Final Report

FEASIBILITY STUDY FOR THE USE OF IOPHENOXIC ACID AS A REFERENCE MARKER FOR PLASMA PROTEINS

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I INTRODUCTION

Iophenoxic acid (IPA), a cholecystographic medium, has been shown to remain in the human blood in association with albumin for many years after its ingestion. Its survival half-life has been calculated as 2.5 years (1). It has also been reported to cross the placental barrier into the fetal blood stream long after ingestion by the mother and to remain in the blood of the progeny (2-4). The compound is apparently unique in its resistance to deiodination, metabolism, and excretion by the human kidney. Since iophenoxic acid seems to remain in the human blood in association with albumin for many years, it may be presumed that it somehow reassociates with the new albumin molecules that are synthesized as others are metabolized (1).

For these reasons, IPA seemed to be a logical candidate for use as an indicator on experimental animals for measuring certain physiological parameters, such as intra- and extravascular albumin pool and plasma volume. Therefore, experiments were designed to investigate the nature of interaction of IPA with albumin, its metabolism in the monkey, rabbit and dog, and its possible effect on the turnover rate of serum albumin. This report describes our findings on the high affinity constant of albumin with IPA; the survival half-lifes of IPA in the monkey, rabbit, and dog; the metabolism of serum albumin-IPA complex; and the possible use of IPA to measure intravascular albumin and volume as well as total body albumin.

II SUMMARY

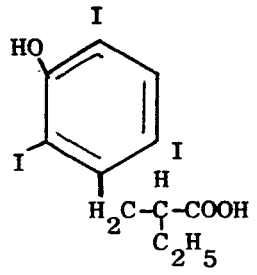
Equilibrium dialysis experiments of human and bovine albumin with iophenoxic acid revealed an apparent association constant of about 10^7 M^{-1} which is much higher than other small molecules interacting with albumin.

Animal experiments have shown that the survival half-time of iophenoxic acid in monkeys, rabbits, and dog was 65-84, 23-32, and 28 days, respectively. This compound was preferentially bound to serum albumin, did not seem to be extensively metabolized, and there was no apparent effect on the metabolism of serum albumin. The biological half-life of serum albumin in monkeys was found to be approximately 20 days and its turnover .15-.18 g/kg/day. Iophenoxic acid seemed to be reassociated with the new albumin molecules synthesized as others were metabolized, and equilibrated with the total exchangeable intra- and extravascular albumin pools. The feasibility of using this compound as a possible indicator for measuring certain physiological parameters in experimental animals was also investigated. The total body and intravascular albumin mass as well as the intravascular volume for monkeys, rabbits and dog were measured following the injection of IPA and the values obtained were within the range of values reported by other investigators except for the total body albumin pool of rabbits.

III MATERIAL AND METHODS

Properties of Iophenoxic Acid

Iophenoxic acid, α-ethyl-β-(2,4,6-triiodo-3-hydroxyphenyl)propionic acid (IPA), was kindly provided by Dr. M. Tanabe (SRI) who obtained it from Schering Corporation.



Iophenoxic acid

To verify the identity and purity of the compound, the UV, IR, and NMR spectra, melting point, and apparent pK of the phenolic group (titration curve) were determined.

The ultraviolet absorption spectrum of IPA in 95% ethanol revealed a maximum at 295 mμ which shifted to 323 mμ in 1 N NaOH and had a molar absorptivity of 3060 and 6790 M^{-1} cm^{-1}, respectively. The IR and UV spectra of IPA were similar to the spectra reported by Astwood (1). The compound melted sharply at 150°C; an apparent pK of the phenolic group was found to be in the range of pH 6.0-6.4 by automatic titration of the salt of the compound with dilute HCl. A pK of 6.3 was reported by Astwood.

The NMR spectrum* revealed that the compound contained (a) no detectable amount of water, (b) 5-substituents in the aromatic ring and one hydrogen, (c) one ethyl and two hydroxyl groups, and (d) 11 hydrogens. These findings correlate well with the structure of iophenoxic acid.

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*The NMR spectrum was performed and interpreted by Mr. Jack Marsh of SRI using a Varian model A-60 spectrometer.

Serum Albumin Preparations

Human, dog, rabbit, and bovine albumin samples were purchased from Pentex Corporation, Kankakee, Illinois. In addition, albumin from rhesus monkeys was prepared by subjecting monkey serum to continuous flow electrophoresis, using a Spinco CP curtain electrophoresis apparatus. The fractions corresponding to the albumin area were pooled, dialyzed thoroughly against distilled water, and lyophilized. The albumin preparations were checked for homogeneity by electrophoresis on cellulose acetate strips. Human, bovine, and dog albumins seemed to be homogeneous under the experimental conditions employed, but the samples from monkey and rabbit were slightly (10 and 5%, respectively) contaminated with α_1-globulin. Each albumin preparation also was analyzed for bound fatty acid content using the titration method reported by Dole (5). The human preparation contained 1.55 mole fatty acid per mole albumin; monkey, 3.29; rabbit, .586; bovine, .368; dog, .120.

Assays of IPA in Equilibrium Dialysis Experiments

A sensitive method for the determination of iodide- as well as iodine-containing organic compounds is based on the catalytic effect of iodide on the ceric-arsenious oxidation reduction reaction (6). The over-all reaction may be written as follows:

$$2\ Ce^{4+} + H_2AsO_3^- \xrightarrow{I^-} 2\ Ce^{3+} + H_3AsO_4$$

The yellow color, due to ceric ion in acid solution, disappears at a rate proportional to the iodide concentration. Under appropriate experimental conditions, this is a first order reaction (7).

Bowden, Maclagan, and Wilkinson (8) originally noted that aromatic iodine ortho to a hydroxy or an amino group readily catalyzed the ceric-arsenious oxidation-reduction reaction. Iophenoxic acid, having two iodine atoms ortho to the hydroxyl group, was added directly to the ceric-arsenious solution and was found to catalyze the reaction at a rate which was proportional to IPA concentration. The IPA in the equilibrium dialysis experiments was determined by following Step 3 of the method cited in reference (7) and analyzing appropriate aliquots

taken from inside the dialysis bag, which contained the albumin solution, as well as from the outside nonprotein solution. Calibration curves were constructed with standard IPA solutions in the presence and absence of albumin and under the same experimental conditions of equilibrium dialysis.

An unsuccessful attempt was made to determine IPA by neutron activation analysis of serum samples and standard IPA solutions.

Equilibrium Dialysis Method

The method employed was that reported by Sterling et al. (9). The concentration of IPA (added to the albumin solution inside the dialysis bag or to the outside buffer solution) usually ranged from 1.5×10^{-5} to 2×10^{-4} M, expressed as the final concentration in the 5 ml of protein solution inside the bag or the 5 ml of nonprotein solution outside the bag. The buffer used for the dialysis experiments was 0.05 M potassium phosphate, pH 7.4, and the albumin solution was 0.1 g/100 ml buffer. IPA solutions were made up by dissolving IPA in dilute KOH and bringing it to the required volume with buffer. The amount of IPA bound to albumin was determined at the end of the equilibrium period (16 hr shaking at room temperature) by subtracting the outside concentration of IPA from the one inside the dialysis bag. The data obtained were analyzed in accordance with Scatchard's equation (10).

$$\bar{V}/A = kn - k\bar{V}$$

$$\bar{V} = \frac{\text{moles IPA bound}}{\text{mole albumin}}$$

A = concentration of free IPA (M)

$$n = \frac{\text{maximum no. of moles IPA bound}}{\text{mole albumin}}$$

k = apparent association constant (M^{-1})

According to the equation, a plot of $\bar{V}/A$ against $\bar{V}$ gives a straight line if k is constant, i.e. if all the binding sites are equivalent or if the protein possesses a single class of binding sites. The intercept on the $\bar{V}/A$ axis would become equal to kn as $\bar{V}$ approached zero, and the intercept on the $\bar{V}$ axis would be equal to n as $\bar{V}/A$ approached zero.

Serum IPA, Albumin, and Total Protein

Serum albumin and total protein were determined according to the method reported by Debro, Tarver, and Korner (11).

One to 10 µl of serum was analyzed for IPA by determining the total iodine content, following ashing of the serum sample in an alkaline medium (7). The endogenous iodine content of 13 pre-IPA serum samples obtained from different monkeys and rabbits was less than .005 µg/10 µl each. Therefore, it was felt that the serum IPA could be analyzed by direct ashing. Calibration curves prepared with IPA standard solution and IPA added to serum and subjected to the same treatment were similar. Each serum sample obtained from monkeys, rabbits, and dog was analyzed three or four different times; values were always within 5-8% of their mean.

Urinary IPA Determination*

Two ml of clear urine was pipetted into a test tube containing 0.1 ml of 50 mg/ml bovine albumin. The urine and albumin were mixed and incubated at 30°C for 1 hr. One ml of this mixture was dialyzed for 16 hr at room temperature against deionized water in 30/32-inch Visking dialysis tubing. The contents of the dialysis tubing were transferred quantitatively to a 10-ml volumetric flask. Aliquots were taken and ashed for IPA. Appropriate standards and controls were incorporated with each run. Eight control urines, collected from the same and other animals prior to injection with IPA, were analyzed by this method. No iodine-like material was found except for about 20 µg/24 hr specimen on two occasions. Standard IPA solutions added to urine and processed the same way showed an approximate recovery of IPA of 80-90%.

Extraction, Isolation, and Identification of Serum IPA from Monkeys

Serum samples from monkeys that had received IPA (M-2, M-4, M-11) were pooled (10 ml) and the proteins were precipitated with 1 ml of 20% TCA. To the precipitate a sufficient amount of 95% ethanol was added

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*For collection of urine from monkeys see "C^{14} Urine", p.9

to solubilize the albumin fraction. The globulins were spun down and again washed with EtOH-TCA mixture (1% TCA in EtOH 95%) to dissolve all the albumin. The initial EtOH supernatant and wash were combined and made alkaline with NaOH pellets. The alcohol was removed in a Rinco evaporator kept at 50°C and under water-vacuum. Water (15 ml) was added to the basic mixture and extracted with 20 ml of $CHCl_3$. The sample was spun to break the emulsion and the $CHCl_3$ layer was discarded. The water phase was made acidic with HCl and extracted with 30 ml of $CHCl_3$. The $CHCl_3$ extraction was repeated and the combined extracts were dried with anhydrous $MgSO_4$. To further purify the IPA, the dried chloroform extract was reduced in volume and was extracted with 10 ml of 1 N NaOH. The aqueous layer was acidified with HCl and the $CHCl_3$ extraction was repeated. The $CHCl_3$ layer was dried with $MGSO_4$, filtered, and evaporated to reduce its volume to about 0.5 ml. Thin layer chromatography was used to identify IPA using the two solvent systems: N-BuOH saturated with water and N-BuOH saturated with 5% NH_4OH on silica gel HF plates. IPA on fluorescent plates is readily detectable under UV light. A large portion of the $CHCl_3$ extract was applied on an analytical silica gel HF (8 x 8 in) plate and the chromatogram was developed in the BuOH-NH_4OH system. The IPA streak was located and scraped off the plate into a flask; the IPA was eluted from the silica gel with acetone and one drop of concentrated HCl. The silica gel was filtered off and the acetone was evaporated at 50°C. The residue was dissolved in methanol and the UV spectra were obtained in acidic and basic methanol solutions and compared with the reference standard IPA solution.

Animals, Injection, and Sampling

During the initial phase of this work different doses of IPA were tried on rabbits and monkeys. For the measurement of physiological parameters in various species a dose of about 2 to 3 mg/kg body weight appears to be appropriate.

Rabbits

Each of three New Zeland White rabbits was placed in a wooden restraining box and a blood sample was taken from it prior to the

injection of IPA. Samples of approximately 1 ml each were taken at appropriate intervals from the marginal ear vein during the first four hours after the injection of IPA. The animals were then transferred to their cages. Blood samples were withdrawn twice daily during the first week and at intervals of 1 to 7 days throughout the observation period. One rabbit (R-3) was found dead in its cage (cause unknown) after the 35th day following the injection of IPA.

Monkeys

Each of the seven male rhesus monkeys (Macaca mulatta), weighing 4.5 to 6.5 kg, was placed in a restraining box and injected iv with IPA. During the first hour of restraint, two to six blood samples were taken from the femoral vein and the animals were returned to their cages. Subsequent samples were obtained at appropriate intervals throughout the observation period.

Urine collection for monkeys M-4, M-5, M-10, and M-11 was begun one day after the IPA injection.

Thirteen days after the injection of IPA, monkeys M-4 and M-11 and two additional monkeys (M-6 and M-8) each were injected iv with 250 μc of reconstituted C^{14}-protein hydrolysate (Schwarz BioResearch, Inc., Lot No. 660). Prior to injection the animals were fasted for approximately 24 hr. They were restrained during the first hour of injection and sampling.

Thereafter, blood specimens were taken at appropriate intervals and 24-hr urine specimens were collected for the estimation of C^{14} activity and IPA concentration when appropriate.

Dog

A male hound dog weighing 28.85 kg was injected iv with 60 mg of IPA and blood specimens were taken at suitable intervals for the estimation of serum IPA concentration. This experiment was performed in collaboration with Dr. Datnow of Ames Research Center.

The serum samples obtained from the various animals were kept frozen until analyzed for the proper constituent.

Preparation of Serum Protein Samples for Counting and Analysis

Serum samples were fractionated into albumin and globulins according to the method of Debro, Tarver, and Korner (11), with the following modifications. After precipitation of the proteins from 0.1 ml serum with 0.9 ml of 5% trichloroacetic acid (TCA) the precipitate was washed once with 0.5 ml of 5% TCA and the supernatants were pooled and counted for C^{14} in Bray's solution (nonprotein C^{14} activity); 1.0 ml of 95% EtOH was added to the washed precipitate with adequate mixing. After solubilization of albumin was completed the mixture was centrifuged, the precipitate was washed again with 0.5 ml EtOH and the supernatant fractions were pooled. Of the albumin fraction, 0.5 ml was added to 0.5 ml hyamine and 10 ml Bray's solution for counting; .03 to .05 ml of another aliquot was processed for protein determination (3), so that the specific activity (cpm/mg) of the labeled albumin could be calculated.

The precipitate that contained the globulins was dissolved in 1.2 ml of 0.1 N NaOH and a 0.5-ml aliquot was added to 0.5 ml hyamine and 10 ml Bray's for counting; another .03 to .05 ml was processed for protein analysis. Specific activity for total protein was estimated by counting 0.1 ml serum in hyamine and Bray's solution. The specific activity (cpm/mg) of total protein was calculated after subtracting the counts due to the nonprotein fraction.

C^{14} Urine

Monkeys were housed in individual cages equipped with standard urine collection trays. Glass wool plugs were used to filter out food and feces from the urine, which was collected in a plastic bottle. The volume was measured daily and representative samples were placed in 15-ml disposable plastic test tubes and kept frozen until assayed. Samples were thawed at room temperature, thoroughly mixed and centrifuged. One ml of clear urine supernatant was added to 15 ml of Bray's counting fluid and counted in a Nuclear-Chicago liquid scintillation counter. Standard C^{14}-toluene (0.05 ml) was added to the counting mixture to correct for quenching due to the urine.

Analysis of Data

Serum IPA concentration after the 10-15 day period, expressed as μg IPA per ml serum and per mg albumin, were plotted on semilogarithm graph paper as a function of time. A straight, freehand line was fitted to the points.

IPA Metabolism

Half-time was calculated graphically by extrapolation of the straight line to zero time. In Method I, it was calculated from the plot of μg IPA per mg albumin vs. time; in Method II, from the plot of μg IPA per ml serum.

Fraction of Total IPA Injected Eliminated Per Day

The fraction $\frac{.693}{t_{\frac{1}{2}}}$ is derived from the first order exponential equation

$C = C_o e^{-kt}$ where C_o = IPA concentration at zero time

C = IPA concentration at time (t).

The calculated quantity (μg) of IPA eliminated per day = total IPA injected X fraction eliminated per day

The fraction of circulating serum IPA excreted in the urine per day =

$$\frac{\text{urinary excretion of IPA/24 hr}}{\text{mean circulating IPA during same 24 hr (μg/ml) X serum volume}}$$

The mean circulating IPA value is read directly from the plots.

The averaged fraction X circulating IPA at zero time = maximum quantity IPA excretable in urine.

Intravascular Volume and Albumin Mass

$$\text{Volume} = \frac{\text{administered IPA}}{\text{μg IPA per ml serum at zero time}}$$

By Method I

$$\text{Intravascular albumin} = \frac{\text{administered IPA}}{\text{μg IPA per mg serum albumin at zero time}}$$

Zero-time values for volume and albumin were obtained from semilog plots of μg/ml serum and μg/mg albumin during the first 4 hr after the injection of IPA, by extrapolation.

By Method II

Intravascular albumin = avg serum albumin (mg/ml) X intravascular volume

$$\text{Total body albumin} = \frac{\text{administered IPA}}{\mu\text{g IPA per mg albumin at zero time}}$$

Micrograms of IPA per mg albumin was obtained by extrapolating the linear portion of the curve (drawn after 10-15 days) to zero time.

C^{14} Studies

The biological half-life of serum proteins was obtained graphically (12) and the additional C^{14} data were analyzed according to the method of Waldmann and Laster (13).

$$\text{Fraction of body proteins degraded per day} = \frac{.693}{t_{\frac{1}{2}}}$$

Albumin turnover (g/kg/day) =
total body albumin pool (g/kg) X fraction degraded per day.

Fraction of circulating serum protein degraded per day =

$$\frac{\text{urinary } C^{14} \text{ activity in 24 hr sample}}{\left(\text{mean circulating } C^{14} \text{ activity during same 24 hr} \times \text{mg protein per ml} \times \text{intravascular volume}\right)}$$

The mean circulating C^{14} activity value (cpm/mg protein) is read directly from the C^{14} protein plots.

IV RESULTS

A. Equilibrium Dialysis Experiments

A representative set of calibration curves (under the equilibrium dialysis conditions) for the estimation of IPA is shown in Fig. 1. The presence of 0.1 mg of albumin in the final ceric-arsenious mixture considerably suppressed the rate of the reaction. The same type of curve was obtained with either human or bovine albumin. With increasing amounts of albumin the inhibition of the rate of the reaction became greater. Therefore, the determination of IPA inside the dialysis bag (albumin solution) was carried out in the presence of 0.1 mg of albumin.

Preliminary data obtained from equilibrium dialysis experiments, using bovine and human albumin, were analyzed by means of the Scatchard equation and are shown in Figs. 2 and 3. The effect of EDTA* on the equilibrium dialysis experiments with bovine albumin was evaluated since it was reported by Sterling (14) that EDTA prevents thyroxine from forming complexes with metal ions in solution and that, therefore, the interaction of IPA with albumin could be influenced by such a complex formation with contaminating metal ions. It was observed that with or without EDTA (its outside concentration being 6.8×10^{-4} M) the Scatchard's plots were similar when IPA at a concentration range of 4×10^{-5} to 4×10^{-4} M was equilibrated with bovine albumin.

In Fig. 2, IPA at a concentration range of 1.5×10^{-5} to 2×10^{-4} M was equilibrated with bovine albumin by adding the IPA inside or outside the dialysis bag. Both curves produced similar $\bar{V}/A$ and $\bar{V}$ values. This was interpreted to mean that equilibrium was established. The nonlinear relationship of $\bar{V}/A$ against $\bar{V}$ indicates that more than one type of binding site(s) existed in the albumin molecule. By extrapolating the linear portion of the curve ($\bar{V} < 2$) to the $\bar{V}/A$ and $\bar{V}$ axis, an approximate apparent kn_1 value of 1.6×10^{7} M^{-1} and an average n_1 of 2 was obtained, respectively. The quotient $\frac{kn_1}{n_1} = 8.0 \times 10^{6}$ M^{-1} produced the value for k,

*Ethylenediaminetetraacetic acid

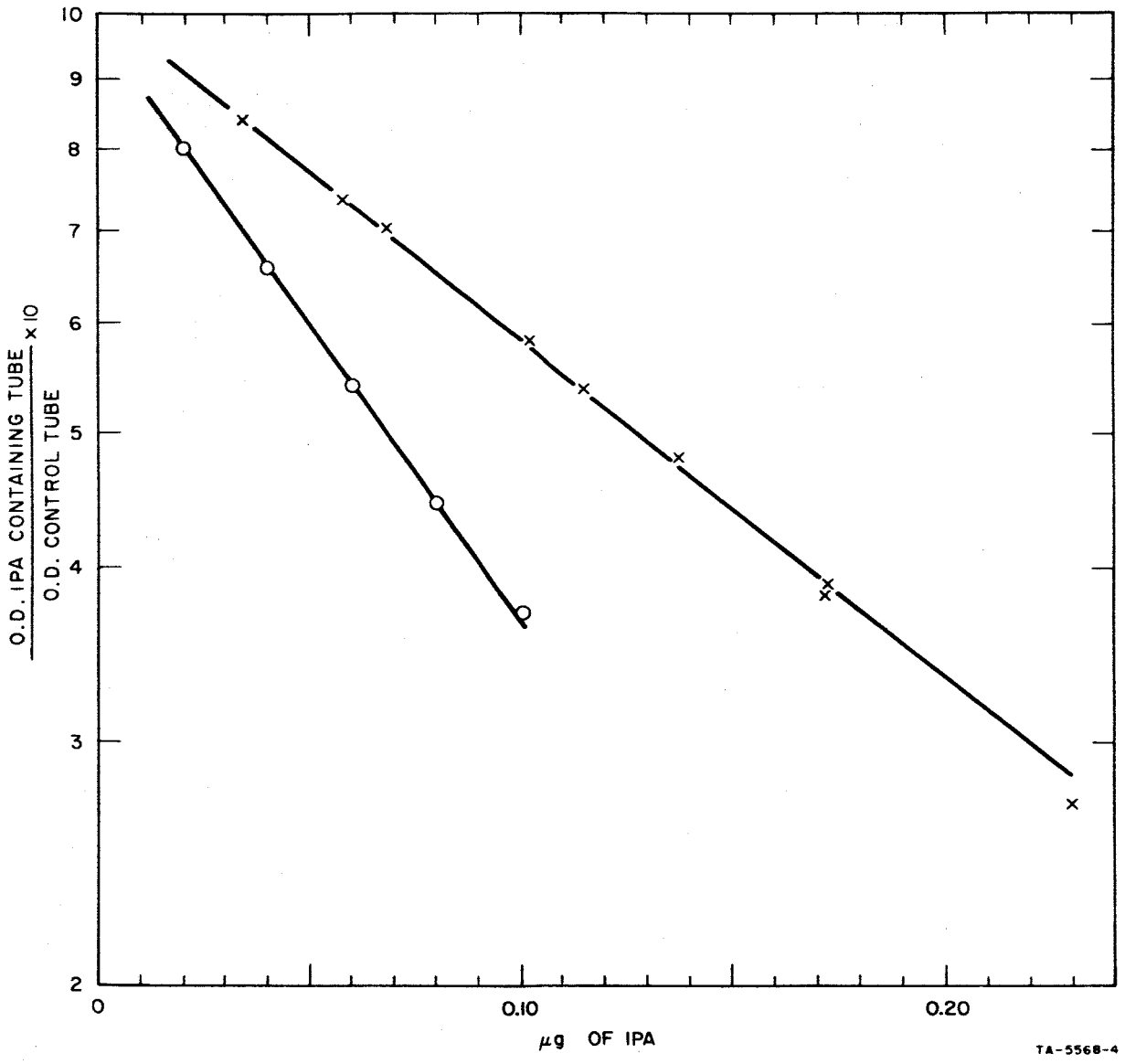

FIG. 1 CALIBRATION CURVES FOR THE DETERMINATION OF IPA. The final reaction mixture contained the appropriate amounts of ceric and arsenious acid (see Methods), the indicated quantities of IPA (o—o), without albumin and with 0.1 mg albumin (x—x).

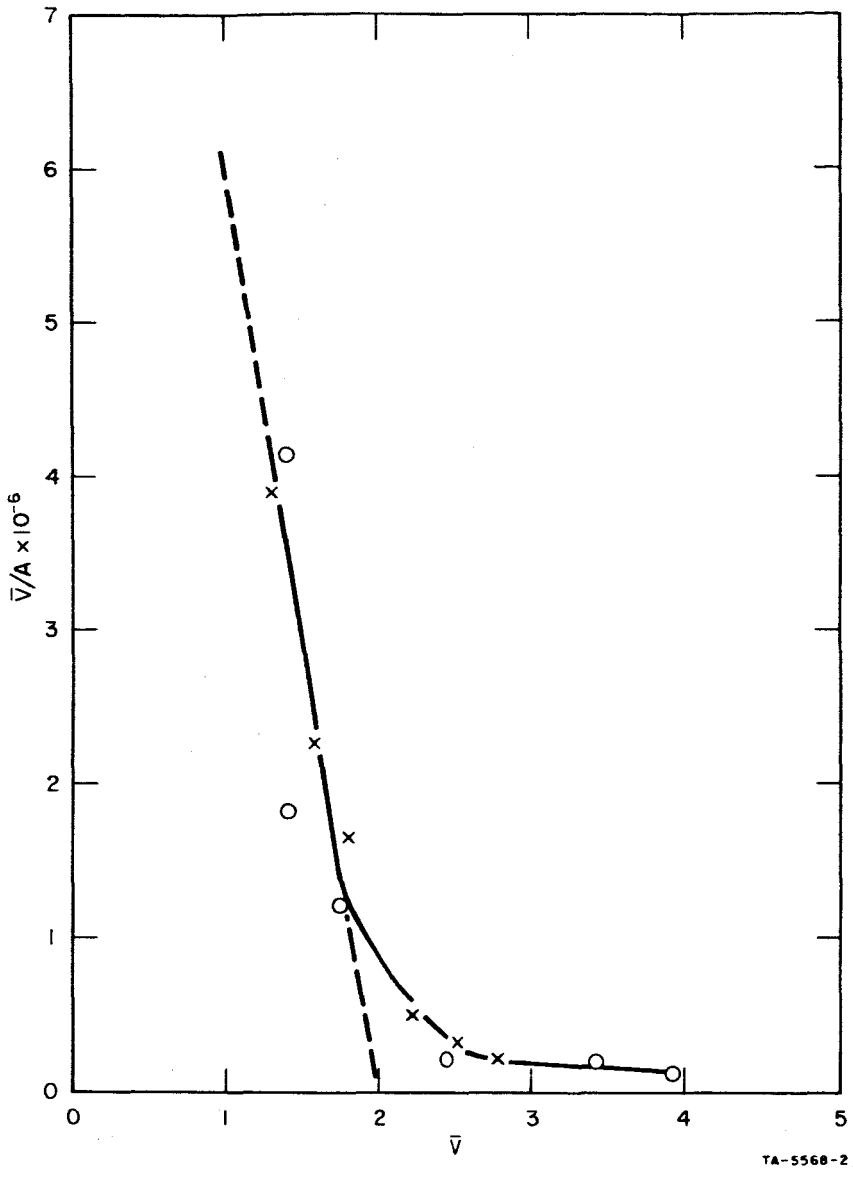

FIG. 2 EQUILIBRIUM DIALYSIS EXPERIMENTS OF IPA BINDING BY BOVINE ALBUMIN. Dialysis conditions: Room temp., 16 hrs shaking; inside dialysis bag, 5.0 ml of 0.05 M potassium phosphate, pH 7.4, 1.0 mg of albumin per ml; outside bag, 5.0 ml of buffer containing 6.8×10^{-4} M EDTA and 1.5×10^{-5} to 2×10^{-4} M IPA (o—o); in the experiment denoted by (x—x) IPA was added to the albumin inside the bag and EDTA was omitted.

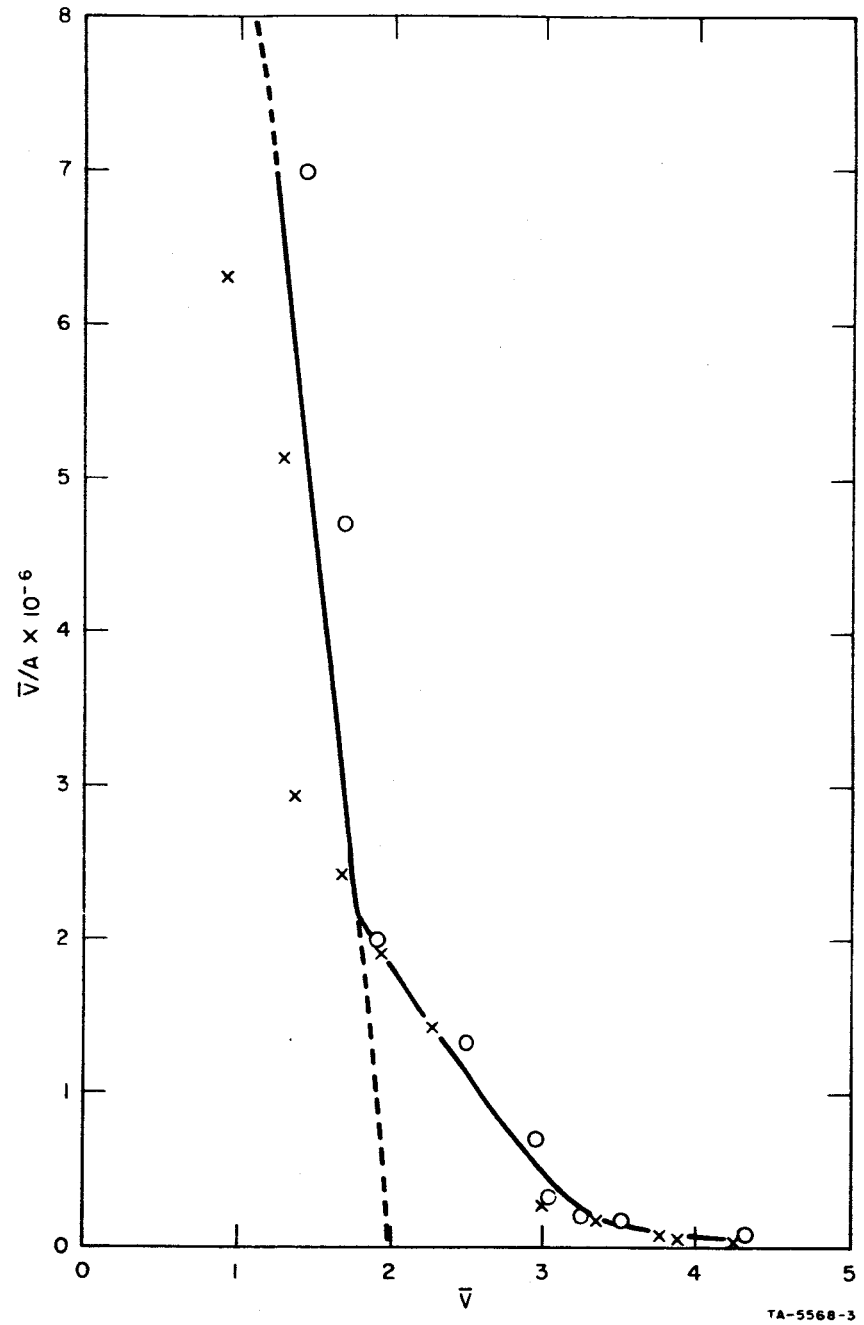

FIG. 3 EQUILIBRIUM DIALYSIS STUDY OF IPA BINDING BY HUMAN ALBUMIN. Dialysis conditions: Room temp., 16 hrs shaking; inside dialysis bag, 5.0 ml of 0.05 M potassium phosphate, pH 7.4, 1.0 mg of albumin per ml; outside bag, 5.0 ml of buffer containing 6.8×10^{-4} M EDTA and 1.5×10^{-5} to 2×10^{-4} M IPA (o—o); in the experiment denoted by (x—x) IPA was added to the albumin inside the bag and EDTA was omitted.

the apparent association constant. Figure 3 shows the plots obtained from dialysis experiments carried out with human albumin and IPA ranging in concentrations from 1.5×10^{-5} to 2×10^{-4} M. IPA was added to the albumin in one set of experiments (x——x) and to the outside of the dialysis bag in another (o——o). The values obtained for the $\bar{V}/A$ and $\bar{V}$ seemed to correlate well at the higher concentrations of IPA ($\bar{V} > 1.8$) while at the lower concentrations of IPA ($\bar{V} < 1.8$) there is some discrepancy. The only difference between the two experiments was that in one the IPA was added inside the dialysis bag and in the other IPA was added to the outside, which contained 6.8×10^{-4} M EDTA. Extrapolation of the linear portion of the curve to the $\bar{V}/A$ and $\bar{V}$ axis produced an approximate kn of $\geq 1.8 \times 10^{7}$ M^{-1} and n = 2, respectively. The apparent association constant of human albumin with IPA seems to be in the range of 9×10^{6} M^{-1} or greater.

B. IPA Metabolism in Rabbits, Dog, and Monkeys

The serum elimination of intravenously injected IPA was followed for 60 to 240 days in three rabbits, one dog, and seven monkeys. Results are shown graphically in Figs. 4 to 13. The experimentally determined rate of disappearance of IPA from the serum of these animals in relation to time seemed to fit the equation for a first order reaction. The semilogarithmic plots, relating μg of IPA/ml of serum and per mg serum albumin to time, all showed a biphasic curve. The initial fall of the serum concentration of IPA is attributed to the distribution of IPA, which is preferentially bound to serum albumin, within the total exchangeable intra- and extravascular albumin pool. The serum IPA level fell during the first 10-15 days and thereafter seemed to follow the linear portion of the decay curve. During this time it was assumed that some sort of an equilibrium had been established between the different albumin compartments of the animal. It is interesting to note that the linear portion of the curve started after days 5, 10, and 16 in rabbit, dog, and monkey, respectively (Figs. 5 to 7). This could mean that the time required for the equilibration of IPA within the various albumin compartments is variable with the different animal species. The initial fall in the concentration of serum IPA

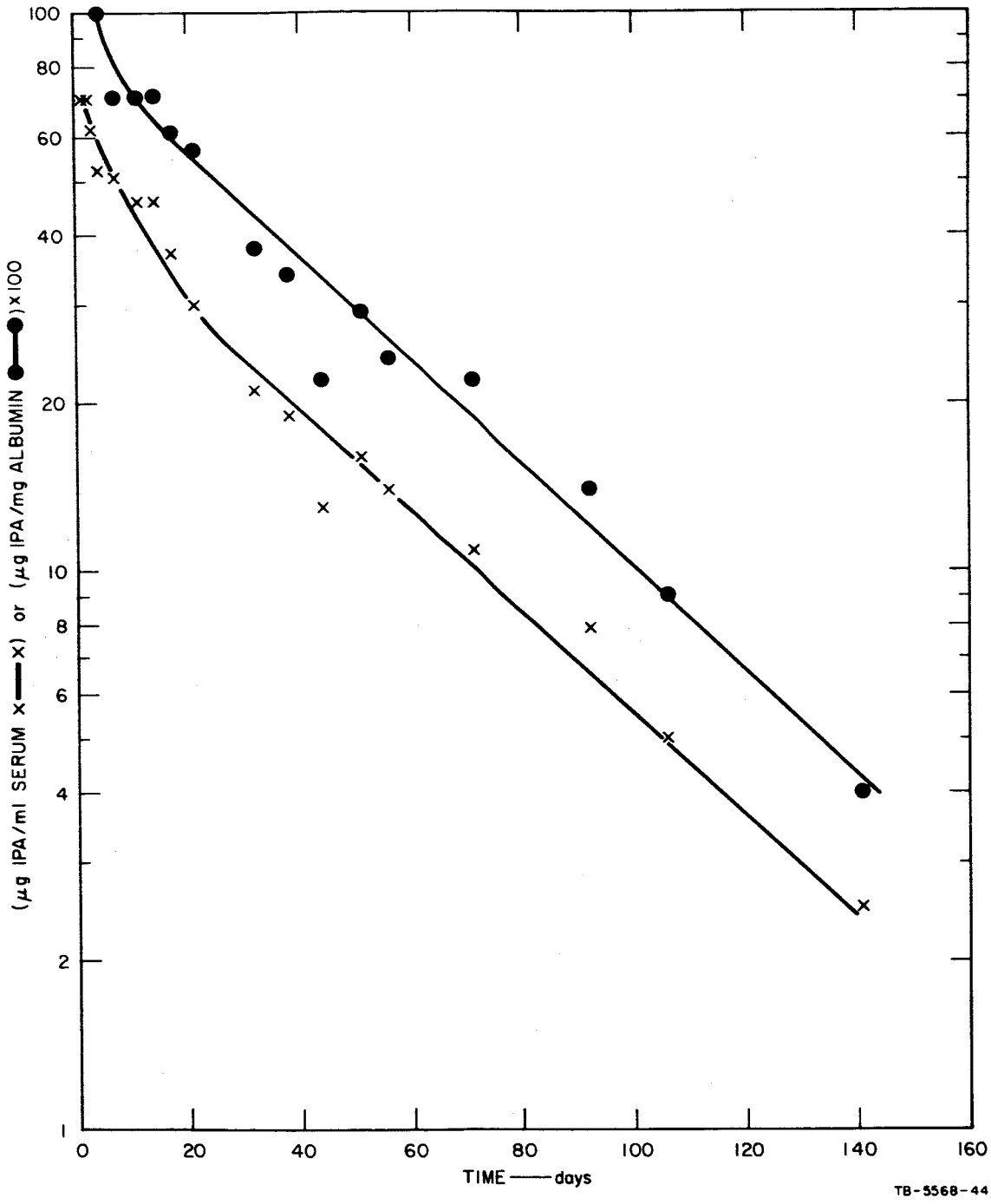

FIG. 4 SERUM IPA CONCENTRATION IN RELATION TO TIME, AFTER THE INTRAVENOUS INJECTION OF RABBIT NO. 2 WITH 12.4 mg IPA/kg

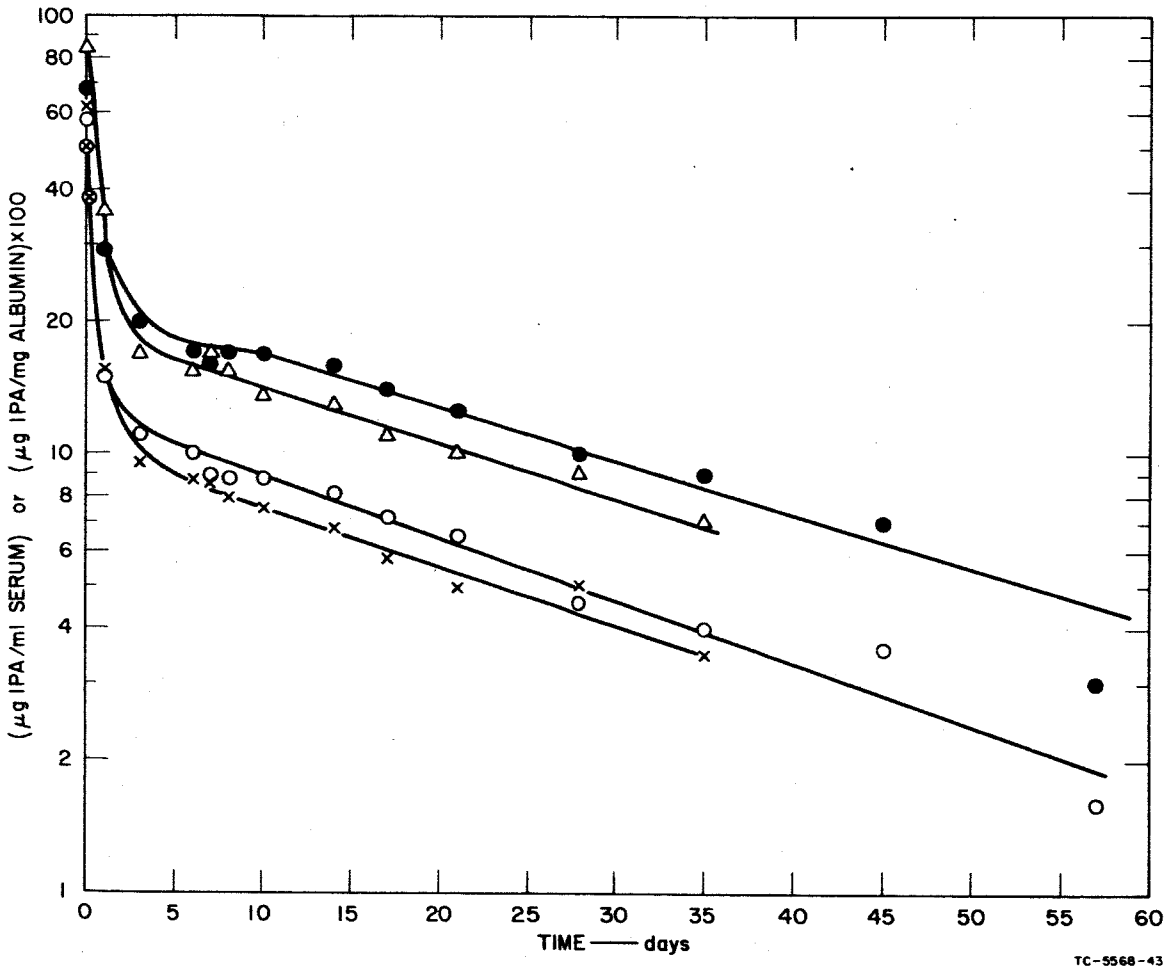

FIG. 5 SERUM CONCENTRATION OF IPA WITH TIME. The concentration is expressed as μg IPA/ml serum or per mg albumin for rabbit No. 3 (x—x or Δ—Δ) and rabbit No. 4 (o—o or ●—●), respectively; both rabbits were injected each with 2 mg IPA/kg.

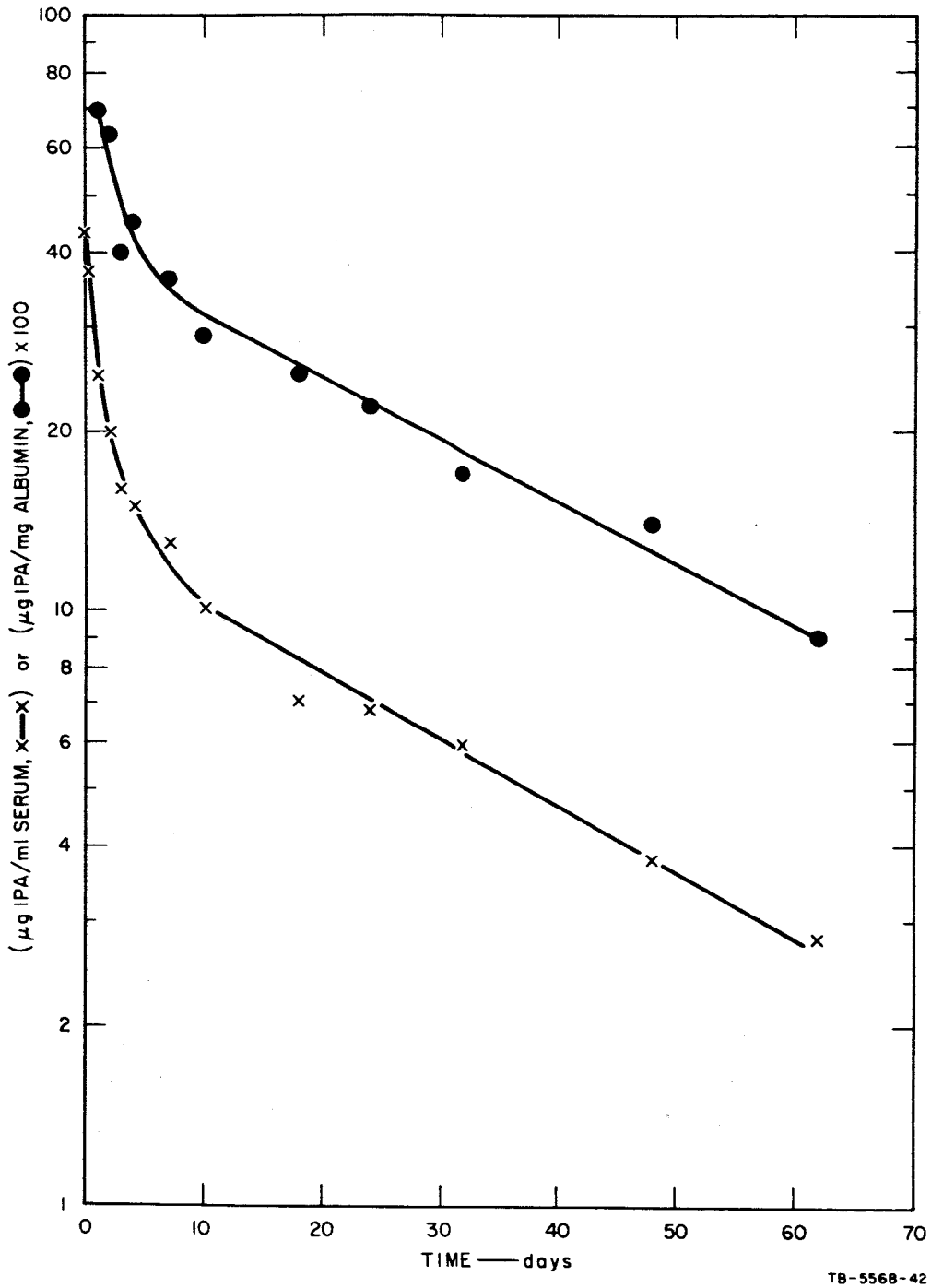

FIG. 6 SERUM IPA CONCENTRATION WITH TIME IN A DOG INJECTED iv WITH 2.07 mg IPA/kg

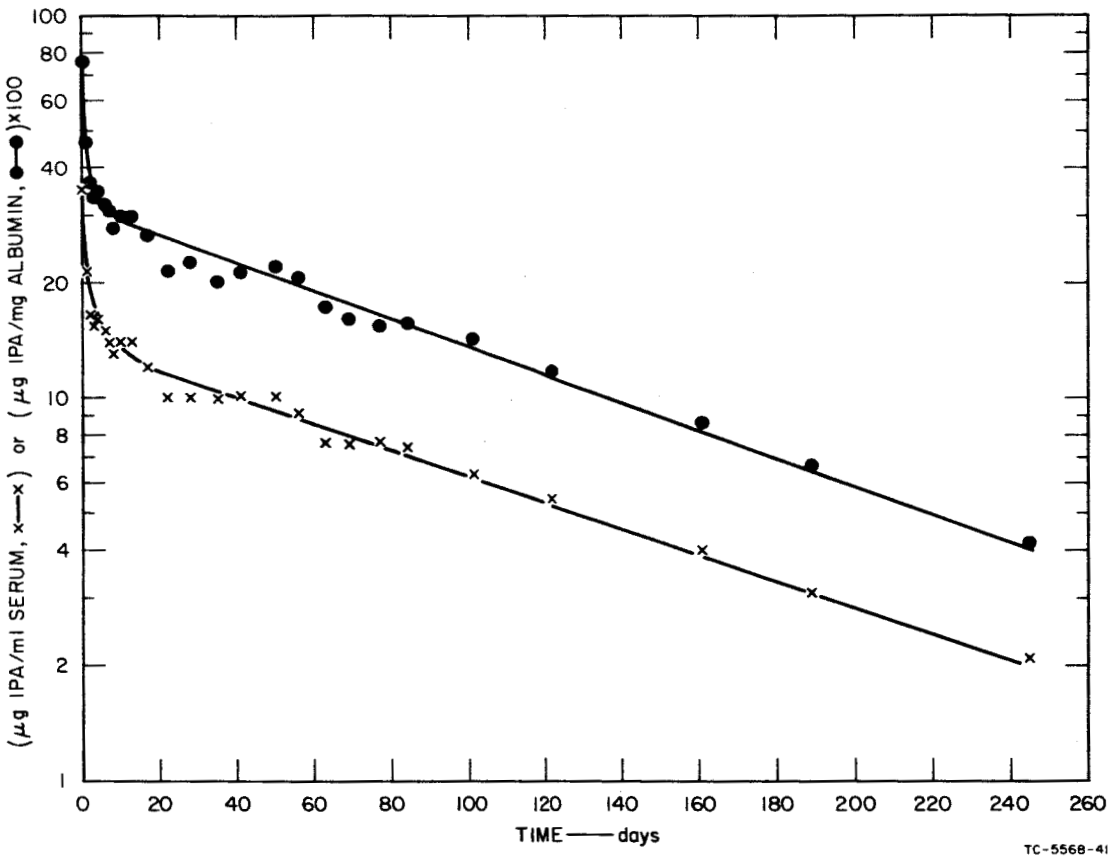

FIG. 7 SERUM CONCENTRATION OF IPA WITH TIME AFTER THE INTRAVENOUS INJECTION OF MONKEY NO. 1 WITH 1.31 mg IPA/kg BODY WEIGHT

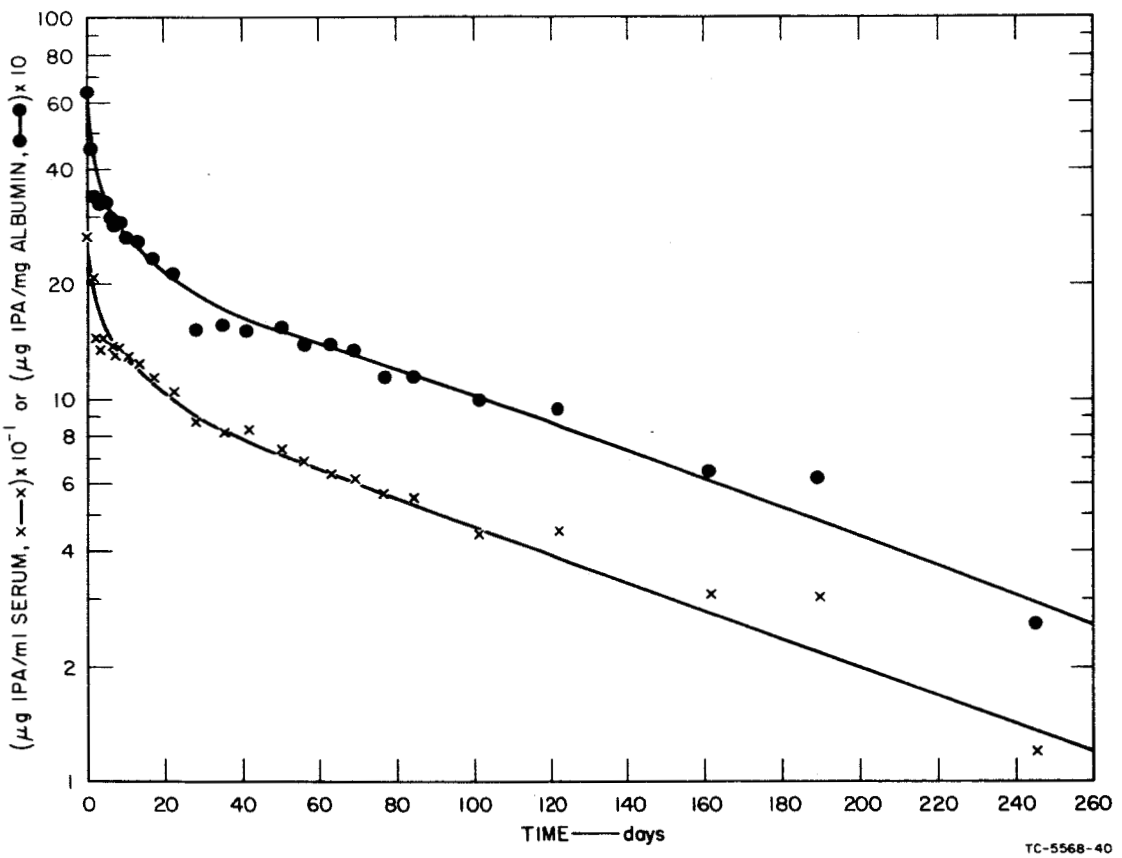

FIG. 8 SERUM CONCENTRATION OF IPA WITH TIME FOR MONKEY NO. 2 INJECTED APPROXIMATELY WITH 18.6 mg IPA/kg

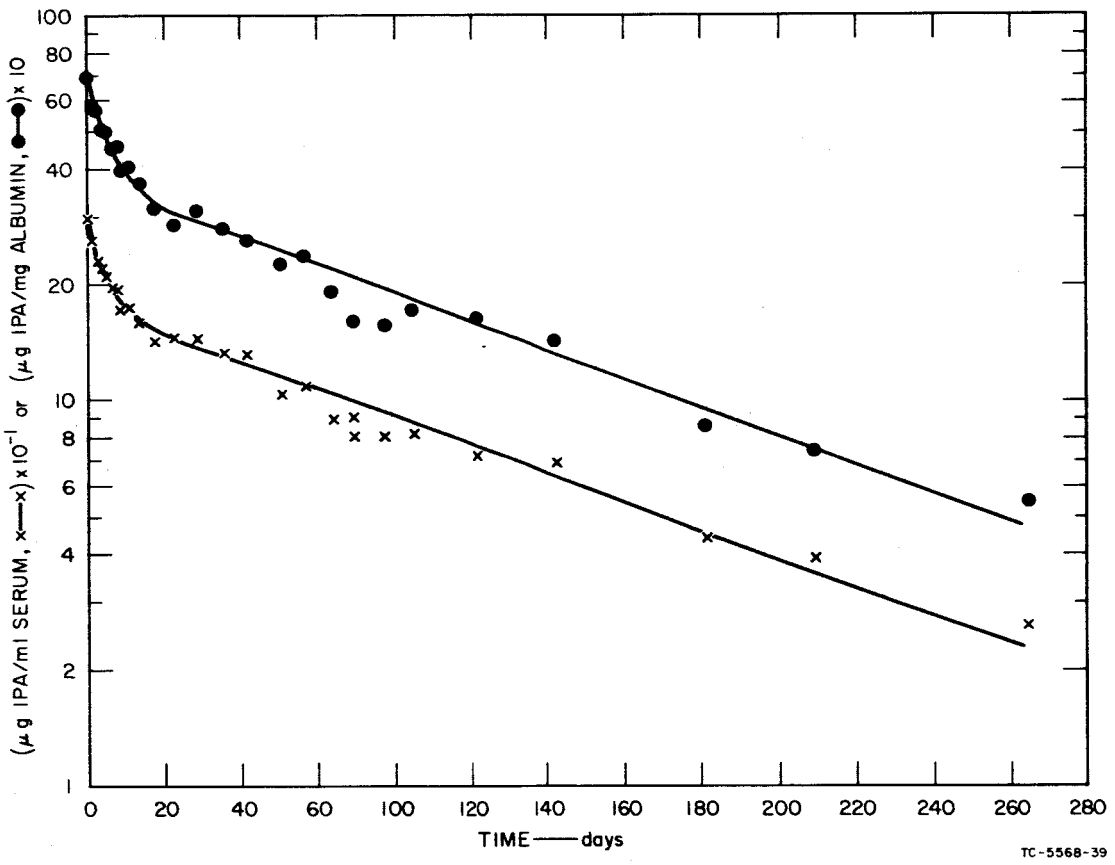

FIG. 9 SERUM CONCENTRATION OF IPA WITH TIME FOR MONKEY NO. 7 WAS INJECTED WITH 24.6 mg IPA/kg

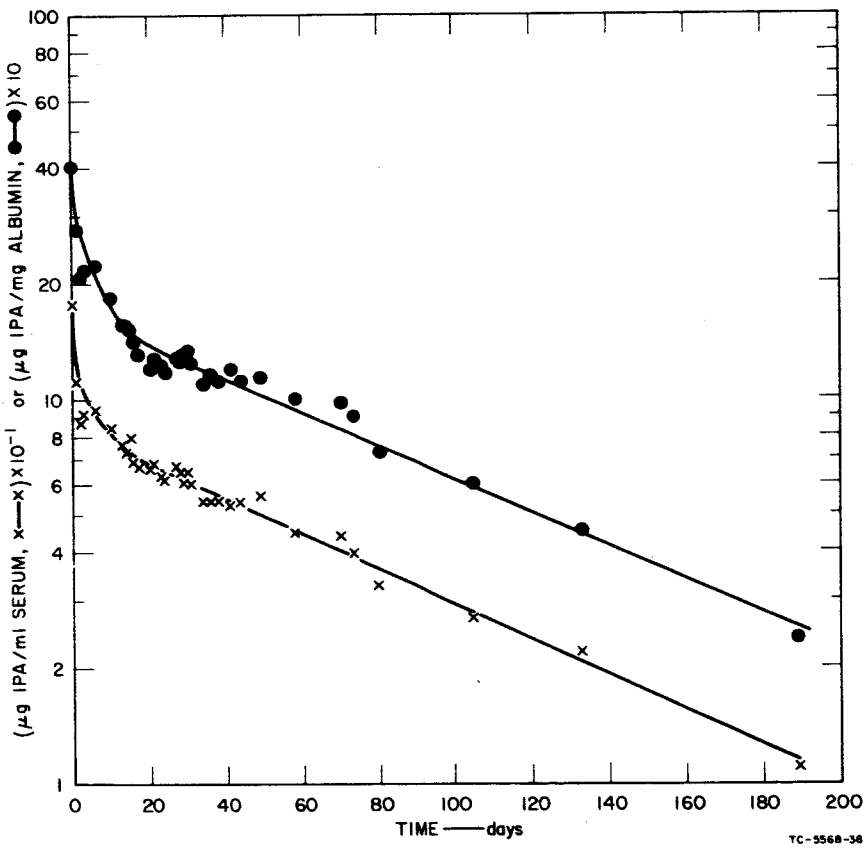

FIG. 10 SERUM CONCENTRATION OF IPA WITH TIME FOR MONKEY NO. 4 INJECTED WITH 8.58 mg IPA/kg

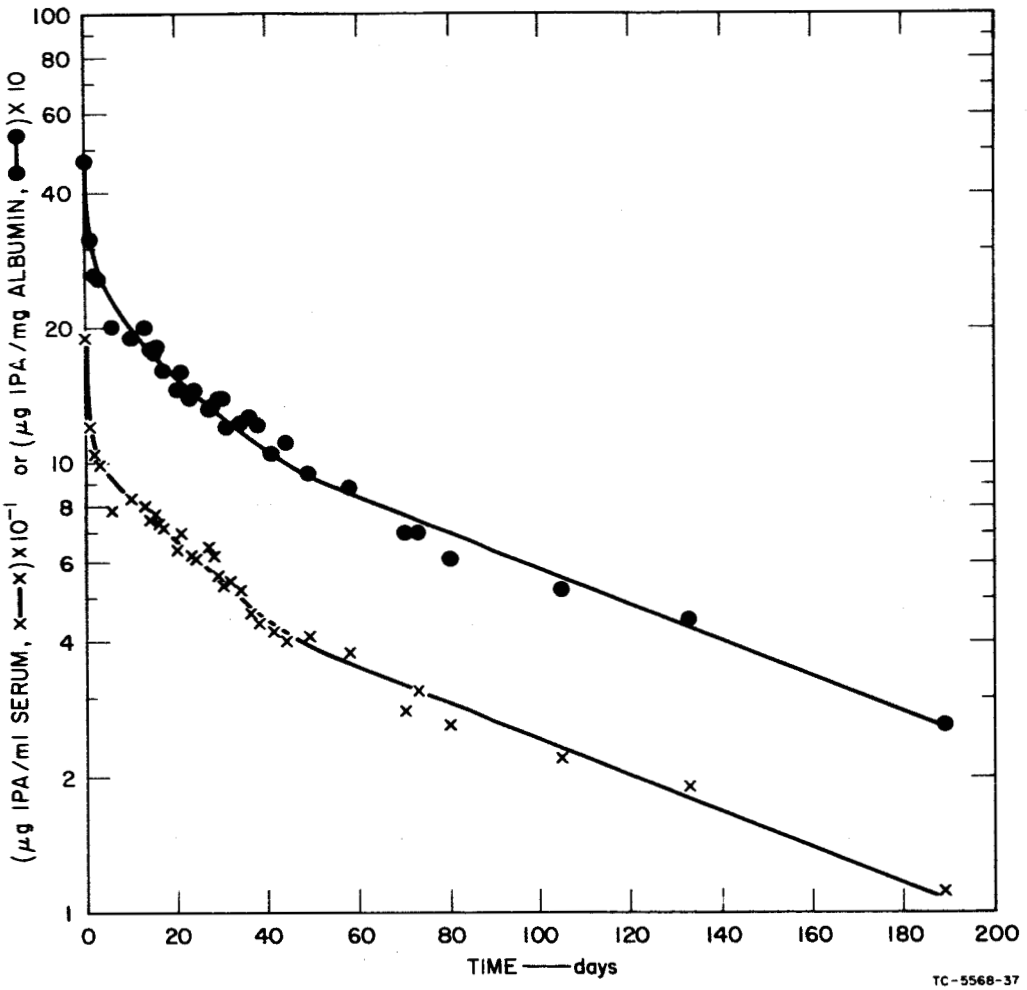

FIG. 11 SERUM CONCENTRATION OF IPA WITH TIME FOR MONKEY NO. 11 INJECTED WITH 8.0 mg IPA/kg

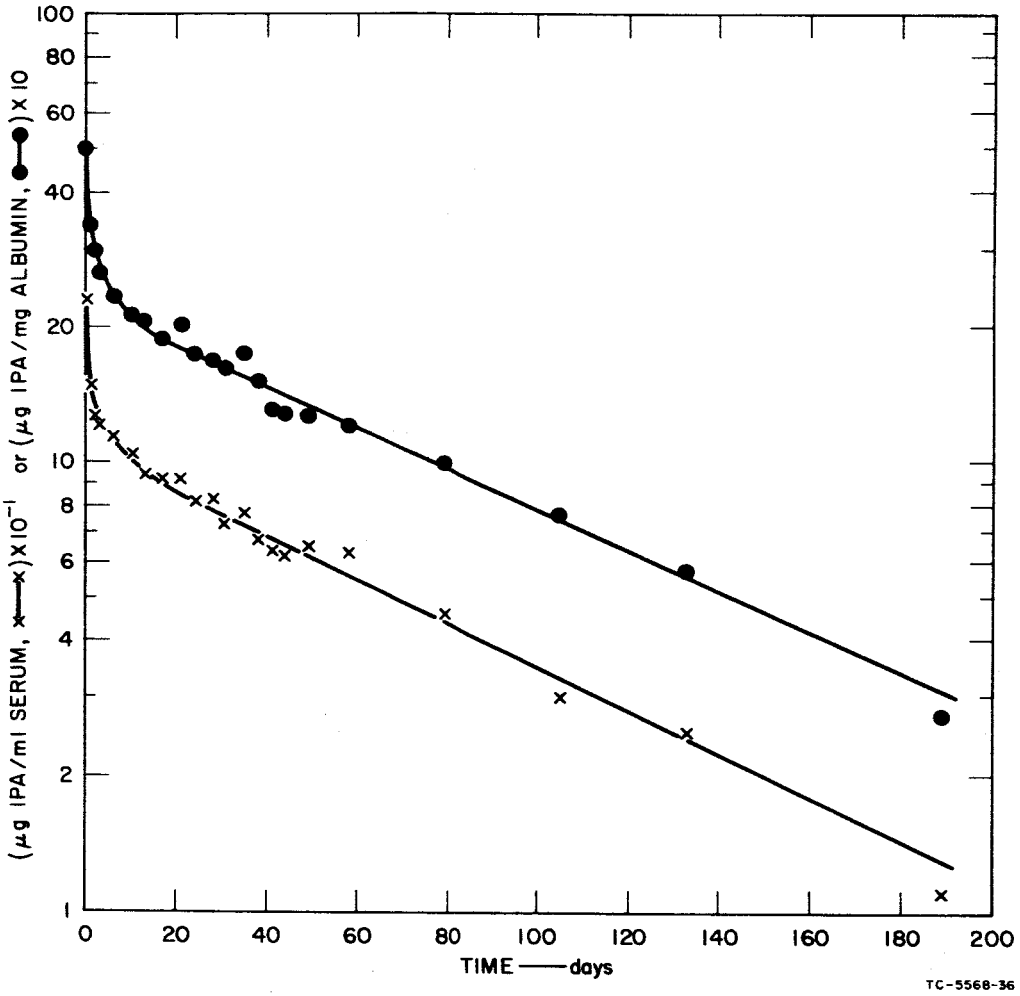

FIG. 12 SERUM CONCENTRATION OF IPA WITH TIME FOR MONKEY NO. 5 INJECTED WITH 9.64 mg IPA/kg

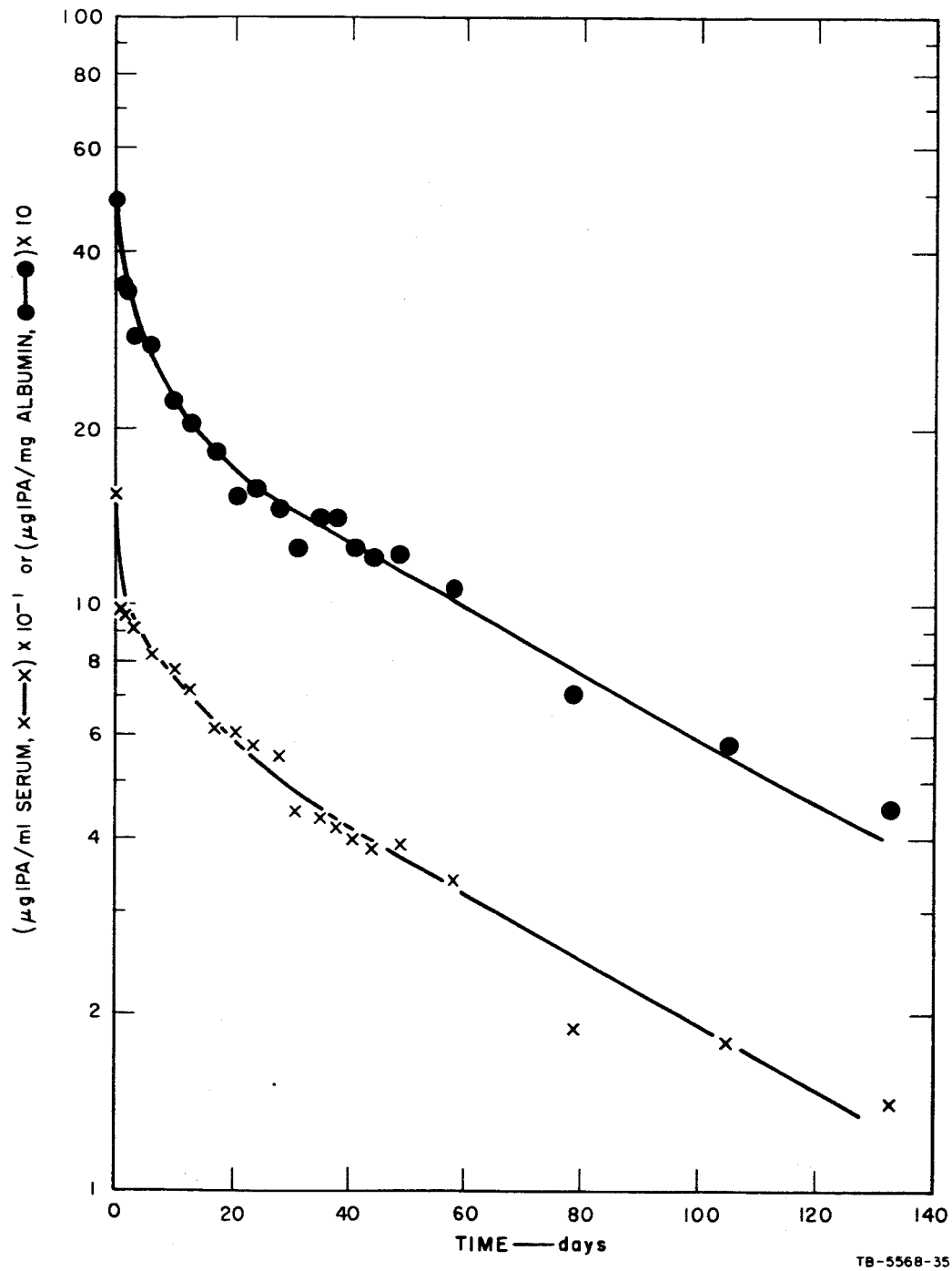

FIG. 13 SERUM CONCENTRATION OF IPA WITH TIME FOR MONKEY NO. 10 INJECTED WITH 10 mg IPA/kg

during the first 10 days was greater in the rabbit than the dog (compare Figs. 5 and 6) although the animals were injected each with 2 mg IPA per kg body weight.

The linear portion of the decay curve in each experiment was used to calculate the survival half-time of IPA, the fraction and the amount of the total IPA injected eliminated per day. The half-life was obtained graphically from the plots of μg IPA/mg albumin (Method A) and per ml serum (Method B) vs. time. Calculation of the other parameters is described in Section III, Analysis of Data. The results obtained from such calculations were summarized in Table 1. Both methods for calculating half-life gave similar values. The half-life of IPA is much greater in the monkey than in either the dog or the rabbit. In the dog, the compound was retained slightly longer (average $t_{\frac{1}{2}}$ = 28 days) than in rabbits R-3 and R-4 ($t_{\frac{1}{2}}$ = 23 days); all three animals were injected with 2 mg IPA/kg body weight. It is possible that there may be no difference between the dog and rabbits: rabbit no. 2, injected with 12.4 mg/kg, produced a higher half-life than either rabbit 3 or 4. In addition, such a difference could be due to variation within the species. The half-life of IPA in the monkeys ranged from 66 to 84 days except for monkey M-10, which produced a half-life of 54 days. The weight of this animal had increased by 2.1 kg at the end of the observation period. However, if the shorter half-life of IPA in monkey M-10 were due to weight gain, the half-lifes for monkeys M-1, M-7, and M-11 should have been similarly affected, since these animals also gained 1 to 1.5 kg. Monkey no. 10 had a low serum albumin concentration (3.2 g/100 ml serum) as compared to an average value of 4.5 g/100 ml for the other six monkeys.

The fraction of the total IPA injected eliminated per day, calculated from the expression $0.693/t_{\frac{1}{2}}$, was found to be approximately .01 for the monkeys and .023 to .03 for the rabbits and dog (Table 1). This fraction represents the percentage of the IPA eliminated per day. The actual quantity eliminated per day is given in the next column. In monkeys M-4, M-5, M-10, and M-11 the amount of IPA-like material excreted in the urine was determined (see Table 2) and the fraction of the circulating

Table 1

THE METABOLISM OF IPA IN THE MONKEY, RABBIT, AND DOG

Animal Species and No.	Body Wt (kg) At Time of Injection of IPA	Body Wt (kg) End of Observation Period	Amount of IPA Injected (mg/kg)	IPA Survival Half-Time (Days) Method A	IPA Survival Half-Time (Days) Method B	Fraction of Total IPA Injected Eliminated/Day ($Day^{-1} \times 10^2$)	Calculated Quantity of IPA Eliminated/Day (μg/day)	Fraction of Circulating Serum IPA Excreted in the Urine ($Day^{-1} \times 10^2$)*	Maximum Quantity of IPA** that Could be Excreted In the Urine (μg/day)
Monkey									
M-1	5.67	6.41	1.32	82	86	.825	62	-	-
M-2	5.10	5.67	18 ***	84	82	.835	-	-	-
M-7	6.01	7.48	25	83	82	.840	1260	-	-
M-4	5.83	5.90	8.57	71	67	1.00	500	1.04 ± .30	216
M-5	5.19	5.78	9.65	67	64	1.06	530	1.4 ± .40	254
M-10	5.1	7.20	9.80	53	54	1.30	650	.59 ± .24	92
M-11	6.25	7.23	8.00	75	76	.918	460	1.4 ± .70	200
Rabbit									
R-2	3.08	4.80	12.4	33	32	2.30	875	-	-
R-3	4.40	-	2.0	24	23	2.95	260	-	-
R-4	3.95	4.4	2.0	25	21	3.01	238	-	-
Dog	28.85	-	2.06	29	27	2.48	1490	-	-

* Mean ± SD

** IPA-like material

*** This value is only approximate due to difficulties encountered during the iv injection of IPA.

Table 2

URINARY EXCRETION OF IPA-LIKE MATERIAL DURING A 73-DAY PERIOD FOLLOWING THE INJECTION OF IPA IN MONKEYS

Time After Injection (days)	μg IPA-Like Material/24-Hr Urine Specimen				Urinary IPA/24-Hr (% Total IPA in Serum on The Same Day)			
	M-4	M-5	M-10	M-11	M-4	M-5	M-10	M-11
2	224	299	-	325	1.1	1.7	-	2.3
3	280	334	73	-	1.4	1.9	.49	-
4	-	345	180	426	-	2.0	1.2	3.4
5	277	164	-	296	1.4	.92	-	2.1
6	276	214	130	272	1.4	1.3	.89	2.0
7	290	-	94	274	1.5	-	.66	2.0
8	142	229	-	240	.75	1.4	-	1.8
13	195	150	72	203	1.1	.96	.54	1.6
18	-	230	85	173	-	1.6	.69	1.4
21	231	-	-	244	1.4	-	-	2.1
23	-	219	-	284	-	1.5	-	2.4
28	81	292	86	222	.52	2.3	.78	2.0
33	149	113	35	155	.99	.85	.34	1.4
35	145	-	-	140	1.0	-	-	1.4
38	-	148	47	-	-	1.2	.78	-
41	129	-	-	110	.97	-	-	1.1
43	-	166	44	98	-	1.5	.49	1.0
48	109	152	44	65	.85	1.4	.52	.70
53	119	122	39	76	.98	1.2	.50	.86
58	119	154	38	55	1.0	1.6	.51	.65
61	-	-	-	64	-	-	-	.78
63	89	101	22	42	.83	1.1	.32	.52
68	54	88	29	24	.52	1.0	.45	.31
71	-	-	-	83	-	-	-	1.1
73	-	114	18	-	-	1.4	.30	-

serum IPA excreted per day was computed. It is designated IPA-like material (see Section III, "Assays of IPA in Urine") since it was not isolated and identified. From the fraction excreted in the urine and the total amount of IPA in the intravascular volume (calculated by extrapolation of the linear portion of the curve to zero-time and the total serum volume) the maximum amount of IPA that could be excreted in the urine was computed and found to be 216, 254, 92, and 200 μg/day for monkeys M-4, M-5, M-10, and M-11, respectively.

Except for monkey no. 10, the difference between the calculated quantity of IPA eliminated per day and the maximum quantity of IPA excreted in the urine was in the order of 260 to 286 μg/day. It is possible that this quantity of IPA could be excreted in the stool, since a certain amount of serum albumin is lost normally into the gastrointestinal tract (15,16). The low amount of IPA excreted in the urine of monkey M-10 could not be explained unless the animal had some sort of gastroenteropathy.

The urinary excretion of IPA-like material for monkeys M-4, M-5, M-10, and M-11 is presented in Table 2. The percentage of the total IPA in serum on the same day (see methods for calculation) excreted in the urine more or less is constant for each animal. The percentage values for monkeys M-4, M-5, M-10, and M-11 were 1.04 ± .30; 1.4 ± .40; .59 ± .24; and 1.4 ± .70, respectively. The standard deviation of the mean was quite large for each animal, possibly due to incomplete 24-hr collection of the urine. The determination of urinary creatinine could have helped to smooth out this variation but unfortunately it was not done.

C. Extraction, Identification, and Electrophoresis of Serum IPA

The isolation and identification of IPA from the blood of monkeys seemed to be appropriate although Astwood had previously isolated and characteristized the compound from human subjects (1).

The extracted material from the pooled serum of monkeys M-2, M-4, and M-11 was subjected to thin layer chromatography using two solvent systems; it was identical to the reference marker IPA (Fig. 14). In

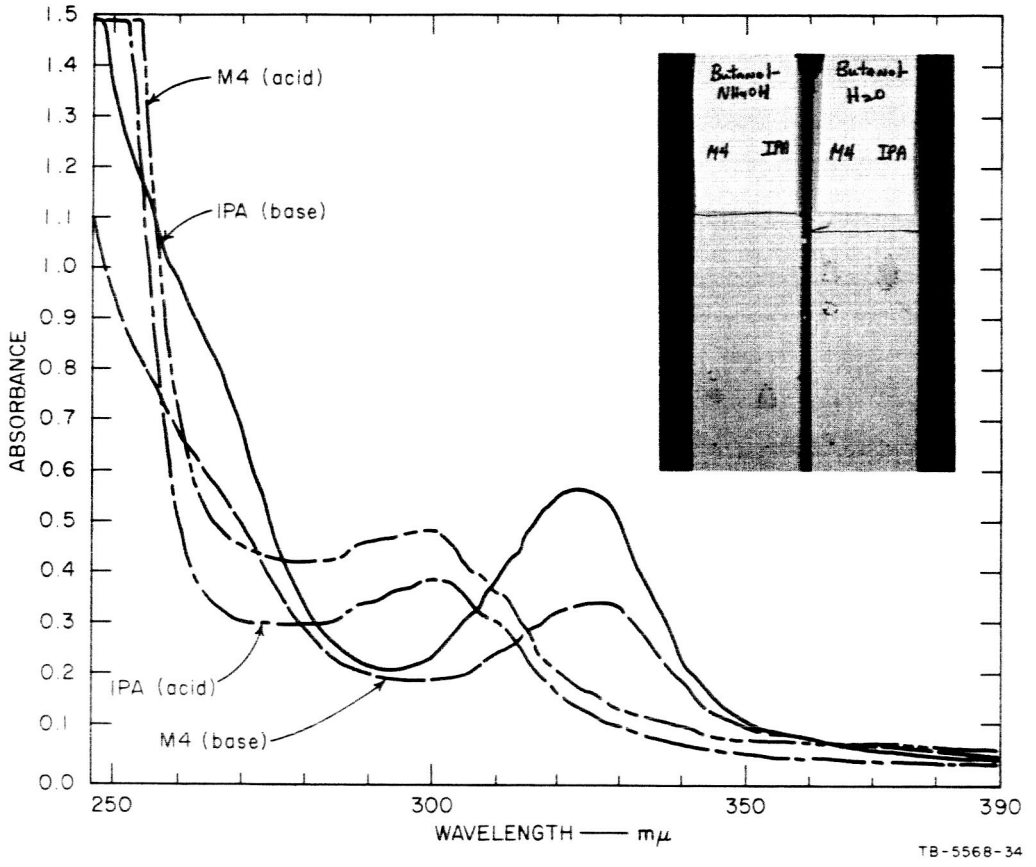

FIG. 14 ULTRAVIOLET SPECTRUM AND CHROMATOGRAM OF EXTRACTED IPA FROM THE SERUM OF MONKEY NO. 4 INJECTED WITH IPA

addition, the compound was eluted from the silica gel plate and found to contain iodine (by adding an aliquot of the eluate to ceric-arsenious mixture). It produced in acidic and basic solutions UV spectra which were identical to those for the reference standard IPA. The spectra of IPA have been well described by Astwood (1).

Pooled sera obtained from monkey M-7 after the injection of IPA and control serum to which IPA was added in vitro were also separately subjected to chloroform extraction after a preliminary butanol extraction of serum. Thin layer chromatography produced findings similar to those noted above. It can safely be stated that IPA does not seem to be appreciably metabolized in monkeys. However, the final proof should await the isolation and identification of this compound from urine.

It has also been found that IPA in serum exists preferentially in association with the albumin fraction.

A serum sample from monkey M-5, obtained 41 days after IPA injection, was subjected to electrophoresis on cellulose acetate and polyacrylamide gel. In the Gelman electrophoresis system one strip was stained for proteins and another was stained with a mixture of ceric and arsenious acids. A white band against a yellow background which fades with time appeared in the albumin fraction, indicating the presence of an iodine-containing compound in this area. A control serum sample (before injection of IPA) from the same monkey did not show this. The association of IPA to albumin did not seem to influence the electrophoretic mobility of this protein.

Free IPA solution (50 μg/ml) was applied at the origin of another strip, and was found to move toward the anode faster than the albumin-IPA complex. The same serum sample was subjected to electrophoresis using polyacrylamide gel as the supporting medium. After electrophoresis, part of the gel was stained for protein pattern and in another portion the various proteins were eluted and assayed for IPA. Only the fractions corresponding to the albumin region contained IPA. In addition to

electrophoresis, it was observed that about 80% of the serum IPA was associated with the albumin fraction, which was isolated by the TCA/EtOH fractionation method (11).

D. C^{14} Studies

To study the influence of IPA on the metabolism of serum albumin, four monkeys (two preinjected with IPA) were injected with C^{14}-reconstituted protein hydrolysate. Figures 15 and 16 show typical semilogarithmic plots of specific radioactivity of albumin over 50 to 65 days. Maximum incorporation of the isotope into serum proteins was observed 4 hr after the injection of the C^{14} amino acid mixture. The curves are biphasic. The initial fall in specific activity (1 to 10 days) was attributed to the distribution of labeled albumin within the total exchangeable intra- and extravascular albumin pool, as well as to degradation. Thereafter, the linear experimental decay curve resulted from progressive dilution of labeled albumin by unlabeled molecules. The slope is now due only to the degradation of the labeled albumin. Similar plots were made for total serum protein and the globulins and are shown in Figs. 17 to 20. In each case the biological half-life of serum protein, albumin, and globulins was obtained graphically from a linear semilogarithmic plot which resulted after the first ten days. Table 3 shows the half-lifes of serum proteins for monkeys M-4 and M-11, M-6 and M-8. There is no apparent difference between the albumin half-life of monkeys M-4 and M-11 (preinjected with 50 mg IPA) and monkeys M-6 and M-8 (controls). It may be concluded, therefore, that IPA does not directly or indirectly alter the degradation of albumin.

The daily urinary excretion of C^{14} was determined and is shown in Figs. 21 and 22. After the first seven days the pattern of excretion was similar for all four animals. This is best demonstrated by the values obtained for the fraction of circulating protein degraded per day as shown in Table 3. This constant relationship, when urinary activities were expressed as fraction of serum activities for the same day, persisted throughout the observation period and after the first seven days. In addition, the albumin turnover in these animals was calculated

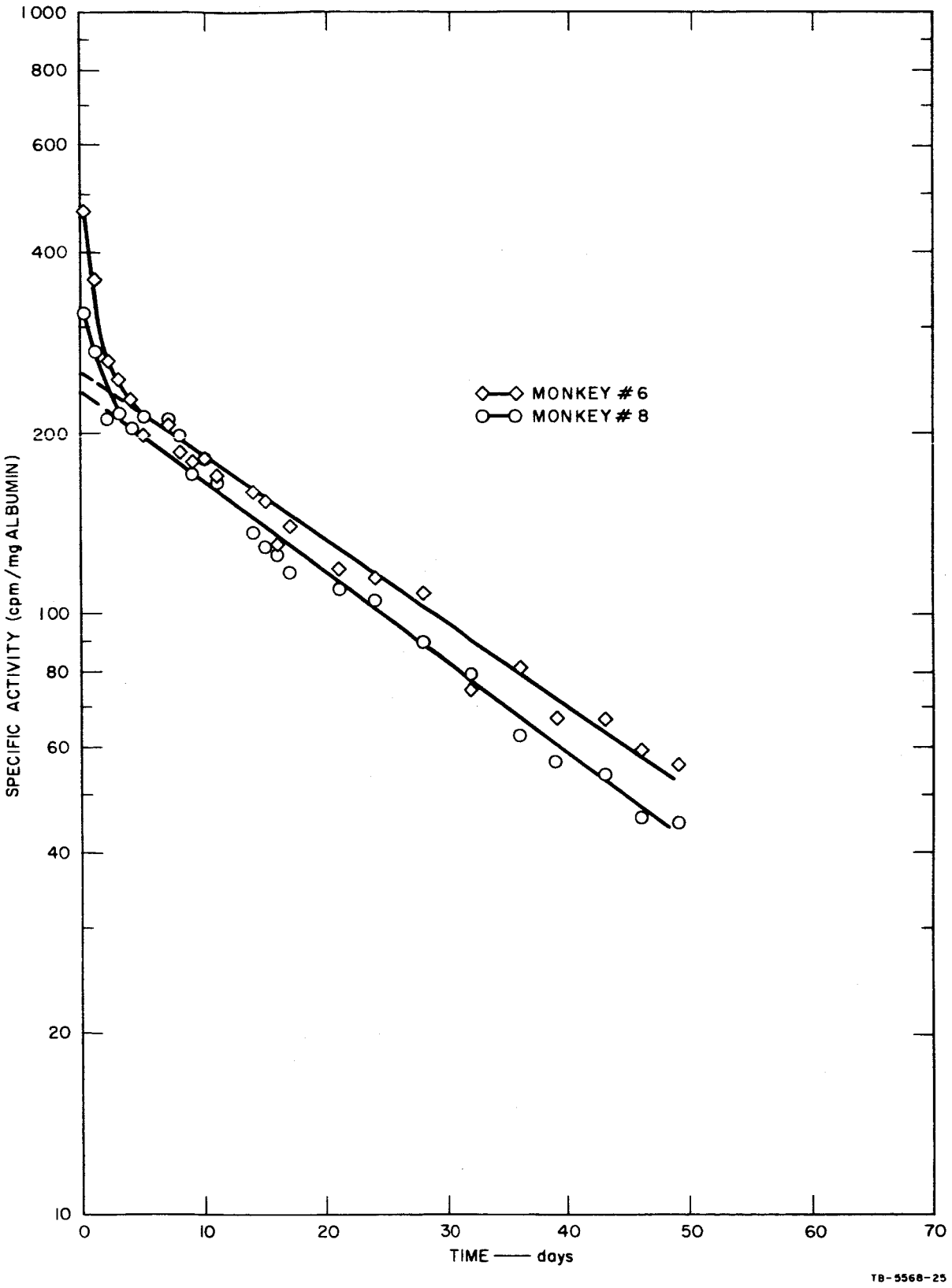

FIG. 15 SPECIFIC ACTIVITY OF SERUM ALBUMIN IN RELATION TO TIME
Animals injected with C^{14} — reconstituted protein hydrolysate

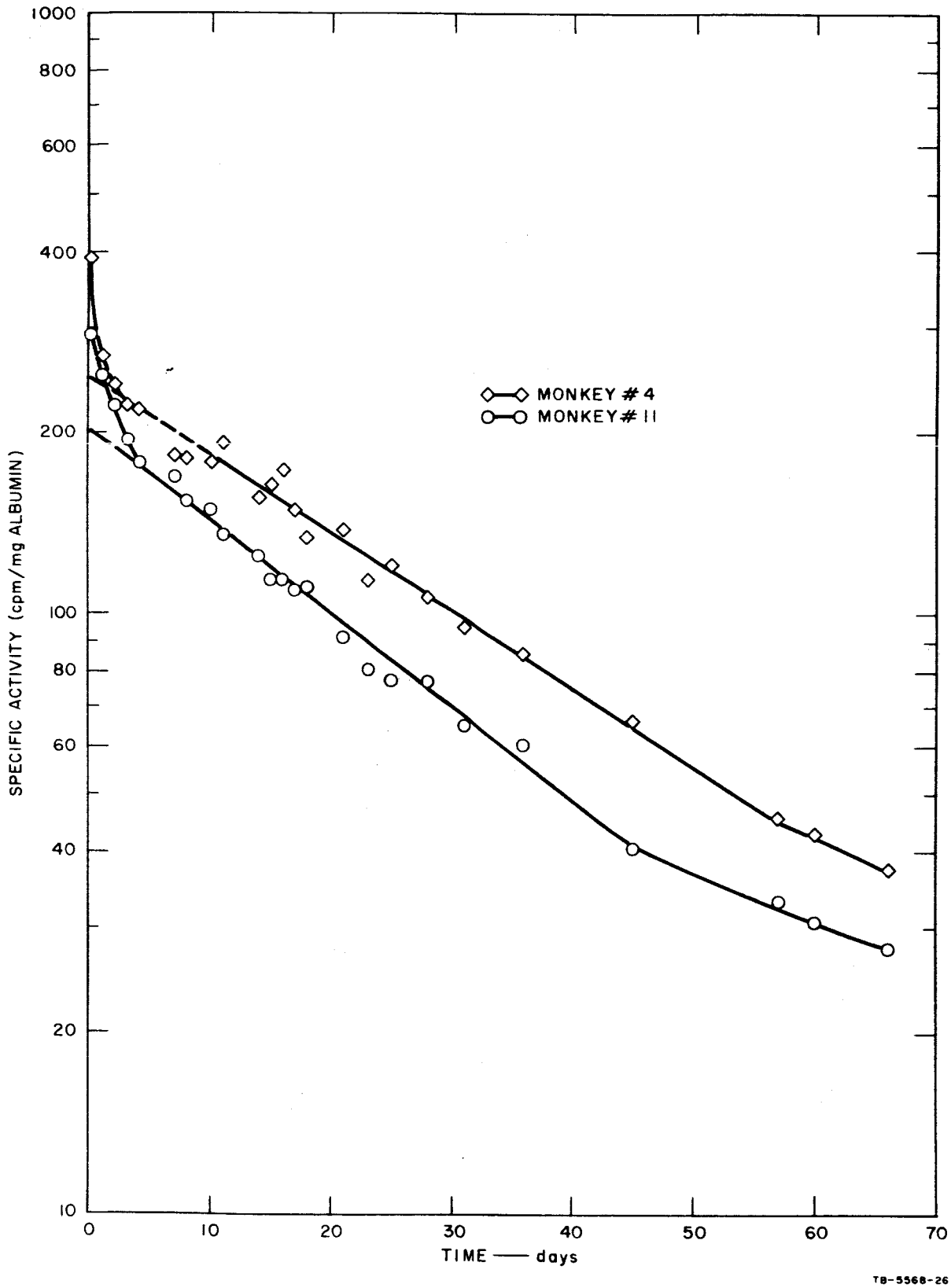

FIG. 16 SPECIFIC ACTIVITY OF SERUM ALBUMIN IN RELATION TO TIME
Animals preinjected with 50 mg IPA, 13 days before injection with C^{14}

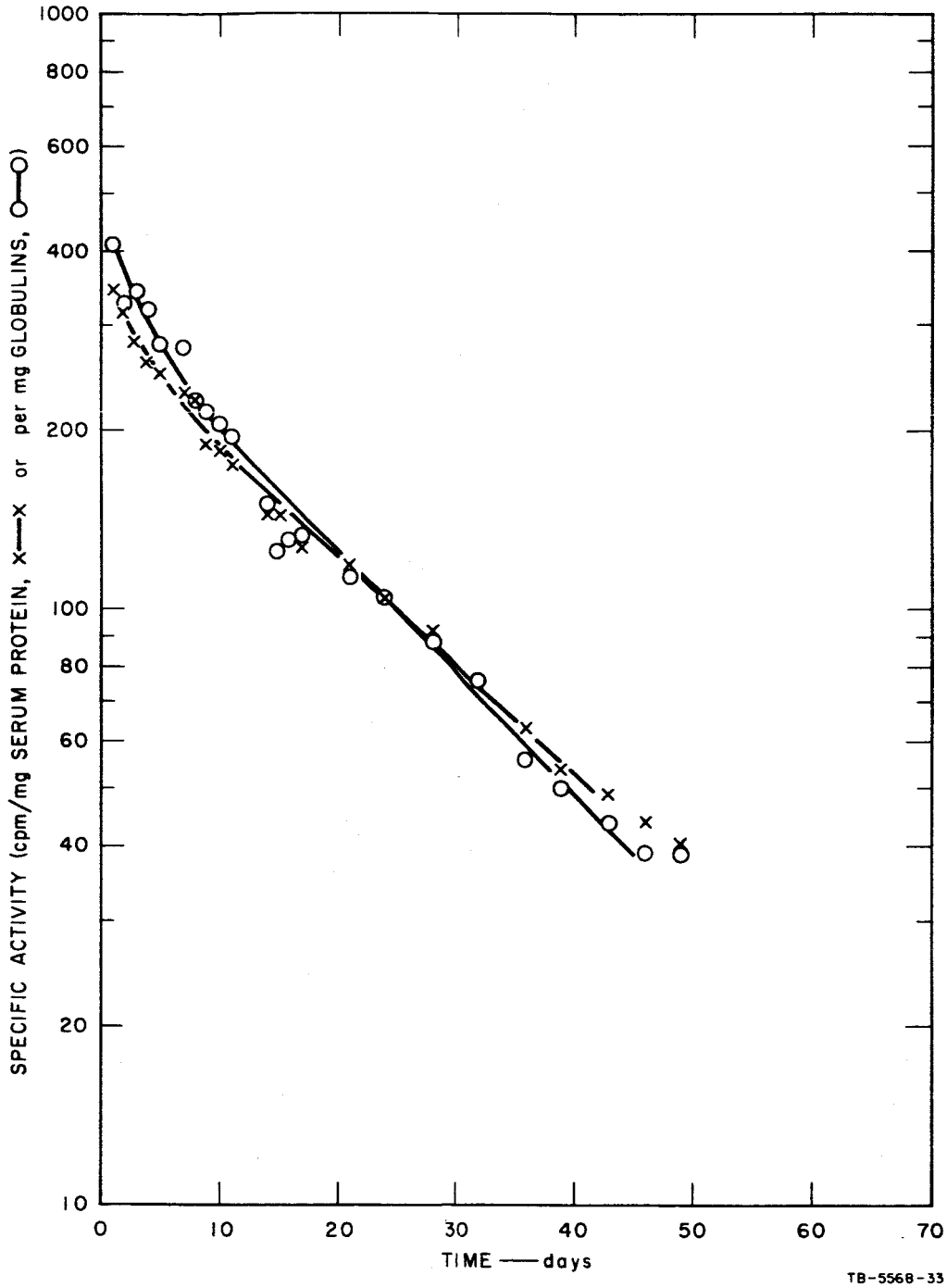

FIG. 17 SPECIFIC RADIOACTIVITY OF SERUM PROTEINS WITH TIME AFTER THE INTRAVENOUS INJECTION OF MONKEY NO. 8 WITH C^{14}-PROTEIN HYDROLYSATE

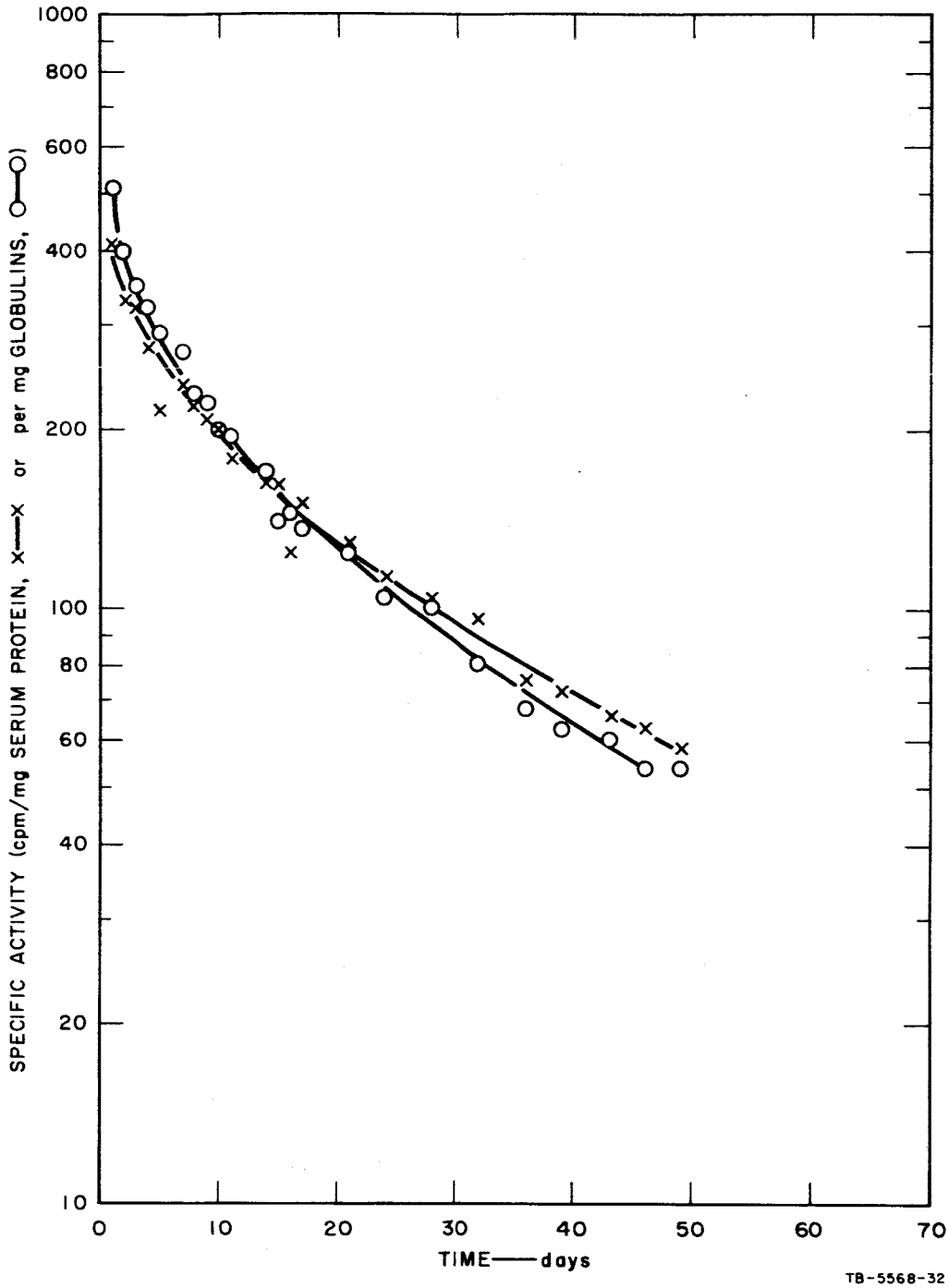

FIG. 18 SPECIFIC RADIOACTIVITY OF SERUM PROTEINS WITH TIME AFTER THE INTRAVENOUS INJECTION OF MONKEY NO. 6 WITH C^{14}– PROTEIN HYDROLYSATE

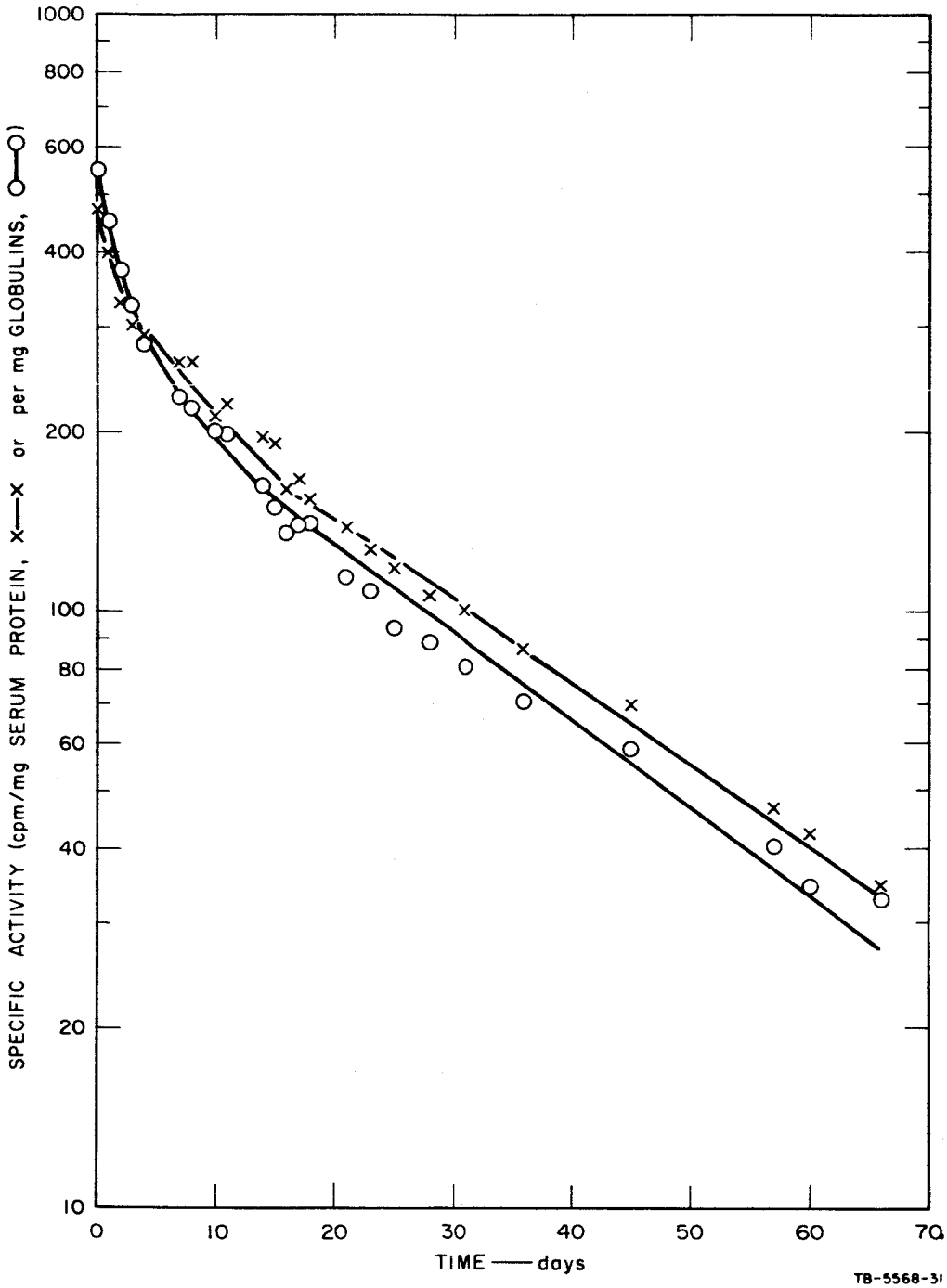

FIG. 19 SPECIFIC RADIOACTIVITY OF SERUM PROTEINS WITH TIME AFTER THE INTRAVENOUS INJECTION OF MONKEY NO. 4 WITH C^{14}–PROTEIN HYDROLYSATE

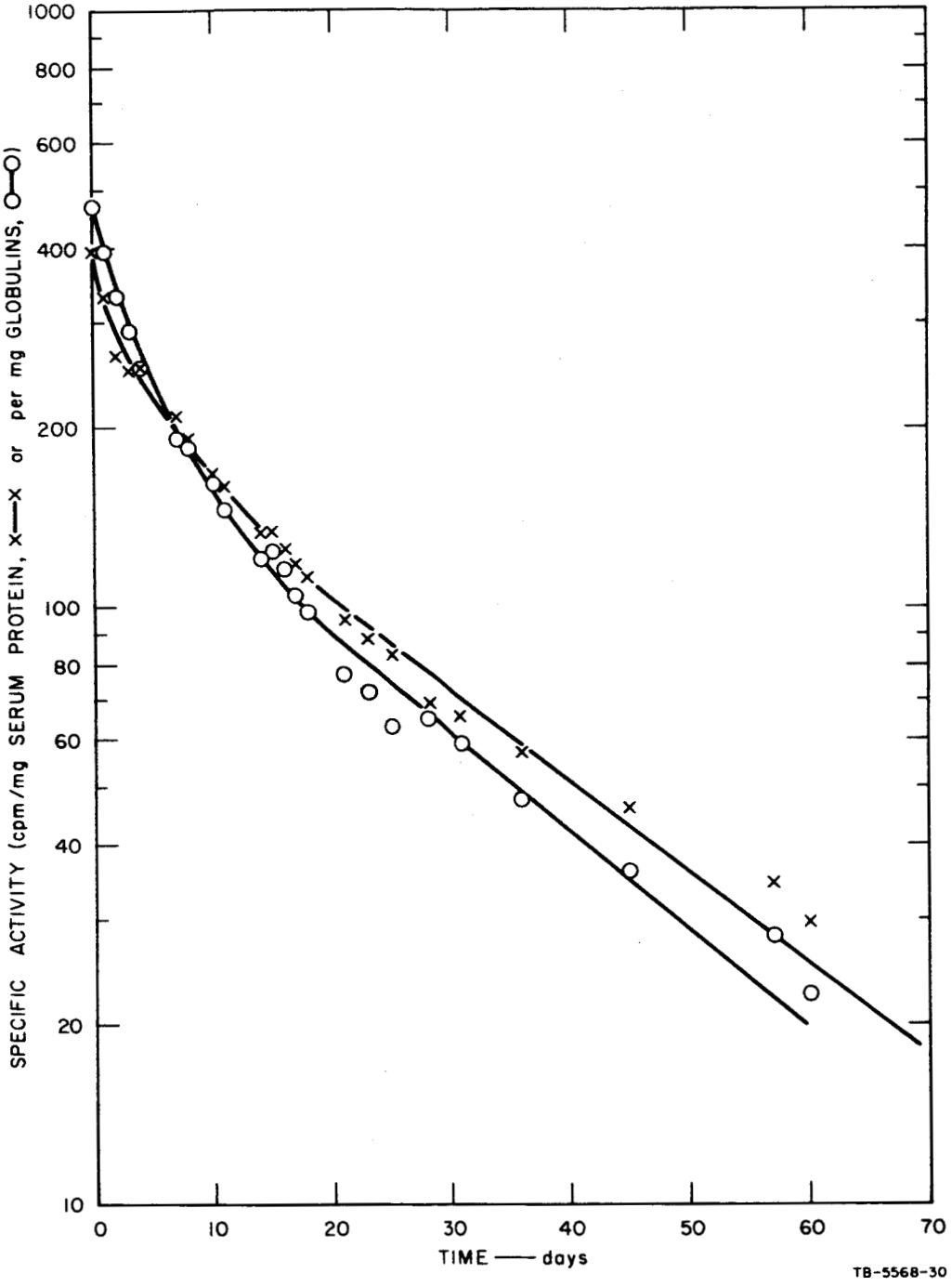

FIG. 20 SPECIFIC RADIOACTIVITY OF SERUM PROTEINS WITH TIME AFTER THE INTRAVENOUS INJECTION OF MONKEY NO. 11 WITH C^{14}-PROTEIN HYDROLYSATE

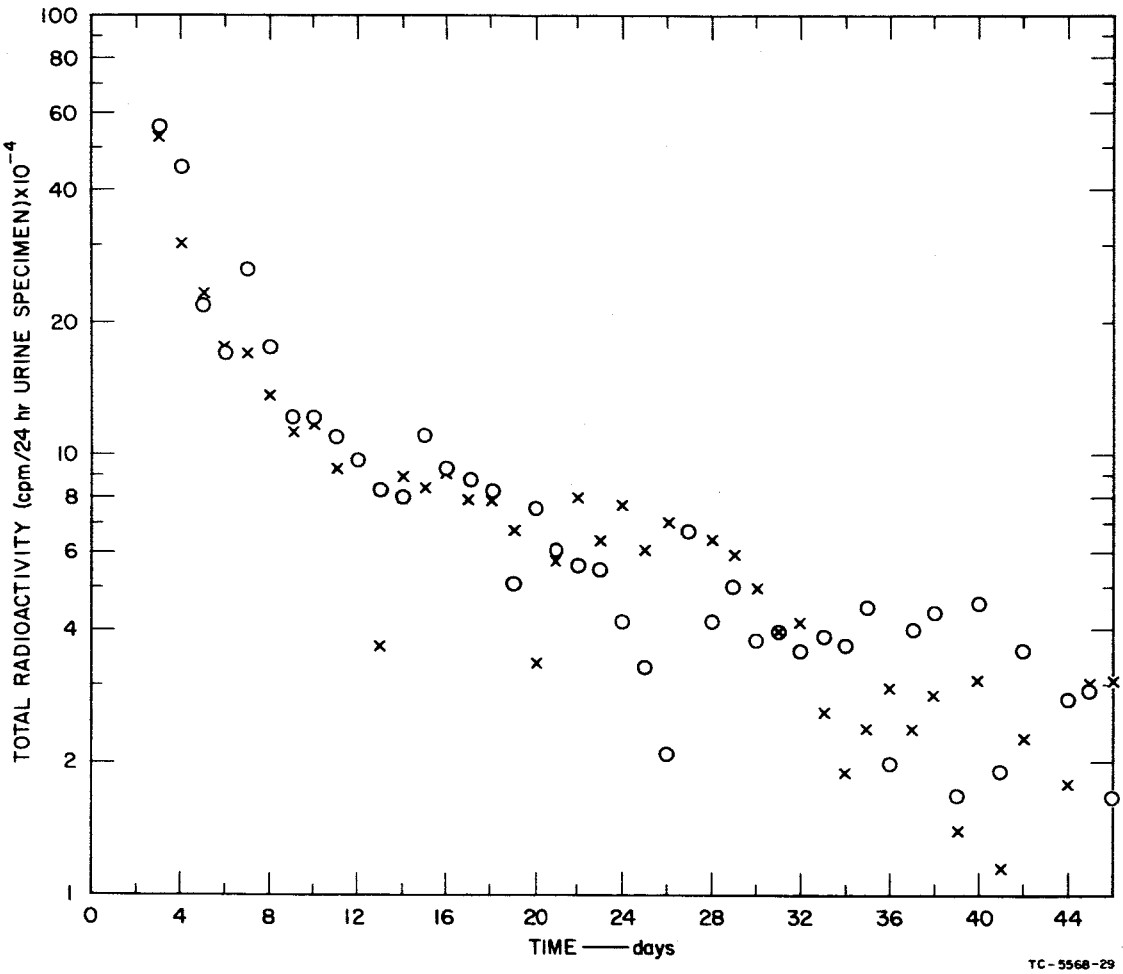

FIG. 21 URINARY C^{14}-RADIOACTIVITY CURVES FOR MONKEYS NO. 6 (x—x) AND NO. 8 (o—o)

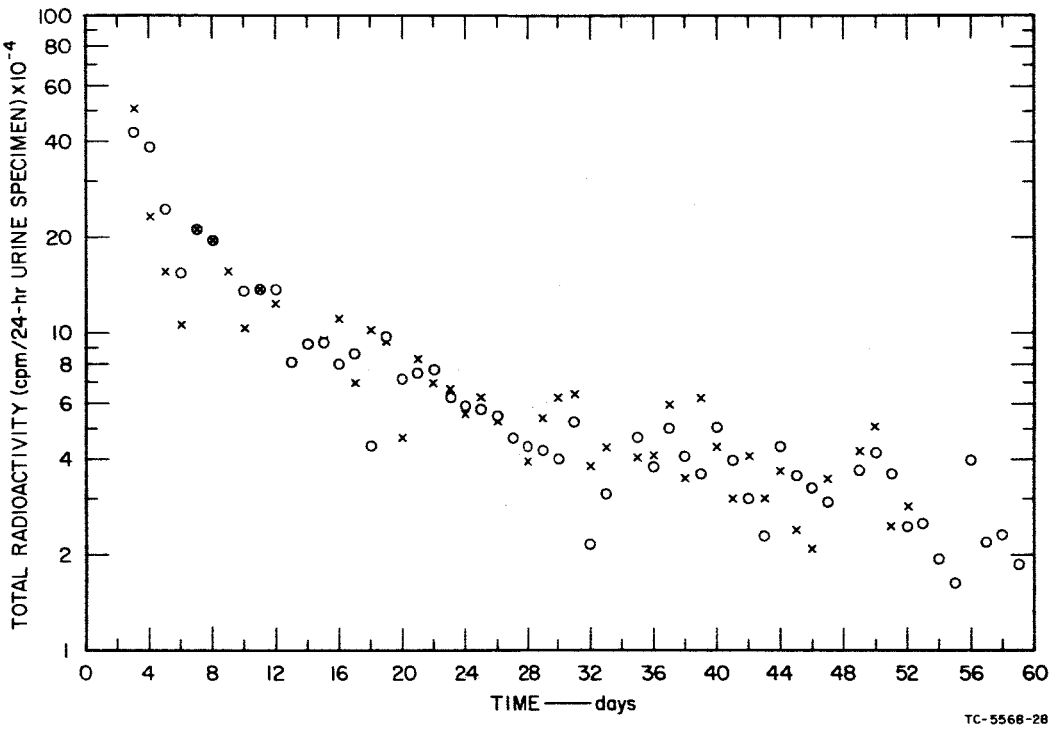

FIG. 22 URINARY C^{14}–RADIOACTIVITY CURVES FOR MONKEYS NO. 4 (x—x) AND NO. 11 (o—o)

Table 3

THE METABOLISM OF SERUM PROTEINS IN MONKEYS PREINJECTED WITH IPA PRIOR TO C^{14}-PROTEIN HYDROLYZATE INJECTION

	Preinjected With 50 mg IPA		Controls	
	M-4	M-11	M-6	M-8
Apparent Biological Half-Life of Serum Proteins (days)				
Total Protein	21.5	19.5	21.5	18
Albumin Fraction	23	20	22	20
Globulin Fraction	20.5	18.7	19	15
Fraction of Body Proteins Degraded ($day^{-1} \times 10^2$)				
Total Protein	3.22	3.55	3.22	3.85
Albumin	3.01	3.46	3.15	3.46
Globulins	3.38	3.71	3.65	4.62
Albumin Turnover Rate (g/kg/day)	.154	.184	.157*	.173*
Fraction of Circulating Protein Degraded ($day^1 \times 10^2$)	2.55 ± .72	3.88 ± 1.0	2.40 ± .89**	2.83 ± .92**

* Total body albumin not known but assumed to be 5 g/kg.

** The serum volume for the calculation of this parameter was assumed to be 40 ml/kg.

at .154 to .184 g/kg/day. It can be seen that there is no difference between monkeys M-4 and M-11 (preinjected with IPA) and M-6 and M-8 (controls).

E. Physiological Measurements Using IPA as an Indicator

Since IPA is preferentially bound to albumin when injected into monkeys, does not seem to influence the metabolism of albumin and is apparently equilibrated within the albumin compartments, it was felt that this compound could be used as an indicator for measuring intravascular volume and total body albumin pool. Such measurements were computed from the plots of Figs. 4 to 13, as described. The results are summarized in Table 4. The intravascular serum volume of the monkeys ranged from 33 to 44 ml/kg except for monkey M-1, which produced a value of 29 ml/kg. These volumes are comparable to 36.4 ± 3.98 ml/kg reported by Gregersen et al. (17) and 31 to 63 ml/kg (average 44.7) given in reference (18). The intravascular volumes found for the rabbits and dog are also comparable to the ones reported in the literature (18-19). The total circulating or intravascular albumin was calculated by two different methods; both seemed to be in close agreement. The values obtained for the total body and intravascular albumin pool of monkeys and dog seem to be comparable to the total albumin reported for human subjects (13,16), except for monkey M-7, in which the total body albumin may have been overestimated due to the high dose of IPA (150 mg). In rabbits the intravascular albumin mass seems to fall within the range reported by S. Cohen et al. (20), but the total body albumin is overestimated.

F. Effect of IPA on the Growth of Pseudomonas saccharophilia

P. saccharophilia was grown at 30^{o} for 6 hr in a medium containing 0.033 M, pH 6.8 phosphate buffer, 0.2% sucrose, and some inorganic ions. Under these conditions, the culture showed an increase in protein from 0.25 mg/ml to 0.932 mg/ml in medium.

IPA added to the growth medium inhibited the growth of the organism. The concentration of IPA required for 50% inhibition of growth was between 3.5×10^{-5} M and 7.0×10^{-5} M.

Table 4

APPARENT PHYSIOLOGICAL MEASUREMENTS IN EXPERIMENTAL ANIMALS USING IPA AS AN INDICATOR*

Animal Species and No.	Serum Albumin g/100 ml (Average Value)	Intravascular Serum Volume (ml/kg)	Total Circulating Serum Albumin (Intravascular) (g/kg)		Total Body Albumin Pool (g/kg)
			Method I	Method II	
Monkey (*M. mulatta*)					
M-1	4.70	28.8	1.44	1.35	4.14
M-7	4.45	40.6	1.99	1.80	6.60
M-4	4.45	42.4	1.85	1.88	5.13
M-5	4.40	33.3	1.48	1.46	4.30
M-10	3.26	43.5	1.46	1.42	4.65
M-11	4.40	38.1	1.55	1.68	5.33
Rabbit (New Zealand White)					
R-2	5.20	37.4	1.82	1.94	14.2
R-3	5.00	29.8	1.50	1.49	10.5
R-4	5.20	31.6	1.67	1.64	8.85
Dog	4.30	42.3	1.54	1.83	5.20

* See Table 1 for amount of IPA injected.

To examine the extent and nature of binding of IPA to bacterial protein, the cells were lysed and the soluble protein was precipitated by adding ammonium sulfate to saturation. The precipitated protein was dialyzed against water overnight. The amount of IPA bound to the bacterial protein varied from 0.14 μg to 1.45 μg per mg of dialyzed protein. IPA, when tested at a final concentration of 7×10^{-4} M, showed no inhibitory action against sucrose phosphorylase activity of a crude bacterial extract.

V DISCUSSION

The results obtained from the equilibrium dialysis studies of the binding of IPA with human and bovine albumin were analyzed by means of the Scatchard equation and found to be nonlinear. If all binding sites were equivalent and independent, a plot of $\bar{V}/A$ as a function of $\bar{V}$ should produce a linear relationship. Since deviations from linearity occur when binding takes place at more than one set of sites with different association constants (10,14,21), the deviation could be due to the existence of more than one type binding of IPA with albumin. By extrapolation of the linear portion of the curves ($\bar{V} < 2$), an approximate apparent association constant of $\geq 8 \times 10^{6}\ M^{-1}$ was obtained indicating a firm binding of IPA with albumin. An association constant of this magnitude represents a firmer binding than that of many small molecules known to be bound to albumin (9-11), but slightly less firm binding than the interaction of human albumin with the free fatty acids of serum (22).

The half-life of IPA in the monkey, rabbit, and dog was found to be 65-84, 23-32, and 28 days, respectively. Such differences among the species could be due to (a) firmer binding of IPA to the monkey serum albumin, (b) lack of specific albumin subfraction with a high affinity to IPA in rabbit and dog serum proteins, or an insufficient quantity of this particular fraction, and (c) metabolism of IPA by the dog and rabbit, forming products with less affinity for the albumin. In an experiment in which monkey, rabbit, dog, and human albumin preparations were equilibrated with .25 and .5 mole IPA per mole, albumin failed to reveal any significant difference with respect to the moles of IPA that were bound to one mole of albumin. However, it is felt that there may be a difference in the actual association constant of IPA with albumin for different animal species that could account for the longer half-life of IPA in the monkey and the very long half-life in humans. Astwood has reported a half-life of IPA of 2.5 years in humans (1) and Anton reported a half-life of 7 days for the guinea pig and dog (23). The association constant of this compound with albumin could best be determined by using I^{131} or C^{14}-labeled IPA.

It has been reported that serum albumin is heterogeneous (24 and 25) and the possibility that IPA could be bound to a specific albumin subfraction could not be excluded. From the results presented here one could assume that IPA is not metabolized by the monkey, although identification of the urinary IPA-like material would be appropriate, while for the dog and rabbit this assumption may not hold. In the three out of four monkeys the amount of IPA excreted in the urine per day, expressed as percentage of the circulating serum IPA, ranged from 1-1.4, which corresponded to about 200-250 μg/day--the maximum quantity of IPA that could be excreted in the urine. From the half-life and the total amount of IPA given to these animals $\left(\frac{.693}{t_{\frac{1}{2}}} \times 50 \text{ mg IPA}\right)$ it was calculated that the quantity of IPA eliminated per day was approximately 500 μg. The difference between this quantity and that found in the urine is about 250 μg, which is eliminated daily but not in the urine. This amount is either metabolized, excreted in the gastrointestinal tract along with albumin, stored in certain tissues, or lost during the urine collection period by absorption in the feces. The last possibility can be excluded, since all three animals gave consistent excretion rates for IPA. It has been reported by Laster et al. that with human subjects given I^{131}-albumin, the amount of I^{131} found in stool is about 0-0.4% of body's albumin-I^{131} content (16). It is possible therefore that the unaccounted IPA may follow the albumin leaking into the gut. If 100 mg of albumin leaks into the gastrointestinal tract of the monkey per day, this amount may be equivalent to 150-200 μg IPA lost per day since IPA is bound to albumin at a ratio of 1.5 to 2 μg/mg as determined from the plots extrapolated to zero time.

Since IPA was found to be preferentially bound to the serum albumin fraction, eliminated slowly from the circulation, identical to the original compound when injected into monkeys, and without an apparent effect on albumin metabolism, it was felt that such a compound could be used to measure plasma volume as well as intra- and extravascular albumin. Calculations of these physiological parameters are presented in Table 3. They were based on the following assumptions: (1) IPA attains an equilibrium between the intra- and extravascular compartments, (2) the

albumin-IPA complex is proportionally and evenly distributed between the albumin compartments, (3) IPA does not interfere with the sites at which albumin synthesis and degradation occur, and (4) IPA is evenly bound and distributed within the serum albumin fractions. The values obtained for intravascular volume and total and intravascular albumin mass fall within the range reported by other investigators using Evans blue dye and C^{14}- and I^{131}-labeled albumin (16-20). These values are not unequivocal, and require further confirmation by comparison with other indicators. The values obtained for the total albumin mass in rabbits seem to be overestimated, probably because of a considerable loss of albumin into the gastrointestinal tract (26).

VI ACKNOWLEDGMENTS

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