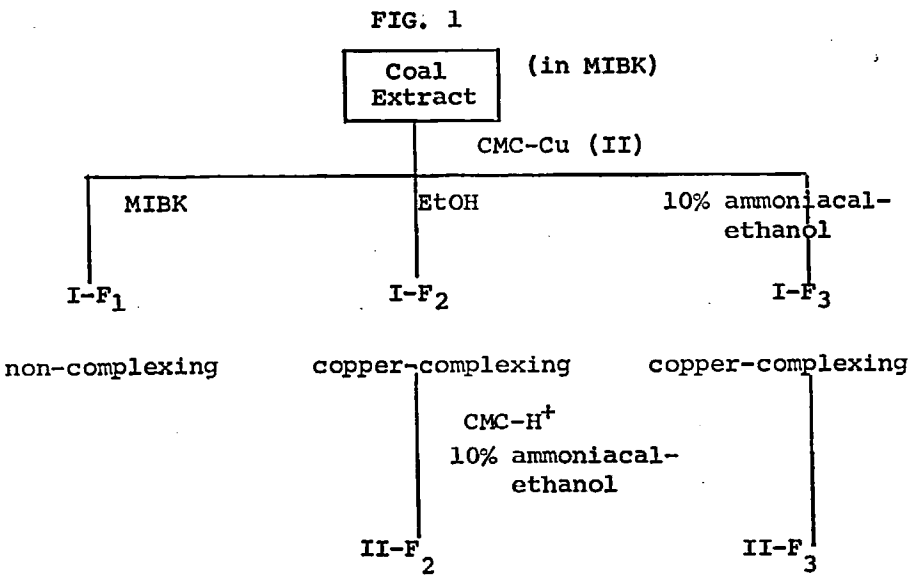
Results

The organic extract from the Utah coal contained material equivalent to 58-70 mg/g of coal, and the Pennsylvania extract contained material equivalent to 12-15 mg/g of coal. The organic extracts were free of metals while the water extracts contained metals.

In order to establish the presence of metal-binding agents in the extracts, the whole organic extract from each coal was chromatographed on carboxymethyl cellulose in Cu(II) form (CMC-CuII). This not only achieved separation of the extracts into non-complexing and copper-complexing fractions, but also provided a measure of the copper-binding ability.

As shown in Figure 1, elution with MIBK gave I-F_1, a

FIG. 1



non-complexing fraction containing hydrocarbons, polynuclear aromatics and weakly binding ligands; elution with EtOH gave I-F_2, a copper-complexing fraction consisting of protonated ligands eluted as non-charged copper-chelates, L_nM; and elution with 10% ammoniacal ethanol gave I-F_3, a copper-complexing fraction made up of stronger binding ligands eluted as ionizable complexes of the type $CuL_n^{+2}{}_{2x}^{-}$. The free ligands were obtained by passing these fractions over a CMC-H^+ column. The copper remains on this column while the free ligands are eluted from the column.

The amount of copper removed by each fraction and the weight of each fraction are shown in Table 1. In the case of the Utah extract, the I-F_1 fraction (42.9 mg) was about 70% of the total extract while the corresponding fraction from the Pennsylvania coal was 78% of the total extract. The total copper binding capacity was approximately 400 ug/g for the Utah extract versus 40 ug/g for the Pennsylvania extract. The weight of material and the copper-binding capacity for the Utah coal is 10-fold greater than that of the Pennsylvania. On the basis of the weight of the extract or fraction however, the copper-binding capacity is nearly the same for both coals (Table 1).

Analysis of these fractions for benzo(a)pyrene demonstrated that B(a)P is present in the I-F_1 fraction and that the amounts in these coals are greatly different. The Utah coal contained 75 times more B(a)P than the Pennsylvania coal (52 ug/g coal and 0.7 ug/g, respectively).

TABLE 1

Copper Removed from CMC-CU(II) Column

Sample	Eluent	Wt. mg/g- equiv.	Cu Removed ug/g equiv.[a]
UTAH:			
Whole extract		64.4	---
I-F_1[b]	MIBK	42.9	6.0
I-F_2	95% EtOH	11.3	244
I-F_2	10% NH_3 EtOH	10.2	128
PENNSYLVANIA:			
Whole extract	---	13.6	---
I-F_1[c]	MIBK	10.6	3.8
I-F_2	95% EtOH	1.8	25.3
I-F_3	10% NH_3-EtOH	1.2	11.7

[a] Corrected for column blank. [b] BaP 52 ug/g equiv.

[c] BaP 0.7 ug/g equiv.

In order to test the effects of the coal extract fractions on the function of a metal-requiring biological system, we have examined their ability to uncouple oxidative phosphorylation of rat liver mitochondria, a system containing copper and iron metalloenzymes. The results presented in Table 2 show that the whole extract, I-F_1, and II-F_2, all completely uncouple at low levels while the II-F_3 gives partial uncoupling at the levels tested - 0.1 - 0.2 g eq./sample. Complete uncoupling is taken to be the same effect as that caused by 1.9 X 10^{-5}M 2, 4 - dinitrophenol (DNP), a known uncoupling agent.

TABLE 2

Effect of Coal Extract Fractions on Uncoupling Oxidative Phosphorylation of Rat Liver Mitochondria

Sample	g equiv./sample	Conc., ug/ml*	uncoupling**
UTAH:			
Whole Organic Extract	0.005	107	+ +
$I\text{-}F_1$	0.01	143	+ +
$II\text{-}F_2$	0.05	188	+ +
$II\text{-}F_3$	0.10	340	+ -
Water (Metal Free)	0.20	101	+ -
PENNSYLVANIA:			
Whole Organic Extract	0.02	91	+ +
$I\text{-}F_1$	0.05	176	+ +
$II\text{-}F_2$	0.10	60	+ +
$II\text{-}F_3$	0.20	80	+ -
Water (Metal Free)	1.0	470	+ -

* Amount of extracted material divided by the 3 ml volume

** + + Complete uncoupling is a reduction of the respiratory control index (RCI) value to 1.0. + - partial uncoupling is a 50% or greater reduction of the RCI in comparison to control value.
- - no uncoupling

DISCUSSION

The data which we have presented establish that extraction of Pennsylvania and Utah coal with organic solvents removes organic chemicals, that part of these agents

are metal-complexing ligands and that the metal-complexing ligands cause an uncoupling of oxidative phosphorylation in rat liver mitochondria.

The most striking differences between the Pennsylvania and Utah coals are that the Utah whole extract and fractions contain 6 - 10 times more material than the corresponding Pennsylvania fractions and that there is a 10 fold difference in the copper-binding capacity between these coal samples.

The biological effects of the fractions from both coals were assessed using uncoupling of oxidative phosphorylation of rat liver mitochondria as the criterion for inhibitory activity. The whole extract and the I-F_1 fraction probably cause blockage of the surface due to the hydrophobic nature of these materials. However, the results with II-F_2 and II-F_3, which are water soluble materials, establish that the metal-binding ligands in these coal samples can adversely affect mitochondrial oxidative phosphorylation. Since the Utah fractions contain about 6 - 10 times more material than the corresponding Pennsylvania fractions and since the doses on a g-equivalent basis required to produce the same effect for both coals were nearly the same, it appears that on a weight basis the Pennsylvania fractions contained the stronger inhibitors. On the other hand, however, it may be that the Utah fractions contained more inactive material.

Metal-binding ligands may act by reducing the absorption and availability of essential metals, increasing the rate of excretion of essential metals or increasing the availability of toxic metals. These agents could cause alteration of the function and/or synthesis of metalloenzymes by acting as substrate competitors or in other ways by interacting with the metal moiety of the enzymes. In addition, chelating agents can react *in vivo* with metals to form toxic chelates.

ACKNOWLEDGMENTS

This study was supported by grants from USPH ES-00159 and NIOSH OH-00355.

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Received August 17, 1973

Accepted August 28, 1973