methods also stained small clumps of material arranged in a concentric pattern around the nucleus, as did AF. The periodic acid–Schiff reaction gave only a faint generalized pink cast to the cytoplasm. Sudan Black B (saturated solution in 70 percent alcohol) demonstrated bound lipid in coarse droplets of variable size sparsely distributed throughout the cytoplasm, but most often clumped at one pole of the neuron. The pattern of staining by these methods was distinctly different from that of AF in alternate serial sections, and was observed as often in normal ganglia. In a few cells, the pattern of Sudan Black B staining resembled that seen with AF, but the number of cells stained were so few compared to the number of AF-positive cells that we could not conclude that the same structures were stained. Neurons in sections in which the oxidation with sulfuric acid and potassium permanganate was omitted were not stained with AF (although the melanin in pigment cells in these ganglia stained with or without prior oxidation).

In the blastema, regenerating nerve fibers stained homogeneously with AF (Fig. 1B). These delicate, wavy fibers, approximately 0.3 μ in diameter, were found among the mesenchymal cells beyond the original level of transection of the peripheral nerve. On occasion, they could be traced from the blastema into the epidermis or proximally to the large nerve bundles. Slight bulbous enlargements were observed at the ends of some of these fibers, probably representing end bulbs or growth cones (Fig. 1B). Staining of the regenerating fibers was observed at 14 to 28 days after amputation or nerve deviation. No staining was detected within the fibers of the larger peripheral nerves proximal to the level of transection. Connective tissue fibers stained with AF were seen in the blastema, especially surrounding arteries and muscle bundles. These could be distinguished from nerve fibers as they occurred in a branching and anastomosing network composed of unevenly stained bundles of variable size. Nerve fibers, in contrast, were uniformly small and evenly stained along their length; they occurred singly, pursued a wavy course, and could often be traced to their origin from the large peripheral nerves containing myelinated fibers.

The electron microscope revealed large membrane-bounded granules in sensory ganglia and within nerve fibers in the blastema during limb induction. In ganglion cells a few granules were found throughout the perikaryon and associated with the Golgi complex (Fig. 1C), and were most numerous in the region of the axon hillock and proximal portion of the axon. In the blastema, the end bulbs of the regenerating nerve fibers contained many granules (Fig. 1D), whereas proximal regions had few. The granules were large, averaging 1700 Å in diameter (range 1000 to 2500 Å), and were composed of moderately dense material. Granules of this size were rarely found in normal ganglion cells or normal peripheral nerves, although smaller dense vesicles occur. The granules observed during limb induction were identical to those described in regenerating nerves (*8*) which were distinguished from dense-core vesicles containing catecholamines.

Secretory activity of neurons apparently changes during limb regeneration or induction. Because this change in secretory activity occurs during a physiological process in which a hormonal substance is believed to function, it could reflect the formation and transport to the peripheral tissues of a trophic substance. This process occurs in neurons heretofore not considered capable of hormonal activity.

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Alveolar Macrophages: Reduced Number in Rats after Prolonged Inhalation of Lead Sesquioxide

Abstract. *A decreased number of alveolar macrophages was found in washings from lungs of rats inhaling small particles of lead sesquioxide for 3 to 12 months, as compared with control animals exposed to filtered air. This result contrasts with that reported by others for animals given massive exposures to various dusts for short periods of time. Because the concentrations of lead were comparable to those observed in some industrial (150 $\mu g/m^3$) or urban (10 $\mu g/m^3$) environmental conditions, the results may be significant in terms of human lung clearance processes after such exposures.*

Phagocytosis by alveolar macrophages is considered to be an important step in removal of dust particles or bacteria from the respiratory tract. For this reason the number and activity of alveolar macrophages are thought to be important aspects of pulmonary defense. The technique of harvesting alveolar macrophages from excised mammalian lungs has been used to obtain a quantitative estimate of the number of these cells (*1, 2*). With this technique we have demonstrated significant decreases in the number of alveolar macrophages that were washed from the lungs of rats after prolonged inhalation of low concentrations of very small particles of lead sesquioxide.

In contrast, LaBelle and Brieger (*2*) found that intratracheal injection of carbon particles caused a tenfold increase in the number of cells they obtained with lung washings. After termination of the exposure the rate of clearance of particles was proportional to the number of alveolar macrophages recovered in other comparable animals. Other investigators (*3, 4*) have reported obtaining increased numbers of alveolar macrophages after massive exposures to dusts for short periods of time, and accelerated clearance of dust from the lungs of animals similarly exposed.

Young adult male and female rats (Controlled-Flora, Greenacres Farm) were exposed continuously (24 hours per day) in three separate chambers (one a control chamber) to different concentrations of lead aerosol for periods up to 1 year. The particulate lead compound was generated by burning

vapor of commercially pure tetraethyl lead with natural gas. The desired concentrations of lead in the atmosphere were obtained by controlling the volume of vaporized tetraethyl lead introduced into the burner. The combustion products (tetraethyl lead was not among those in detectable quantity) were diluted with filtered air and passed into inhalation chambers that were housing the rats.

The particulate material in the inhalation chambers was identified as lead sesquioxide (Pb_2O_3) by x-ray diffraction analysis. Particle size distribution, determined from electron photomicrographs of the particles collected by an oscillating-wire thermal precipitator, showed a median size of 0.06 μ with 99 percent of the particles (by count) less than 0.1 μ. The mass median aerodynamic size was 0.18 μ as measured by the Goetz aerosol spectrometer.

Chemical analysis for lead, based on atomic absorption spectrophotometry, was conducted regularly on samples collected by filtration of the air through Millipore filters. The concentrations of lead sesquioxide in the inhalation chambers [rooms 7.5 by 12 by 10 feet (2.3 by 3.7 by 3 m)] averaged approximately 150 and 10 μg of lead per cubic meter of air for the two test atmospheres. The standard error of the average concentration of lead in these two rooms was $\pm$ 15 percent. In the control chamber, ambient air was filtered and analyses showed less than 0.2 μg of lead per cubic meter of air. In the control chamber lead was probably present in forms other than the sesquioxide.

The method for obtaining alveolar macrophages was originally described by Gersing (*1*) and used extensively by LaBelle and Brieger (*2*). We have used a modification of this procedure, described by Brain and Frank (*5*), in which several washings with isotonic saline solution are used. Aliquots of the washings were counted in hemocytometers to obtain the total number present. We have washed the lungs repeatedly for nine times rather than twelve, as done by Brain and Frank. In our experience, the rate of recovery of cells has decreased sharply by the ninth rinse, and the final results are not significantly changed by omitting the subsequent washes. In addition, we determined the proportions of various types of cells from slides prepared by combining the washings and centrifuging them. A smear of the cell button was air-dried, fixed in Bouin's solution, and stained with hematoxylin and eosin.

Table 1. Number of alveolar macrophages (in millions of cells per gram of lung) washed from lungs of rats exposed to inhalation of particles of lead sesquioxide. Each value is the mean and standard error for the group. The number in parentheses below each value is the number of rats in each group.

Time of exposure (mo.)	Concentration in air ($\mu g/m^3$)		
	0.2	10	150
3	3.6$\pm$0.27 (14)	1.5$\pm$0.20 (5)	1.5$\pm$0.19 (5)
6	3.2$\pm$0.15 (15)	1.7$\pm$0.18 (5)	1.2$\pm$0.14 (11)
12	3.2$\pm$0.25 (12)	1.1$\pm$0.16 (11)	1.3$\pm$0.10 (9)

Table 1 gives the mean and standard error of the number of cells obtained by washing after 3, 6, and 12 months of exposure in the control and two test chambers. The number of cells obtained from the lungs of rats breathing the control atmosphere remained fairly constant at about 3.3 million per gram of lung (*6*). These control results do not differ from the value of 3.4 million per gram of lung for 3-month-old rats of this strain kept in the usual animal quarters in the laboratory, where the average atmospheric lead concentration is generally 1 $\mu g/m^3$. The lungs of rats inhaling the lead sesquioxide aerosol (10 and 150 $\mu g/m^3$) yielded alveolar macrophages in numbers significantly lower than the control groups ($t < .01$) after 3, 6, and 12 months of such exposure. The decrease of approximately 60 percent was about the same at both concentrations of lead, and there was no further reduction after 3 months' exposure. Preliminary studies now under way indicate that less than 1 week of exposure to the higher concentration (150 $\mu g/m^3$) is required to cause this maximum reduction.

The morphology of cells washed from the lungs of the control rats was quite similar to that described for unexposed animals by Collet *et al.* (*7*). Approximately 55 percent of these were typical macrophages (10 to 15 μ), each with a well-defined eccentric nucleus (occasionally two or three nuclei) and a granular, eosinophilic, abundant cytoplasm. The remaining 45 percent consisted of smaller cells (5 to 10 μ) containing very little cytoplasm. Most of these cells appear similar to lymphocytes, while some have irregular nuclei and more closely resemble polymorphonuclear leukocytes. Collet and coworkers believe that these smaller cells are the precursors of the mature, functional alveolar macrophage.

The population of cells washed from the lungs of rats subjected to the inhalation of particles of lead sesquioxide, in addition to being reduced in number, was predominantly (85 percent) of the type that contained abundant cytoplasm. The cytoplasm of these cells frequently contained large, pale-staining vacuoles, and often the nucleus took the stain poorly. Fifteen percent of the cells were the smaller cells with reduced cytoplasm. Occasionally a cell with an irregularly shaped nucleus was seen.

If the hypothesis of Collet *et al.* is accepted, then one may speculate that the reduction in the proportion of small cells in the total population reflects an accelerated development of functional phagocytic cells, and consequently a drain on the precursor cells. The reduced number of total cells may be the result of a new equilibrium between cell production and cell removal. It is also possible that there is greater fragility of the alveolar macrophages and increased susceptibility to lysis during washing, as a result of phagocytosis of the particles or from a direct effect of lead on the cell itself.

The concentrations of lead particles to which the animals in the two test groups were exposed in these experiments (10 and 150 $\mu g/m^3$) have special significance because they are within the range encountered in certain environmental situations. The concentration of lead in air near certain industrial operations often will be as high as 150 $\mu g/m^3$ throughout the working hours (*8*). The threshold limit value established for inorganic lead by the American Conference of Governmental Industrial Hygienists (*9*) is 200 $\mu g/m^3$ for daily exposure during a 40-hour week. The concentration of lead in ambient air of the urban environment is reported to average 0.8 $\mu g/m^3$, although certain localities on occasion will reach 10 $\mu g/m^3$ (*10*).

The significance of our results is not yet clear, primarily because it is not known whether changes observed reflect a change in the capability of lungs to deal with dusts or inhaled bacteria and viruses. The reports of other studies with acute exposures (*1–3*) suggest that decreased numbers of phagocytes may result in a reduced rate of pulmonary clearance of particles. Comparison of our data with previous reports (*2*) indicates that the effect on macrophages may be specific for lead; however, it is possible that the response

is nonspecific and that it occurs as a result of inhalation of very small particles.

The extent to which this biological response is reversible will be an important consideration in determining the potential hazard from long-term exposure. In addition, the development of a dose-response relationship at some lower levels and duration of exposure will serve to verify the public health significance of this study. Further work is needed to settle these questions.

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Operant Controlled Neural Event: Formal and Systematic Approach to Electrical Coding of Behavior in Brain

Abstract. *Traditional studies of electrophysiological correlates of behavior contain inherent high variability resulting from the arbitrary choice of behaviors, brain locations, and wave parameters. The operant control of neural events is a formal and systematic approach to the study of prespecified parameters and components of brain activity as they encode behaviors. Two studies in which the electrical activity of brain was the criterion for reinforcement demonstrate the acquisition, under such operant control, of two mutually exclusive behaviors or states which selectively alter evoked potential components.*

The purpose of this report is to raise some reservations regarding traditional approaches to the study of neural correlates of behavior and to offer a strategy for investigating behaviorally relevant bioelectrical activity of brain. Electrical activity of the brain has been of continuous interest in studies of neural correlates of behavior, especially learning (*1*, *2*), in which bioelectrical activity of brain in the form of cortical evoked potentials, single cell discharge, spontaneous electroencephalogram, d-c shifts, impedance changes, and the like has been related to changes in behavioral state accompanying learning or differential performance.

Although a variety of such changes has been described, replicability has been a recurrent problem, and the variable, transient, unreliable, and arbitrary character of such responses is well recognized (*1*); this character is consistent with any arbitrarily selected collateral response system (heart rate changes, galvanic skin response, and other).

We are concerned with the apparent simplicity of these studies and feel that reexamination of such correlative paradigms leads to the conclusion that the understanding and control in such experiments may be less than is believed, for a number of reasons as follows.

1) The inherent variability of spontaneous behavior contributes substantially to the variability of the bioelectric response to a so-called "neutral" stimulus, resulting in less than complete confidence in the knowledge of the conditioned stimulus.

2) The choice of a given behavior is arbitrary in reference to a chosen brain location. The fact that electric responses to stimuli can be recorded from many widely separated areas of the brain has encouraged the placement of large arrays of electrodes to compensate for the lack of specific information by force of number of placements in locating brain responses that may be relevant to the specific behavioral paradigm.

3) Transient responses may be expected, and the process of establishing a particular functional connection may at some specific time in training be considered complete. Continuation of the behavioral task may depend upon processes not necessarily occurring at the original location. Thus, the component nature of complex behavior may be both multiple and sequentially represented in brain.

4) The arbitrary choice of behavior also takes into account in acquisition or performance only one or a few endpoint responses, and not the infinitely complex and unknown (possibly not parallel in time) set of collateral responses that are occurring in the conditioning paradigm (*3*) and that have unknown individual and conjoint influences on the bioelectric response. Most important is that behavior is rarely described accurately on a time base comparable to the base used to describe the momentary fluctuations in excitability as reflected in the bioelectric response. Correlation of momentary and discrete bioelectric events, therefore, with multiply determined molar behaviors may be in error by one or more orders of magnitude (days compared to milliseconds).

5) Bioelectric response parameters for evaluation is often not prespecified and awaits the empirical outcome of an experiment. Parameter specification for correlation with molar behavior is necessarily arbitrary and may be unrelated to the major response system under conditioning control by the animal.

Therefore, knowledge of the effective stimulus, of the actual response being conditioned, of the relevant recording site, and of relevant parameters of the dependent variable probably contributes substantially to the variability of results in studies of bioelectric correlates of behavior.

A modified approach to the study of the behaviorally significant bioelectric events is described here. This approach provides for the formal, sequential, and systematic study of bioelectric response parameters which either separately or conjointly are relevant to or encode learned behavior. Available techniques (*4*, *5*) are used and these methods are applied to functional bioelectric coding.

Several findings have been important for our renewed belief that the sequen-