ENVIRONMENTAL RESEARCH 15, 232–241 (1978)

Coal: Response of Cultured Mammalian Cells Corresponds to the Prevalence of Coal Workers Pneumoconiosis

ROBERT T. CHRISTIAN AND JUNE NELSON

Department of Environmental Health, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45267

Received February 24, 1977

The growth of mammalian cells is inhibited when leachates of coal are incorporated into the growth medium. Coal samples in these experiments are exposed to water, calf serum, or tissue culture medium under physiological conditions of temperature and pH. The coal sample from a mine whose miners had a very high incidence of coal workers pneumoconiosis, "Black Lung Disease," inhibited the growth of cells much more than coal from another mine whose miners showed a much lower prevalence of the disease. The cellular growth inhibition was related linearly to the logarithm of the concentration of the coal leachates. Several metals in the leachate were determined in an attempt to determine the toxic substances leached from the coal.

INTRODUCTION

Coal Workers Pneumoconiosis (CWP) is caused by the inhalation of coal dust (Morgan, 1971) and not by other factors in the miner's environment such as rock dust. Collis (1928) and Gough (1940) showed that coal trimmers working with washed coal in ships developed a similar disease in an environment in which coal was the only common factor. In 1951 Heppleston stated that CWP is distinct from silicosis and postulated that it is caused by a "mechanical overloading of the lung with dust." Two recent monographs support the general belief that coal particles play an important role in the pathogenesis of CWP (Key, *et al.*, 1971 and Selikoff, *et al.*, 1972). Corn (1973) studied the relationship between the particle size of the coal in the mine and the incidence of CWP. The chemical composition, as well as the particle size, was known to differ but the complexity of the chemical composition of the coal made it difficult to study. Two factors, therefore, may be important in the etiology of the disease: size may govern deposition of particles in the lung; and chemicals released from coal particles may be toxic to lung cells.

In order to study the effect of chemicals released from coal we proposed a model in which coal is deposited in the lung as a function of its aerodynamic mass and leached by fluids covering the lung cells. This model depends on the coal remaining in the lungs for a sufficient time to permit leaching. Gibb *et al.* (1975) recently reported that the residence time of coal in animals is surprisingly long so that there may be sufficient time for the chemicals to be released into lung fluids. To simulate the *in vivo* process coal particles were leached at physiological temperature and pH with liquids similar to those in the lung (water serum, and tissue culture medium). The biological effect of these readily leached chemicals was determined in a mammalian cell culture system.

0013-9351/78/0152–0232$02.00/0

Two bituminous coal samples were used in this study, one from a mine in Pennsylvania (PA), where miners exhibited a high incidence of CWP, and the other from a mine in Utah (UT), where the incidence of the disease was much lower (Lainhart, 1969). The coal samples were selected, characterized, and supplied by investigators at the National Institute of Occupational Safety and Health. Samples of these coals are being studied by a number of investigators (Elia, Murthy and Petering, 1973; Nord, and Bingham, 1973; Sorenson, Kober and Petering, 1974; Morman, Lewis and Wagner, 1975) in an effort to elucidate factors in the coal that may be responsible for the difference in the incidence of CWP in miners working in these two mines.

MATERIALS AND METHODS

The coals were micronized to particles of approximately 3 μm in diameter. The gross composition of the micronized Utah (Carbon County) and Pennsylvania (Cambria County) coal samples are found in Table 1. This data was furnished by the National Institute of Occupational Safety and Health.

One g of coal was leached either with 100 ml of double distilled water, fetal calf serum (Grand Island Biological Company–GIBCO), or Eagle's Minimal Essential Medium (MEM) (GIBCO). The liquids containing coal particles and matched controls were held at 36.5°C for 7 days with intermittent shaking. Particulate coal was removed by passing the liquid through a 0.22-μm membrane filter under pressure. The same procedure was used for the matched controls. Complete growth medium was prepared with only one component of the medium exposed to coal, the water, fetal calf serum, or MEM. The aqueous extract was used as the solvent for powdered MEM. Similarly, coal-exposed fetal calf serum was added to unexposed MEM; unexposed calf serum was added to coal-treated MEM. The media for the controls were handled in exactly the same way except that the components were not exposed to coal.

In a preliminary experiment, cells were exposed directly to coal particles to determine the effect of coal particles on cell growth. Sterilized coal particles (1 g of coal in 100 ml of medium) were suspended in freshly prepared MEM containing 1% fetal calf serum. Cells were added to the coal medium mixture and grown in

TABLE 1
GROSS COMPOSITION OF COAL SAMPLES

	Utah (Carbon County) (percent)	Pennsylvania (Cambria County) (percent)
Moisture	2.2	0.6
Ash	8.0	17.8
Sulfur	0.9	3.2
Hydrogen	5.6	4.0
Carbon	72.9	70.7
Oxygen plus nitrogen	11.0	3.7
Volatile matter	36.3	17.2
Free silica	0.5	4.0

culture for six days. Medium was renewed by removing the medium–coal mixture and adding freshly prepared MEM containing 1% coal. Cell growth was determined by removing the media, washing the cells, and measuring total protein.

Cell Growth

Earle's L-cells (Clone 929) were grown for six days in the medium containing 1% fetal calf serum. The medium was renewed twice during this period. At four points, six cultures from each series were removed, washed, and total protein was determined by the Lowry technique (Oyama and Eagle, 1956). Total protein was the routine method used to determine growth. However, cell number, cell size, and DNA have been used in these and similar experiments. These different measurements gave similar results but total protein was employed for convenience. Growth was measured as the increase in total protein and plotted as a percentage of the maximum protein concentration in the experiment. The percentages were transformed using the arc sine transformation and regressed against time.

Determination of Metals

Control medium and medium exposed to coal were analyzed for Ni, Zn, Cu, Fe, Mg, Ca, and Mn by atomic absorption spectrophotometry. The difference in metal concentration between control and coal-exposed medium was assumed to be the amount leached from the coal.

RESULTS

Coal Particles

Because coal particles are presumably the cause of CWP in miners, the first experiment was designed to determine the effect of particles of the two coals on growth of mammalian cells in culture. One g of PA or UT coal particles was added to 100 ml fresh MEM and placed directly on the cells. The coal particles completely covered the cell sheet and depressed the growth of the culture. The PA coal depressed the growth to a greater extent than the UT coal. Since the medium leached the chemicals from the coal during the six-day growth period, the effect of particles and the effects of leachates could not be differentiated. Therefore, this procedure was unsatisfactory. In subsequent experiments, the coal particles were removed by filtration and only the effects of the leachates were determined.

Leachates of Coal

The water, serum, and MEM leachates of both PA and UT coal depressed the growth of cells. The PA leachates depressed growth to a greater extent than UT coal leachates (Figs. 1 and 2). It is interesting that the results from a cell culture system paralleled the epidemiological evidence of CWP exhibited in the miners. Leachates of coal from the mine showing the greater incidence of CWP in the miners were the most cytotoxic. The results were qualitatively similar whether coal was leached with water, serum, or MEM or when the experimental design was varied. This was a consistent finding. Serum appeared to extract more inhibitory chemicals than either water or MEM because serum comprises only 1% of the growth medium while water and MEM comprise approximately 95 and 99% of the growth medium, respectively.

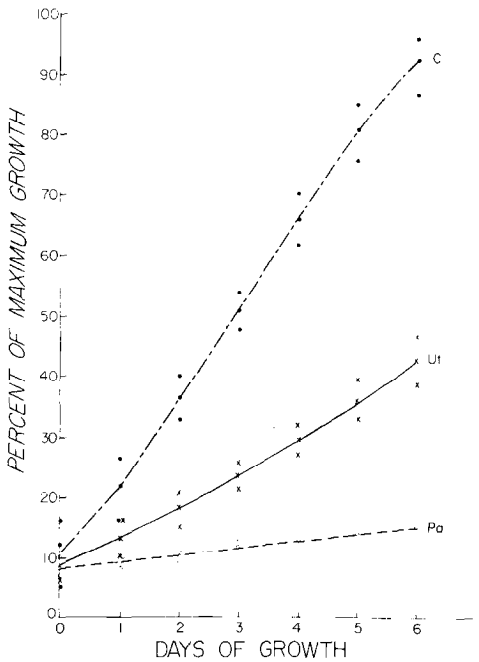

FIG. 1. Growth curves for L-cells exposed to distilled water leachates of coal particles (1 g of coal/100 ml of distilled water). Growth was measured as the increase in total protein and plotted as a percentage of the maximum protein concentration. The percentages were transformed using the arc sine transformation and regressed against time.

Dose Response

Response curves were plotted in order to more clearly define some of the properties of substances leached from coal. The MEM leachates of PA coal inhibited cell growth to the greatest extent, so dose response relationships were determined with this leachate. The leachate was serially diluted (1 ml of leachate in 9 ml of control medium). The rates of cellular growth in undiluted, 1/10 and 1/100 dilutions of the leachate gave a dose–response relationship (Fig. 3). The growth in the 1/1000 dilution of the leachate was the same as control growth, while the growth for the 1/10,000 dilution was slightly greater than that of controls. One possible explanation for this stimulation of cell growth is that MEM containing 1% fetal calf serum is a minimal growth medium for L-cells. It is possible that substances leached into medium can be utilized by cells to enhance their growth. The substances leached from coal may be trace metals and other inorganic and organic chemicals. Trace metals have been shown to be necessary for the growth of L-cells (Thomas and Johnson, 1967).

A dose–response curve was obtained for this data by plotting the average protein on the sixth day of growth for the dilutions as a percent of the control growth. As shown in Fig. 4A, there is an approximately linear relationship for the undiluted, 1/10, and 1/100 dilutions of the extract. The dose–response relationship makes it possible to determine the contribution of the components of the leachate to the toxicity of the complete leachate.

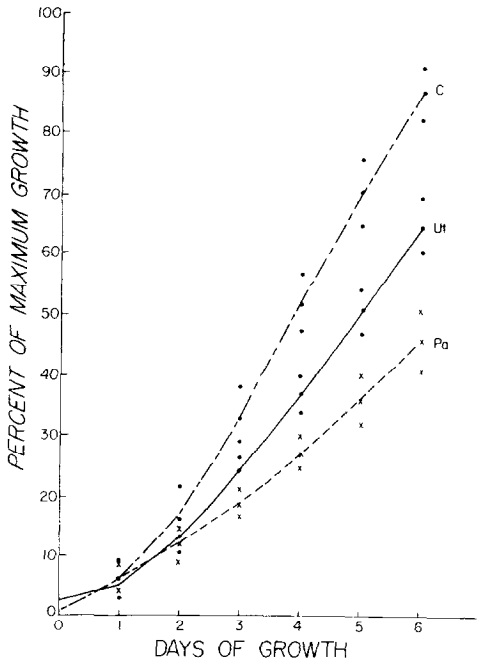

FIG. 2. Growth curves for L-cells exposed to serum leachates of coal particles (1 g of coal/100 ml of fetal calf serum). Growth was measured as the increase in total protein and plotted as a percentage of the maximum protein concentration. The percentages were transformed using the arc sine transformation and regressed against time.

Metals

The first components of coal leachates investigated were metals. Nord and Bingham (1973) reported that coal contains appreciable quantities of metals and suggested that certain metals found in coal might contribute to the pulmonary response to coals. The metals of interest are those that may be easily leached from coal since these are the ones that would be expected to be leached from the coal particles in the miners' lungs. Metals that are found in high concentrations in coal and are of known biological significance were determined in the leachate (Table 2). The metals calcium, copper, iron, magnesium, maganese, nickel, and zinc were determined by atomic absorption spectrophotometry. Because MEM and fetal calf serum contain low concentrations of some of these metals and others may be added to the leachates during the exposure and filtration process, the concentration of these metals in control media, treated in the same manner except for the exposure to coal, were compared to the concentrations found in the media used in leaching the coal particles. The difference between these concentrations was assumed to be contributed by the coal.

Analysis of the concentration of the metals in the coal and in the medium used to leach the coal reveals that PA coal contains more nickel (Ni) and iron (Fe) than the UT coal but the Fe was not easily leached from the PA coal. The Ni on the other hand was leached into the medium and appears in much greater concentration in the PA leachate than in the UT leachate.

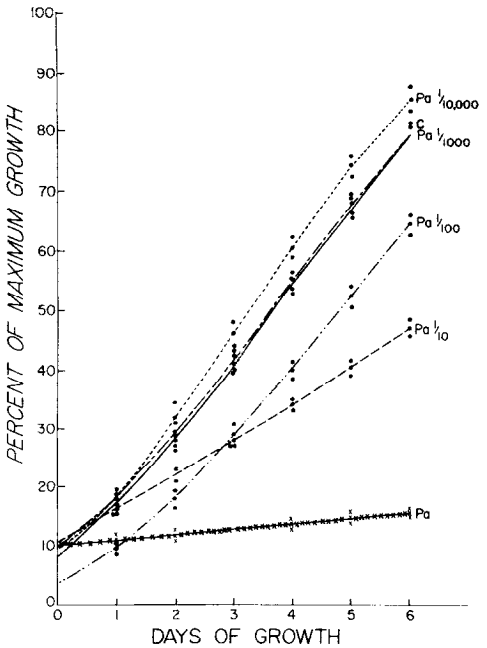

FIG. 3. A family of growth curves for L-cells grown in various dilutions of medium exposed to coal.

In an attempt to determine how much of the cytotoxicity of the PA coal leachate may be due to Ni, a dose–response curve for $NiCl_2$ was compared to the dose–response curve for PA coal leachate (Fig. 4A). A comparison of dose–response curves for Ni (as $NiCl_2$) and the PA coal leachate plotted on its Ni content are shown in Fig. 4B. The difference between these dose–response curves was analyzed by parallel line bioassay analysis (Finney, 1952). The results indicate that 2.7 (95% confidence limits 2.0 to 3.5) times as much Ni would have to be present in the coal leachate for Ni to be responsible for the cytotoxicity exhibited by the leachate. These results are based on the assumption that the chemical form of Ni in the leachate is equivalent in toxicity to that of $NiCl_2$. If this assumption is correct, other organic and/or inorganic components in the coal contribute to its cytotoxicity.

DISCUSSION

Our hypothesis is that chemicals leached from coal particles in the miners' lung are responsible, at least in part, for coal workers pneumoconiosis. The etiology of this, as well as other chronic human diseases, has proved quite difficult to determine. The first objective of this report is to determine the effect of coal on mammalian cells in culture. The second objective is to show the usefulness of these *in vitro* methods in determining the biological effect of environmental substances such as coal.

This work has shown that substances can be leached from coal under physiological conditions and that these leachates inhibit the growth of mammalian cells in culture. These coal leachates invariably inhibit the growth of cultured cells

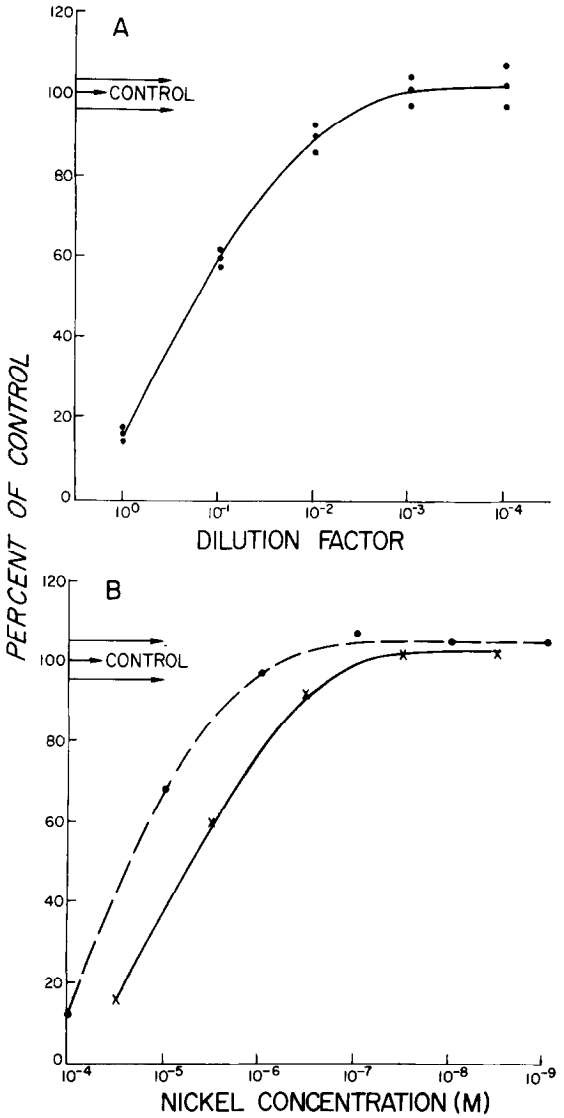

FIG. 4. Dose–response curves of the average growth based on protein synthesis and plotted as a percentage of average control growth where the average control growth was adjusted to 100%. (A) Dilutions of the media extracts of Pennsylvania coal. (B) The broken curve represents the response to several concentrations of nickel chloride. The solid line is the Pennsylvania coal extract plotted on the nickel concentration of each dilution.

whether the cells are the continuous line mouse cells reported here, normal human lung cells (WI-38) or primary human (amnion) cells in their first passage *in vitro*. The inhibition is seen from early logarithmic phase (two days of growth) through stationary phase (14 days of growth). Growth is inhibited by the coal leachates whether the growth is measured by total protein (as reported here), cell number, or DNA. Therefore, growth inhibition of cultured cells by leachates of coal is a

TABLE 2

ANALYSIS OF METALS LEACHED FROM COAL

Sample	Ni	Zn	Cu	Fe	Mg	Ca	Mn
Media[a]							
Control	<0.54 (<0.05–<1.0)	0.04 (0.02–0.06)	0.32 (0.20–0.48)	<0.21 (<0.1–<1.0)	21.1 (18.0–26.5)	57.14 (50.0–70.0)	<0.04 (<0.01–0.07)
1% PA[b]	2.86 (0.2–8.2)	0.05 (0.03–0.07)	0.33 (0.04–0.5)	<0.23 (<0.1–<1.0)	22.1 (20.0–28.0)	57.4 (52.0–70.0)	<0.05 (0.01–0.1)
1% UT[b]	<0.41 (<0.05–<1.0)	0.05 (0.03–0.06)	0.29 (0.26–0.32)	<0.33 (<0.1–<1.0)	20.75 (17.0–25.0)	57.25 (50.0–65.0)	<0.03 (0.01–0.06)
Coal[c]							
PA	1000	19	7.5	24,800	550	1,200	22
UT	140	10	23.5	3,100	610	5,300	25

[a] These metal concentrations are reported as microgram per milliliter of media and are the averages of seven replicates for control and 1% PA and four replicates for 1% UT with the ranges of values in parenthesis.

[b] One g of coal was leached with 100 ml of media for seven days at 36.5°C.

[c] The concentration of metals in the coal are expressed as microgram per gram of coal. The data were taken from Sorenson *et al.* (1974).

consistent finding in this laboratory. It is not surprising that mammalian cells react to toxic substances. The mammalian cell is not a simple biological system. It is a complex feedback system representing the basic unit of life. It is the smallest self-sustaining part of the whole animal. A basic reproducible reaction of this complex unit to a toxic substance may be similar to the initial lesion in the cells of the human lung which initiates the disease.

The PA coal is from a mine whose miners had a very high incidence of CWP. Leachates of this coal completely stop the growth of the cells. Even a 1/10 dilution of this leachate depresses the growth below 50% of the growth of the controls and a 1/100 dilution still depresses the growth of the cells. This leachate is very toxic in the sensitive cell culture bioassay system. The UT coal, on the other hand, is from a mine where CWP is not a significant problem. While it depresses the growth of the cells it appears to be an order of magnitude less toxic to the cells than the PA coal leachate. The parallelism between the human epidemiology and the biological reaction in cultured fibroblasts is very interesting and could have very important implications. Further studies are needed to determine the relevance of this observation to the natural disease.

The two coal samples were chosen by investigators at National Institute of Occupational Safety and Health on the basis of the prevalence of the disease in miners working those mines. The samples were then collected, micronized, and distributed to a number of interested investigators. They have maintained an adequate supply of these coals for reference and future studies. Studies that have been carried out on these samples include: metal content (pulmonary function changes); inhalation toxicology; and effect on macrophages. These two coal samples are, therefore, better characterized than most samples of environmental interest. There is a need for detailed studies of other coal samples but the samples cannot be compared to the PA and UT coal samples used in this study unless the incidence of CWP for the miners who worked in the mine, as well as other critical

chemical and physical data, are obtained. Preferably, these studies would be conducted on coal samples obtained in mines where the miners demonstrate an intermediate prevalence of CWP. These results may then be compared with the results presented here on coal associated with high and low incidence of the disease. The cell biologist can do little on this problem until other well-documented coal samples become available.

Complex substances, such as coal, are a part of the man's environment but are very difficult to study because of their complexity. However, the biological effect of these types of materials may be studied by the *in vitro* cell culture system described in this paper. The sensitivity and reproducibility of the cell culture system was demonstrated by studies on dilutions of the MEM leachates of PA coal. The dose–response curve generated from these experiments indicates that there is a straight line relationship between concentration of leachates and biological effect. This relationship suggests that the cell culture system may be used to indicate the toxicity of the leachate and the toxicity of chemical fractions of the leachate. Using a scheme in which the leachate is fractionated by chemical techniques and the biological activity of the fractions determined, the fraction that shows the greatest biological activity can be refractionated until the compounds that are responsible for the biological effect are identified.

The first attempt to identify the active agents in these coals was the determination of the biological effect of Ni present in the PA coal in a much higher concentration than in the UT coal. It was also leached into the MEM efficiently. The dose–response curve for Ni as $NiCl_2$ indicates that Ni may contribute to the cytotoxicity of the leachate, assuming that the chemical form of Ni in the leachate is equivalent to the toxicity of $NiCl_2$. If this assumption is correct, other organic and/or inorganic components in the coal contribute to its cytotoxicity. A chemical fractionation scheme has been adapted from Elia *et al.* (1973) for the isolation of organic components of coal. These fractions will be tested in the cell culture bioassay system and will be reported in future communications.

The *in vitro* techniques presented in this paper appear to be useful in the study of the cytotoxicity of these two different coals. They may be useful in the study of other toxic mixtures in man's environment.

ACKNOWLEDGMENTS

We thank the late Dr. Lester Scheel of NIOSH for his helpful suggestions throughout the project, Mr. Sylvan Witherup for his assistance with the statistical data, Mr. Dominik Schneider for his excellent technical support and to Dr. Peter Gartside for statistical analysis. Our particular thanks to Dr. Victor J. Elia for editing this manuscript.

This study was supported in part by grants from National Institute of Occupational Safety and Health No. OH 00356 and National Institute of Environmental Health Sciences No. ES 00159.

REFERENCES

Collis, E. L. (1928). Effects of dust upon coal trimmers. *J. Ind. Hyg.* **10**, 101–110.

Corn, M., Stein, F., Hammad, Y., Manekshaw, S., Bell, W., and Freedman, R. (1973). Coal mine dust characterization for health related investigations. *Amer. Ind. Hyg. Assoc. J.* **34**, 279.

Elia, V. J., Murthy, L., and Petering, H. G. (1973). Isolation of metal binding agents from coal dust and their effects on mitochondrial function. *Environ. Lett.* **5**, 237–248.

Freeman, R., and Hartstein, A. M. (1973). Physical and chemical properties of respirable coal dust from two U. S. mines. *Amer. Ind. Hyg. Assoc. J.* **34**, 279–285.

Finney, D. J. (1952). "Statistical Methods in Biological Assay." Hafner Press, New York.

Gibb, F. R., Beiter, H., and Morrow, P. E. (1975). Studies of coal dust retention in the lungs utilizing neutro-activated coal. U. S. Atomic Energy Report No. V, 3490–679.

Gough, J. (1940). Pneumoconiosis in coal trimmers. *J. Pathol. Bacti.* **51**, 277–285.

Heppleston, A. G. (1951). Coal workers' pneumoconiosis. *Arch. Ind. Hyg. and Occup. Med.* **4**, 270–288.

Key, M. M., Kerr, L. E., and Bundy, M. (Eds.) (1971). "Pulmonary Reactions to Coal Dust." Academic Press, New York.

Lainhart, W. S. (1969). Roentgenographic evidence of coal workers' pneumoconiosis in three geographic areas in the United States. *J. Occup. Med.* **11**, 399–408.

Morgan, W. K. C. (1971). Coal workers' pneumoconiosis. *Amer. Ind. Hyg. Assoc. J.* **32**, 29–34.

Morman, W. J., Lewis, T. R., and Wagner, W. D. (1975). Maximum expiratory flow-volume studies on monkeys exposed to bituminous coal dust. *J. Appl. Physiol.* **39**, 444–448.

Nord, P. J., and Bingham, E. (1973). Biological availability of certain metals in coal. *In* "Trace Substances in Environmental Health" Vol. VI. (D.D. Hemphill, Ed.), University of Missouri, Columbia. pp. 341–345.

Oyama, V., and Eagle, H. (1956). Measurement of cell growth in tissue culture with a phenol reagent (Folin-Ciocalteau) *Proc. Soc. Exptl. Biol. Med.* **91**, 305–307.

Selikoff, I. J., Key, M. M., and Lee, D. H. K. (Eds.) (1972). Coal workers' pneumoconiosis. *In* "Annals of the New York Academy of Sciences," Vol. 200. New York Academy of Sciences, New York.

Sorenson, J. R. T., Kober, T. E., and Petering, H. G. (1974). The concentration of Cd, Cu, Fe, Ni, Pb, and Zi in bituminous coals from mines with differing incidences of coal workers pneumoconiosis. *Amer. Ind. Hyg. Assoc. J.* **35**, 93–98.

Thomas, J. A., and Johnson, M. J. (1967). Trace-metal requirements of NCTC clone 929 strain L-cells. *J. Natl. Canc. Inst.* **39**, 337–345.