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PRELIMINARY STUDIES ON THE DISAPPEARANCE OF BOTULINUM TOXIN
FROM THE CIRCULATING BLOOD OF RHEBUS MONKEYS

by

James L. Stookey
C. Spencer Streett
Diane F. Ford

Experimental Medicine Division
Directorate of Medical Research

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US Army Edgewood Arsenal
CHEMICAL RESEARCH AND DEVELOPMENT LABORATORIES
Edgewood Arsenal, Maryland

FOREWORD

The work described in this memorandum was authorized under Subtask IC622401A097-09-03, Prophylaxis and Mechanism of Action of C. Botulinum (U). This work was started in October 1964 and completed in May 1965.

In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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PRELIMINARY STUDIES ON THE DISAPPEARANCE OF BOTULINUM TOXIN FROM
THE CIRCULATING BLOOD OF RHESUS MONKEYS

I. INTRODUCTION.

This experiment was to determine the blood serum disappearance curve of parenterally injected Type A botulinum toxin in mature rhesus (Macaca mulatta) monkeys. This might give us a better understanding of the relationship between symptoms and absorbed toxin. The study was also designed to determine whether this disappearance curve is altered in monkeys that have survived previous exposure to the toxin.

II. PROCEDURE.

The 7 adult rhesus monkeys used in these experiments weighed between 3.9 and 4.2 kg and were fully conditioned, having resided in this colony for over 2 yr. Both sexes were used randomly, except that when 2 monkeys were given the toxin at the same time, they were of the same sex and their weights and body conformations were as similar as possible. The subcolony was completely free of tuberculosis, as proved by periodic intradermal eyelid injection of Koch Old Tuberculin (KOT) and subsequent autopsies.

A stock suspension of 1 gm of partially purified botulinum toxin powder (3.3×10^9 MU*/gm), Type A, in 100 ml of gelatin buffer (pH = 6.8) was prepared. This suspension was further diluted with buffer to prepare the final test solution, which was intended to contain about 5,000 MU/ml. This toxin solution was bioassayed in mice each day a monkey was given an injection so that an accurate measure of the potency of that day's solution, in MU's, was available. Therefore the actual dose given was not known until the bioassay was completed. Mice used for all bioassays were acclimated and maintained at 72°F. The assay consisted of injecting each serial dilution (at least 6) of the toxin-containing solution (0.2 ml/mouse) intraperitoneally into 10 Swiss albino female mice weighing from 18 to 22 gm. (These mice came from a homogeneous, disease-free colony.) The LD50 (i.e., MU) was then computed, based on dosage and cumulative mortality after 4 days, by the method of Bliss.¹

Just prior to injection of toxin, the monkeys were restrained in an upright sitting position in an adjustable primate restraint chair (figure 1). The hair over all potential bleeding sites was clipped and the skin cleaned. A single intravenous injection of the toxin was made into the right saphenous vein over the gastrocnemius muscle by means of a 3-way stopcock, double-syringe system. After the needle was inserted in the vein and flushed with saline, the toxin-containing solution was injected. The toxin-containing syringe was filled with saline and the contents injected, insuring complete injection of the measured amount of toxin.

* MU = mouse intraperitoneal LD50.



FIGURE 1

RESTRAINT CHAIR FOR MONKEYS

The first (10 min) and second (2 hr) post-injection blood samples were taken from the left femoral vein (in the region of the groin). Subsequent blood samples (6 hr, 12 hr, time of death, and varying intervals) were taken from either the left or right femoral vein, or other selected sites, but never from the site of the toxin injection.

The monkeys were removed from the chair and returned to their individual cages at the end of 7 hr because the majority were showing definite signs of botulism, and the head restraint of the chair interfered with the clearing of excess nasal and oral secretions. Subsequent bleedings were either performed in the cage or, if necessary, after the animal had been temporarily returned to the restraint chair.

TABLE 1
DILUTIONS OF SAMPLED MONKEY SERUM USED
FOR MOUSE INOCULATION

<u>Dilution</u>	<u>Volume of serum</u>	<u>Volume of buffer</u>
	ml	ml
Undiluted	3.0	0
1:1	1.5	1.5
1:3	0.75	2.25
1:9	0.3	2.7
1:50	0.06	2.94
1:100	0.03	2.97

All blood samples for bioassay of toxin level were heparinized, cooled, centrifuged, and the serum diluted with buffered gelatin (pH 6.8) just prior to mouse inoculation. Bioassay of the serum toxin level was the same as that for determining the dose given the animals. Table 1 lists the dilutions of serum and buffered gelatin used for the mouse bioassay. In calculating the total amount of toxin in the blood, a plasma volume of 36.4 ml/kg was assumed.²

Whole blood and serum extracted from the same sample were bioassayed and the results were similar. It was further determined that monkey whole blood is not toxic to mice.

The hematocrit value of each blood sample was determined by the microcapillary tube method using International nonheparinized tubes (diameter = 1.3 to 1.5 mm, length = 75 mm).

Gross postmortem examinations were performed on all monkeys and, where indicated, a microscopic examination was conducted.

III. RESULTS AND DISCUSSION.

The first 3 monkeys were given the toxin on different days. The last 4 monkeys were studied in pairs, both animals being given the toxin and bled on the same schedule.

A. Serum Toxin Levels After Intravenous Botulinum Toxin.

The results of the 3 single-monkey experiments are illustrated in figure 2. The doses for all 3 monkeys were mixed separately from the same stock solution on different days. Although equal or similar doses were intended, the bioassay showed that total doses were actually 8,379 MU, 12,105 MU, and 31,712 MU. Since bioassay of the serum of the animal that received 31,712 MU never showed more than an extrapolated 4,200 MU in this monkey's circulating blood, it is possible that an error occurred during the original bioassay.

B. Effect of an Earlier Exposure on Serum Toxin Levels.

This original stock solution of toxin was exhausted after the first 3 monkeys were tested, and a new solution was mixed. After being mixed with buffer, this solution was frozen and the same volumes from the same bottle were administered to the next 4 monkeys, which were studied in pairs. The doses given these two sets of monkeys were very similar (14,816 MU and 14,544 MU), and the amount found in their sera 10 min after injection ranged from 11,400 to 15,400 MU. The single variable was the new batch of Type A botulinum toxin stock solution. Apparently the stock solution of toxin used in this portion of the experiment aged and tended to lose its potency. Storing the solution in the frozen state, however, seemed to retard this aging process. No adverse effects from freezing the solution were noted.

The main purpose of the second series of experiments was to determine if the rate of disappearance of toxin from the sera of monkeys that had previously survived a lethal dose of botulinum toxin would differ from that of previously unexposed monkeys. The two previously exposed monkeys, No. 65 and 86, weighed 2.9 kg and 3.2 kg, respectively, in August 1963 when No. 65 received 42,700 MU/kg and No. 86 received 46,296 MU/kg of Type A toxin intragastrically.* Both monkeys exhibited typical symptoms of botulism, but recovered spontaneously and remained in good health.

* The intragastric LD50 for the rhesus monkey is 30,696 MU/kg.³

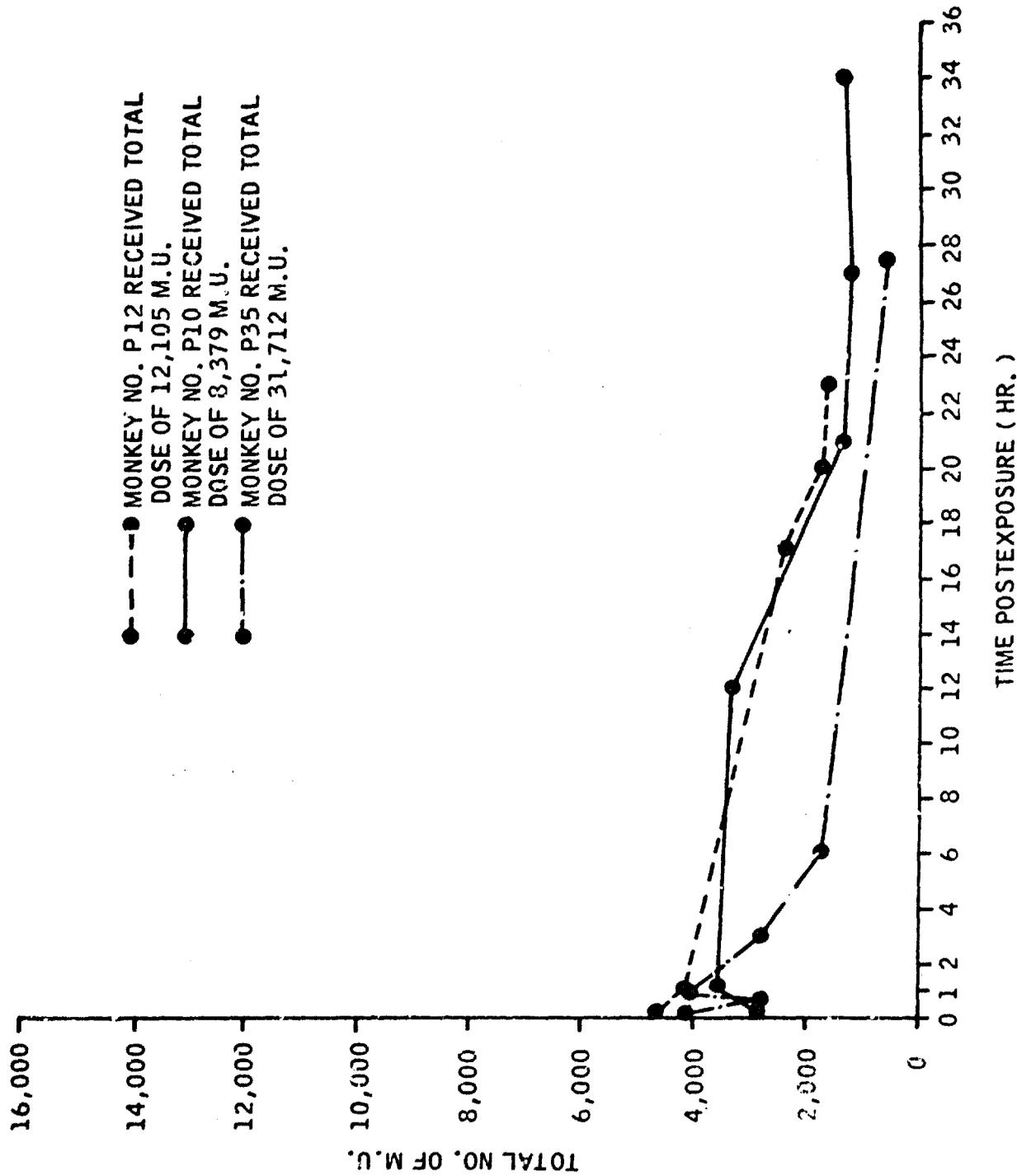


FIGURE 2

SERUM TOXIN LEVEL AFTER INTRAVENOUS INJECTION OF BOTULINUM TOXIN

No gross variation between the rates of disappearance of toxin for the previously exposed and the previously unexposed monkeys was observed (figure 3). The doses used were probably too overwhelming and death (occurring 15 hr post-exposure) too quick to permit an immune response to develop. The relatively high blood loss through sampling also contributed to the early deaths of these animals. All animals still had a measurable level of toxin (30% to 60% of the dose) in their blood at the time of death.

High doses of toxin were used so that toxin in each blood sample could be measured, even in some of the lower serum dilutions. The intravenous LD50 of Type A botulinum toxin in rhesus monkeys is 40 MU/kg,³ so these monkeys were receiving an average of 95 LD50's.

The amount of blood from a single sampling necessary to furnish enough serum for all dilutions was 12 ml, and each monkey's blood was sampled a total of 5 times. Considering vascular and skin seepage, the total blood loss was more than 65 ml. The total blood volume of the rhesus monkey is 74.1 ml/kg.² Since these test primates averaged 4 kg, they lost almost one-third of their circulating fluids over a 15- to 34-hr period.

C. Hematocrit Values After Toxin.

The hematocrit value of each blood sample was determined to gauge the degree of anemia and dehydration. One control monkey (P-28, female, 4.2 kg) was placed in the restraint chair and bled exactly as if it had received the toxin. At the end of 26 hr the control experiment was terminated and the animal, apparently in good health, was returned to its cage. All hematocrit readings are shown in figure 4. Normal hematocrit for the Macaca mulatta is 39.6%.² The packed cell volume of monkeys that received toxin dropped much more swiftly and further than did that of the control. One possibility to account for this phenomenon is that red blood cells pool in the muscles because the neuromuscular action of the toxin causes the muscles to lose tone. Another possibility is that extra fluids may be gaining access to the circulating blood. These conjectures require further investigation.

The hematocrit values for monkeys No. 65 and 86, which had previously survived large doses of botulinum toxin, tended to level out while those of their counterparts continued to drop. Since all these animals expired early, this may not be significant; however, it may be an indication of an early immune response.

D. Signs and Symptoms.

The signs and symptoms exhibited by all 7 monkeys used in this series of experiments were identical to, or closely paralleled, those described by Herrero, et al.³ Immediately following injection of toxin, the monkeys were observed for lethargy or loss of consciousness. None of these monkeys exhibited any noticeable depression in activity and, indeed, protested quite vigorously to restraint and bleeding 10 min after toxin injection.

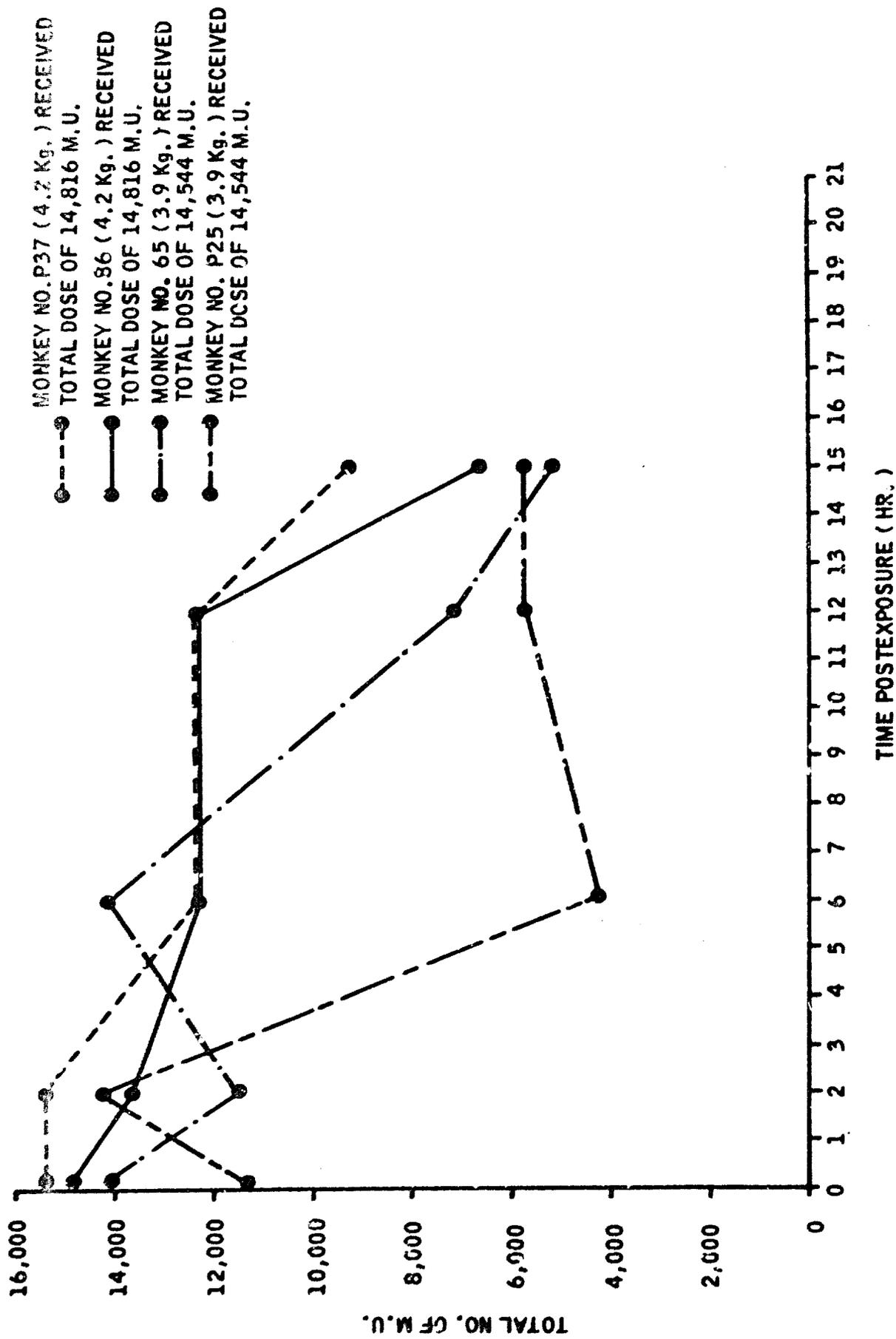


FIGURE 3

SERUM TOXIN LEVEL AFTER INTRAVENOUS INJECTION OF BOTULINUM TOXIN
 (Monkeys 86 and 65 were survivors of a previous exposure to the toxin)

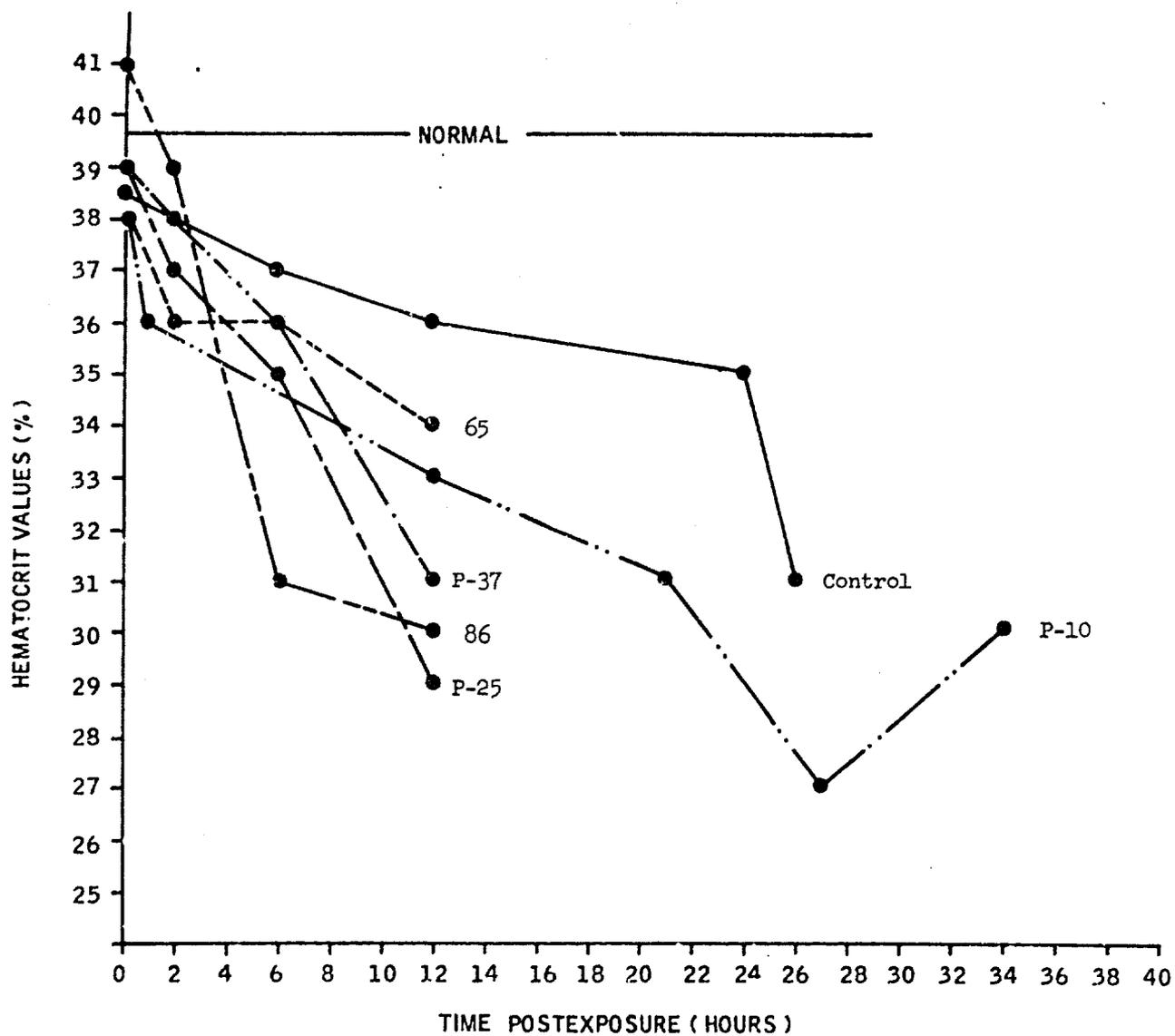


FIGURE 4

HEMATOCRIT VALUES AFTER INTRAVENOUS EXPOSURE TO BOTULINUM TOXIN
 (Monkeys 86 and 65 were survivors of a previous exposure to the toxin)

E. Pathological Findings.

Gross and microscopic examination of necropsied tissues revealed no remarkable changes.

IV. CONCLUSIONS.

From this study it is concluded that a dose of 95 LD50's of botulinum toxin, type A, administered intravenously to monkeys, kills the animals before the toxin has disappeared from the blood and probably too swiftly for an immune response to occur. Future studies of disappearance rate and of immune response should, therefore, be made with a smaller dose of toxin.

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13. ABSTRACT Rhesus monkeys (including two survivors of previous exposures to botulinum toxin) were given Type A botulinum toxin intravenously, and disappearance of the toxin from the serum was measured. These animals were given about 95 LD50's and died while there was still from 30% to 60% of the dose in the serum. Hematocrit values dropped much more swiftly and farther than did those of a control monkey bled on the same schedule.			
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