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Without the guidance of Dr. David Seligson, the support of Dr. Gilbert Glaser, the assistance "beyond the call of duty" of Dr. James Prichard and Dr. Brian Gallagher and the help of numerous individuals in the Neurology and Clinical Chemistry laboratories, this research could not have been accomplished.

## INTRODUCTION

Neurologic dysfunction is prominent in uremia. Apathy, drowsiness, coma, psychosis, acute mania, parasthesias, muscular weakness, twitching and convulsions may occur. Cardiac disturbances are also common. Many of these disorders can be reversed or prevented by dialysis. This fact supports the hypothesis that the uremic syndrome is caused--at least in part--by the accumulation of one or more dialyzable substances in the blood. Post-treatment dialysis fluid is thought to contain these toxic substances and has been utilized for their study.

Numerous aromatic acids--including derivatives of nicotinic[1-3] and benzoic[4] acids--have been discovered in uremic dialysates. Some phenolic acids from patients in acute renal failure depress cerebral enzyme reaction rates *in vitro*.[5] Of nine phenolic acids studied by Ross and Wooten, greatest overall inhibition of rat cerebral enzymes was achieved with 3,4-dihydroxybenzoic acid.[6] Two substituted derivatives of this compound have been found in human uremic dialysates.[4] (The unsubstituted dihydroxy acid would not have survived the chromatography procedure and was not itself identified.) A normal constituent of human urine,[7] 3,4-dihydroxybenzoic acid itself would be expected to accumulate in renal failure.

Faced with such *in vitro* evidence, it seemed appropriate to study *in vivo* the possible role of certain aromatic acids in the genesis of central nervous system derangement in uremia. Because of its marked

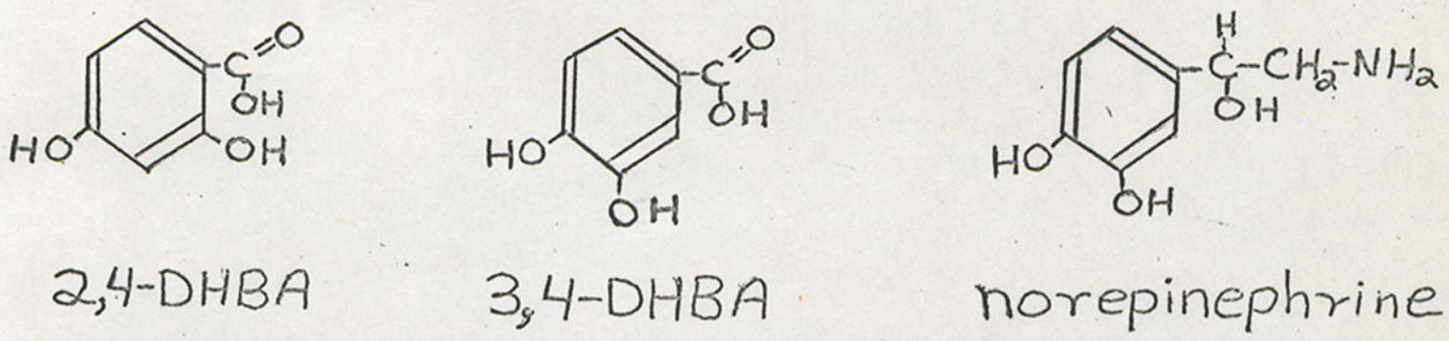


Figure 1: Structural resemblance of 3,4-dihydroxybenzoic acid (3,4-DHBA) to its 2,4 isomer, 2,4-dihydroxybenzoic acid (2,4-DHBA) and to norepinephrine.

inhibition of rat cerebral enzymes and in view of its close structural (Fig. 1) and, presumably, metabolic relationships to compounds of importance in neural function (norepinephrine and dopamine),[4] 3,4-dihydroxybenzoic acid (3,4-DHBA) was selected for extensive testing. To evaluate the specificity of the 3,4-configuration, 2,4-dihydroxybenzoic acid (2,4-DHBA), a compound as yet not found in uremics, was likewise tested. Normal saline solution (NSS) was also used as a control. By injecting these (and occasionally other) substances into rats in acute renal failure and then monitoring selected parameters--EEG, EKG, behavior, certain blood chemical concentrations, duration of life and seizure threshold--it was the objective of this study to examine the possibly causal relationship between the accumulation of specific aromatic acids and certain signs of neurologic dysfunction in uremia. The effects of 3,4- and 2,4-dihydroxybenzoic acids were thus superimposed upon the usual manifestations of acute renal failure in rats and evaluated by comparing rats receiving the acids to control uremics receiving saline.

## MATERIALS AND METHODS

Electrode Implantation: Male and female albino rats (Charles River) weighing 200-250 grams were used. Four permanent cortical electrodes were implanted under ether anesthesia in holes drilled 3 mm from the coronal and 4 mm from the sagittal sutures in each of the frontal and

parietal bones. Each electrode set consisted of a small stainless steel screw joined by a short piece of enamel-coated copper wire to an Amphenol female connector. Electrodes were screwed into the skull far enough to rest on the dura mater and their extracranial portions covered with dental cement. The female connectors were inserted into a short strip of plastic connector mounting which, when fixed to the skull by dental cement, formed a permanent, light-weight plug for easy coupling to the recording cable. Precordial electrodes were implanted subcutaneously through a ventral midline thoracic incision at the time either of cortical implantation or later of ureteral ligation.

Ureteral Ligation: Bilateral ureteral ligation was performed under ether anesthesia via midline abdominal incision three to five days after electrode implantation. Each ureter was doubly tied with silk ligatures and transected. All rats in a set were ligated at one operating session and, except for injection with different substances, were treated identically for the duration of the experiment. Rats were routinely maintained without food and water following ligation, except that those living more than 24 hours after injection were given brief access to water twice daily. Rats were weighed before implantation, before and after ligation and injection and daily thereafter.

Injection: Standard isotonic solutions of 3,4-DHBA and 2,4-DHBA containing 150 mEq/L of the acid was the sodium salt at about pH 7.0 were prepared.

Commercial normal saline solution (containing 150 mEq/L of both sodium and chloride) was used. Sixteen hours after ureteral ligation, each rat received by intraperitoneal injection a standard dose and volume (750 uEq of anion per 100 gm of body weight) of 3,4-DHBA, 2,4-DHBA or saline. Times were recorded in terms of "hours after injection;" for example, ligation occurred 16 hours before injection at Hour -16.

Recording: The EEG and EKG were recorded simultaneously from the two or three rats in a set--each rat in its own large glass fishbowl, unanesthetized and restrained only by a light, flexible recording cable--on a standard 8-channel Grass electroencephalograph with input time constants set at 0.3 seconds. All recordings were bipolar (frontal-frontal, frontal-parietal, parietal-parietal). At least two (and sometimes four) channels were used for each rat at any given time. Baseline EEG recordings were made at least twice before ureteral ligation and once just before injection. The spontaneous EEG was recorded for 2-3 minutes every two hours (with more frequent and longer recording at crucial periods) for the first 18 hours after injection and every 4-8 hours thereafter. In addition, the final six sets of rats were subjected to photic stimulation with a Grass stroboscopic photic stimulator during part of each recording session. Several flash rates (1, 6, 10, 15 and 20 f/sec for 20 seconds each, alternating with 20-second light-free intervals) were used routinely to induce latent seizure activity. Cardiac rate and rhythm were also monitored during recording sessions. All EEG tracings were reviewed by individuals with special training in electroencephalography (J.P. and others).

Behavioral Evaluation: Behavior was evaluated grossly and quantitatively. The occurrence of the following was determined by gross observation: paresis, prostration, hypersensitivity to sound, tremors at rest, tremors on effort, prolonged muscle spasms, myoclonic jerks and grand mal convulsions. To quantify behavior more objectively, the "square test" was used. Rats of a given set were placed, one at a time, in the middle of a 12-inch square floor tile. The time required by each rat to get all four feet simultaneously outside the square was the sole output of this simple test. A maximum of 120 seconds was recorded as the final result for a given hour.

Time of Death: Time of death (in hours after injection) was recorded for all animals. When actual demise was not observed, the estimated hour of death--based on last known time alive, degree of rigor mortis and gross body temperature--was noted.

Blood Chemistry: In selected animals, blood was drawn from the heart and submitted to the clinical chemistry laboratory of the Yale-New Haven Hospital for analysis by the routinely used techniques. Serum urea, sodium, potassium, bicarbonate and chloride were determined in all cases; serum transaminase (SGOT), clacium and phosphate were measured on occasion. "Delta," the level of unidentified anions, was calculated by subtracting measured anions ($HCO_3$ + Cl) from measured cations (Na + K + 5), the added 5 mEq/L representing estimated serum calcium. The concentration of 3,4-DHBA in the serum of experimental animals was

determined by ultraviolet spectrophotometry. The serum was treated with trichloroacetic acid and the protein-free filtrate diluted with distilled water. Serial dilutions of the standard 3,4-DHBA solution were used to determine the acid's absorption peaks and extinction coefficient.

Seizure Threshold:= Seizures were induced simultaneously in selected experimental and control rats by exposure to an atmosphere of the volatile convulsant, hexafluorodiethyl ether (Indoklon), using the technique and apparatus recently devised by Gallagher, Prichard and Glaser.[8] Male and female albino rats weighing 130-180 grams were used. Three experimental rats and one control (saline or normal) were placed singly in each quadrant of an air-tight plastic box (volume 10.3 liters) sectioned with 1 cm square wire mesh. The convulsant ether was delivered by infusion pump at a constant rate (40 ul per minute) to the chamber where it volatilized from filter paper. Time in seconds was measured from the start of infusion to the onset of both the first myoclonic jerk and the first generalized tonic-clonic seizure. These endpoints were generally clear and unequivocal.

Statistical Analysis: Statistical significance was determined by the "t test".[9] A probability of less than 5% ($p < 0.05$) was accepted as the limit of significance. Standard error (SE) was calculated as an indicator of the variation of sample populations around the mean. "Normal range" for blood chemical determinations was defined as the mean $\pm$ 2SE of 6 samples from normal animals.

## EXPERIMENTS AND RESULTS

EXPERIMENT #1: Sixteen sets of rats with cortical and precordial electrodes underwent ureteral ligation and 16 hours later received the standard dose of 3,4-DHBA, 2,4-DHBA or NSS. Behavior (including the square test), EEG (with photic stimulation in the last 6 sets) and EKG were monitored prior to ligation and injection and every two hours during the first 18 hours after injection and every 4-8 hours thereafter. Time of death was recorded. Blood chemical determinations were performed on animals which died during observation.

Time of Death: In this experiment 16 rats received 3,4-DHBA, 12 2,4-DHBA and 12 saline. (See Table 1 and Fig. 3.) Both benzoic acids were markedly toxic. Rats injected with either acid lived less than half as long as saline controls ($p < 0.01$); 2,4-DHBA rats died slightly sooner than 3,4-DHBA animals, but the difference was not significant.

Behavior: Marked behavioral differences distinguished the three groups (Table 1). At some point in the uremic course, all animals became paretic (consistently displaying signs of generalized weakness) and later prostrate (lying sprawled, unable to achieve an upright posture or raise head). These signs of clinical deterioration occurred much earlier in rats receiving the benzoic acids than in saline controls (Fig. 3). Both paresis and prostration occurred earlier in 2,4-DHBA than in 3,4-DHBA rats, but the differences were not significant.

Table 1: Incidence and Time of Onset of Behavioral, EEG and EKG Phenomena.

| | 3,4-DHBA | | 2,4-DHBA | | NSS | |
|---|---|---|---|---|---|---|
| | incidence* | time of onset (hrs after inj) | incidence** | time of onset | incidence*** | time of onset |
| BEHAVIOR | | | | | | |
| death | 100% | 20.9±6.4 | 100% | 17.1±5.1 | 100% | 44.3±12.3 |
| paresis | 100% | 12.2±4.1 | 100% | 5.9±2.2 | 100% | 30.3±14.5 |
| prostration | 100% | 17.6±5.6 | 100% | 10.9±3.6 | 100% | 40.3±11.0 |
| $T_{120}$ (square test) | 100% | 19.3±5.7 | 100% | 12.9±4.5 | 100% | 35.9±11.5 |
| tremors at rest | 33% | 16 | 0% | -------- | 25% | 42 |
| tremors on effort | 60% | 15 | 50% | 10 | 33% | 32 |
| spasms | 20% | 12 | 0% | -------- | 8% | 31 |
| myoclonic jerks | 73% | 9 | 33% | 8 | 8% | 13 |
| grand mal convulsions | 28% | 14 | 8% | 5 | 8% | 13 |
| hyper to sound | 40% | 6 | 25% | 9 | 40% | 22 |

*Fifteen rats in 3,4 group, except for photo-induced burst (6) and arrhythmias (11).
**Twelve rats in 2,4 group, except for photo-induced burst (5) and arrhythmias (11).
***Twelve rats in NSS group, except for photo-induced burst (7) and arrhythmias (9).

(Continued on following page)

Table 1: Incidence and Time of Onset of Behavioral, EEG and EKG Phenomena. (Continued from previous page)

| | 3,4-DHBA | | 2,4-DHBA | | NSS | |
|---|---|---|---|---|---|---|
| | incidence* | time of onset (hrs after inj) | incidence** | time of onset | incidence*** | time of onset |
| EEG | | | | | | |
| spontaneous bursts | 73% | 8 | 33% | 6 | 25% | 10 |
| photo-induced bursts | 83% | 5 | 20% | 6 | 0% | -- |
| generalized seizure | 28% | 14 | 8% | 5 | 8% | 13 |
| slowing | 28% | 16 | 17% | 10 | 25% | 40 |
| increased fast | 20% | 9 | 8% | 4 | 8% | 31 |
| | | | | | | |
| EKG | | | | | | |
| arrhythmias | 27% | 11 | 54% | 8 | 33% | 48 |

*Fifteen rats in 3,4 group, except for photo-induced burst (6) and arrhythmias (11).
**Twelve rats in 2,4 group, except for photo-induced burst (5) and arrhythmias (11).
***Twelve rats in NSS group, except for photo-induced burst (7) and arrhythmias (9).

The "square test" was used with the last 8 sets of rats (Fig. 2). Prior to ligation, all three groups left the square in about 10 seconds; after 16 hours of acute renal failure, all animals continued to move out rapidly. Within 2 hours after injection, however, 2,4-DHBA animals had become noticeably slower, and by Hour 4 the difference between the 2,4-DHBA group (58 secs) and the 3,4-DHBA (22 secs) and NSS (20 secs) groups had become so marked as to be statistically significant ($p < 0.05$). After Hour 4, 3,4-DHBA rats also became progressively slower so that by Hour 12 there was a distinct difference between 3,4-DHBA (73 secs) and saline (25 secs). (The sharp drops of average times displayed in Fig. 2, e.g., at Hour 6 for 2,4-DHBA, are mathematical artifacts, as they reflect the deaths of those rats with the most retarded mobility and highest times.)

$T_{120}$, the time when a rat first required the full 120 seconds on each of three successive trials, was calculated for all three groups. On the average, rats receiving 2,4-DHBA were essentially non-mobile within 13 hours and 3,4-DHBA within 20 hours after injection, whereas saline rats continued to leave the square until almost 36 hours after injection. This difference between saline and the benzoic acids was statistically significant ($p < 0.01$). Thus, by use of this simple test, the effects of 3,4-DHBA, 2,4-DHBA and saline on development of mobility retardation were clearly distinguished from one another.

Certain behavioral phenomena were detected in some animals but not in others; times of onset also differed. (The complete data are presented in Table 1; selected portions are displayed graphically in

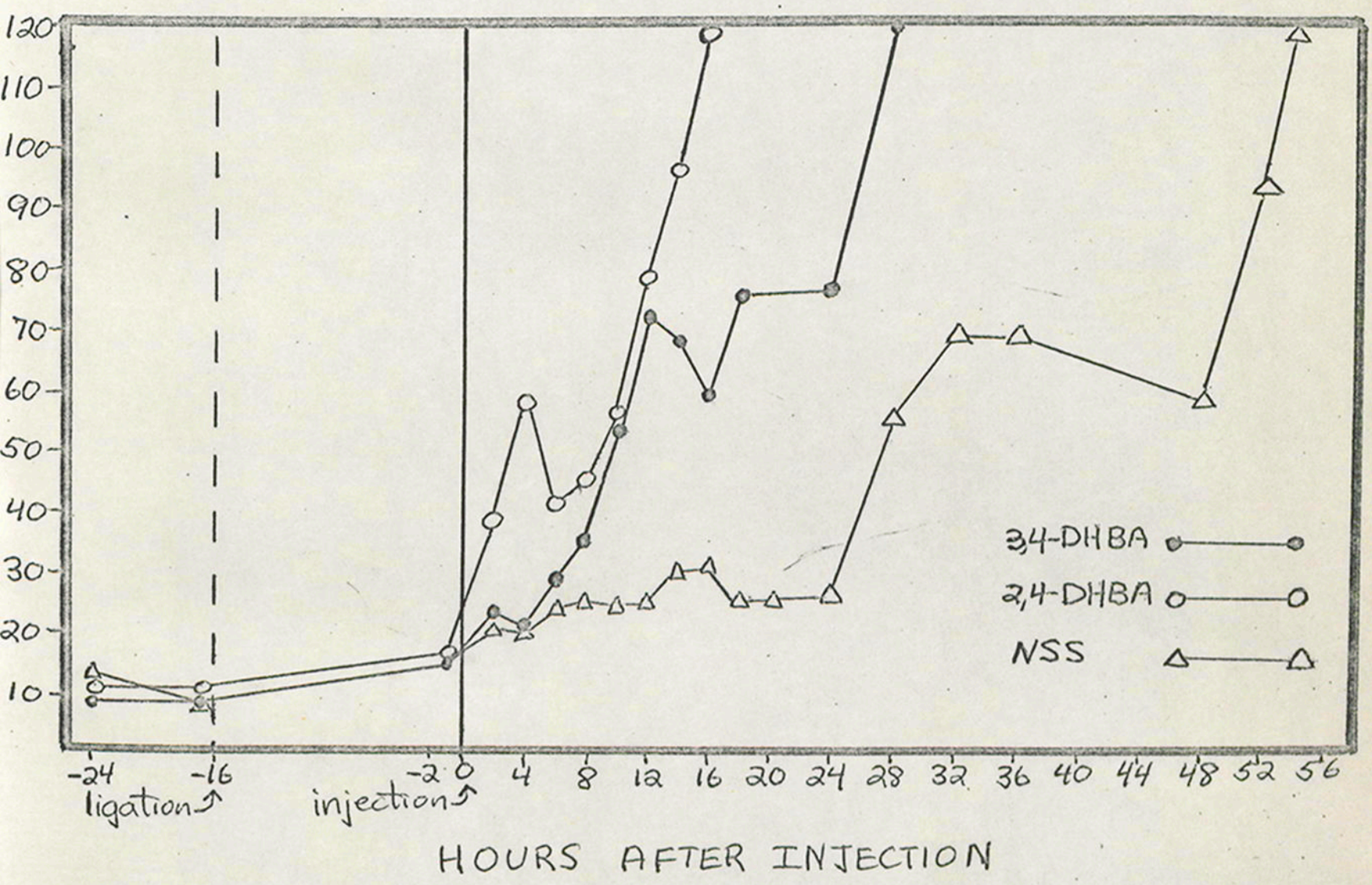


Figure 2: Results of "square test". Marked retardation of mobility occurred first in the 2,4-DHBA group, shortly thereafter in the 3,4-DHBA rats, and much later in saline controls.

Figures 3 and 4.) Tremors on effort (generalized trembling while attempting gross bodily movement or while moving; regarded here as a sign of weakness and neuromuscular depression) occurred in one-third of saline animals and in the majority of 3,4-DHBA and 2,4-DHBA animals. On the average, these appeared earliest in 2,4-DHBA, then in 3,4-DHBA, and much later in saline animals. To be distinguished from tremors on effort were tremors at rest (generalized quivering unassociated with attempted movement; regarded here as evidence of neuromuscular irritability). Tremors at rest were not observed in 2,4-DHBA rats, but were noted in one-third of 3,4-DHBA and one-fourth of NSS animals, much earlier in the former. This discrepancy between the 3,4 and 2,4 isomers represented a significant difference ($p < 0.05$).

Similarly not observed in 2,4-DHBA rats were prolonged muscle spasms (tonic contractions lasting more than one second, unassociated with a grand mal convulsion, usually without EEG changes; likewise regarded as evidence of hyperexcitability). Such spasms were noted in 20% of 3,4-DHBA rats and occurred much earlier in these than in the single saline animal with spasms. Myoclonic jerks (intermittent, brief contractions, lasting less than one-half second, with or without EEG changes) occurred in 75% of 3,4-DHBA animals, a significantly greater incidence ($p < 0.05$) than in either of the other groups. Grand mal convulsions (with tonic and clonic contractions followed by post-ictal depression) also were observed more frequently in the 3,4-DHBA group. Hypersensitivity to sound (manifested by exaggerated startle responses to repeated hand claps) was elicited more frequently in 3,4-DHBA than in 2,4-DHBA animals, and earlier in course of 3,4-DHBA than saline animals.

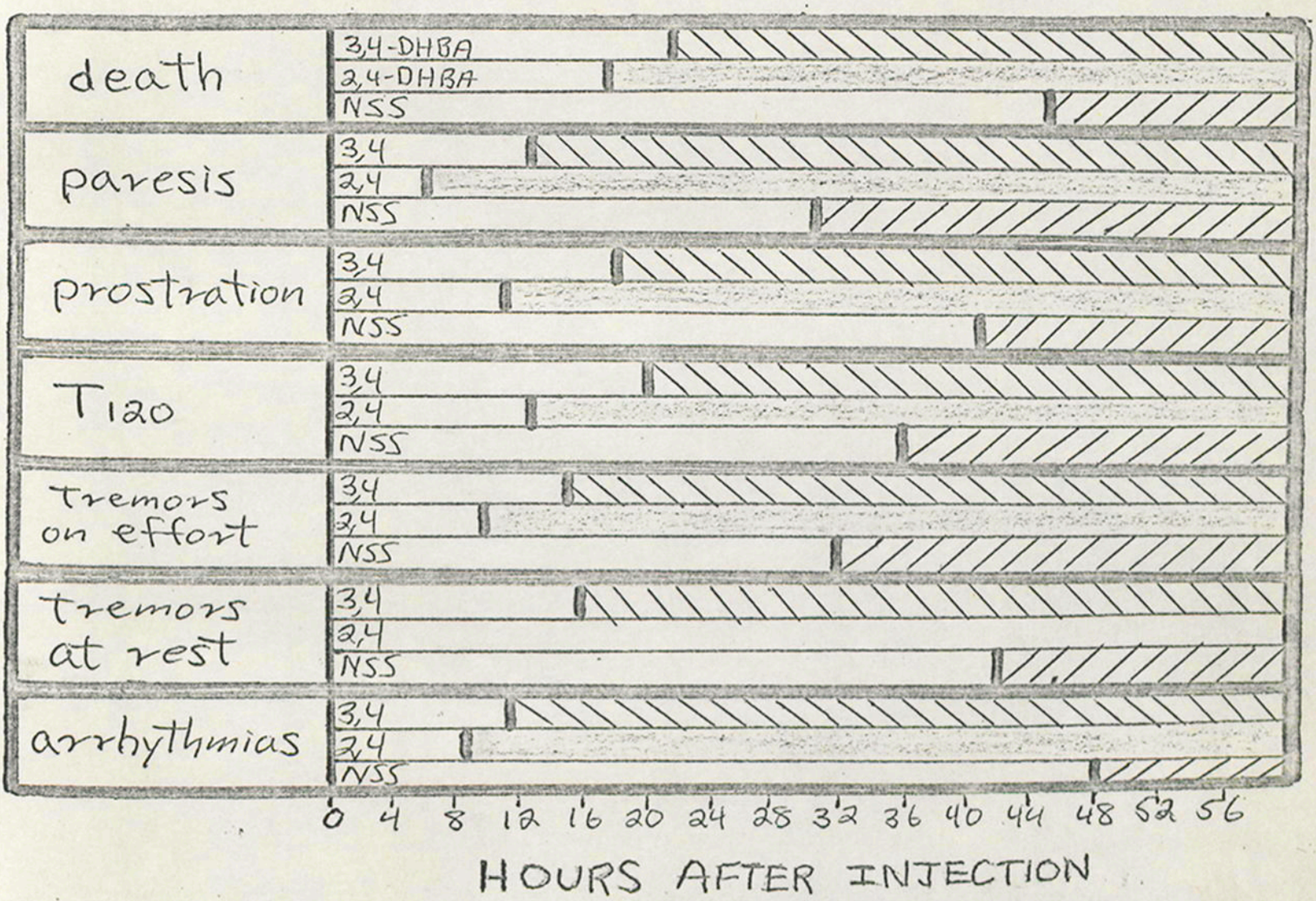


Figure 3: Average time of death and of onset of selected signs of clinical deterioration, all of which (except for tremors at rest) occurred earliest in 2,4-DHBA rats, shortly thereafter in 3,4-DHBA animals and much later in saline controls.

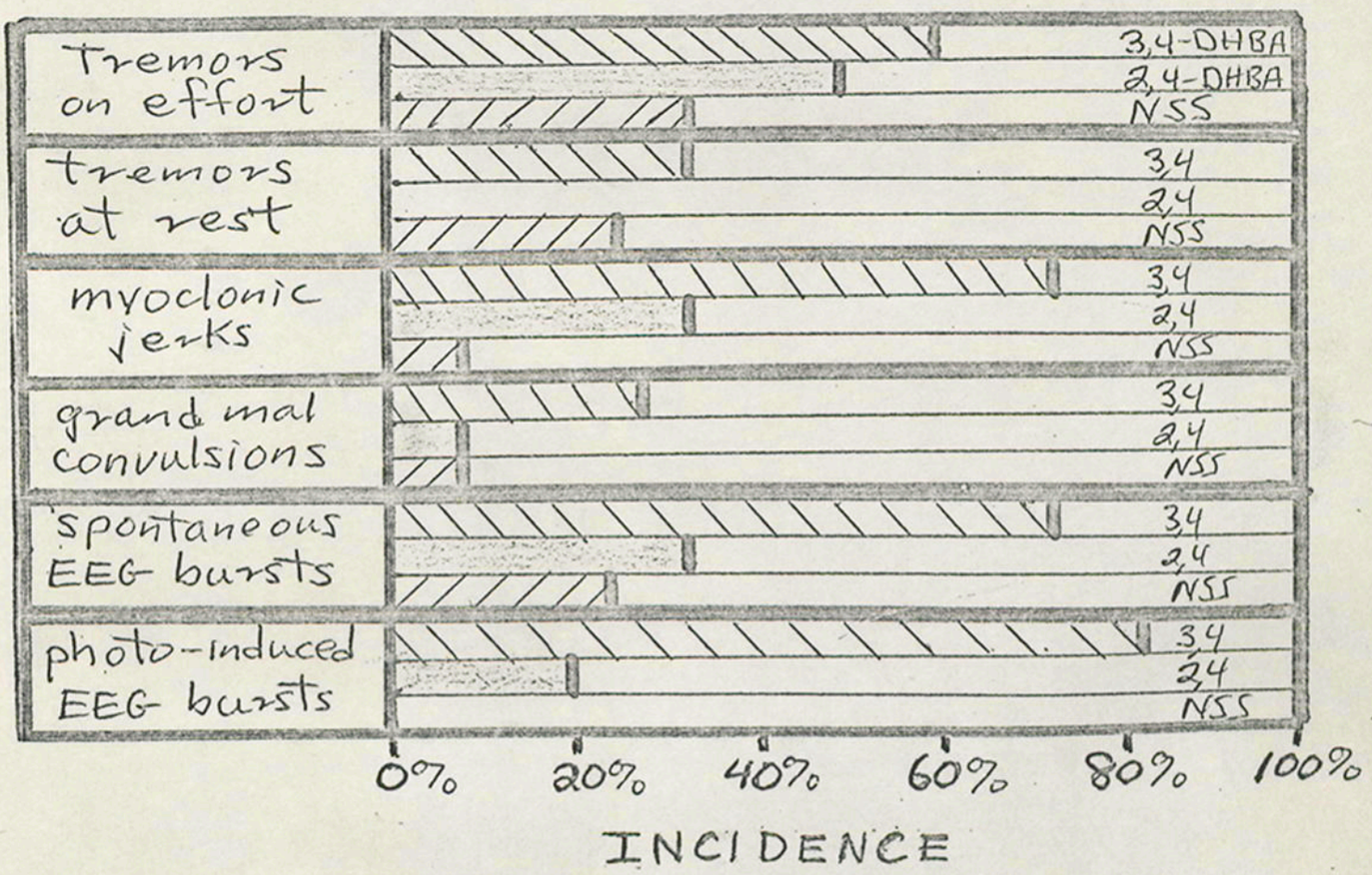


Figure 4: Percent of rats in each group demonstrating selected signs of neuromuscular excitability, with the greatest incidence in all categories observed in the group receiving 3,4-DHBA.

It should be noted that all of these behavioral phenomena were observed more frequently and/or earlier in the 3,4-DHBA group than in saline controls. Furthermore, signs of neuromuscular hyperexcitability--myoclonic jerks, grand mal convulsions, tremors at rest and hypersensitivity to sound--were noted more frequently in 3,4-DHBA than in 2,4-DHBA rats. On the other hand, signs of neuromuscular depression--paresis, tremors on effort, prostration and mobility retardation ($T_{120}$)--occurred earliest in rats receiving 2,4-DHBA, somewhat thereafter in those receiving 3,4-DHBA, and generally much later in saline controls.

EEG: The basic EEG pattern of the awake, unrestrained rat in this study (Fig. 5A) was very similar to that reported by Morris and Glaser.[10] This consisted of 60-160 uV, 30-50 cps fast activity superimposed upon 150-230 uV, 4-7 cps irregular waves in the anterior-posterior leads, often with bilateral synchronization. The anterior and posterior side-to-side recordings were of lower voltage and showed considerably less slow and more regular 30-50 cps activity. Periods of 150-450 uV, 9-12 cps irregular sharp waves occurred during sleep and when the rats appeared drowsy; at these times the basic patterns could be restored by sudden auditory stimuli. Photic stimulation at different frequencies often induced synchronous "driving" of EEG activity, occasionally in the form of distinct spike and wave patterns.

Electroencephalographic findings in the uremic animals are summarized in Table 1. No consistent change in the basic EEG pattern

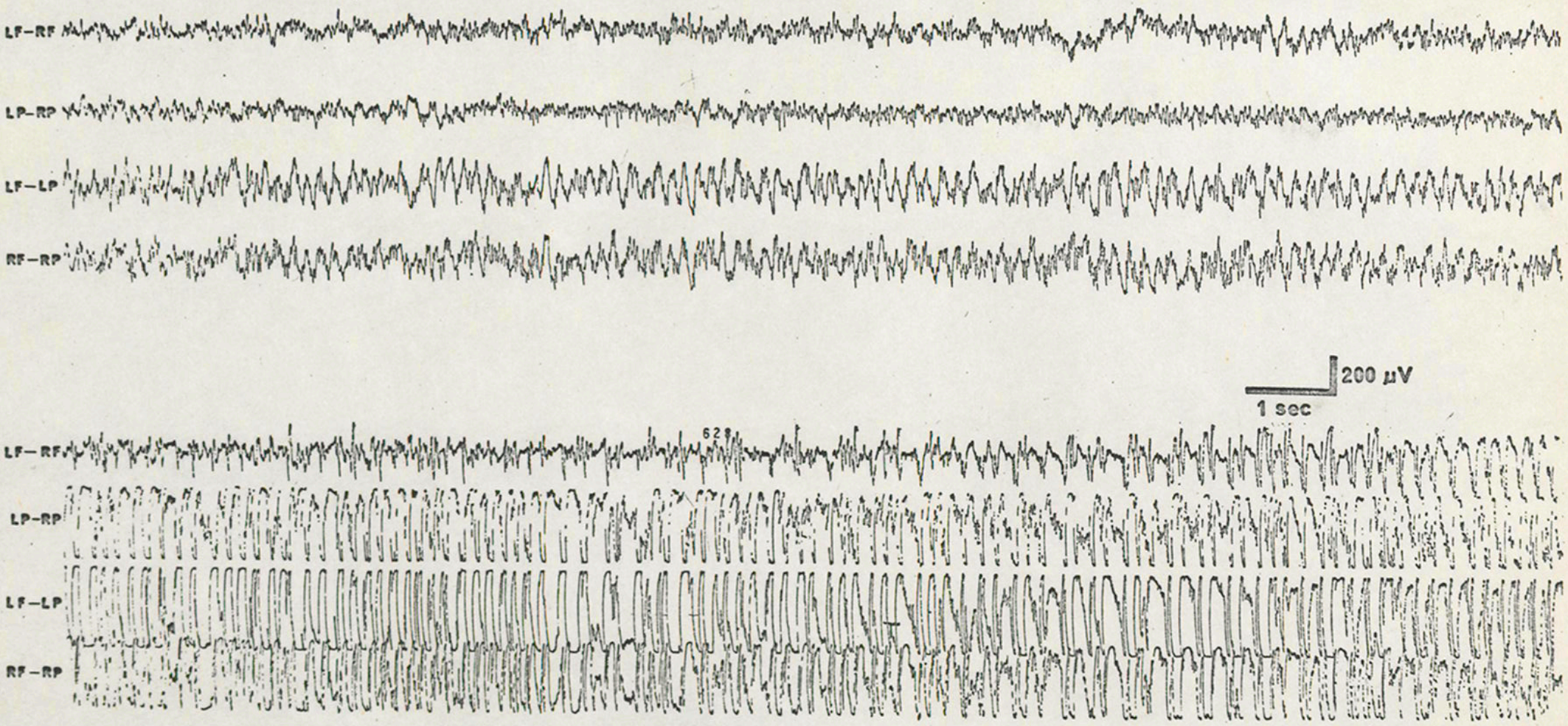


Fig. 5: A: Saline control rat (#44) 12 hours after injection. Recording unchanged from prior to ligation. Typical of normal rat EEG.

B: 3,4-DHBA rat (#43) 12 hours after injection. Onset of first generalized seizure. Note spread of seizure discharge to the frontal leads (LF-RF). (Paper speed 30 mm/sec. L, left; R, right; F, frontal; P, parietal).

was detected over the course of the experimental uremia studied here. In most cases, the basic background pattern within the hour before death was nearly indistinguishable from that recorded prior to ligation or injection. However, marked slowing (with decreased amplitude of fast activity in all leads and appearance of 100-200 uV, 1.5-4 cps waves in the anterior-posterior leads) was observed at some point in the course of about one-quarter of all animals, much earlier in animals receiving either benzoic acid than in saline controls.

Seizure phenomena, manifested behaviorally as grand mal convulsions and myoclonic jerks, were also demonstrated electroencephalographically. All grand mal convulsions were accompanied by generalized electrical seizure activity (Fig. 6B). The tonic phase was characterized by regular, 500 uV, 6-8 cps activity (often with spike and wave configuration); during the clonic phase, short segments of this regular activity were interrupted by intervals of very low amplitude, fast activity; post-ictal behavioral depression was associated with marked flattening of the EEG tracing.

Another manifestation of abnormal electrical activity were spontaneous bursts of spikes and slow waves (Fig. 6). On many occasions these paroxysms accompanied myoclonic jerks (Fig. 6B) but often they occurred without observable physical counterparts (Fig. 6A). Characteristically, such bursts lasted 1-2 seconds and were marked by stereotyped 400-500 uV, 6-8 cps waves, often preceded by or interspersed with single spikes of similar amplitude. Unlike the high amplitude bursts accompanying sleep and dozing, these paroxysms were not abbreviated by auditory stimuli.

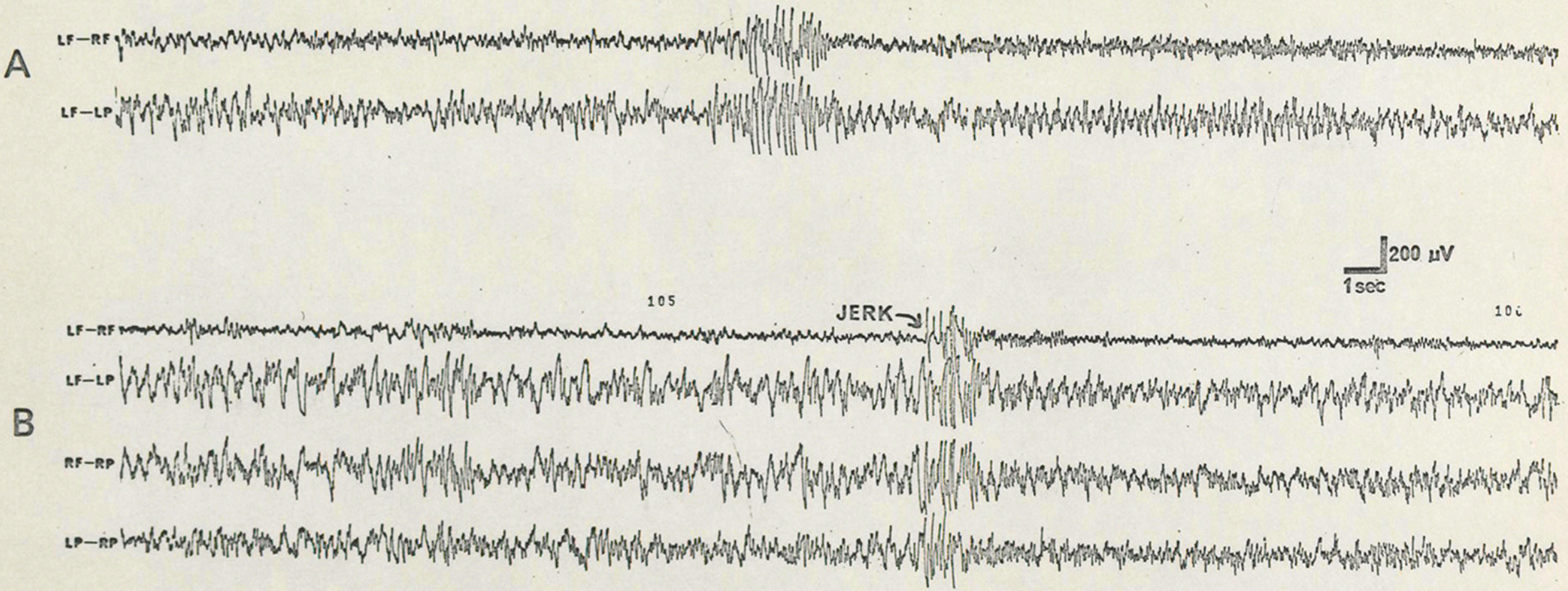


Fig. 6: A: 3,4-DHBA rat (#429) 4 hours after injection. Spontaneous burst of spikes and slow waves without a myclonic jerk.

B. 3,4-DHBA rat (#416) 13 hours after injection. Spontaneous burst of spikes and slow waves beginning with and lasting 0.4 seconds after a single myoclonic jerk. (Paper speed 15 mm/sec. L, left; R, right; F, frontal; P, parietal).

The incidence of such spontaneous bursts in 3,4-DHBA rats was 73%, significantly greater ($p < 0.05$) than in the 2,4-DHBA and saline groups. (Occasional bursts were recorded during control periods prior to ligation and/or injection in 7 of the 40 rats studied with the EEG. Two of these 7 (one 3,4-DHBA and one saline) demonstrated a definitely increased frequency of bursts following injection, and these two rats were regarded in the results as having spontaneous bursts. The remaining five rats (one 3,4-DHBA, one 2,4-DHBA and three saline) showed no increased frequency of bursts following injection and were regarded in the results as not having spontaneous bursts.)

Photic stimulation was used in the last six sets of animals. At some point in the post-injection course of five (of 6) 3,4-DHBA rats, but in only one (of 5) 2,4-DHBA and none (of 7) saline animals, photic stimulation was accompanied (or followed within 3 seconds) by bursts of slow waves and spikes (Fig. 7B). These were often similar in configuration to spontaneous bursts in the same animal and were distinct from the "driving" phenomenon mentioned above. (In two 3,4-DHBA rats and in the single 2,4-DHBA rat, the bursts were apparently "induced" by the photic stimulation, inasmuch as spontaneous bursts had not yet or never occurred. In three 3,4-DHBA rats, however, it was impossible to clearly distinguish bursts induced by from bursts coincident with photic stimulation.) None of the flash rates used was singularly effective at inducing these paroxysms, nor were the bursts accompanied by gross behavioral changes. As with spontaneous bursts, the incidence of strobe-induced paroxysmal activity was significantly greater ($p < 0.05$) in

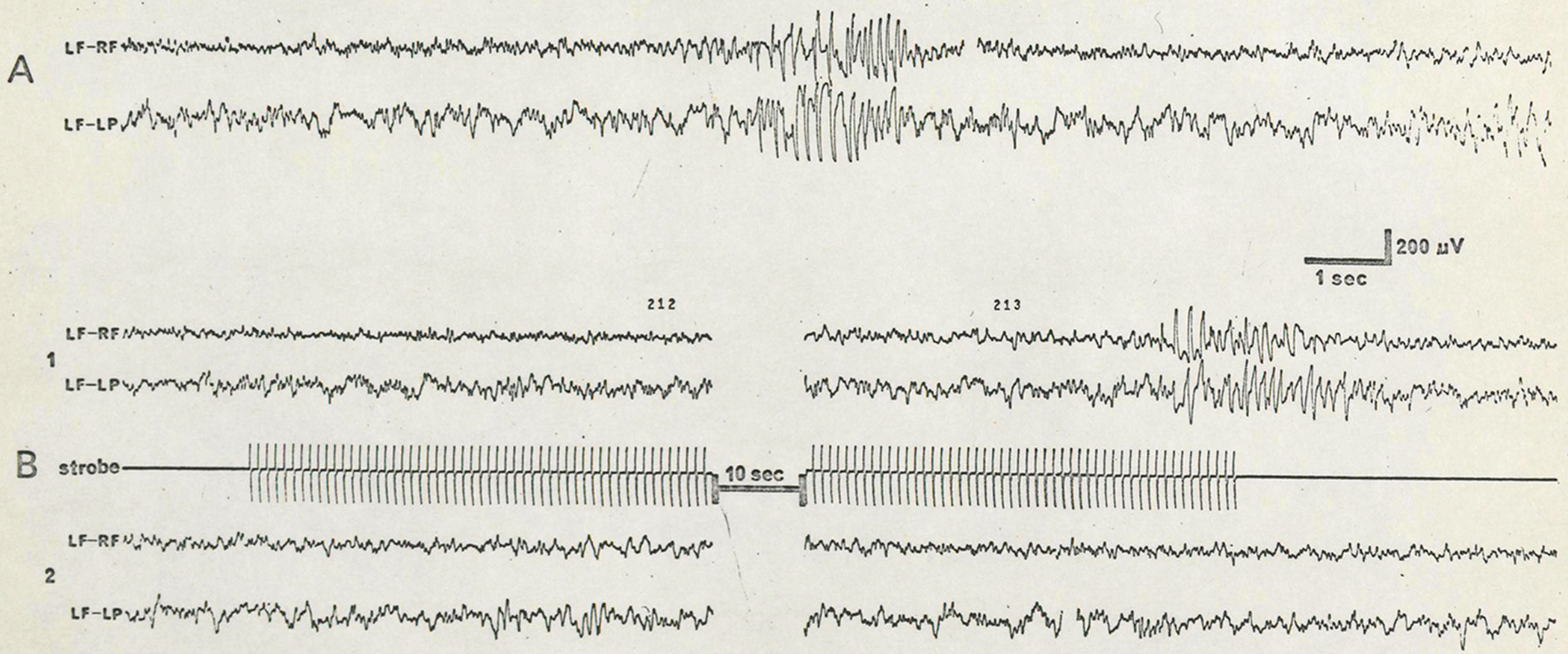


Fig. 7: A: 3,4-DHBA rat (#429) 4 hours after injection. Spontaneous burst of spikes and slow waves recorded at faster paper speed than in Fig. 6A. Compare burst to Fig. 7B.

B: Rat 1: 3,4-DHBA rat (#425) 2 hours after injection. Burst of spikes and slow waves lasting 1.5 seconds occurring near end of 20 second period of photic stimulation at 10 f/sec.

Rat 2: 2,4-DHBA rat (#426) 2 hours after injection. No change in EEG during or after period of photic stimulation. (Paper speed 30 mm/sec. L, left; R, right; F, frontal; P, parietal).

3,4-DHBA than in 2,4-DHBA and saline animals; however, the difference between 2,4-DHBA and saline was not significant.

EKG: The electrocardiogram revealed marked changes in rate and rhythm over the uremic course. Changes in heart rate are displayed graphically in Fig. 8. Prior to ligation, the average rates in all three groups were essentially the same (about 400 beats/min). After 16 hours of acute renal failure, all three groups manifested a similar lowering of heart rate by about 65 beats/min. Immediately following injection, rates dropped in all three groups. In saline animals, the bradycardia was relatively moderate and transient. In 3,4-DHBA and 2,4-DHBA animals, however, the bradycardia was more severe and persistent. Heart rates in both groups fell steadily during the first 6 hours following injection (3,4-DHBA falling 116 beats/min; 2,4-DHBA dropping 100). (In the 2,4-DHBA group, a gradual rise in average rate over the next ten hours reflected the deaths of five rats with very low heart rates; similarly, the rapid fall at Hour 15 in the 3,4-DHBA group represented the deaths of three rats with high rates.) In the 3,4-DHBA group, the average rate remained essentially stable from Hour 6 through Hour 12 and then rose sharply by 40 beats/min over the next two hours. Unlike the changes in 2,4-DHBA rats explained above, this sudden elevation in average heart rate was not a mathematical artifact; no animals had died in the interval. Indeed, it was generally true that the death of a 3,4-DHBA rat was preceded by a marked rise in heart rate (often temporally associated with the appearance of exaccerbation of signs of neurologic hyperexcitability),

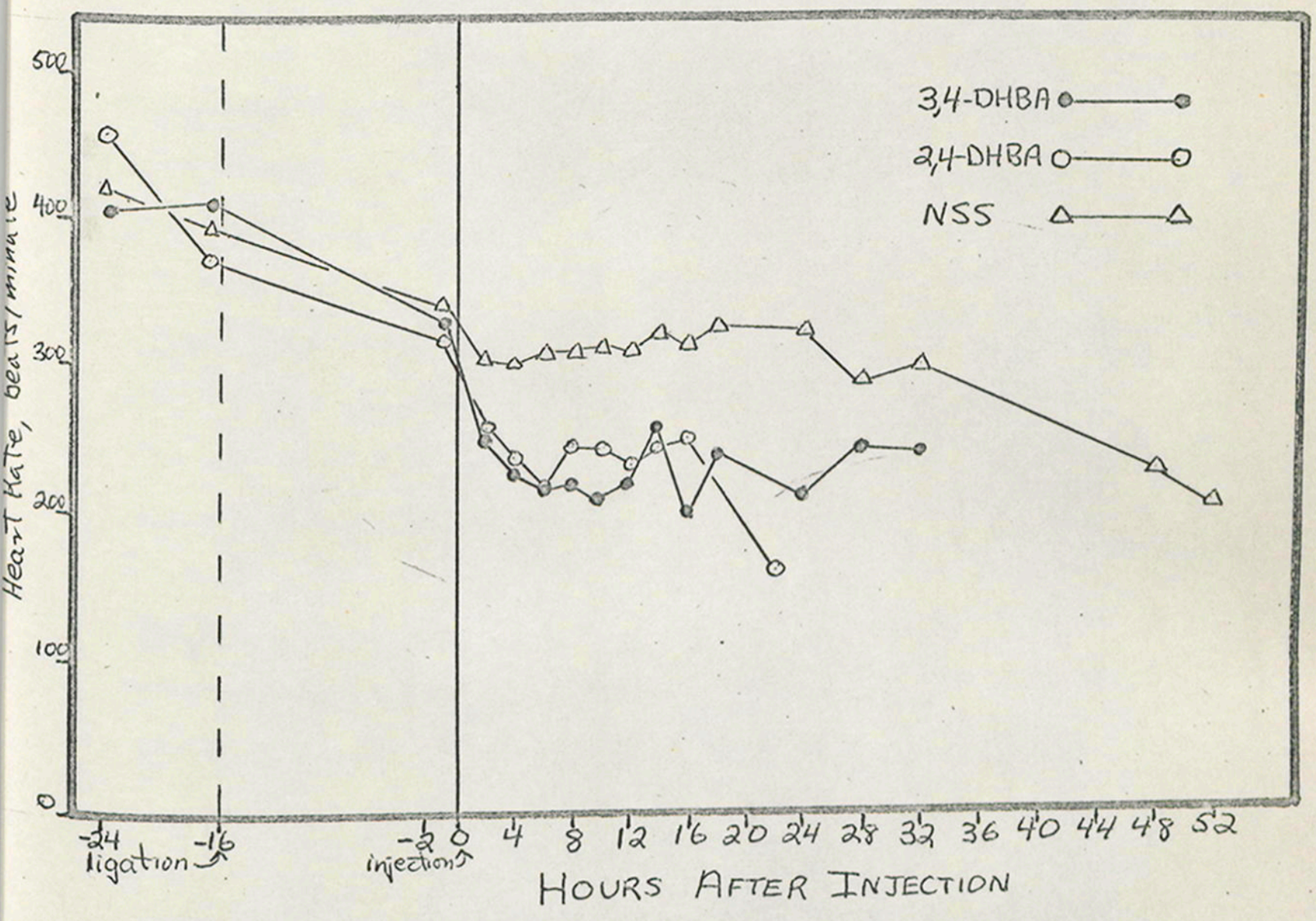


Figure 8: Average heart rates before and after ligation and injection. Compare transient, mild bradycardia in saline controls to the persistent, marked bradycardia which followed injection of either dihydroxybenzoic acid.

whereas the demise of a 2,4-DHBA rat characteristically followed a progressive fall in heart rate over several hours. Arrhythmias--manifested as an irregular ventricular beat on the EKG--preceded death by several hours in more than one-quarter of the animals in each group, most commonly (54%) in rats receiving 2,4-DHBA.

Blood Chemistry: Results of terminal blood chemical determinations will be presented with results from interval blood studies in Experiment #4.

Weight: Rats receiving 3,4-DHBA weighed an average of 210 grams prior to ligation and 218 grams at death, with a weight gain of +8 grams. Comparable data for 2,4-DHBA rats were 214, 226 and +12 grams; and for NSS, 212, 211 and -1 grams.

Controls: Two sets of implanted but non-ligated rats were injected with the standard doses of 3,4-DHBA, 2,4-DHBA or NSS. None died or showed any notable changes in EEG, EKG, behavior or square test.

EXPERIMENT #2: Time of death was the sole output of this study, which compared the overall toxicity of 3,4-DHBA with several substances related either to 3,4-DHBA itself or to uremia. Groups of 4-6 non-implanted rats were ligated and 16 hours later injected with the standard dose of one of the following: 3,4-DHBA, NSS, urea, creatinine, benzoic acid,

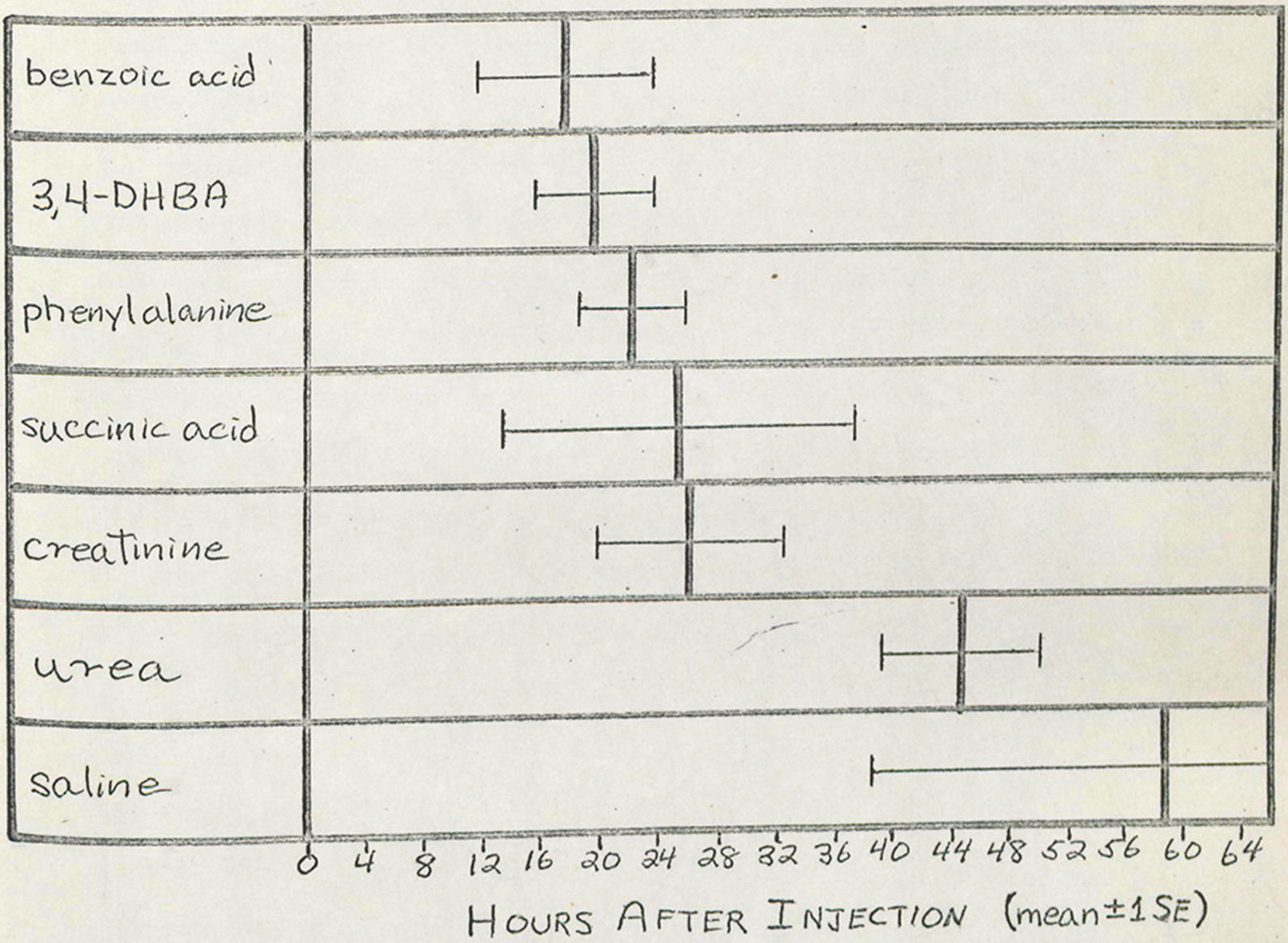


Figure 9: Average times of death of groups in Experiment #2, demonstrating early death in rats given one of several compounds (including 3,4-DHBA), compared with late deaths in rats given urea or saline.

phenylalanine and succinic acid. All solutions contained 150 uM/ml of test substance, but--because of differences in solubility, acidity and degree of dissociation--there were unavoidable variations in sodium concentration, tonicity and pH. Animals were checked briefly every 4-8 hours following injection to determine time of death.

The results of this experiment (Fig. 9) reveal that, in terms of lethal effect, 3,4-DHBA was clearly more toxic than one substance associated with renal failure (urea), perhaps somewhat more toxic than another (creatinine), but was within the same range of toxicity as benzoic acid and phenylalanine. Rats receiving urea and saline were clearly distinguished by their long survival from other compounds tested ($p < 0.05$), but the latter were not separated by significant differences. In Experiment #3, most rats given a full dose of 3,4-dihydroxycinnamic acid were dead within 6 hours.

Experiment #3: The results of Experiment #1 suggested alterations in central nervous system excitability. As an independent check on these findings, changes in seizure threshold were determined by exposing animals to an atmosphere of convulsant ether. Sixteen hours after ureteral ligation, rats were injected with the standard dose of one of several substances (3,4-DHBA, 2,4-DHBA, saline, urea, creatinine, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, and 3,5-dihydroxybenzoic acid) or with one-half the standard dose of one of three substances (3,4-DHBA, 3-hydroxybenzoic acid and 3,4-dihydroxycinnamic acid). Six

hours later seizures were induced and the times of onset of myoclonic jerks and generalized tonic-clonic convulsions recorded.

The results of this experiment are displayed in Figure 10. Seizure threshold in acutely uremic rats six hours after injection with saline solution was essentially the same as in normal rats. Compared to these saline controls, myoclonic jerks (Fig. 10a) and generalized convulsions (Fig. 10b) occurred significantly earlier ($p < 0.05$) in rats receiving standard doses of 4-hydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 3-hydroxybenzoic acid and 3,4-DHBA. One half the standard dose of 3-hydroxybenzoic acid also lowered the seizure threshold, but 3,4-DHBA was not effective at this lower dose.

On the other hand, myoclonic jerks began significantly later ($p < 0.05$) in rats receiving full doses of 2,4-DHBA and creatinine than in saline controls; generalized convulsions in these groups began either within the same range as saline (2,4-DHBA) or later (creatinine). Myoclonic jerks were somewhat (but not significantly) delayed in rats receiving urea. The standard dose of 3,4-dihydroxycinnamic acid generally killed rats within six hours, but one-half the standard dose significantly delayed the onset of both jerks and generalized convulsions. Using 1/10 and 1/100 the standard dose, none of the compounds tested (3,4-DHBA; 3-hydroxybenzoic acid and 3,4-dihydroxycinnamic acid) produced significant alteration in seizure threshold.

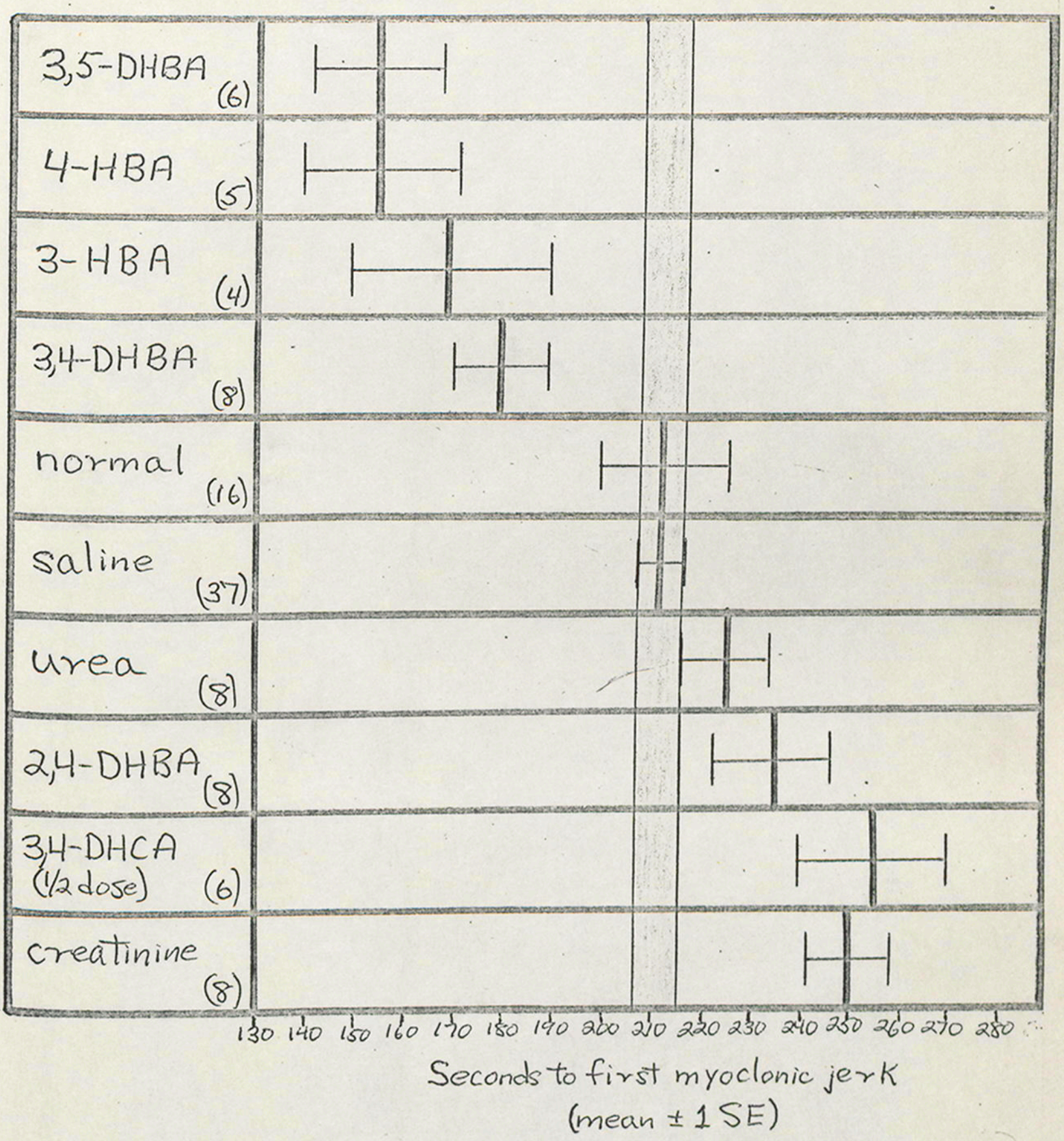


Figure 10a: Seizure threshold (seconds of Indoklon administration until first myoclonic jerk) in groups of rats given one of various compounds. Compared to normal rats, some compounds increase and some decrease susceptibility to seizures. (DHBA=dihydroxybenzoic acid; HBA=hydroxybenzoic acid; DHCA=dihydroxycinnamic acid. Number of rats in each group given in ( ).) Shaded area represents values for saline controls.

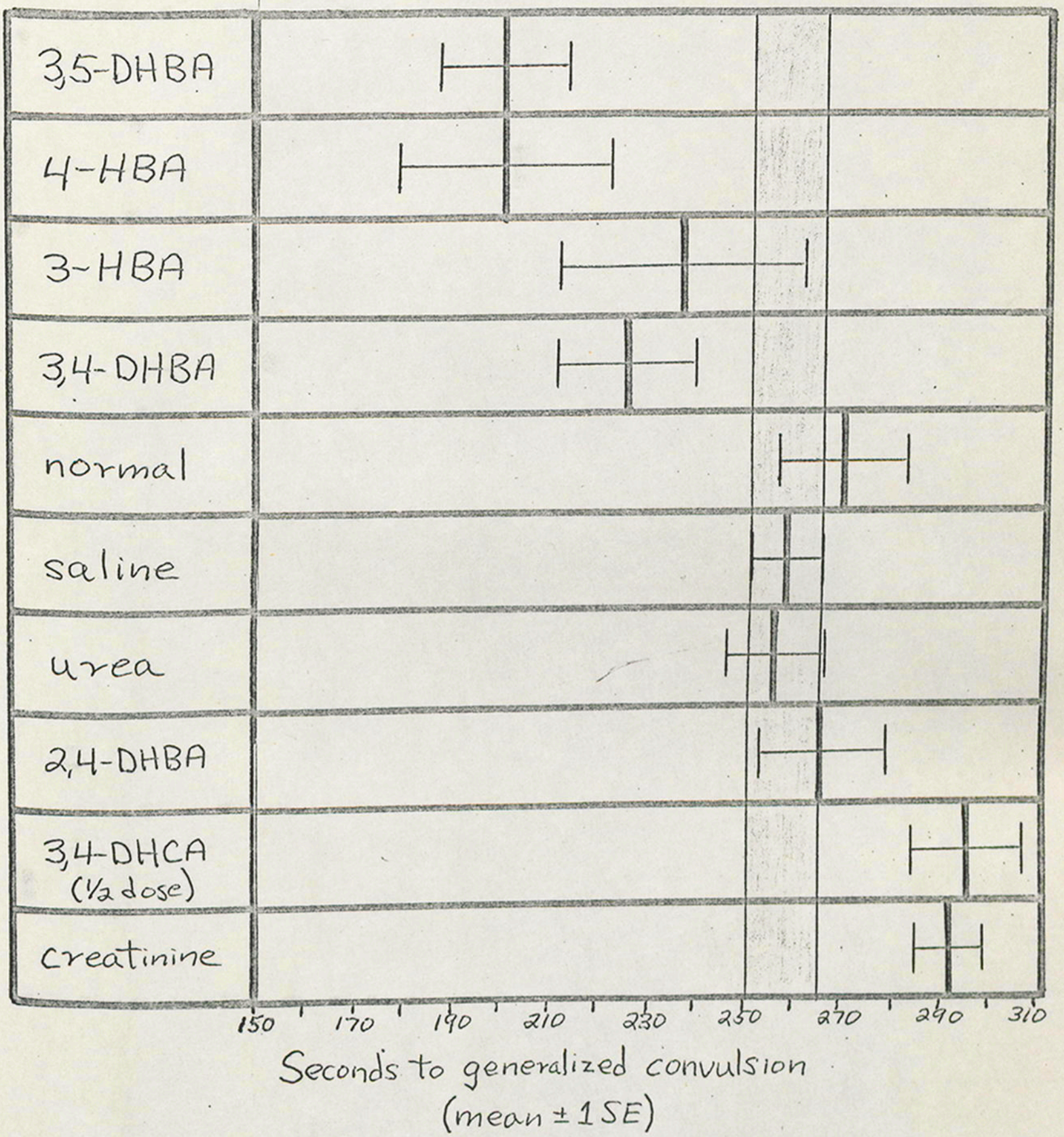


Figure 10b: Seizure threshold (with generalized convulsion as endpoint) in same groups represented in Fig. 10a.

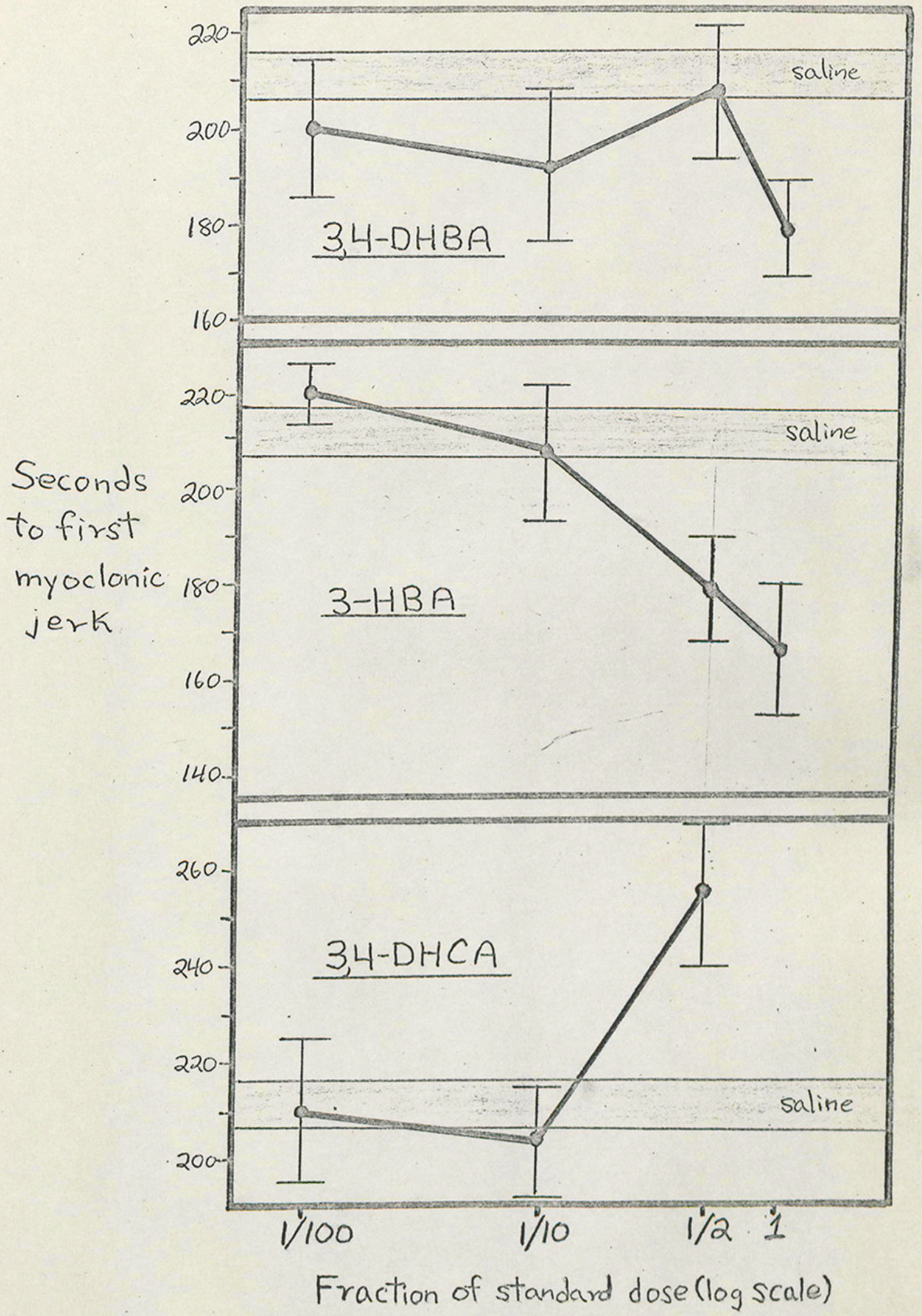


Figure 10c: Seizure threshold measurements (myoclonic jerk as endpoint) demonstrating dose-effect relationship. Unlike 3,4-DHBA, 3-HBA (3-hydroxybenzoic acid) and 3,4-DHCA (3,4-dihydroxycinnamic acid) altered threshold at one-half the standard dose. Shaded areas represent threshold values in saline controls.

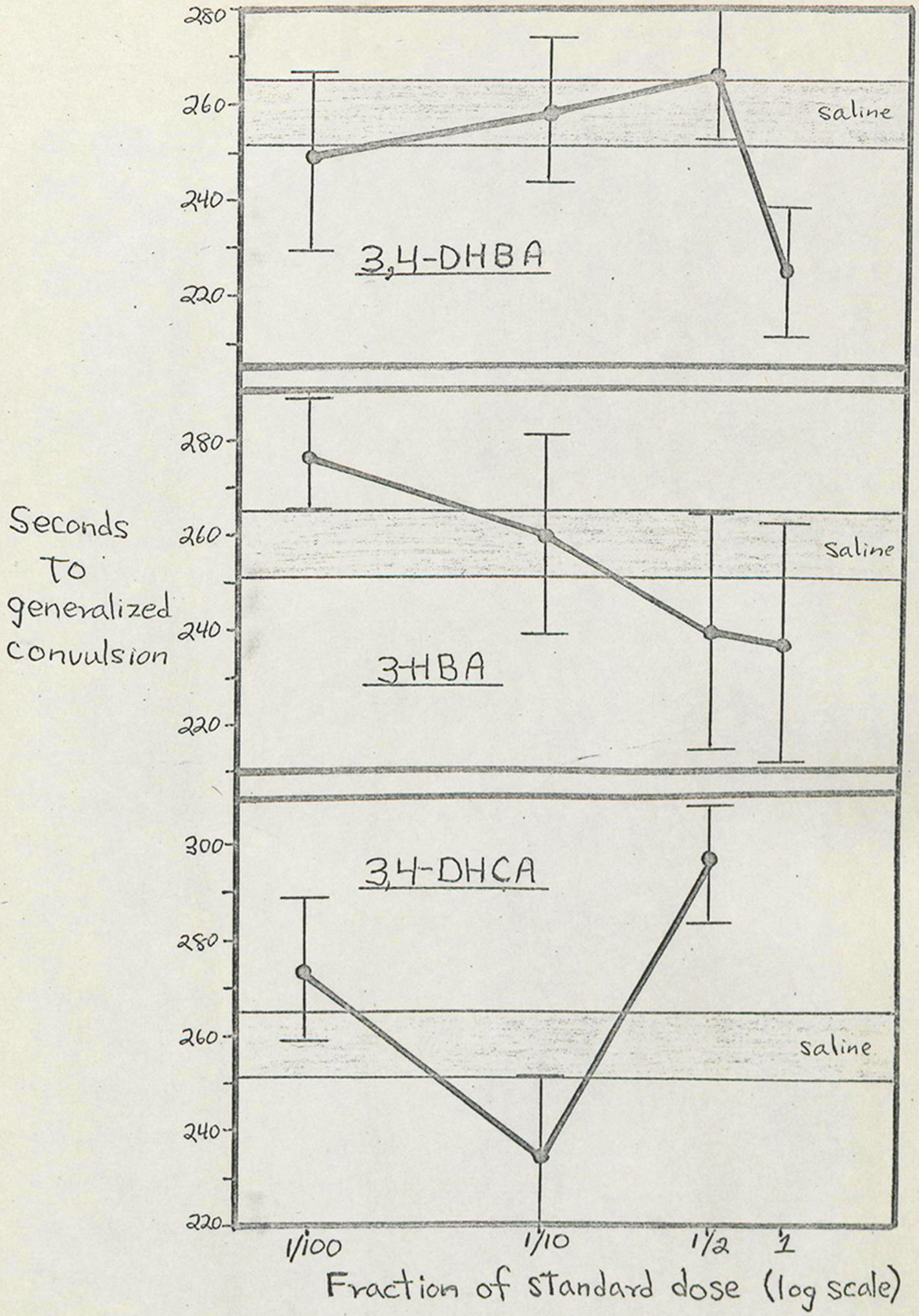


Figure 10d: Seizure threshold measurements (generalized convulsion as endpoint) demonstrating dose-effect relationships. As in Fig. 10c, 3,4-DHBA and 3-HBA tend to lower threshold, whereas 3,4-DHCA raises it markedly.

EXPERIMENT #4: To study changes in blood chemistry following injection, nonimplanted rats were ligated and 16 hours later injected with 3,4-DHBA, 2,4-DHBA (Hour 4 only) or NSS. At 4, 10 and 30 (NSS only) hours after injection, sets of rats receiving each substance were killed by exsanguination and their blood submitted for chemical determinations. The results of this experiment will be presented with blood chemistry results from Experiment #1, in which terminal chemical determinations were performed on blood from ten (of 16) 3,4-DHBA, six (of 12) 2,4-DHBA and seven (of 12) saline rats. Normal rats (Hour-24) and ligated but non-injected rats (Hour-1) provided baselines. The data are presented in detail in Table 2 and Figure 11 a-i.

Viewed together, interval and terminal determinations demonstrated various chemical changes during the experimental uremia. At time of death, all animals in all three groups were hyperkalemic, hypocapneic, hypochloremic and azotemic; all had markedly elevated delta (unidentified anion) levels. Some chemical parameters behaved similarly in all three groups, whereas others differed.

Sodium: Slightly elevated 16 hours after ligation, serum sodium (Fig. 11a) varied considerably following injection. At Hour 4, serum sodium was normal in saline animals, moderately elevated in 3,4-DHBA and markedly elevated in 2,4-DHBA rats. Ten hours after injection, sodium was down to normal in the 3,4-DHBA group but the saline rats were hypernatremic. At death, sodium was somewhat elevated in both benzoic acid groups, but not significantly higher than the saline group.

Table 2: Blood Chemical Determinations (Interval and Terminal).*

| TIME, hours after injection | SUBSTANCE | Na, mEq/L | K mEq/L | $HCO_3$, mEq/L | Cl, mEq/L | Delta, mEq/L | BUN, mgm% | Ca, mEq/L | $PO_4$, mEq/L | SGOT, units/ | No. of Rats Used |
|---|---|---|---|---|---|---|---|---|---|---|---|
| -24 | normal | 139±2.9 | 5.1±0.6 | 25.4±2.0 | 101±2.7 | 23±4.2 | 221±6 | 4.9±0.1 (2) | 2.3±0.3 (2) | 100 (2) | 6 |
| -1 | none | 143±2.8 | 7.2±2.0 | 19.2±4.9 | 99±1.3 | 37±3.6 | 98±8 | 5.3±0.7 (4) | 5.1±0.5 (4) | 353±27 (4) | 6 |
| 4 | 3,4-DHBA | 148±3.2 | 6.9±0.9 | 17.0±2.4 | 82±3.9 | 61±2.9 | 136±9 | 3.0±0.1 (5) | 5.9±1.4 (3) | 246±40 (4) | 6 |
| 4 | 2,4-DHBA | 157±4.1 | 9.6±1.4 | 13.2±2.0 | 91±1.1 | 69±5.7 | 151±9 | 3.5±0.1 (4) | 6.6±0.6 (5) | 380±56 | 6 |
| 4 | NSS | 139±3.0 | 7.1±1.7 | 16.9±1.4 | 90±3.3 | 42±5.0 | 131±10 | 4.7±0.5 (5) | 4.9±0.8 (5) | 280±36 (5) | 6 |
| 10 | 3,4-DHBA | 140±3.6 | 6.8±1.1 | 13.3±3.0 | 85±6.0 | 44±6.0 | 141±25 | 3.6±0.1 (3) | 8.0±0.1 (3) | 299±60 | 4 |
| 10 | NSS | 147±2.9 | 7.8±1.1 | 15.9±3.7 | 106±2.8 | 37±5.0 | 158±19 | 4.4±0.7 (5) | 5.5±0.2 (5) | 238±33 (5) | 6 |
| 30 | NSS | 143±3.5 | 14.4±0.1 | 8.3±0.7 | 104±0.6 | 50±3.6 | 281±10 | 5.4±0.1 (2) | 7.0±0.2 (2) | 247±61 (3) | 3 |
| at death | | | | | | | | | | | |
| 20.9 | 3,4-DHBA | 145±5.2 | 16.8±4.2 | 12.4±5.1 | 81±6.2 | 74±9.5 | 211±43 | 4.8 (1) | 13.7 (1) | 800 (2) | 10 |
| 17.1 | 2,4-DHBA | 148±4.7 | 12.6±4.4 | 13.6±2.0 | 85±6.2 | 68±5.3 | 215±61 | ------- | ------- | >400 (1) | 6 |
| 44.3 | NSS | 139±9.0 | 17.5±3.6 | 9.2±3.9 | 94±7.9 | 59±7.9 | 329±53 | ------- | ------- | 306 (3) | 7 |

*Values given represent mean±ISE, in most cases based on the number of rats noted in right-hand column. When fewer rats contributed to the mean, the number of rats used is recorded in parentheses ( ) beside the value itself.

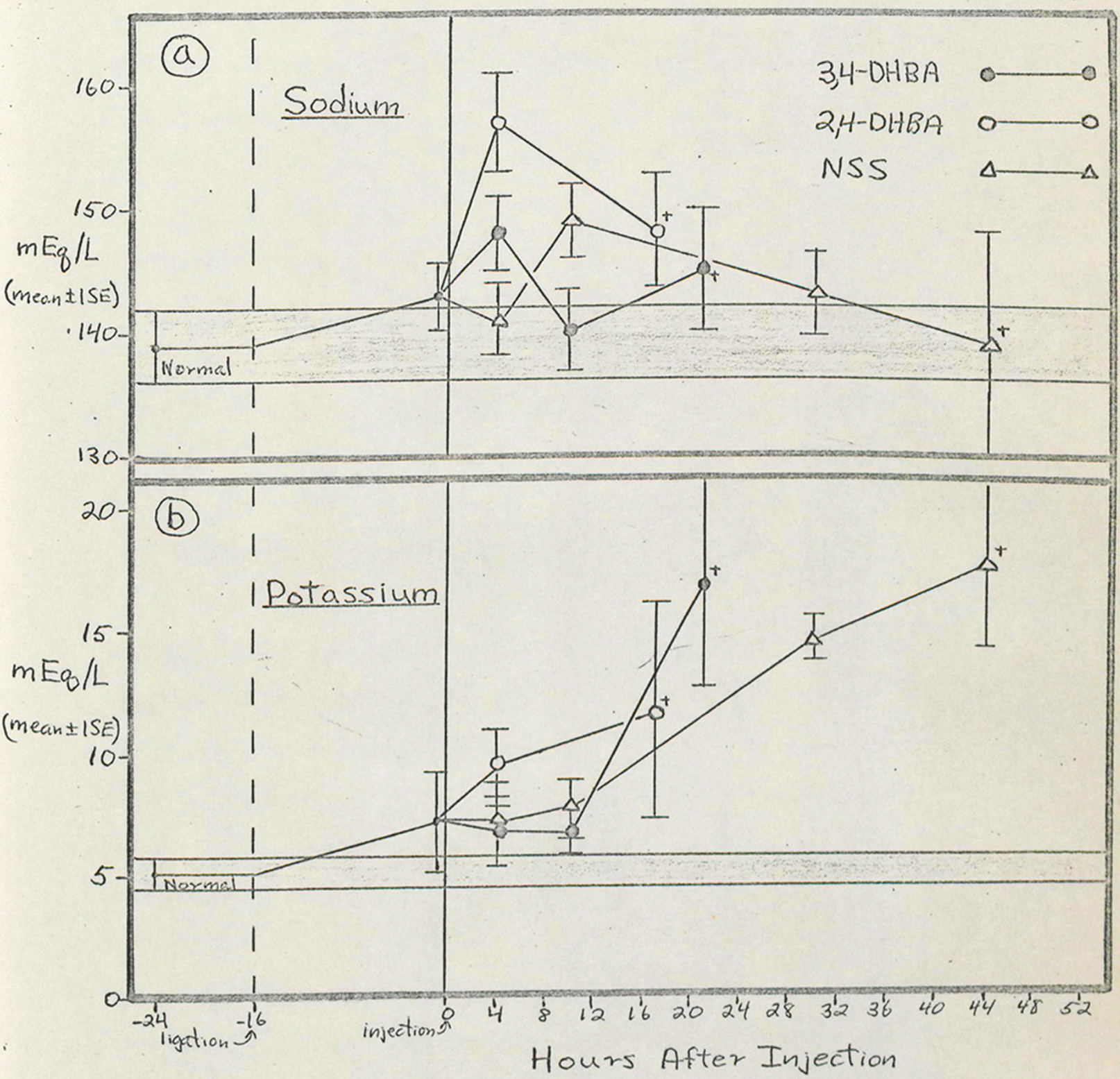


Figure 11a-b: (a) Serum sodium before and after ligation and injection in rats given 3,4-DHBA, 2,4-DHBA or NSS.* In this and following graphs, terminal values are marked with (†). Shaded area represents normal range.
(b) Serum potassium in same groups as above. Note early rise with 2,4-DHBA and marked terminal hyperkalemia in all groups.

*Note initial elevation following injection.

Potassium: Serum potassium (Fig. 11b) rose steadily following ligation. Following injection, 2,4-DHBA rats demonstrated a somewhat more rapid rise in potassium than the 3,4-DHBA and saline groups, which remained essentially stable for several hours. These differences were not statistically significant. At death all three groups were severly hyperkalemic, but serum potassium was somewhat lower in the 2,4-DHBA rats than in the other groups.

Bicarbonate: Serum bicarbonate (Fig. 11c) fell progressively following ligation, somewhat more rapidly in rats receiving 2,4- and 3,4-DHBA, but not significantly so. At death, 3,4 and 2,4-DHBA groups were similarly hypocapneic (12-13 mEq/L), and saline animals were even more so.

Chloride: Serum chloride (Fig. 11d) was low-normal 16 hours after ligation. After injection, chloride fell rapidly in all 3 groups, but rose again to high-normal and above-normal levels in saline animals, dropping below normal again at death. In rats receiving dihydroxybenzoic acid (especially 3,4), chloride dropped progressively following injection and was well below normal at death.

Delta: Unidentified anions (Fig. 11e) had risen above normal by 16 hours after ligation. Following injection delta continued to rise gradually in NSS animal, reaching 55 mEq/L at death. Delta rose precipitously following injection of 3,4 and 2,4-DHBA and was very high (70-75 mEq/L) at death. In 3,4-DHBA rats, delta had fallen well below the Hour 4 level by Hour 10, but then rose again.

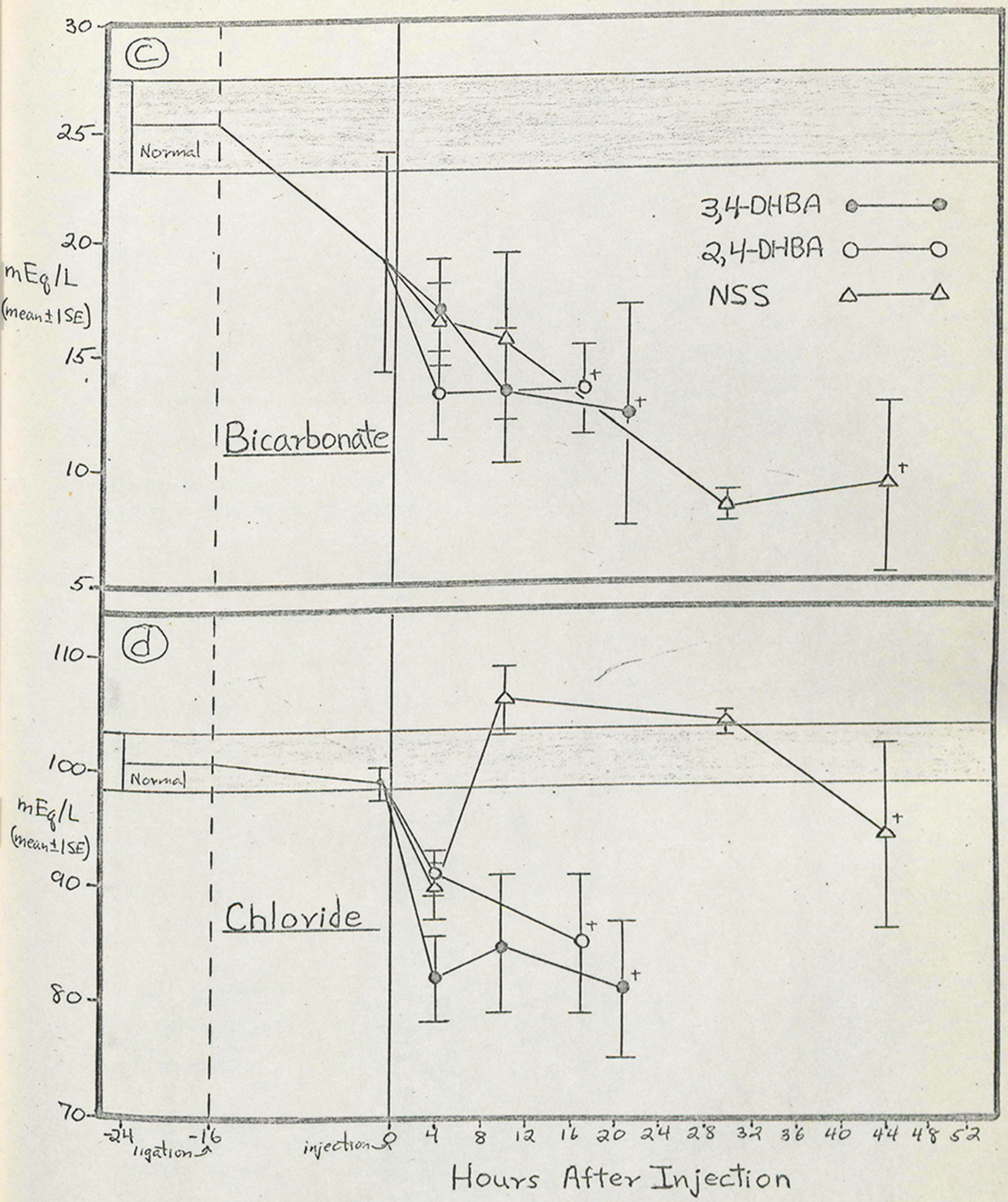


Figure 11c-d: (c) Serum bicarbonate, as in Fig. 11a. Note progressive fall following ligation.
(d) Serum chloride. Note progressive fall following administration of "unidentified anions", compared with eventual elevation with saline.

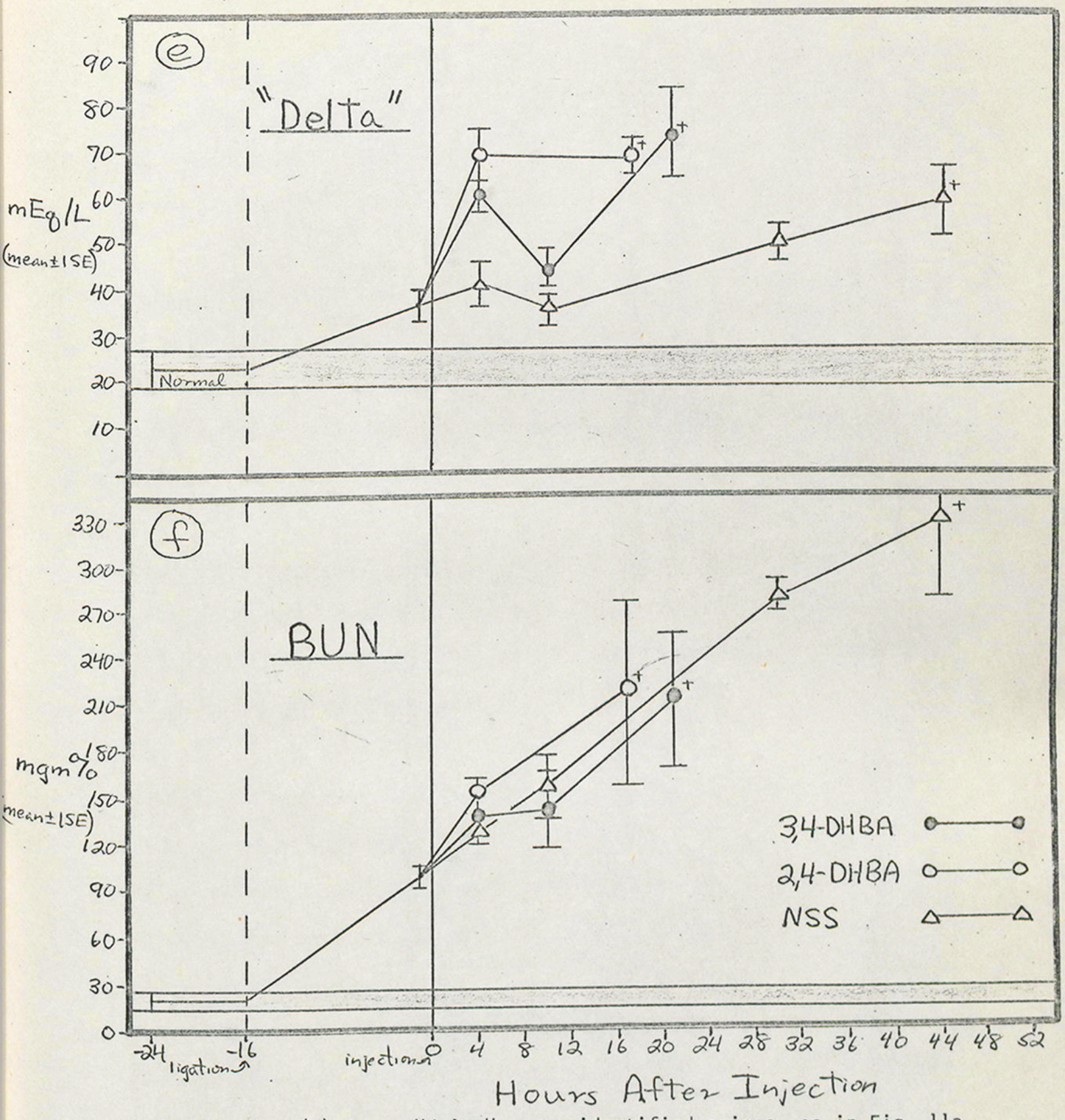


Figure 11e-f: (e) Serum "delta", or unidentified anions, as in Fig. 11a. Note immediate, rapid rise in 3,4-DHBA and 2,4-DHBA groups and gradual, progressive rise in saline controls.

(f) Blood urea nitrogen. Note progressive elevation following ligation at a remarkably constant rate, little affected by nature of injection.

Urea: Blood urea nitrogen (Fig. 11f) rose steadily following ligation at a rate of about 5 mgm% per hour. This rate was essentially unaffected by injection with any of the test substances, and the saline group--which died the latest--had the highest BUN at death.

Calcium: Serum calcium (Fig. 11g) fell somewhat following injection of saline, but was above normal at death in the saline group. In both the 3,4- and 2,4-DHBA groups, calcium fell precipitously from 5.2 to 3.0-3.5 mEq/L during the 4 hours after injection, but (in 3,4-DHBA rats at least) appeared to rise towards normal by the time of death.

Phosphate: Serum phosphate (Fig. 11h) rose rapidly following ligation, and continued to rise very rapidly in rats injected with either 3,4 or 2,4-DHBA. In rats receiving saline, phosphate stabilized briefly after injection, but then it resumed its steady rise.

SGOT: Serum transaminase activity (Fig. 11i) was clearly above normal 16 hours after ligation. Following injection of saline, SGOT activity diminished over the first 10 hours and then remained essentially stable (elevated but below pre-injection levels). Rats receiving 3,4-DHBA demonstrated a similar initial drop, but then rose again and terminally (in the only animal tested) was very high (800). SGOT activity continued to rise following injection of 2,4-DHBA and a single terminal measurement was very high (>400).

Of the ten 3,4-DHBA rats with terminal blood chemistry studies, seven developed myoclonic jerks and three did not. Of the six 2,4-DHBA rats with terminal studies, two developed jerks. Comparison of

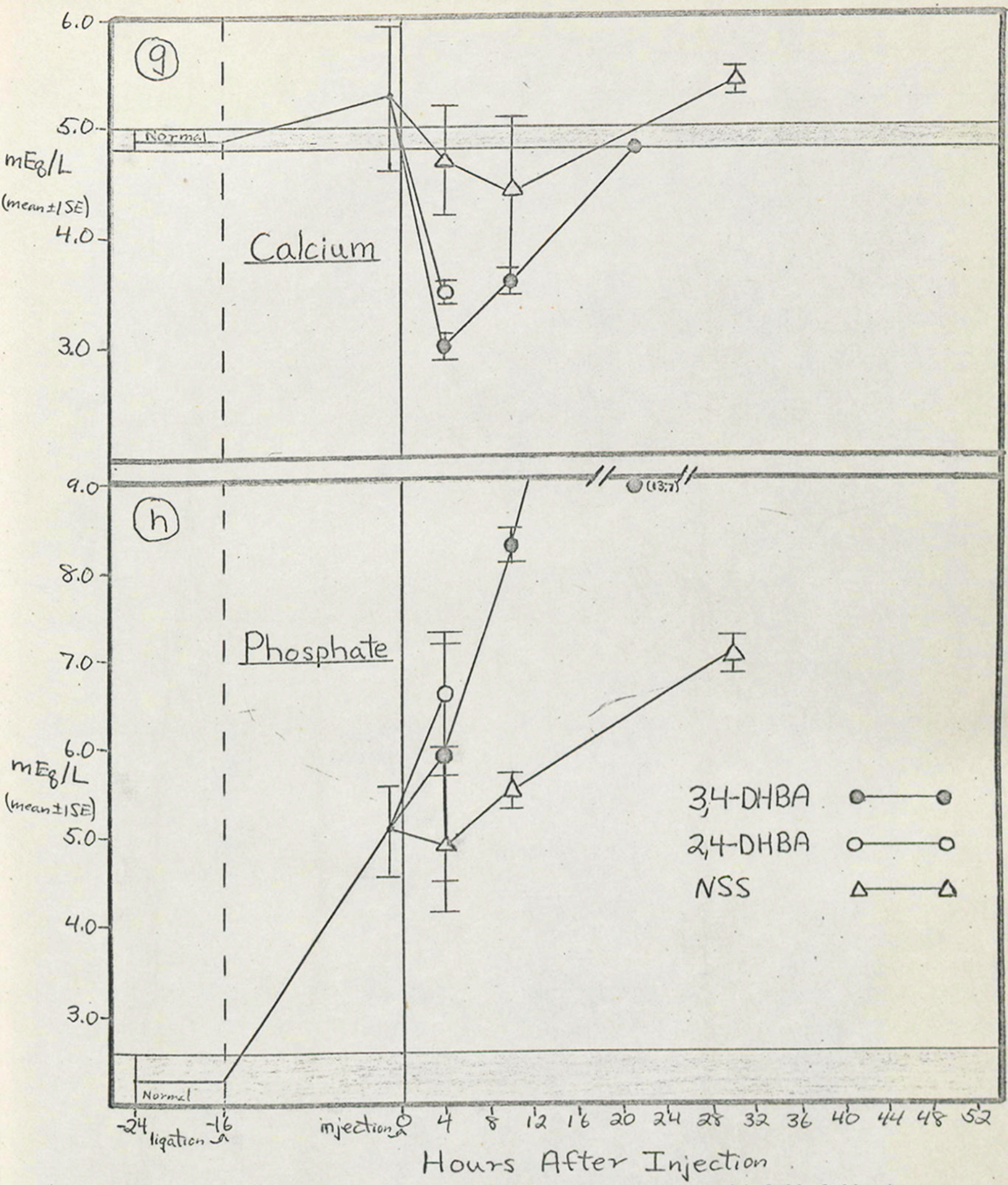


Figure 11g-h: (g) Serum calcium as in Fig. 11a. Note rapid fall following injection of either dihydroxybenzoic acid, compared to essentially normal levels in NSS rats.

(h) Serum phosphate. Note rapid rise following injection, with some acceleration after injection with either acid.

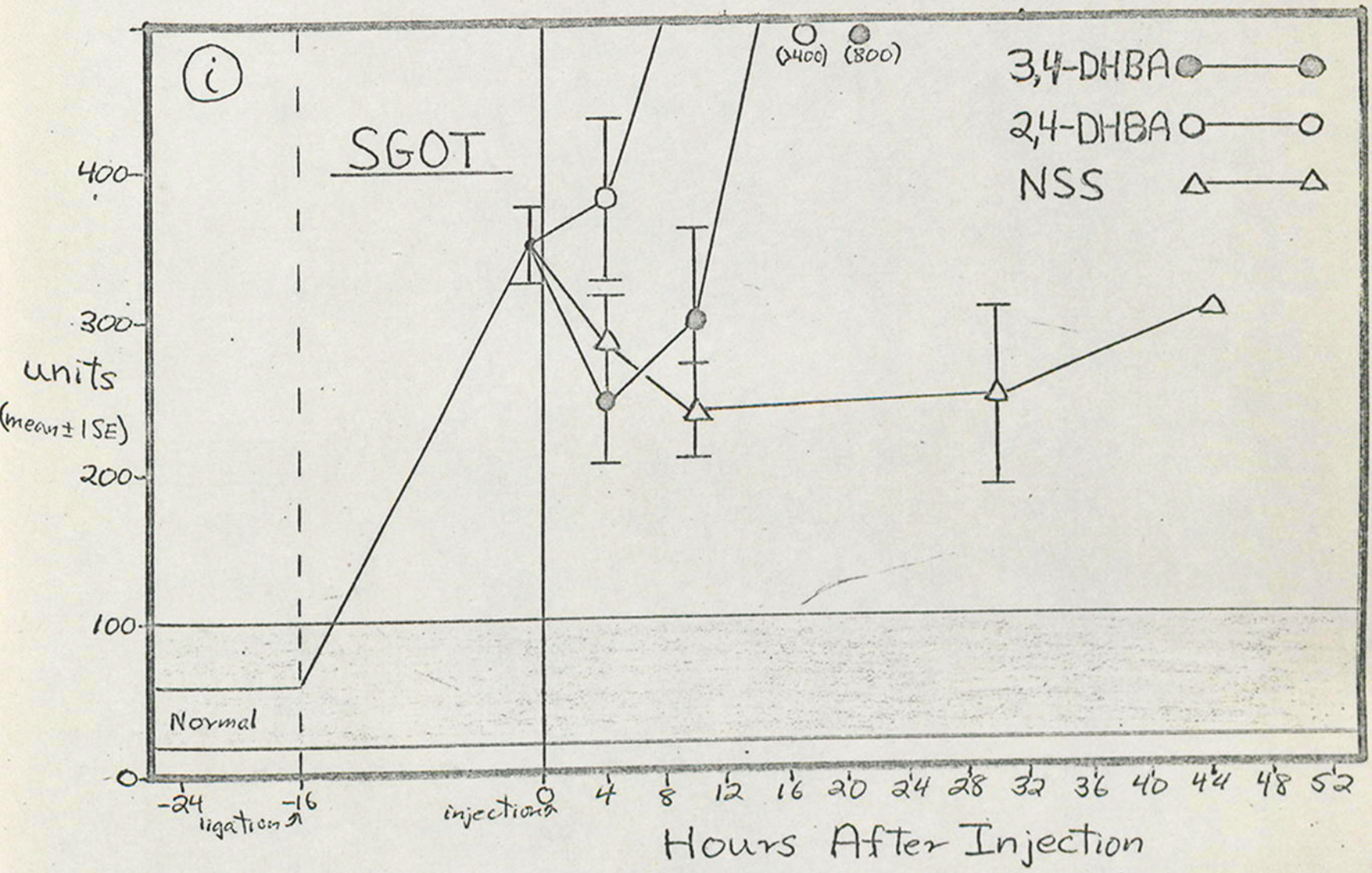


Figure 11i: Serum glutamic-oxaloacetic transaminase activity, as in Fig. 11a. Note rapid rise following ligation, and continued rise with 2,4-DHBA, delayed rise with 3,4-DHBA, and plateauing with NSS.

average urea and electrolyte levels in both "jerks" and "no jerks" groups revealed differences which, except for urea, were in the same direction and of similar magnitude in both 3,4-DHBA and 2,4-DHBA groups (Table 3). Animals with myoclonic jerks--compared with those with none--had at death slightly higher sodium and chloride, and moderately higher potassium and delta. Consequently, they were relatively hyperosmotic. Bicarbonate was markedly lower in 3,4-DHBA rats with jerks and moderately lower in the 2,4-DHBA group. BUN was lower in 3,4-DHBA rats with jerks but higher in the 2,4-DHBA group.

EXPERIMENT #5: The purpose of this experiment was to measure the concentration of 3,4-DHBA in the serum of experimental animals. Sixteen hours after ligation three non-implanted rats were injected with the standard dose of 3,4-DHBA and 10 hours later exsanguinated. At 290 mu, a 1:4000 dilution of the standard (150 mEq/L) solution of 3,4-DHBA had an absorbance of 0.34. Protein-free filtrates from the three 3,4-DHBA rats gave absorbances of 0.07, 0.06 and 0.085. These are equivalent to concentrations of 8.3, 6.7 and 9.4 mEq/L, or a mean concentration of 8.1 ± 1.2 mEq/L. Inasmuchas serum from a single saline rat at Hour 20 did not absorb light at this wavelength, it is reasonable to expect that these absorbances reflected either injected 3,4-DHBA or a closely related metabolic product.

Table 3: Comparison of Terminal Blood Electrolyte and Urea Levels in Rats With and Without Myoclonic Jerks.

| | 3,4-DHBA | | 2,4-DHBA | | |
|---|---|---|---|---|---|
| jerks | yes | no | yes | no | Comment |
| no. of rats | 7 | 3 | 2 | 4 | |
| sodium | 147 | 142 | 151 | 147 | higher with jerks |
| chloride | 82 | 79 | 90 | 83 | higher with jerks |
| potassium | 17.8 | 14.5 | 16.0 | 11.0 | higher with jerks |
| delta | 74 | 58 | 67 | 61 | higher with jerks |
| bicarbonate | 9.6 | 19.1 | 10.9 | 14.9 | lower with jerks |
| BUN | 206 | 221 | 286 | 180 | not consistent |

## DISCUSSION

These data suggest that 3,4-dihydroxybenzoic acid is toxic when administered to rats with acute renal failure; that, as studied here, it contributes to the occurrence of certain phenomena characteristic of clinical uremia; and that the effects of this phenolic acid can be distinguished from those of its 2,4 structural isomer. By implication, abnormal accumulations of 3,4-DHBA might contribute to the occurrence of signs and symptoms--particularly those of neurologic hyperexcitability--in human uremia.

The pathogenesis of uremia has been exhaustively investigated and discussed. Many biochemical abnormalities have been detected in uremics and nearly all have been indicted as potentially toxic. Urea, creatinine, amino acids, various organic acids and bases, sulfate, phosphate, chloride, potassium, magnesium, acidosis, hyperosmolarity, water intoxication and permeability changes have all been implicated.[11] Yet no single biochemical or physiological abnormality has been correlated consistently and unequivocably with the signs and symptoms of uremia. As a consequence, many investigators have concluded that uremia results not from a single factor but from the cummulative effects of innumerable abnormalities (none grossly toxic by itself) which develop in renal failure. This concept of "summation of sub-minimal stimuli" is now widely accepted.[12]

Some work has been done to differentiate the effects of these "sub-minimal stimuli," including several studies which compared the

toxicity of various substances on *in vitro* enzyme systems.[12,13] As noted earlier, differential inhibition of rat cerebral enzyme systems by derivatives of benzoic, hippuric and cinnamic acids has been demonstrated.[5] Concentrations of phenolic acids approximately 100 times greater than found in uremic plasma were required to demonstrate consistent inhibition. A subsequent study showed that glycine conjugation of several benzoic acid derivatives (a potential *in vivo* mechanism of detoxication) had variable effects on the degree of cerebral enzyme inhibition produced *in vitro* by high concentrations of these compounds (again about 100 times the uremic plasma level).[6] A subordinate finding--but one of relevance to the present study--was the considerable toxicity of 3,4-dihydroxybenzoic acid relative to other hydroxy- and methoxybenzoic acids. Succinate oxidation, anaerobic glycolysis and glutamic acid decarboxylation in rat brain slices were all markedly inhibited by 3,4-DHBA. (Of interest in the previous study was the notable toxicity of other 3,4 substituted phenolic acids, especially 4-hydroxy-3-methoxy-cinnamic acid.[5])

The possible role of phenols (of which 3,4-DHBA is one) in the pathogenesis of uremia has been reviewed by Schreiner and Maher.[11] Blood phenols are primarily derived from the action of intestinal bacteria on protein derivatives containing aromatic amino acids, are absorbed from the gut, conjugated in the liver and excreted primarily by the kidneys. Normally, total human plasma phenols--consisting of various amino acids, phenols and aromatic hydroxy acids--do not exceed 0.1 mg%. Marked elevations of free and conjugated phenols have been

detected in uremia (total phenols 1.43-4.21, free phenols 0.93-2.94 mg% in 17 patients with BUN of 47-570 mg%). Generally, phenol levels have been found to correlate with uremic symptoms, most notably with the presence (and often the degree) of central nervous system depression.[14-18] Some later studies, however, have failed to demonstrate a clear correlation between blood phenols and either BUN or the clinical picture.[19,20]

The present study was undertaken to evaluate in vivo the potential contribution of the single phenolic compound, 3,4-dihydroxybenzoic acid, to the pathogenesis of uremia. This compound was selected because: 1) it is excreted in considerable amounts (46-116 mg/day) in the urine of normal humans on regular diets;[7] 2) metabolic derivatives of the acid have been identified in human uremic dialysates; 3) the compound inhibits several rat cerebral enzyme systems in vitro; and 4) the compound has a close structural and (presumably) metabolic relationship to compounds of probable importance in central nervous system function (norepinephrine and dopamine).[4] The 2,4 structural isomer was studied to help distinguish specific effects of the 3,4 configuration. The neurologic effects of these compounds in rats in acute renal failure were the primary focus of the study, but certain cardiac phenomena were also investigated.

As studied here, both 3,4-DHBA and 2,4-DHBA were markedly toxic in terms of longevity: saline controls lived almost twice as long after injection. In equal doses, 3,4-DHBA killed acutely uremic rats much sooner than phenylalanine, succinic acid and creatinine. Therefore, mole

for mole, 3,4-DHBA appears to be more toxic than urea and, with the exception of 3,4-dihydroxycinnamic acid, at least as toxic as the others. In terms of hastening death, 3,4-DHBA and 2,4-DHBA were about equally toxic, with the latter slightly more so.

Behavioral phenomena observed here can be categorized as signs of neuromuscular depression (weakness, prostration, tremors on effort) and neuromuscular hyperexcitability (tremors at rest, muscle spasms, myoclonic jerks, grand mal convulsions and hyperexcitability to sound). Both acids hastened the onset of neuromuscular depression, 2,4-DHBA somewhat more rapidly than 3,4-DHBA. In fact, using the square test as a non-specific indicator of overall neuromuscular depression, a statistically significant difference between the two isomers was clearly demonstrated: mobility of 2,4-DHBA rats was retarded much earlier than that of 3,4-DHBA animals. Phenomena of neuromuscular hyperexcitability demonstrated even more clearly the differences between 3,4-DHBA and 2,4-DHBA. Tremors at rest, myoclonic jerks, muscle spasms and grand mal convulsions were all noted more frequently in 3,4-DHBA than in 2,4-DHBA or saline rats; in respect to tremors and jerks, the differences were statistically significant. Moreover, two of these phenomena (tremors and muscle spasms) were observed actually less frequently in 2,4-DHBA rats than in saline controls.

As a whole, these findings suggest that administration of 3,4-DHBA contributed not only to early weakness, prostration and demise, but also to neuromuscular hyperexcitability. In comparison, 2,4-DHBA produced much less irritability, and perhaps even depressed some of those parameters of neuromuscular activity which 3,4-DHBA excited. Electroencepha-

lographic and seizure threshold data support this hypothesis. The incidence of both spontaneous and strobe-induced paroxysmal seizure activity was significantly greater in 3,4-DHBA than in 2,4-DHBA and NSS animals. Generalized electrical seizures (accompanying grand mal convulsions) also were documented more often in 3,4-DHBA rats. Susceptibility to seizures induced by hexafluorodiethyl ether was clearly increased in rats given 3,4-DHBA and clearly diminished in those given the 2,4 isomer.

Electroencephalography has been used infrequently to study uremia experimentally and more often to evaluate the syndrome clinically. In cats with intact renal function, intravenous infusion of 20% urea was followed by spindling and seizure discharge of cortical leads.[21] Generalized slowing of background frequencies has been reported in uremic patients.[22] EEG deterioration--bursts of high voltage, rhythmic delta waves--occuring during hemodialysis has been attributed to the development of cerebrospinal fluid-blood osmotic gradients and rapid water shifts.[23] In 13 acutely uremic patients, loss of background activity and appearance of predominant delta rhythm were detected; however, EEG alterations did not correlate with either clinical or chemical changes.[24]

Kiley and Hines found a close correlation between EEG frequency distribution and mental status (the lower the frequency, the more obtunded the patient).[25] Although these changes were not specific for uremia, the authors concluded that the EEG provides a more precise and sensitive means of evaluating the severity of uremia than do blood chemical levels or simple psychological testing. Jacob and colleagues evaluated 22 uremic

patients (14 chronic, 8 acute) and described several prominent but non-specific abnormalities. In all but two patients they noted poor regulation and slowing of background activity and bursts of paroxysmal, bilaterally synchronous slow waves; of less frequency were paradoxical responses to eye opening, abnormal arousal responses, and myoclonic responses to photic stimulation.[26] EEG deterioration during hemodialysis seemed related to falling plasma osmolarity; there was no correlation between EEG changes and BUN.

In the present study electroencephalography was of limited value as a monitor of the general degree of uremic toxicity. Paroxysmal seizure phenomena were the exception; here the EEG contributed considerably in detecting and documenting differences between 3,4-DHBA and 2,4-DHBA. Otherwise, behavioral observation was a more reliable indicator of clinical status than was the EEG. Although marked slowing occurred at some time in about 25% of all animals irrespective of group, no consistent change in the basic EEG pattern was detected over the course of acute uremia. The absence of consistent abnormality in the corticogram when rats were obviously lethargic, paretic and prostrate suggests that this depression resulted either from central nervous system depression lower than and not reflected in the rat's small, relatively undeveloped cortex; or from peripheral neuromuscular toxicity; or from diminished peripheral perfusion and/or impaired oxygenation secondary to congestive heart failure. EEG evidence of central system dysfunction, therefore, was not manifested by consistent generalized slowing and disorganization, but was restricted to paroxysmal seizure phenomena and transient slowing in some animals.

Behavioral and electroencephalographic findings--augmentation of neuromuscular excitability of 3,4-DHBA and depression of excitability by 2,4-DHBA--were corroborated by responses to the convulsant ether, Indoklon. Seizure threshold measurements clearly distinguished three groups of substances. Compared to normal animals, neither saline nor urea significantly altered seizure threshold. Compounds in the second group (which, in addition to 3,4-DHBA, included 3- and 4-hydroxy and 3,5-dihydroxybenzoic acid acids) made rats more susceptible to artificially induced seizures. Compounds in the third group (which, in addition to 2,4-DHBA, included creatinine and 3,4-dihydroxycinnamic acid) diminished susceptibility to Indoklon-induced seizures.

Seizure threshold determinations thus demonstrated that neither 3,4-DHBA nor 2,4-DHBA is unique in its effect on neuromuscular excitability. Moreover, it appears that 3,4-DHBA is less potent than some related compounds in changing seizure threshold, and that the 3,4-dihydroxy configuration is not requisite for lowering threshold. Of the many substances which accumulate in renal failure, it appears that some lower and some raise seizure threshold, while some (for example, urea) have no effect. These findings are intriguing inasmuch as the uremic syndrome is characterized neurologically by signs of both hyperexcitability and depression.

The elevation of seizure threshold by creatinine is of special interest in view of a recent clinical study which correlated elevation of serum creatinine in uremic patients with decreased conduction velocity

in peripheral nerves.[27] Perhaps the diminished susceptibility to seizures produced by creatinine (and other compounds) resulted from impaired central and/or peripheral neural conduction.

Of special interest is the marked difference between the effects of 3,4-DHBA and 3,4-dihydroxycinnamic acid. By replacing -COOH with $-CH_2CH_2COOH$, marked lowering is exchanged for marked elevation of seizure threshold. Similarly, 3,4-DHBA and 2,4-DHBA differ only in the position of a single hydroxyl group, yet their effects on seizure threshold appear to be in opposite directions. It is clear that molecular structure is an important determinant of effect, but a consistent structure-action relationship is not yet apparent.

Also of interest--and likewise unexplained--is the paradoxical lowering of seizure threshold by one-tenth dose of 3,4-dihydroxycinnamic acid, inasmuch as one-half the standard dose produced marked elevation of threshold (Fig. 10d). Perhaps this comparatively potent compound produced both peripheral neuromuscular depression and central excitation; with the former predominating at high doses. Or perhaps the delayed onset of seizures with high doses resulted from serious impairment of cardiac or pulmonary function (see discussion below), with consequent delayed transport of the epileptogenic stimulus to the brain. Such systemic effects might not predominate at lower doses.

The technique used here for measurement of seizure threshold has both advantages and disadvantages. In its favor are "minimal handling, maximal observation and capability of simultaneous determination of threshold in experimental and control animals."[8] On the other hand,

the epileptogenic stimulus--presumably a critical level of ether in brain tissues--is delivered by an indirect route (airways, lungs, blood, brain) and is dependent on pulmonary ventilation, alveolar exchange and cardiac output for the production of seizures. Since brain levels of Indoklon were not measured in this study, the possible effects of increased or decreased ventilation, exchange and cardiac output in altering seizure threshold cannot be dismissed.

Impairment of these cardiorespiratory functions might have contributed to prolonging the onset of seizures in rats receiving 2,4-DHBA, 3,4-dihydroxycinnamic acid and creatinine. (Indeed, in Experiment #1 arrhythmias were detected earlier and more frequently in 2,4-DHBA than in 3,4-DHBA rats; the degree of bradycardia produced by both compounds, however, was the same). It seems less likely that administration of 3,4-DHBA and other toxic compounds facilitated transport of Indoklon to the brain by increasing cardiac output and ventilation. Whatever the effects of test compounds on cardiorespiratory function, the concurrence of behavioral, EEG and seizure threshold findings favors the validity of the seizure threshold measurements as indicators of cerebral excitability.

Electrocardiography revealed marked effects on the heart by both dihydroxybenzoic acids. The transient, moderate bradycardia occuring in the saline group probably reflected rapid expansion of intravascular volume following injection. Superimposed upon this in 3,4-DHBA and 2,4-DHBA animals was a more severe and persistent bradycardia. Early arrhythmias were also prominent in these animals, especially in the 2,4-DHBA

group. These findings are consistent with those of earlier studies. The cardiotoxicity of uremic serum and of phenols was demonstrated initially by Mason.[18] Raab showed that blood and heart muscle from uremic patients contained excessive amounts of catechol compounds, that uremic serum produced marked bradycardia and standstill in isolated frog hearts, and that analogous results could be reproduced using various known catechol and phenol compounds.[28]

Recently Bailey et al reported four uremic patients with cardiomyopathy--congestive failure, pericarditis, arrhythmias, arrest, cardiomegaly and EKG changes--which cleared after 30-60 days of intensive hemodialysis; according to the authors, these results suggest the presence of myocardial suppressant substance(s) in the blood or uremic patients.[29] Although bradycardia is not a commonly recognized cardiovascular manifestation of uremia, both Raab[28] and Hess[30] have discussed bradycardia and increased cardiac susceptibility to inhibitory vagal stimuli in uremic patients. In this regard, the gradually progressive bradycardia of the saline controls in the present study--with onset at ligation and progressing until death--is of interest. Because of its rapid onset following injection, the bradycardia produced here by both dihydroxybenzoic acids probably resulted from direct action on the heart itself, but central increase of vagal tone might have contributed. Hyperkalemia was an unlikely cause of bradycardia here since potassium levels were near normal at Hour 4 when bradycardia was marked.

The relative tachycardia which generally occurred preterminally in 3,4-DHBA rats and its temporal association with the onset of aggravation

of signs of neuromuscular irritability support the hypothesis that 3,4-DHBA (but not 2,4-DHBA) contributed to hyperexcitability. Once again, 3,4-DHBA seems to have had both a depressant effect (perhaps because it is a phenol) and an excitant effect (perhaps because of its structural similarity to catecholamines).

Alterations in blood chemistry detected in this study closely resemble those of human uremic acidosis. Following ureteral ligation, rats in all three groups became progressively hyperkalemic, hypocapneic, hypochloemic, hyperphosphatemic and azotemic; the level of unidentified anions increased. So universal was marked terminal hyperkalemia that hyperkalemic cardiomyopathy may well have been the ultimate cause of death in a majority of animals.

Blood chemical changes were evaluated in order to determine whether behavioral EEG, EKG and seizure threshold phenomena might have resulted from changes in blood chemical factors. Can the varied effects of 3,4-DHBA, 2,4-DHBA and NSS be attributed to blood chemical alterations produced by injection of these substances?

At no time (either at interval evaluation or terminally) were there statistically significant ($>$ 2SE) differences among the three groups with respect to serum potassium, bicarbonate and urea nitrogen. Compared to 3,4-DHBA at Hour 4, 2,4-DHBA rats had significantly higher sodium, chloride and SGOT; otherwise there were no significant blood chemical differences between the two benzoic acid groups. Both groups experienced rapid fall of serum calcium and elevation of phosphate; since the magnitude of these changes was comparable in both groups, hypocalcemia and hyperphosphatemia cannot be invoked to explain the comparatively striking

neuromuscular hyperexcitability of the 3,4-DHBA rats. Hypocalcemia secondary to hyperphosphatemia (itself secondary to enhanced cellular destruction and protein degradation caused by the injected benzoic acids) might have accounted for these findings. Hypocalcemia may have contributed directly to the early demise of both acid groups.

Compared to saline controls, both benzoic acid groups at Hour 4 had significantly higher sodium, higher delta, lower calcium; and at Hour 10, lower chloride, higher phosphate and higher SGOT. The elevation of serum sodium in both benzoic acid groups 4 hours after injection (and a similar rise in saline animals at Hour 10) probably resulted from influx into the vascular space of a solution relatively hypertonic in sodium (mean serum sodium prior to injection, 143 mEq/L; normal saline solution, 154 mEq/L; benzoic acid solutions, at least 150 mEq/L, with NaOH added to bring pH to 7.0-7.4). If, as serum determinations suggest, 2,4-DHBA rats received a greater sodium load, then this might have contributed to more severe cardiac dysfunction in the 2,4-DHBA group. Since the sodium concentration of the injected solutions was not measured, this cannot be ruled out. More rapid elevations of potassium (not statistically significant) and SGOT (significant) in rats injected with 2,4-DHBA are consistent with earlier myocardial damage.

The rapid rise in delta in both benzoic acid groups undoubtedly reflected rapid movement of the acids, "unidentified anions" by definition, into the intravascular space, with consequent displacement of serum chloride. The minimal fall in delta at Hour 10 in NSS rats probably reflected dilution of the serum with injected saline solution.

The drop in chloride in NSS rats at Hour 4 is unexplained, but the elevation at Hour 10 is consistent with injection of solution containing 154 mEq/L of chloride. The progressive fall of bicarbonate and the gradual fall of chloride after Hour 10 reflect the reciprocal elevation of unidentified anions.

There is no evidence that water intoxication--which can produce restlessness, twitching, muscle cramps, convulsions and coma in human patients--contributed in any way to phenomena observed here. Terminal and interval serum sodium levels were normal or above normal throughout, and weight changes (slight increase in 3,4-DHBA and 2,4-DHBA rats and minimal decrease in saline animals) were consistent with the interval of relative dehydration between injection (10 ml in 200 Gm rat) and death.

The determining factor in terms of behavior, EEG, EKG, seizure threshold and demise appears to have been whether a dihydroxybenzoic acid was administered and, if so, which one. It is of interest, however, that 3,4-DHBA and 2,4-DHBA rats which developed myoclonic jerks--when compared to their fellows without jerks--were, at death, relatively hypernatremic, hyperkalemic, hyperosmotic, hypocapneic and hyper-delta; there was no similar consistent difference in BUN. The secondary contribution of any or all of these factors cannot be discounted.

"Delta," the level of unidentified anions, was first correlated with the clinical uremic course by Hyman.[31] Determined by subtracting ($Cl+HCO_3$) from (Na+K+Ca), delta is an indirect measure of the sum of serum protein, organic acids, phosphates and sulfates. In normal human sera, delta is about 24 mEq/L; in uremia, protein may drop somewhat but organic acids, phosphate and sulfate fractions rise, and delta rises to

the range of 30-40 mEq/L.[32] Seligson found that serum organic acids, normally about 6 mEq/L, ranged from 6 to 26 mEq/L in uremic patients, with the highest elevations in symptomatic uremia.[33] Salisbury and colleagues have reported two patients in whom exaccerbation of the uremic course was associated with a rise in unidentified anions above 25 mEq/L but was independent of changes in serum cations, urea, creatinine and uric acid.[34] They interpreted their data as supporting the concept that central nervous system manifestations in uremia are caused by the accumulation of unknown, low molecular weight anions.

In the present study, delta was 23 mEq/L in normal rats and 59 mEq/L at death in saline controls, which represents an increase of 26 mEq/L of unidentified anions over 60 hours of acute renal failure. In 3,4-DHBA and 2,4-DHBA, delta at death was 74 and 68 mEq/L, respectively, representing gains of 45-51 mEq/L over about 36 hours of renal failure, or about 9-15 mEq/L more than saline controls over a considerably shorter interval.

A fraction of the augmented delta consisted of the injected acid itself. Spectrophotometric measurements of 3,4-dihydroxybenzoic acid ten hours after injection revealed concentrations of about 8 mEq/L of the free dihydroxybenzoic acid. At the same time, delta for the 3,4-DHBA group was 7 mEq/L greater than for the NSS group; this difference may represent the direct contribution of injected 3,4-DHBA to total unidentified anions following equilibration. This value is consistent with distribution through total body water (if total body water = 70% of body weight, 750 mEq/100 gm body weight (the standard dose) = 10.1 mEq/L of total body water), or with binding to tissue or plasma proteins

(in the latter case, actual blood levels of acid would be higher than those measured in the serum), or with metabolic alteration to compounds with different electro-magnetic absorbtion properties. Perhaps all three mechanisms had a role in lowering the serum concentration of the acid which, shortly after injection (as implied by delta values at Hour 4), must have been about 16 mEq/L.

A portion of the rise in delta in uremia is due to elevation in sulfate and phosphate. Sulfate was not measured in this study. From measurements of phosphate, it appears that serum phosphate rose more rapidly and to higher levels in 3,4-DHBA and 2,4-DHBA rats than in saline controls. A more rapid accumulation of phosphate (and perhaps also a sulfate) would have contributed to the rapidly achieved and sustained elevation of delta. SGOT, which was elevated in all the uremic animals (perhaps as a result of the surgical trauma of ureteral ligation), was consistently higher in 3,4-DHBA and 2,4-DHBA animals after Hour 4. Both phosphate and SGOT elevations suggest accelerated cell destruction and protein degradation in animals receiving either dihydroxybenzoic acid.

The objective of this study was <u>not</u> to demonstrate toxicity of 3,4-dihydroxybenzoic acid at levels known to accumulate in uremia. Rather, it was to distinguish, by using high concentrations, which of the many signs and symptoms of the uremic syndrome might be <u>contributed</u> to by the accumulation of this specific compound. <u>In vitro</u> studies[6] of these substances used concentrations of 10-16 mEq/L, approximately 100 times the concentration of free aromatic hydroxy acids (1.56 mg/100 ml or about 0.1 mEq/L) measured in uremic plasma.[35] The <u>in vivo</u> study

reported here used similarly high concentrations (8 mEq/L by spectrophotometry), which is 80 times the uremic concentration of free aromatic hydroxy acids and 27 times the total blood phenols (4.6 mgm/100 ml, or about 0.3 mEq/L using mol wt of 150).[22] However, this dose, as large as it is, represents but one-third the concentration of total unidentified organic acids known to accumulate in symptomatic human uremia (26 mEq/L),[33] and is less than one-fourth the total elevation of unidentified anions in control rats in this study.

Although the present study had documented the in vivo toxicity of 3,4-dihydroxybenzoic acid and related compounds, it has done little to clarify the mechanism by which this toxicity is effected. There is some chemical evidence of direct cellular damage. There is also evidence of a marked effect--either direct or indirect (vagal)--on the cardiac pacemaking system. The neurologic consequences of administration of 3,4-DHBA are consistent with in vitro evidence of cerebral enzyme inhibition, although the exact mechanism remains unclear. And although it is apparent that all phenolic compounds (indeed, all dihydroxybenzoic acids) do not produce identical effects, a clarification of structure-action relationships awaits further study.

The possibility of interference with catecholamine metabolism remains unsubstantiated. This hypothesis finds support, however, in work of Axelrod and Laroche, who showed that ortho-methylation of catecholamines (on which their metabolic inactivation depends) is inhibited by catechol (1,2-dihydroxybenzene).[37] This compound (which, but for a single carboxyl group, is identical to 3,4-DHBA) has been shown to sensitize various structures to injected catecholamines and to sympathetic

nerve stimulation. Perhaps 3,4-dihydroxybenzoic acid contributes to neuromuscular hyperexcitability in a similar manner.

It is likely--as both the gross test of relative toxicity and the seizure threshold study suggest--that 3,4-dihydroxybenzoic acid is neither the most nor the least toxic substance to accumulate in uremic plasma. If it represents but the mean toxicity of the entire group, then a pathogenetic role for organic acids in uremia would seem highly probable. If it is more toxic than most, then accumulation of much smaller amounts than used here might contribute disproportionately to various signs and symptoms of uremia. Low concentrations to potentially toxic substances--at concentrations too low to assume significance in in vitro or acute in vivo experimentation--might prove particularly noxious if, as recent work with rats by Fishman and Raskin suggests, there occurs in uremic encephalopathy a non-specific increase in brain permeability.[36] This would enable greater accumulation in brain of the dialyzable compounds that accumulate in uremia, compounds which normally have limited ability to penetrate the intact blood-brain barrier.

Extrapolation from experimental studies in rats to human uremia is difficult. It is possible, however, that accumulations of 3,4-dihydroxybenzoic acid in the blood of uremic patients may contribute to signs and symptoms of neuromuscular hyperexcitability, in addition to possible contributions to overall depression and cardiomyopathy. Implications for management are also unclear. It is known, however, that caffeic acid--a constituent of coffee--is excreted in the urine catechol fraction, largely as 3,4-DHBA.[7] Although 3,4-dihydroxybenzoic acid is certainly not the single uremic toxin, it seems clear that this phenolic

acid may well contribute to uremic intoxication. Moreover, its contribution may derive both from its effects as a phenol (primarily depression) and its evidently more specific effects (primarily neuromuscular hyperexcitability) as a benzoic acid with hydroxyl groups in the 3 and 4 positions.

SUMMARY

From previous investigations it is known that derivatives of 3,4-dihydroxybenzoic acid accumulate in uremic patients and that the phenolic acid itself is particularly inhibitory to rat cerebral enzymes. In the present study, solutions of 3,4-dihydroxybenzoic acid, its 2,4-structural isomer, normal saline and other substances were injected into rats in acute renal failure and several parameters--EEG, EKG, hebavior, certain blood chemical concentrations, longevity and seizure threshold--were monitored. The results show that, as studied here in high doses, 3,4-dihydroxybenzoic acid is definitely toxic. Like the 2,4 isomer, it hastened paresis, prostration and death and contributed to cardiac dysfunction (bradycardia and arrhythmias). Unlike the 2,4 isomer, it contributed to the exaccerbation of signs of neuromuscular hyperexcitability (myoclonic jerks, spasms, grand mal seizures, spontaneous and strobe-induced electrical seizure activity and lowered seizure threshold). All animals developed chemical evidence of metabolic acidosis, but electrical and behavioral differences between the three groups (3,4-dihydroxybenzoic acid, the 2,4 isomer and saline) were not clearly correlated with differences in urea and electrolyte concentrations.

In terms of hastening death, 3,4-dihydroxybenzoic acid was clearly more toxic than urea and saline, comparable in toxicity to phenylalanine, creatinine and benzoic acid, and less toxic than 3,4-dihydroxycinnamic acid. Seizure threshold, measured by exposure to a convulsant ether, was lowered by 3,4-dihydroxybenzoic acid (and several other mono- and dihydroxybenxoic acids found in uremics), raised by 2,4-dihydroxybenzoic acid (as well as by creatinine and 3,4-dihydroxycinnamic acid), and essentailly unchanged by saline and urea.

In high concentrations, 3,4-dihydroxybenzoic acid is definitely toxic to rats in acute renal failure. Although extrapolation is hazardous, it seems possible that abnormal accumulation of this phenolic acid (and similar compounds) might contribute to human uremic intoxication, particularly to signs and symptoms of neuromuscular hyperexcitability.

The possibility that 3,4-dihydroxybenzoic acid interferes with catecholamine metabolism remains an attractive but as yet unsubstantiated hypothesis.

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