THE EFFECTS OF CHRONIC ORAL COBALT EXPOSURE ON PASSIVE AVOIDANCE PERFORMANCE IN THE ADULT RAT

A Thesis
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ABSTRACT

The Effects of Chronic Cobalt Exposure on

Passive Avoidance Performance

in the Adult Rat. (April, 1984)

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Adult rats were tested for step-down passive avoidance following exposure to either water contaminated with $CoCl_2$ (Group Co) or uncontaminated distilled water (Group X). Behavioral analyses included acquisition and retention performance data and a test for analgesic tolerance. determinations of tissue co levels were also made following termination of the behavioral analyses. Exposure to 20 mg Co/kg body weight/day, for 60 days, produced significantly greater step-down passive avoidance latencies compared to controls during retention testing. Analyses of passive avoidance acquisition data, hot-plate test results and body weights taken just prior to testing produced no group differences. Significant accumulations of Co were found in blood, brain and testes of treated animals. Contrary to previous findings using an equivalent exposure regimen in the food, no testicular atrophy or morphological disruption was found. Behavioral perturbations are discussed in terms of emotionality and possible depletion of gamma-aminobutyric acid reserves in the CNS.

DEDICATION

This text is dedicated to Michael John Freeman whose exhuberance in life, generosity in love, and untimely death have inspired me to make the best of all three.

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INTRODUCTION

Behavioral toxicology is a unique new field which integrates several old ones: Toxicology, with its emphasis on pathology; behavioral pharmacology, and its concern for the effects of drug use; experimental psychology, with its search for the causes of behavioral anomaly; and finally ecology with its emphasis on the interaction between organisms and their environments.

Behavoiral toxicology gains its primary impetus from the fact that it has something unique to offer the field of toxicology [33]. Toxicology studies have traditionally been used to obtain safety standards used in setting acceptable exposure levels. These studies often rely on criteria of death and tissue pathology, indicative of massive and irreversible damage. However, it has become increasingly apparrent in recent years that these criteria are inadequate, because many substances evoke effects at the functional level (i.e. they disrupt performance) which often precede overt tissue damage. Thus, it is in searching for ways to test these functional perturbations and in characterizing the resulting behavioral syndromes, that the behavioral scientist can make a contribution in the field of toxicology.

Behavioral neurotoxicants (substances which disrupt central nervous system functions and the effects of which are manifested at the behavioral level) gained widespread attention in 1973. This occurred as a result of the discovery that methyl-n-butylKetone, an ostensibly innocuous chemical solvent used as an ink-thinner and machine cleaner, disrupted neuromuscular control, producing weakness and a loss of coordination in workers in the area in which it was used [2]. Workers reported weakness in the hands and feet and difficulty in

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grasping heavy objects. Some suffered a sharp loss of weight, others lost control of their hands, and still others could barely even walk. This was a key episode in that it resulted in a shift in thinking about the relevance of behavioral problems to toxicology. Scientists began to understand that the adverse health impact of environmental chemicals could and should be guaged by how people feel and function, and not solely by traditional criteria.

The field has undergone a rapid expansion since the 1973 incident. The National Institute of Occupational Safety and Health now lists over 200 chemicals which, based on neurological and behavioral effects are condidered dangerous enough to warrant a reccommended limit on exposure, called a Threshold Limit Value-TLV [2]. Today, more than 20 million people work with one or more neurotoxic chemicals, many of which are released in ostensibly clean environments such as electronics labs and operating rooms. Moreover, many of these chemicals interact adversely with common substances such as alcohol, producing even greater risk of toxicity and behavioral anomaly [4]. Thus, neurotoxic risk is a widespread problem with serious implications which have only recently come to light.

Many poisonings, before they engender overt clinical symptoms, may be heralded by vague, subjective psychological complaints, which are attributable to any number of factors [33]. Furthermore, since the first warning signs are typically subtle they tend to go unnoticed. The ontogeny of further symptoms is usually very slow, taking months or even years to develop [4]. Thus behavioral neurotoxicants are pervasive and their effects may be difficult to detect and quantify, making the need for adequate behavioral measures of these effects even more compelling. Behavioral toxicology paradigms are uniquely capable of fulfilling these needs.

BEHAVIORAL TOXICITY OF HEAVY METALS

A major subdivision of behavioral toxicology is defined by the area of heavy metal toxicity [11]. This area has recently become a popular research topic, both because of the ubiquity of some metals such as lead and because of the potential hazards of consuming excessive doses of even essential trace metals [30]. Metal exposure is implicated in a wide variety of syndromes, ranging from hyperactivity in young children to Alzheimer's disease [30].

Metal exposure is known to produce three effects which are particularly relevant to the purposes of this study. These are, increased reactivity to aversive stimuli (emotionality), reduced neurotransmitter levels and functional disruption and atrophy in the testes.

Emotionality

First, there is a tendency for metal exposure to cause increased emotionality, as indexed by performance in aversive situations [19-22]. More specifically, triethyl lead causes an increased startle response in exposed rats to both an aversive tone and a puff of air in the face [31]. These investigators also found that lead exposure facilitated two-way active avoidance performance in these animals. These results were not due to heightened pain sensitivity because lead-exposed animals actually exhibited analgesic tolerance on hotplate and tail-flick tests.

Chronic inorganic lead exposure also affects schedule-controlled behavior in rats, with lower levels of lead resulting in increased lever-press responding and higher levels suppressing lever-pressing [5]. Elsewhere, lead-exposed animals showed increased lever press suppression on a conditioned emotional response (CER) test [22]. CER tests are used to index emotionality,

or reactivity to aversive stimulation. Specifically, ongoing appetitive behaviors are monitored in response to presentation of a disrupting or aversive cue.

In a similar study looking at the effects of chronic oral cadmium (Cd) exposure on emotionality, subjects fed 5 mg/kg Cd displayed increased CER suppression relative to both controls and animals given 1 mg/kg Cd [21]. Cadmium-exposed subjects have further been found to exhibit greater step-down passive avoidance (whereby an animal avoids shock by passively remaining on an elevated platform), as measured by number of descents during acquisition [19]. Thus, the proposition that metal exposure increases emotionality is well documented.

Neurotransmitter effects

There is also a tendency for metal exposure to decrease neurotransmitter and transmitter-related enzyme levels in the central nervous system. Intrapertoneal injections of cobalt induced depletion of dopamine, norepinephrine and serotonin concentrations in various regions of the rat brain [12]. Cobalt injections into the substantia nigra of rats have also manifested decreased brain levels of dopamine, gamma-aminobutyric acid (GABA) and glutamic acid decarboxylase [29]. Other metals have similar effects. For example, aluminum, manganese and cadmium have all been found to inhibit L-glutamate and GABA transport in rats [35]. Thus there are strong indications thata metal exposure causes disruption in neurotransmitter functioning.

Neurotransmitter depletion has also been linked to anxiety [3]. Thus these depletion effects represent a potential substrate governing metal-induced emotionality.

Testicular dysfunction

A third adverse effect of metal exposure concerns the tendency of these substances to cause testicular dysfunction in male animals.

Over thirty metallic and rare earth salts were found to exert some degree of antitesticular effect in both rats and mice [15]. More specifically, silver, copper, tin, nickel, and mercury all produced acute and chronic changes in the histology of the rat testes and interfered with spermatogenesis when administered subcutaneously [13]. These effects have been found to be attenuated by zinc, following organic metal exposure [23].

BEHAVIORAL TOXICITY OF COBALT

In contrast to some other metals, such as lead, the neurotoxic properties of cobalt have not been extensively researched. It is somewhat paradoxical to think of cobalt as having neurotoxic properties since it is an essential trace element and a component of vitamin B_{12} [24]. Cobalt is also well-absorbed following oral ingestion [24], and unlike lead can cross the blood-brain barrier [20]. Furthermore, metallothionein, a protein complex which binds with other metals and limits their distribution following absorption, does not do so for cobalt [20]. Thus the role of cobalt in nutrition may have prevented defense mechanisms from evolving for it, making increased doses particularly dangerous for this metal.

Given that cobalt is readily absorbed following exposure, one might wonder how excessive quantities of this metal may be contracted. Cobalt is absorbed through both the respiratory and gastro-intestinal tracts and it is present in a number of fairly common products [30]. It is used to make high temperature alloys for jet engines, in ceramic pigments, fast-drying paints, and printing inks. Radioactive cobalt is employed in the treatment of some forms of cancer [30].

One of cobalt's primary exposure modes, however, is through dusts from the production and use of cemented carbides. These carbides are bonded with cobalt powder to make extremely tough drilling bits and cutting edges. When these products are then used in

the machining operations of grinding, cutting, sawing, and drilling, they give rise to fine dusts which then enter the oropharyngeal cavity and are ingested [4].

Cobalt's neurotoxic properties have recently spurred a number of studies, particularly in relation to its epiliptogenic properties. Various types of cobalt brain implants (i.e., cobalt rods, gelatin pellets, etc.) are known to cause epileptic seizures in many animals, from monkeys to rats [8,14,26]. This seizure activity has been shown to be accompanied by significant reductions in the levels of neurotransmitters and their synthesizing enzymes (acteylcholinesterase, tyrosine hydroxylase, and aromatic acid decarboxylase) including a 50% reduction in the amount of GABA and glutamic acid decarboxylase (GAD). These reductions were accompanied by a comparable decrease in GAD-positive (GABA-ergic) terminals at the sites of seizure foci relative to homotopic contralateral non-epileptic cortex in monkeys. Cobalt has also been shown to interfere with neuromuscular transmission in the frog, ostensibly by competing with calcium uptake presynaptically [32]. Thus, cobalt is known to cause epilepsy which is correlated with neurotransmitter reductions, and to disrupt peripheral neurotransmitter function as well.

Recent findings concerning the neurobehavioral toxicity of inorganic cobalt suggest that chronic oral exposure to higher doses of the toxicant may enhance reactivity to aversive stimulation [22]. Compared to controls, adult rats given 20 mg/kg Co daily in their food for 69 days lever-pressed at a significantly slower rate than both controls and animals given 5 mg/kg Co. These investigators further assessed cobalt's effects on emotionality via a CER test. Though results were in the expected direction, they were not statistically significant. This is in direct contrast to previously cited findings

indicating that lead and cadmium exposures do cause increase CER suppression in treated animals. This could indicate that conditioned suppression tests are not sensitive to the effects of cobalt on emotionality, thus these investigators suggested that other parameters might produce quite different results.

The finding, previously cited, that Co decreases GABA is especially relevant to the present project. Decreases in the level of GABA in the central nervous system have been linked to anxiety and emotional reactivity in animals and humans [27]. Thus the purpose of the present study is to examine further the role of cobalt in the promotion of emotional reactivity in animals.

The experiment involved a step-down passive avoidance task in which subjects were required to remain on an elevated platform in order to avoid receiving electric shock. This procedure is commonly used in behavioral pharmacology to test anxiolytic (anxiety-producing) drugs [17,28]. It is especially appropriate for the purpose of examining anxiety in animals in that graduated increases in shock levels are known to increase passive avoidance acquisition and retention performance [25]. Thus, to the extent that Co-induced emotionality alters the functional aversiveness of shock by virtue of GABA depletion, Co-treated animals should exhibit enhanced avoidance relative to controls.

METHOD

Subjects

Subjects were 16 male Sprague Dawley rats approximately 80 days old and weighing 150-200 g at the start of the experiment. Following a 2-day acclimation period, all animals were placed on a 20 g daily diet (Purina lab chow, Ralston Purina Co., St Louis, MO.) which was maintained throughout the experiment. Simultaneously, eight

randomly selected subjects began receiving Co (as $CoCl_2$) through the water at a level adjusted to bring the exposure amount to 20 mg Co/Kg rat body weight/day (See below).

Preparation of Fluid

Co levels in the water were increased, according to avaerage daily consumption, as the animals gained weight, in order to maintain the 20 mg/kg regimen. Water levels began at 133 ppm Co computed for a 200 g rat drinking 30 ml of water daily and were gradually increased to the final value of 171 ppm Co computed for a 300 g rat consuming 35 ml of water daily.

Apparatus

For the behavioral testing, the experimental chamber consisted of a 25X28X31 cm Coulborn E10-10 modular operant cage housed in a 41X46X56 cm Coulborn E10-20 universal cubicle. This apparatus was modified to accomodate step-down passive avoidance acquisition and retention training through addition of a 5X10X15 cm wooden block. The cubicle door was left open for the duration of each subject's session to facilitate viewing. With the house lights on, the room lights were turned down and noise was minimized to assure that the subject would not be distracted. The chamber floor consisted of stainless steel grid rods spaced 2 cm apart and continuously electrified by a Coulborn E13-08 grid floor shock generator. During retention training, latency to step completely (all 4 paws) off the platform was measured by a handheld stopwatch.

Animals were tested for analgesic tolerance, using a hot-plate apparatus, the day following termination of behavioral testing. The hot-plate apparatus consisted of a slide warming tray (Clinical Scientific Equipment Co, Melrose Park, IL.) with the temperature dial set to remain at a relatively constant 120°F (app.50°C). Actual plate temperature was monitored by a Fluke 2100A digital readout thermometer

(Seattle, Wash) and was recorded for each animal upon placement in the apparatus. A 76X38X89 cm^3 clear plastic chamber with a hinged top and an open bottom was placed on the slide-warming tray surface. A 40W light was mounted above the apparatus. Response latencies were recorded to the nearest .01 sec by a Gerbrand G1280 digital electronic timer. The apparatus was located in a test room isolated from the animal holding room.

For the tissue analyses, samples were dried in a 120 F oven then ashed overnight in a muffle furnace maintained at a constant 500 F. Following quantitative transfer (dissolving ashed samples in acid, diluting them and pipeting them from crucibles used for ashing to volumetric flasks), samples were analyzed for Co concentration via a Varian Techtron Aa-175 atomic absorption spectrophotometer using an open flame technique

Procedure

All animals were exposed to the respective control or Co-contaminated water for 57 days prior to the beginning of acquisition training.

Acquisition training. Subjects across groups were run in a counterbalanced order with Co-1 run first, the control 1 (X-1), then Co-2, etc.... Each training session lasted 30 minutes. The chamber was thoroughly washed with a soap solution following each subject's session. Timing for each session began when the animal was placed on the platform, with a 1.5 mA shock continuously qpplied to the grids below. The platform was located in the of the corner chamber, adjacent to the back and left walls. This was done to prevent contamination by the competing thigmotactic (perimeter-seeking) response which occurs with a centrally placed platform [10]. The distance from the top of the platform to the grid floor was 10 cm. Thus animals were shocked continuously contingent upon each platform

descent until returning to the platform. The number of platform descents (defined here as touching the grids with 1 or more paws), recorded manually by the experimenter, functioned as the dependent measure.

Retention testing. Twenty-four hours following acquisition training, each subject was again placed on the platform, but no shock was delivered to the grid flooring. The initial step-down latency (the amount of time to completely step off the platform onto the grid flooring) was the dependent measure for retention. If a subject failed to descend from the platform within a 5-minute period, latency was recorded as 5 minutes and the animal was removed from the testing chamber. Subjects were taken immediately to the home cage following this initial step-down performance.

Analgesic tolerance. Twenty-four hours after retention testing, a hot-plate test was conducted to test for analgesic tolerance. Each animal was placed on the hot plate surfaace and latency to perform a paw-lick response to a front or hind paw was recorded to the nearest .01 sec as paw-lick latency (PLL). If a paw-lick response was not observed within 90 sec, the test was terminated and PLL recorded as 90 sec. Each animal was returned to the home cage immediately following hot-plate testing.

Concentration of Co in the tissues. Determinations of tissue Co were begun 24 hr. following the test for analgesic tolerance. Subjects were rendered unconscious with CO and sacrificed via decapitation. Blood, whole brain (left hemisphere), and testicle (left) samples were collected and stored at -60°C until analysis. Following ashing, all tissue samples were dissolved in a mixture of 1 ml hydrochloric acid and 5 drops (app. .5ml) of nitric acid, then heated. This mixture was quantitatively transferred to a 5 ml volumetric flask and appropriately diluted with distilled, deionized

water. Co residues (g/g, wet weight) were then determined by open flame atomic absorption spectrophotometry.

One control subject was discarded following behavioral testing due to equipment failure.

RESULTS

Overt signs of toxicity such as ataxia, tremors, seizures and paralysis were not observed in any subject during the coarse of the experiment.

Fluid intake

Average weekly Co consumption in the treatment group was computed in order to verify the exposure regimen. Results are reported in Figure 1. Actual Co consumption ageed closely with the desired 20 mg/kg regimen. Water consumption in both the treated animals and control animals, continuously monitored, is also reported in tabular form in Figure 1. A Treatment X Weeks one-way repeated measures analysis of variance, performed on these data, revealed significant differences between groups in water consumption ($F(1,1)=68.3$, $p<.01$).

Insert Figure 1 about here

Body weight

Body weights for all animals (obtained just prior to testing) revealed means of 340 g and 347 g for the control and treatment groups, respectively. No differences were evident between the two groups. An independent samples t-test performed on these data revealed no significant differences between groups ($t = .90$, $p>.05$).

Acquisition training

No differences between groups were observed in number of descents during acquisition training. Means for the two groups were

5.9 and 4.2 for the control and treatment groups respectively. An independent samples t-test performed on these data confirmed this observation ($t = 1.5$, $p > .10$). Results, in terms of individual platform descents, are depicted graphically in Figure 2.

Insert Figure 2 about here

Retention testing

Findings from the analyses of retention data indicated that cobalt animals exhibited significantly longer latencies to descend completely from the platform than did their control counterparts. Mean latencies to step off the platform 24 hr. after axquisition were 53.6 sec for the control group and 237 for the treatment group. The data were analyzed by a two-sample rancomization test suggested by Good [9]. This test has been recommended for use in behavioral toxicology [6], and is sensitive to differences in means and to increased variability in a group caused by the presence of both responders and non-responders. With theta set at the recommended value of .67, the resulting p-value was .045.This indicated that the probability that the treatment group would show this value when considered against the background of all possible permutations of control and treated values was low anad unlikely to be due to chance. So, for this test, Co-treated animals were less likely to descend from the platform and thus exhibited enhanced retention relative to controls. These results, in terms of individual latencies, are depicted graphically in Figure 3.

Insert Figure 3 about here

Analgesic tolerance

No group differences in analgesic tolerance were indicated. Figure 4 summarizes the PLL's obrtained from each group on the hot-plate test. The means for each group were 55 sec and 46 sec for the control and treated animals, respectively. An independent samples t-test performed on these means failed to show significant differences between groups (t =.84, p>.05). Apparantly, treated animals and untreated controls were equally responsive to painful stimuli.

Insert Figure 4 about here

Concentration of Co in the tissues

The results of the atomic absorption spectrophotometry analyses on tissues of subjects from each group are presented in Figure 5. Independent samples t-tests of Co concentrations (g/g) indicated that Co residues were significantly higher in the treatment group blood, brain, and testes than in control tissues (all ps<.01).

Insert Figure 5 about here

Testicular Morphology

In view of findings from a previous study [20] in which Co-exposure (through the food) caused dramatic changes in the morphology of male rat testicles, similar results were expected in this study. Yet, no differences in morphology of the testicles between control and treated animals were observed. This point may be clarified through inspection of Figure 6, which is a collection of pohotographs of cross-sections of seminiferous tubules of food-exposed versus water-exposed control and Co-treated animals.

Insert Figure 6 about here

DISCUSSION

Findings from this study confirmed the hypothesis that chronic oral Co exposure enhances emotional reactivity in rats. Though passive avoidance acquisition performance was unaffected, animals exposed to 20 mg/Kg Co were less likely than controls to descend from a safe platform onto an electrified grid floor during retention testing. These differences were observed to occur coincident with significant accumulations of Co in the tissues of the treated animals. No group differences were observed in either analgesic tolerance or body weights.

The group differences reported for passive avoidance retention testing are compatable with previous findings linking metal exposure with heightened anxiety levels. Presumably, shock exposure occasions an enhanced negative emotional state in treated animals relative to controls which may be mediated by the effects of metal exposure on gamma-aminobutyric acid (GABA) levels in the CNS [27,28]. GABA is thought to be the major inhibitory neurotransmitter in the brain [3,7] and low GABA levels are known to dispose animals and humans toward anxiety. Since Co has previously been shown to lower GABA levels in the brain by as much as 40% [29], there is good reason to believe that Co exposure results in increased anxiety. In so far as increased anxiety levels may be correlated with increased reactivity to aversive stimuli, shock would be expected to be functionally more aversive for an animal suffering from reduced GABA levels. This, in turn would result in an increase in aversive motivation following exposure to shock. Given that a reduction in aversive motivational cues [18] negatively reinforces remaining passive (in this case, remaining on the platform) it follows that the greater the aversive motivation, the

greater the potential for negative reinforcement and the better should be avoidance responding. So, to the extent that Co exposure reduces the bioavailability of GABA, then an increase in passive avoidance responding should be observed in Co-treated animals relative to controls, as was the case in this study.

Two possibilities exist for differential reactivity to shock to occur without reference to the above hypothesis. First, changes could occur at the level of the sensory receptor (in the abscence of GABA depletion) such that Co-treated animals would experience a more intense response to pain than controls. This, in effect, would mediate motivational differences in the abscence of any changes in GABA levels. However, results from the hot-plate test militate against this hypothesis. Specifically, Co-treated animals were equally as responsive to painful stimuli as were untreated controls. Differential reactivity could also occur in the abscence of motivational differences as a function of changes in electrical resistance resulting from differing body weights across groups. These electrical resistance changes could function to lower the experienced shock level in the animals with greater body weights. So, for example, if treated animals weighed less than their control counterparts (perhaps resulting from cobalt-induced malaise), the level of shock they experienced would in fact be greater than that of control animals. This could result in an increased step-down latency for treated animals. Evidence arguing against this hypothesis may be gleaned from the body weight analysis. Since there were no differences in body weights across groups, the proposed differences in electrical resistance could not occur.

In light of a previous study [20] in which it was observed that Co exposed through the food resulted in extreme atrophy and cellular disorganization in male rat testicles, similar results were

expected in this study. Despite, equivalent exposure regimens in the two studies, and significantly greater accumulations of Co in the tissues of treated animals relative to controls, no such effects were observed here. These results are somewhat puzzling and would seem to indicate that two independent mechanisms control absorption and/or distribution of this metal when it is administered in water as opposed to food. Alternatively, it is possible that when Co is matrixed in the food it is bound by proteins and thereby remains in the digestive tract for a longer period of time, giving it enhanced opportunity for absorption. These hypotheses are, of neccessity, speculative and more data on the subject are needed.

An additional finding from this study, which may be of importance to future studies of Co oral exposure, concerns the differences in water intake across the two groups. Analyses showed that Co-treated animals drank a significantly reduced quantity of water relative to controls. Although no obvious indications of nutritional defecits (such as reduced body weights or malaise) were observed in this study, this is a factor which should be controlled. Previous studies of Co exposed through the food [34] indicate that taste aversion begins occurring at the level of 100 mg/Kg Co. The results from this study indicate that the taste of Co when matrixed in water may be more salient and thus aversion may occur at much lower levels. This question also merits empirical investigation.

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Figure Captions

Figure 1. (Top) Mean daily Co intake, collapsed across 7-day periods, for animals exposed to 20 mg/Kg daily. (Bottom) Mean weekly water consumption for the control and Co-treated groups.

Figure 2. Individual number of platform descents for Co-treated and control rats during passive avoidance acquisition.

Figure 3. Individual descent latencies for Co-treated and control animals during passive avoidance retention testing.

Figure 4. Individual paw-lick latencies (PLL's) for Co-treated and control animals on the hot-plate test.

Figure 5. Concentration of cobalt in blood, brain and testicles of Co-treated animals. Results are reported as percent of control (X) with 100% equalling mean control group concentrations in that tissue.

Figure 6. Cross-section of seminiferous tubules in A. Co-treated animals given 20 mg/kg Co via diet, and B. Untreated controls. When Co was exposed via water (20 mg/Kg) results were as in B.

FIGURE 1

Average Daily Co Consumption Across Weeks

	week 1	week 2	week 3	week 4	week 5	week 6	week 7	week 8
Co (mg/kg)	24.2	20.9	21.3	20.4	21.0	19.3	18.0	17.0

Average Weekly Water Intake (ml)

	week 1	week 2	week 3	week 4	week 5	week 6	week 7	week 8
Co group	252	249	250	263	248	247	238	211
Controls	276	286	299	320	306	321	327	329

FIGURE 3

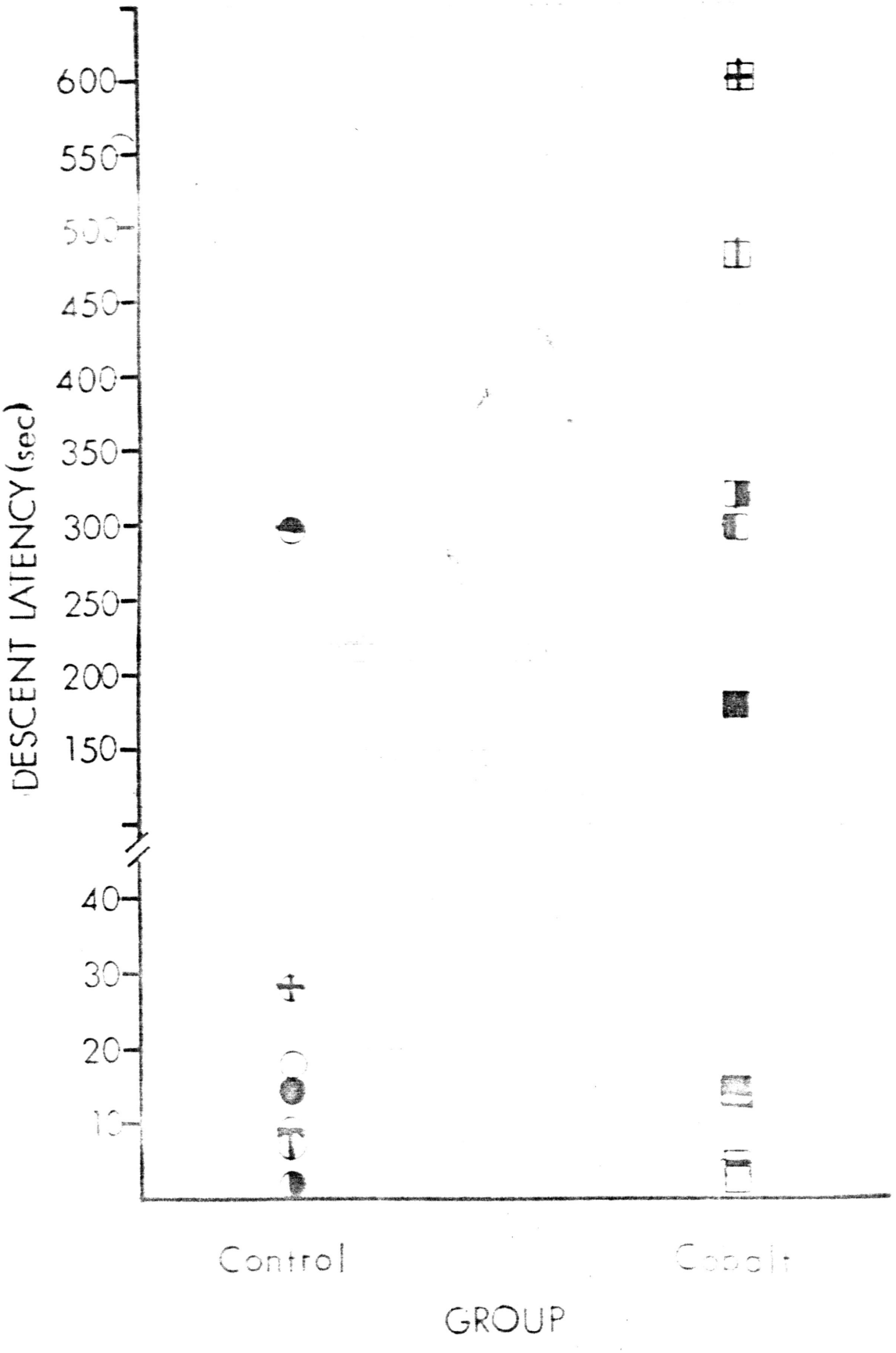

DESCENT LATENCY (sec)
600
550
500
450
400
350
300
250
200
150
40
30
20
10
Control
Cobalt
GROUP

FIGURE 4

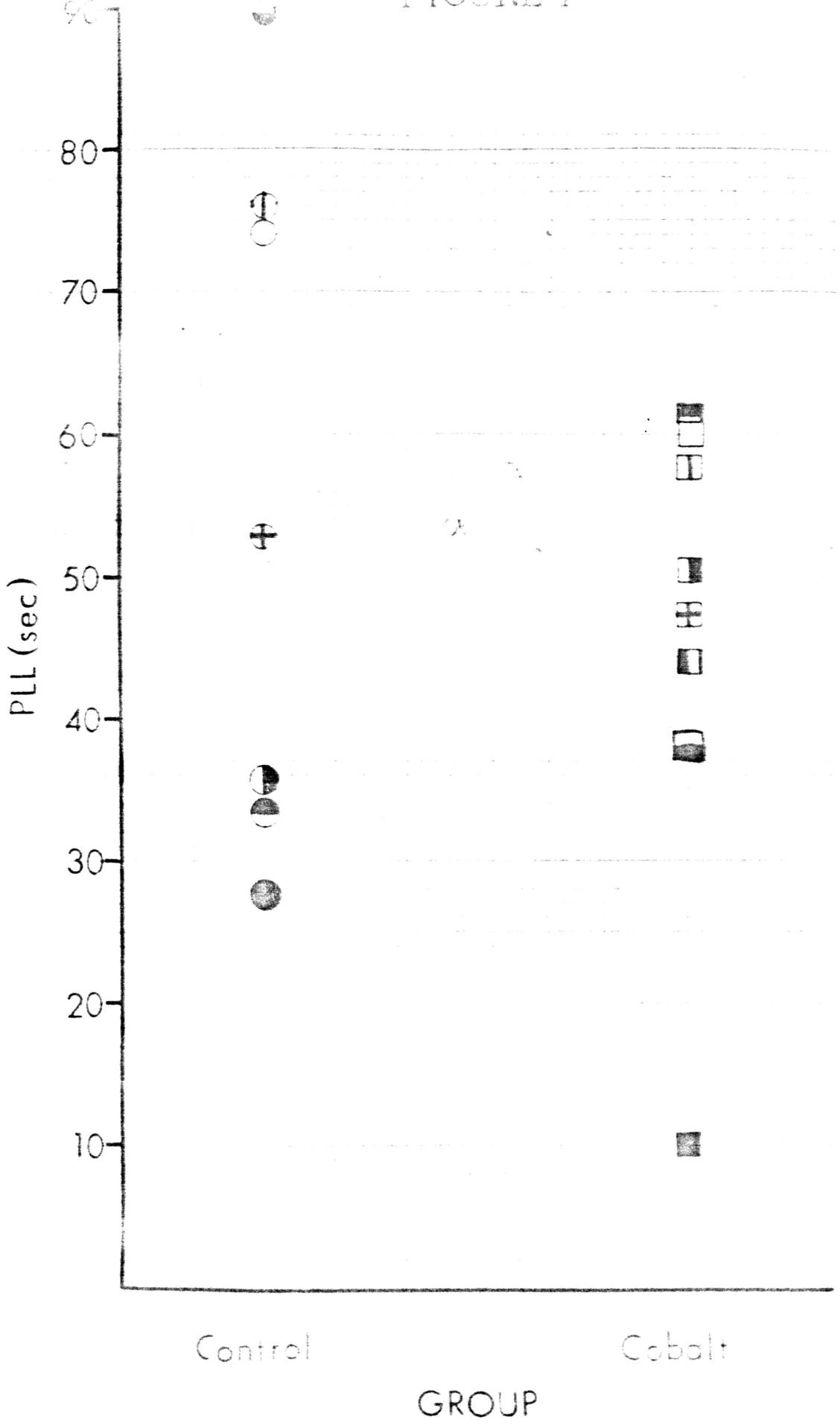


FIGURE 5

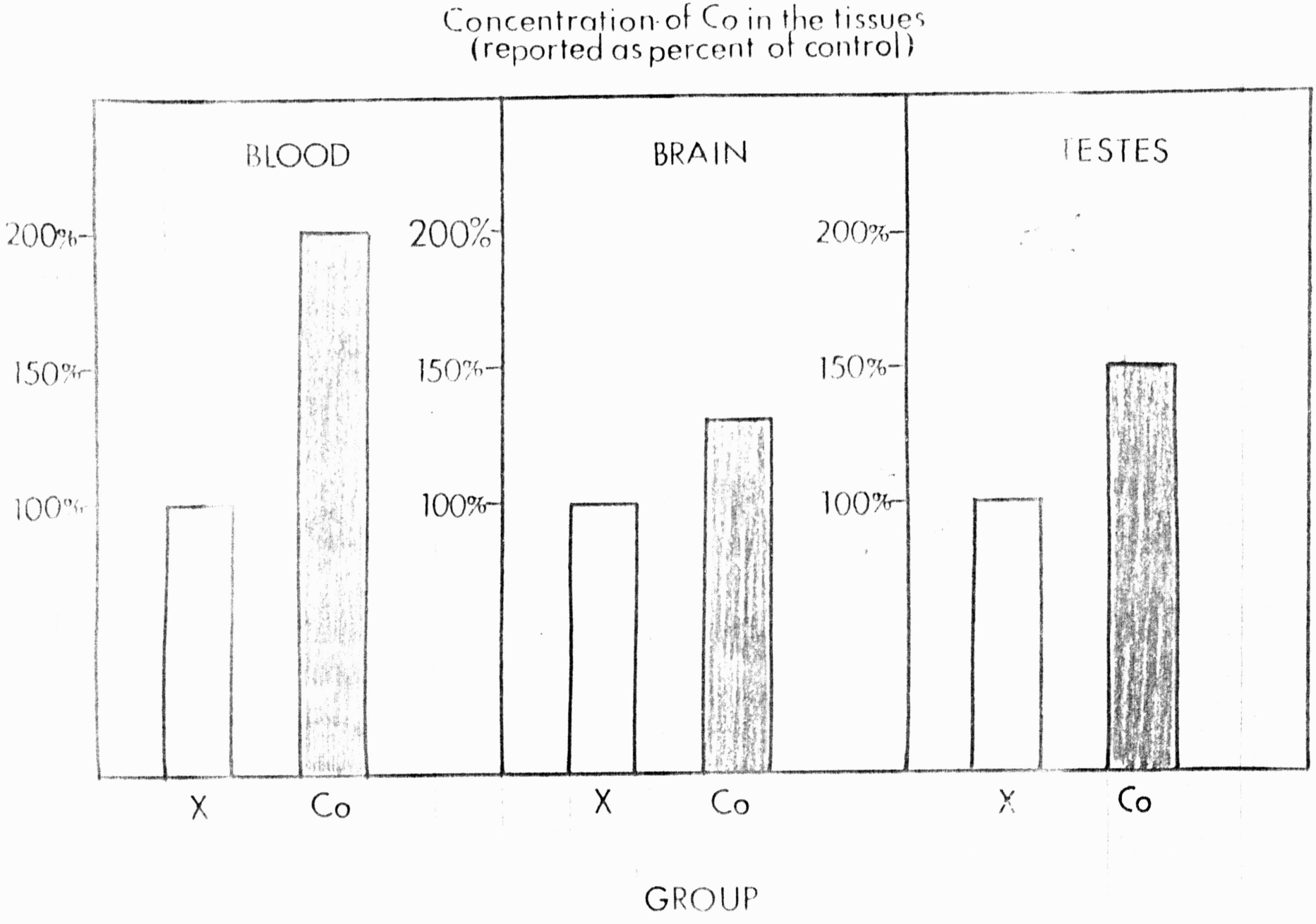
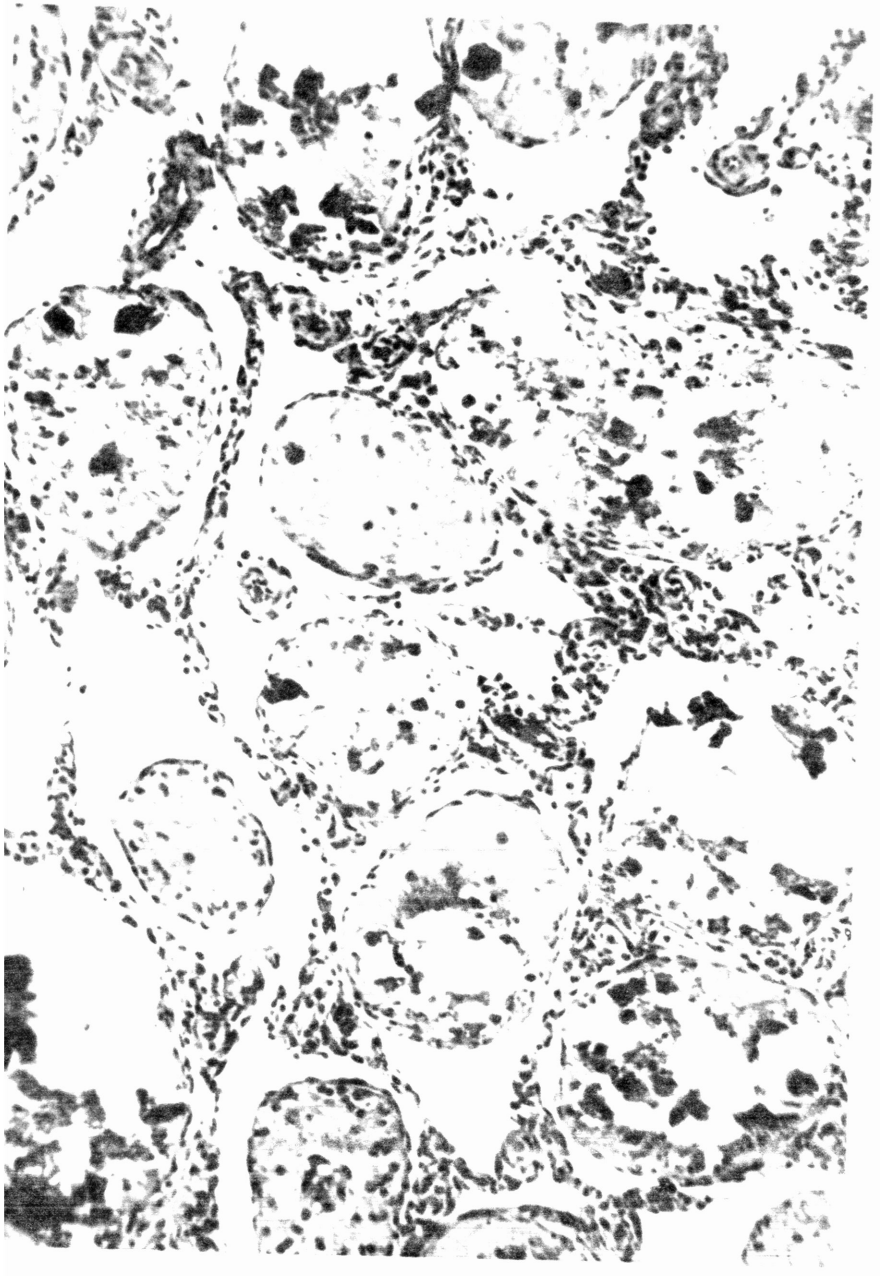
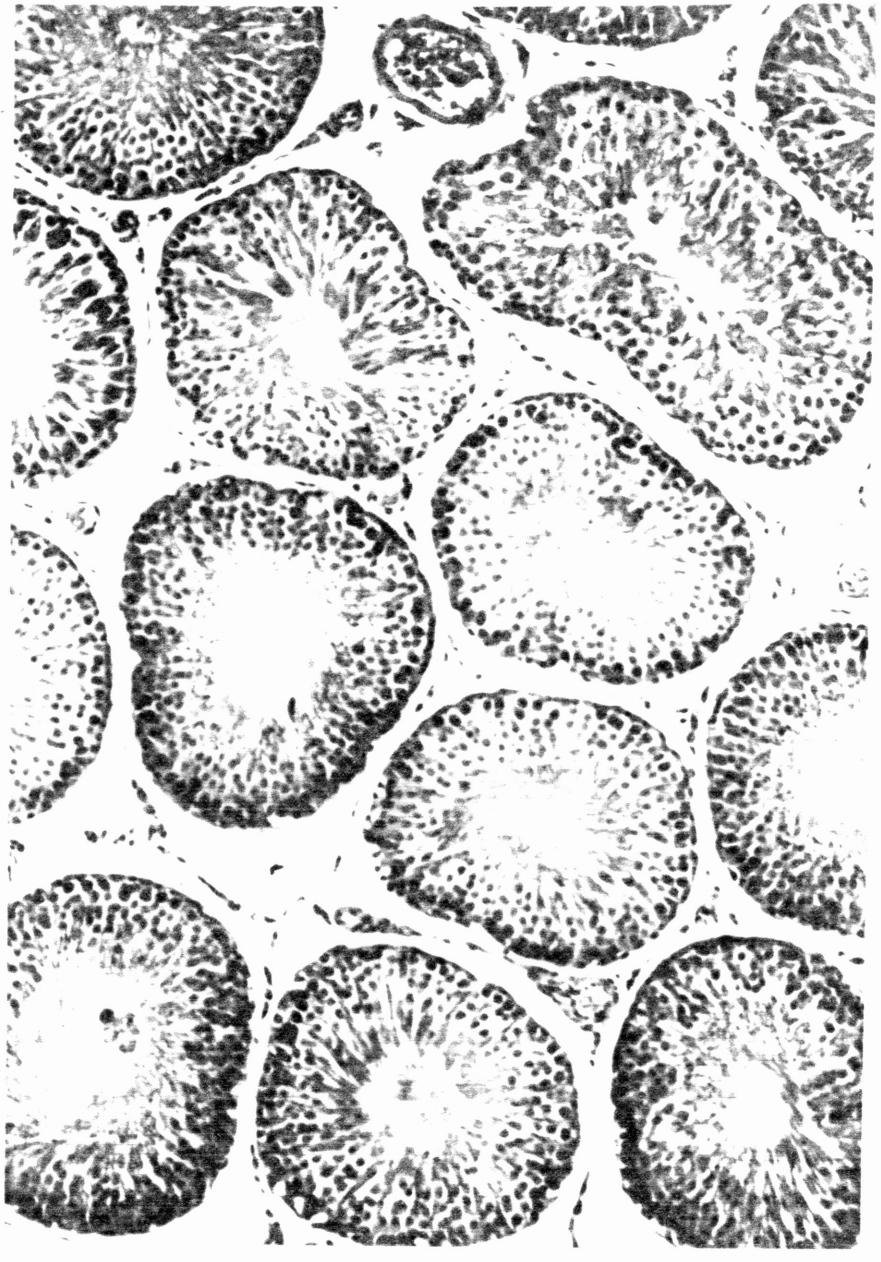

Concentration of Co in the tissues
(reported as percent of control)
BLOOD
BRAIN
TESTES
200%
150%
100%
X
Co
GROUP

FIGURE 6



A



B