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EFFECTS OF COAL COMBUSTION AND GASIFICATION PROCESS
CONTAMINANTS ON THE NEUROMUSCULAR SYSTEM

Sub-Task on Peripheral Nervous System Effects. Final Report

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MASTER

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Sub-Task on Peripheral Nervous System Effects

I. INTRODUCTION

A. Particulate Emissions

This study is a preliminary investigation of the possible toxic effects of flyash particles from an experimental fluidized-bed combustion process at the Morgantown Energy Research Center. Emphasis has been placed on the action of trace metals present on the surface and in the matrix of the particulates emissions, since these elements may be toxic in low dosages.

B. Trace metals effects on the neuromuscular junction.

It is well established that external calcium (Ca^{++}) is essential for neuromuscular transmission (1). In the absence of Ca^{++} from the external medium, nerve impulses continue to invade the terminal but do not evoke transmitter release (2). Many of the di- and trivalent metal ions have been tested for their ability to substitute for Ca^{++} and have been shown to affect evoked and spontaneous transmitter release (3-14). Many of these ions cause a decrease in amplitude of the evoked end-plate potential (e.p.p.), but raise the frequency of spontaneously occurring miniature end-plate potentials (m.e.p.p.s). Several investigators (15-18) have found that the effective concentration necessary to cause an increase in m.e.p.p. frequency is far greater than that which decreases the e.p.p. amplitude.

The neuromuscular junction of the frog was selected to test the effects of flyash particles and pure metal ions, since its general characteristics are well documented and its sensitivity to metal ions is well known. Cadmium (Cd^{++}) was chosen for our investigation because it had previously been reported to be a highly potent inhibitor of evoked release, yet having no significant effect on spontaneous release even at high concentrations (18). In this report we show that the effective concentrations necessary to increase m.e.p.p. frequency are in the same range as those which decrease e.p.p. amplitude.

II. METHODS

A. Biological preparation and test solutions

Conventional intracellular recording techniques were used to study the effects of flyash and Cd^{++} on the electrophysiological events in the sartorius muscle preparation of the frog (Rana pipiens). The preparation was bathed in amphibian Ringer with the composition of (mM): 111 NaCl, 2.5 KCl, 1.8CaCl, 5.0 HEPES buffer (pH 7.4). All CdCl_2 solutions were made up by dissolving the salt in Ringer's. Solutions entered the experimental chamber by gravity and were removed by suction. The temperature of the preparation was not specifically controlled, but was monitored, and remained within 22-25°C for all experiments.

In order to maximize the possible effects of the flyash we eluted the particles under condition which would extract as much of the surface metal contaminants as possible. The elution procedure was as follows: A slurry of 15 mg/ml of flyash in 2N HCl was stirred for 24 hours at room temperature. The slurry was centrifuged at 1,000 for 10 minutes. The supernatant was decanted and its pH and salt content were adjusted with NaOH to approximate frog Ringer. A white precipitate was formed when the pH was increase (probably insoluble metal hydroxides), this was removed by an additional centrifugation. The supernatant was directly applied to the neuromuscular junction preparation.

A digital pulse height window discriminator was used to separate m.e.p.p.s. from noise. The output pulses of the discriminator were counted over 5-minute sampling periods with a frequency meter and monitored on an oscilloscope to insure the accuracy of the count.

B. Baseline measurements of m.e.p.p.s

Fatt and Katz (7) reported that m.e.p.p. frequency was unstable and subject to progressive change. In our experiments the frequency of m.e.p.p.s declined over the entire observation period. Figure 1A is a semilogarithmic plot of the mean frequency of m.e.p.p.s (normalized to the initial frequency) as a function of the time after inserting the micropipette into the muscle fiber. The straight line is a least-squares fit to the data and represents a single-exponential decay with a time constant of 58.3 ± 4.0 minutes (mean $\pm$ S.E., $n = 6$). Immediately after insertion of the micropipette the mean frequency of m.e.p.p.s was $5.7 \pm 1.1/\text{sec}$ (means $\pm$ S.E., $n = 20$). Some investigators consider an initial m.e.p.p. frequency of greater than 3/sec an indication of fiber injury and exclude them from their data. We found no significant difference in the rate of decay of m.e.p.p. frequency for fibers with initial frequencies either greater or less than 3/sec.

Control experiments with normal Ringer solution were done to determine the stability of the preparation undergoing mechanical disturbances from solution changes. The frequency of m.e.p.p.s was measured at least every 15-minutes following penetration. At 30-minute intervals the Ringer solution in the preparation chamber was changed. As in the preparations without solution changes, the frequency of m.e.p.p.s declined over the entire period of observation. Figure 1B is a semilogarithmic plot of the mean frequency of m.e.p.p.s. A single exponential with a time constant of 50.5 ± 6.0 minutes (mean $\pm$ S.E., $n = 8$) was fit to the data. The time constant associated with the m.e.p.p. frequency from preparations with solution changes is not significantly different from the time constant for preparations without solution changes. However, sometimes we did see a transient increase in the slope of the decay curve following a solution change, but the slope returned to the control value within the 15-minute sampling interval.

III. RESULTS

A. General effects of Cd^{++} on m.e.p.p.

The effects of Cd^{++} on the frequency of spontaneously occurring m.e.p.p.s were examined using concentrations of 60-1000 μM . As in control experiments, the m.e.p.p. frequency was measured at least every 15-minute after penetration and solutions were changed in the preparation chamber every 30 minutes. After a 60 to 120 minute control period Cd^{++} was applied to the preparation.

1. Effects of low concentrations of Cd^{++}

Figure 2 is an example of the effect of 80 μM Cd^{++} on the m.e.p.p. frequency in a single preparation. Before the addition of Cd^{++} the m.e.p.p. frequency declined to 7% of the initial value and after the application of Cd^{++} the frequency increases to 24% of the initial value. The maximal effect of Cd^{++} develops over a 30-60 minute period, suggesting that the entry of the ion is a slow process.

2. Effects of high concentrations of Cd^{++}

When high concentrations of Cd^{++} were used ($>100 \mu\text{M}$) we observed a rapid and large rise in the m.e.p.p. frequency to several thousand per minute (Fig. 3 & 4A). However, after approximately 2-3 hours in the presence of Cd^{++} , m.e.p.p. frequency dropped suddenly until only an occasional m.e.p.p. was seen (Fig. 4B). Subsequent washing of the preparation with normal Ringer solution did not restore m.e.p.p. frequency. If Cd^{++} was then reapplied the frequency did not change. We then tried to elicit e.p.p.s by stimulating the nerve electrically. In all cases we were unable to evoke any e.p.p.s. These results suggest that Cd^{++} is capable of

depleting the terminal endings of their neurotransmitter, (see (19) for the action of lanthanum and (20) black widow spider venom).

B. Concentration dependent effects of Cd++ on m.e.p.p.

Figure 5 is a summary of the effects of different concentrations of Cd++ on the m.e.p.p. frequency. All curves show a generally sigmoidal shape suggesting a higher than first-order process is involved. The initial change in frequency depends on the concentration of Cd++, higher concentrations cause the m.e.p.p. frequency to increase faster than lower concentrations do. The maximum frequency attained after a one-hour exposure to Cd++ tends to be greater with higher concentration, however there is a significant difference only between the values obtained with high concentrations (500-1000 μ M) and low concentrations (60-100 μ M), but not within each range. This latter effect is probably due to several factors: 1) inaccuracies in the measurement of high frequencies of m.e.p.p.s due to summation of m.e.p.p.s, 2) continual blocking effects of high concentrations of Cd++ which would tend to lower the frequency.

C. Repeated application of Cd++

Figure 6A is the response of a single neuromuscular junction to repeated short exposures (45 minutes) of the same Cd++ concentration (500 μ M). The rise time of the changes in the m.e.p.p. frequency is successively shorter with each application. This can be seen more easily in figure 6B in which the m.e.p.p. frequencies are normalized with respect to the value of the frequency at the time of each application of Cd++ (F_0) and replotted relative to the time of each application.

D. Effects of Ca++ on Cd++ response

We have looked at the effects of varying the extracellular Ca++ concentration on the increase in m.e.p.p. frequency produced by high concentrations of Cd++ (1.8 to 2.0 mM). The Cd++ response was unaffected by raising the extracellular Ca++ concentration to as much as twice normal or by lowering it to essentially zero (Fig. 7). These results suggest that Cd++ is capable of releasing transmitter directly and is not working through an indirect mechanism which involves extracellular Ca++, such as an increase in the Ca++ permeability.

E. Effects of Cd++ on e.p.p.

Because our results on the effects of Cd++ on the m.e.p.p. frequency have consistently differed from those of other investigators we have looked at the effect of Cd++ on evoked e.p.p.s. Farshaw (18) has shown that 10 μ M Cd++ completely blocked e.p.p.s. Our results agree with those of Farshaw, in that, we have also found that 10 μ M Cd++ blocks e.p.p.s within 15 minutes (Fig. 8).

F. Effect of Flyash on m.e.p.p.s

To test the possible effects of the heavy metal contaminants of flyash on the neuromuscular junction we applied an elution of the flyash to the preparation (see Methods). We observed no change in the frequency of m.e.p.p.s even after 1.5 hours of exposure (Fig. 9). While an effect of the flyash extract on the m.e.p.p.s was not observed, there may still be an effect on the nerve-evoked e.p.p. which is 100 to 1000 times more sensitive to toxic agents (see above). An investigation of the possible effects of flyash on e.p.p.s should be pursued.

IV. DISCUSSION

A. Cd++ effects of e.p.p.s versus m.e.p.p.s

Forshaw (18) has shown that low concentration of Cd++ (10 μ M) can inhibit neuromuscular transmission in the rat diaphragm by reducing the amplitude of the evoked e.p.p.s. However, Cd++ had no effect on m.e.p.p. frequency with concentrations as high as 100 to 500 μ M. Our results show a measureable increase in the m.e.p.p. frequency for Cd++ concentrations as low as 60 μ M. That Forshaw did not observe an effect of such concentrations of Cd++ on m.e.p.p. frequency could be due to a difference in the sensitivity of the rat diaphragm compared to that of the frog satorius.

B. Mode of action of Cd++

Our results indicate that m.e.p.p. frequency is increased by Cd++ over the same range of concentrations which decrease the amplitude of the nerve-evoked e.p.p.s (18). This seemingly paradoxical effect can be most simply explained if we make two assumptions about the mode of action of heavy metal ions, such as Cd++: 1) Cadmium ions enter the presynaptic nerve terminal and affect the release of the transmitter in a manner analagous to Ca++ but with less efficacy, and 2) the sequestering mechanisms which maintain the intraterminal Ca++ concentration at a low level are incapable of sequestering Cd++. Thus when the neuromuscular junction is exposed to Cd++, the terminal will slowly take up the heavy metal ion. This will lead to a gradual increase in the spontaneous release of transmitter as reflected in the increase in the frequency of m.e.p.p.s. When the nerve terminal is stimulated there is a voltage-dependent influx of Ca++. Ca++ must now compete with the less effective Cd++ to release the transmitter. Thus there will be a reduction of the total amount of transmitter released which will be reflected in a decreased amplitude of the endplate potential.

C. Evidence for Cd++ acting on Ca++ sites

It is known that the calcium-dependent release of neurotransmitter is a fourth-order process, i.e. four calcium ions are required for the release of one quantum of ACh (acetylcholine), the

transmitter-content of a single vesicle. If Cd^{++} acts at the same release site as calcium then one would expect the Cd^{++} response to display fourth-order kinetics.

The sigmoidal time course of the change in m.e.p.p. frequency in response to a step increase in the concentration of Cd^{++} is indicative of a higher-order process. Detailed kinetic measurements must be performed to determine the exact order of the process. If the Cd^{++} response is fourth-order then this would be supportive of the theory that Cd^{++} is binding to the release sites that normally bind calcium.

Another indication of a higher-order process is the effects of short repeated application of Cd^{++} . The rise time of the change in m.e.p.p. frequency is successively faster with repeated application of Cd^{++} . This is precisely what would be expected if more than one Cd^{++} is required for the release of one quantum of ACh.

D. Insensitivity of Cd^{++} response to Ca^{++}

If Cd^{++} does bind to the Ca^{++} release sites then why is the Cd^{++} response insensitive to changes in the extracellular Ca^{++} concentrations? This apparent contradiction can be resolved if we consider the following: in the unstimulate state (terminal endings are not depolarized) the Ca^{++} entry is small and slow (low Ca^{++} permeability) and thus the sequestering mechanisms can maintain a low and constant intraterminal concentration of Ca^{++} , even in the face of a rather larger variation in the extracellular Ca^{++} concentration.

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FIGURE LEGENDS

Figure 1. Semilogarithmic plots of the time-dependent changes in m.e.p.p. frequency. (A) Normal Ringer's without solution change. (B) Normal Ringer's with solution changes. Arrows denote the time at which solutions were changed. The ordinate is the mean percentage of the initial frequency ($\bar{X} \pm \text{S.E.}$, $n = 6-8$). Time is plotted on the abscissa in hours.

Figure 2. Semilogarithmic plot of the m.e.p.p. frequency in a single neuromuscular junction. M.e.p.p. frequencies are normalized to initial value and displayed on the ordinate. Time is displayed on the abscissa. The arrow denotes the time at which $80 \mu\text{M CdCl}_2$ was applied to the preparation.

Figure 3. Effects of high concentration of Cd^{++} on m.e.p.p. frequency. The m.e.p.p. frequencies plotted on the ordinate represent the number of m.e.p.p.s counted in each 5 minute observation period. Time after penetration of the junction is on the abscissa. The downward arrow denotes the time at which $1000 \mu\text{M Cd}^{++}$ was applied to the preparation; the upward arrow denotes the time when the preparation was washed with normal frog Ringer.

Figure 4. Long term effects of high concentration of Cd^{++} (1 mM) on the m.e.p.p. amplitude and frequency. (A) M.E.P.P.s recorded 30 minutes after exposure of a neuromuscular junction to Cd^{++} . Frequency of m.e.p.p.s before application was about 1/second. Scales are 0.5 mV/div and 0.1 sec/div. (B) M.e.p.p.s from the same endplate 2 ½ hours exposure, note that the amplitude and frequency have begun to fall. Scales are 0.2 mV/div and 0.1 sec/div.

Figure 5. Summary of the effects of different concentrations of Cd^{++} on m.e.p.p. frequency. Mean m.e.p.p. frequencies ($\bar{X} \pm \text{S.E.}$, $n = 3$) are normalized to the value obtained at the time of application of Cd^{++} (F_0) and displayed on the ordinate. Time measured with respect to the time of application of Cd^{++} is plotted on the abscissa.

Figure 6. Effects of repeated application of Cd⁺⁺. (A). The m.e.p.p. frequencies plotted on the ordinate represent the number of m.e.p.p.s counted in each 5 minute observation period. Time after penetration of the junction is on the abscissa. The downward arrows denote the time at which 500 μ M Cd⁺⁺ was applied to the preparation. The upward arrows denote the times the preparation was washed with frog Ringer. (B) The normalized increase in m.e.p.p. frequency $((F/F_0)-1)$ is plotted on the ordinate, where F_0 is the frequency at the time of application of Cd⁺⁺. The time measure with respect to the time of application of Cd⁺⁺ is displayed on the abscissa.

Figure 7. The effects of Ca⁺⁺ on the (Cd⁺⁺)-induced increase in m.e.p.p. frequency. The normalized m.e.p.p. frequency (F/F_0) is plotted on the ordinate, where F_0 is the frequency at the time of application of Cd⁺⁺. The time measured with respect to the time of application of Cd⁺⁺ is displayed on the abscissa. The filled circles () represent the mean value $(\bar{X} + S.E., n = 3)$ of the data from preparations exposed to normal levels of Ca⁺⁺ and the filled squares () represent the mean value $(\bar{X} + S.E., n=3)$ of the data from preparations exposed to an essentially Ca⁺⁺-free Ringer.

Figure 8. Effects of Cd⁺⁺ (10 μ M) on e.p.p. amplitudes, (A) An e.p.p. before the application of Cd⁺⁺. The superposition of approximately 300 e.p.p.s (B) 0-5 minutes, (C) 5-10 minutes, and (D) 10-15 minutes after the application of Cd⁺⁺. Scales are 2 msec/div and 1 mV/div.

Figure 9. Effects of Flyash elution on m.e.p.p. frequency. The m.e.p.p. frequency plotted on the ordinate represents the number of m.e.p.p.s counted in each 5 minute observation period. Time after penetration of junction is displayed on the abscissa. The upward arrow denotes the time at which the preparation was washed with normal Ringer. The downward arrow denotes the time at which the elution of flyash (see Methods) was applied to the preparation.

Figure 1

Figure 4

Figure 6 B

Figure 8

Sub-Task on Central Nervous System Effects

I. Spontaneous Locomotor Activity of Lead-treated Rats

During the contract period, the wheel running activity of 23 female lead-treated rats, 21 female control rats, 29 male lead-treated rats and 27 male control rats was measured in 7 sequential experiments each lasting 8 weeks. Since the wheel running activity of female rats is greater than that of male rats, the activity data generated by male and female rats must be analyzed separately. The wheel running activity of female control rats was greater each week than that of female lead-treated rats (Fig 1), but only during weeks 2 and 3 was it significantly so. The wheel running activity of male control rats was also greater than that of male lead-treated rats during most of the weeks (Fig 1), but it was significantly greater only during week 5. Thus, control rats have a tendency to run more in activity wheels than do lead-treated rats. With larger sample sizes these differences in wheel running activity may prove to be significant.

During these experiments, we obtained evidence that the level of wheel running activity of rats may be genetically controlled. Therefore, we modified our experimental procedure from the original one in which a litter of 8 rats received lead in their diet while another litter of 8 rats did not to one in which half the siblings of one litter were exchanged for half the siblings from another litter before the start of the experiment. Therefore, half the siblings of each litter received a diet containing lead and half the siblings of each litter received a lead-free diet.

UNDISTURBED ACTIVITY

II An Analysis of Sensory Impairment Following The Administration of Pb

Since the initial project proposal, a screen for sensory alterations produced by coal-gasification by-products has expanded to include an analysis of integrated brain function (Charpentier, J.: In Pain, eds. Soulairec, et al., Academic Press, pp. 171-200, 1968). The new procedure is as rapidly applied as the one proposed earlier, but provided significantly more information. It was possible to analyze for specific brain areas that may be involved in alterations of an animal's sensory perception and integrated reaction. Briefly, the method requires the subcutaneous placement of two needle electrodes at the base of the tail of rats, through which a range of electrical currents (0.15-11.0 mA) are delivered using a constant current generator. The current range is chosen to produce typical responses from the rats that have been shown to be integrated at various levels in the central nervous system. These are progressively: a tail twitch (Tw) characterized primarily as a spinal reflex, startle (St) and flight (Fl) reactions integrated at the level of the mesencephalic reticular formation, and two responses requiring cortical integration, vocalization (V0) and tail biting (Tb). These reactions encompass the full range of behaviors elicited in a normal animal in response to electrical challenge and are easily quantified. Other studies in our laboratory have confirmed that the method distinguishes the level of interruption of sensory stimulation (pain) produced by morphine SO_4 (Charpentier, 1968). In addition a qualitative and quantitative difference in the reaction of the animals after morphine or the anesthetic ketamine has been defined.

The method described has been used to study the effect of the coal gasification by-product, Pb, on the developing rat nervous system. In our laboratory, as reported during the last quarter, Pb has been shown to produce significant behavioral alterations in the developing rat pups. Now, we report that 25-28 day old pups that consume 1-5 percent Pb in their chow appear to have a specific alteration in the response to electrical challenge. Response integrated at the level of the spinal cord (Tw) remains unaltered in Pb pups compared to pair-fed controls. Therefore, sensory information appears to enter the nervous system. Furthermore, since startle and flight reactions are unaltered by Pb, integration at the level of the mesencephalic reticular formation of these responses to electrical challenge appears to be unimpaired. On the other hand, the tail bite reaction of the pups and their vocalization reaction were increased suggesting a lesion to input or output pathways for the cortical integration of these responses.

A second series of experiments suggested that the rat pups had to receive their Pb from mothers' milk in order for the toxicity to assert itself. However, in these studies the animals

were intubated to receive a wide range of doses of Pb (.004% - 3%) and technical difficulties prevented exposure prior to day 5. Therefore, an early Pb exposure was not possible.

The hypothesis that early Pb exposure is responsible for an insult to the developing rat pup was tested in a crossover design experiment. Pups suckling from Pb (40 ppm in milk) mothers were exchanged to control mothers and pups from control mothers were given to the Pb mothers. This exchange was done at either 5, 10 or 15 days. All animals were compared to either normal rat pups not treated with Pb, or pups whose diet was restricted by balancing their mothers food intake with that of control mothers. Eight experimental groups resulted.

1. Control Pups (Mothers ate normal chow and lib)
2. Pairfed Pups (Mothers ate chow based on Pb Mothers daily consumption)
3. Pb Pups switched to Control Mothers at day 5
4. Control Pups switched to Pb Mothers at day 5
5. Pb Pups exchanged as in 3 at 10 days
6. Control Pups exchanged as in 4 at 10 days
7. Pb Pups exchanged as in 3 at 15 days
8. Control Pups exchanged as in 4 at 15 days

All animals were tested at 25-28 days after birth in the electrical stimulation sensory challenge test.

Final Progress Report

The results of the crossover design experiment demonstrated that groups 3, 5, 7 and 4 had significant increases in tail bite and vocalization reaction. The increase in both appeared to be about twice the values obtained in controls. More data would be required to evaluate more precisely the magnitude of the alteration. Control pups exchanged to Pb consuming mothers at day 10 or 15 (groups 6 and 8) were not significantly effected by the exposure. The results were suggestive of a early insult to the C.N.S. developing rat pups that caused alterative in affective reactions to electrical shocks (stress). The nature of the early insult could not be determined. However, one might speculate that natural adult barriers (the blood brain barrier) may not be formed to lead or developing normal pathways may be effected by the low level Pb exposure.