THE EFFECT OF THE SURFACE ACTIVE AGENT PLURONIC F-68 ON RED BLOOD CELL HEMOLYSIS IN VITRO AND DURING CARDIOPULMONARY BYPASS

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ABSTRACT

THE EFFECT OF THE SURFACE ACTIVE AGENT PLURONIC F-68 ON RED BLOOD CELL HEMOLYSIS *IN VITRO* AND DURING CARDIOPULMONARY BYPASS

By

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Pluronic F-68 is a nonionic polyol surfactant which greatly increases the resistance of red blood cells in buffered saline to lysis by mechanical trauma. Further studies revealed a similar effect on red blood cells in plasma. There was no change in the osmotic fragility of red blood cells treated with Pluronic F-68 *in vitro* or *in vivo*. However, an increase in osmotic resistance was observed to occur during the bypass period, irrespective of the use of Pluronic F-68. This was presumed to occur as the result of lysis of less resistant cells in the extracorporeal pump-oxygenator. *In vitro* studies indicated that the inhibition of hemolysis by Pluronic F-68 was dose related. Although the value of Pluronic F-68 during cardiopulmonary bypass is still not conclusively demonstrated, it is most probable that the concentration of Pluronic F-68 (0.35 mg./ml. of blood) in experiments *in vivo* was too low in these animals to have exerted a significant effect.

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Dorothy Tao-Lien Yao

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In memory of my parents

Mr. and Mrs. Yao, Boen Ling

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INTRODUCTION

The maintenance of cardiopulmonary circulation by an artificial apparatus is essential to certain types of cardiovascular surgery. These include the replacement of heart valves and the implantation of artificial heart-assist devices.

Up to the present time there are still many problems associated with cardiopulmonary bypass. In previous clinical and experimental studies it has been demonstrated that blood perfusion systems cause destruction of a fraction of the red cells with the immediate appearance of free plasma hemoglobin. Other red cells, while escaping rapid destruction, may nonetheless sustain a significant degree of injury resulting in shortened cell life span and premature senescence. As a consequence, an extracorporeal perfusion system may result in anemia continuing for days or weeks after an operation. This condition has been characterized by reduction of the packed cell volume and total blood hemoglobin and an increased reticulocyte count. Morphologic changes of the red blood cells has been observed, including anisocytosis, spherocytosis and fragmentation associated with increased mechanical fragility of the red blood cells. In addition, high plasma hemoglobin levels have been associated with impairment of renal function (Brinsfield *et al.*, 1962; Galletti *et al.*, 1965; Kusserow *et al.*, 1965, 1966).

Pluronic F-68 has been used previously to stabilize fat emulsions for intravenous use, to prevent fat embolism and to diminish hemolysis during cardiopulmonary bypass. This investigation was designed (a) to

determine the effectiveness of Pluronic F-68 in reducing hemolysis consequent to experimental surgery in the dog involving the use of cardiopulmonary bypass devices, and (b) to investigate *in vitro* the possible mechanism by which Pluronic F-68 acts to stabilize erythrocytes.

LITERATURE REVIEW

The Pluronic polyols are ethylene oxide-polypropylene glycol condensation products. These comprise a series of closely related polymers with a unique molecular structure and with the general empirical formula: $H\,O\,(C\,H_2\,C\,H_2\,O)_a\,(C\,H\,C\,H_2\,O)_b\,(C\,H_2\,C\,H_2\,O)_c H$. ($CH_3$)

These are nonionic surfactant polyols. Because the hydrophobic-hydrophilic ratio of the Pluronic polyols can be varied by small, controllable amounts, the resulting graded structures provide a wide range of properties. This variability makes it possible to meet particular surface-active requirements (BASF Wyandotte Corporation, Publication, 1971a).

The Physical and Surface-Active Properties of the Pluronic Polyols

Molecular Weight. A unique property of the Pluronic polyols is their unusually high molecular weight compared to many common surface-active agents. The approximate molecular weight of Pluronic F-68 is 8,350.

Taste and Odor. Pluronic F-68 is unique among surface-active agents in being practically tasteless and odorless. Animal feeding tests show that Pluronic F-68 is readily accepted by animals when mixed with feed.

Chemical Stability. Due to the stable ether linkage in the Pluronic molecule, these materials are extremely stable to acids and are not precipitated by most metallic ions. They are also stable in alkaline solutions.

Hygroscopicity. Pluronic F-68 is relatively non-hygroscopic and is free-flowing even after being exposed to air for long periods of time. This is typical of solid Pluronic polyols.

Solubility. The Pluronic polyols are more soluble in cold water than in hot, owing to hydrogen-bonding of water with the many ether oxygen atoms. They are more soluble in dilute mineral acid than in water because of the formation of oxonium ions. Pluronic polyols can be dissolved in organic solvents, in which they are normally insoluble, by the use of a coupling agent.

Surface-Active Properties. Pluronic F-68 in solution has unusual surface-active properties. Its surface tension is lower than that of water combined with the best wetting agents. This characteristic holds over a wide temperature range and is unaffected by change in pH. Pluronic polyols exhibit a strong emulsifying effect and act as excellent defoamers.

Toxicologic Properties. Studies have been made of the acute toxicity of Pluronic F-68 in dogs, rats, mice, rabbits and guinea pigs, to which aqueous solutions were administered by stomach tube. The data indicate that Pluronic F-68 does not produce acute toxic effects. Additional tests, carried out to assess possible chronic oral toxic effects over a period of 2 years, showed no toxic manifestation (Carr, 1951, 1952).

Metabolic Reactions. *In vitro* and *in vivo* experiments with human beings, dogs and rats have shown no alteration in the molecular weight or structure of Pluronic F-68. The Pluronic F-68 was recovered unchanged in the urine; recovery was essentially quantitative within 24 hours after administration. When ingested orally, Pluronic F-68 was recovered

quantitatively from the feces, showing that the Pluronic F-68 is neither absorbed into the body nor is it metabolized (May, 1958). Pluronic F-68 had no effect on the absorption of the anionic dye, phenol red, from the colon of anesthetized rats (Meyer *et al.*, 1957).

Information accumulated since 1952 indicates that the toxicologic properties of these products also vary in a regular and predictable manner, according to the relative size of the polyoxyethylene and polyoxypropylene portions of the molecule and the total molecular weight. The properties of the Pluronic polyols suggest their use in several fields where low toxicity is necessary. These include cosmetics, pharmaceuticals, drugs and certain foods. The effect of grade F-68, one of the oldest and best known of the Pluronic series, has been studied in a number of medical applications (BASF Wyandotte Corporation, Publication, 1971b).

Experimental Use of Pluronic F-68

1. Intravenous infusion of fat emulsions.
2. Complications of cardiopulmonary bypass, including:
   a. Fat embolism
   b. Hemolysis
   c. Blood viscosity
   d. Platelet aggregation and adhesiveness
3. Splenic vascular perfusion.
4. Experimentally induced amniotic fluid embolism.
5. Cardiac assist devices.
6. Hemorrhagic shock.

Intravenous Infusion of Fat Emulsions. Many emulsifying agents have been utilized in the intravenous administration of fat. However, these agents have proved to be unsatisfactory. Soya phosphatides ("lecithin")

were introduced by Stare in 1943 as an emulsifying agent in place of egg phosphatide. Shortly thereafter it was found that the use of a small amount of a co-emulsifier permitted a reduction in the amount of phosphatide required. In 1955 Pluronic F-68 was selected as the co-emulsifier. It is a synthetic, nonionic, extremely water-soluble compound which enhances the physical stability of the emulsion (Meyer *et al.*, 1955; Waddell *et al.*, 1955).

In 1957, Waddell and his co-workers initiated the clinical use of a nonphosphatide (Pluronic F-68-alcohol) emulsion. Their results showed an unexpectedly high incidence of the immediate or "colloid" reaction which was manifested by clinical signs such as back pain, dyspnea, flushing, and abdominal pain. This "colloid" reaction can be eliminated by the infusion of a mixture of Pluronic F-68, alcohol and glycerol prior to the first fat infusion. A fat emulsion stabilized with Pluronic F-68 for intravenous use does not cause this reaction.

Walter *et al.* (1957), in studying the effect of intravenous administration of Pluronic F-68 in the dog, stated that this agent produced no change in the electrophoretic mobility of serum lipoproteins and no prolongation of clotting time of the blood. Experimentally, combined "Phosphatide-Pluronic" preparations given intravenously resulted in an increase in the speed of electrophoretic migration of the lipoproteins and prolongation of blood-clotting time. Since intravenous administration of Pluronic alone caused none of these changes, it was concluded that the soybean phosphatide was responsible for the observed changes.

A side effect frequently reported following daily infusions of intravenous fat emulsion is a significant drop in hemoglobin concentration. The severity of the anemia produced in this way has been correlated with the emulsifier system utilized. Hartwig (1961) carried

out an *in vitro* comparison of hemolysis following incubation of erythrocytes in experimental fat emulsions. Pluronic F-68 consistently produced less hemolysis than any of the other emulsifier systems.

Complication of Cardiopulmonary Bypass

Fat embolism. Pluronic F-68 has both a nonionic quality and excellent emulsifying properties and has been employed to stabilize parenteral fat emulsions. Adam *et al.* (1959) reported a study testing the effectiveness of Pluronic F-68 in preventing the formation of fat emboli during prolonged cardiopulmonary bypass. Their results indicated that fat emboli and lipuria can be largely prevented if a nonionic surface-active agent (Pluronic F-68) is employed. They also demonstrated that Pluronic F-68 has no effect either physiologically or on the function of the pump-oxygenator.

Wright *et al.* (1963) indicated that fat globulinemia which develops during cardiopulmonary bypass can be minimized by the addition of Pluronic F-68 to the circulation.

Hemolysis. O'Neill and Collins (1965) designed a group of experiments to evaluate the effects of various agents on hemolysis occurring *in vitro* during the use of the bypass apparatus. Pluronic F-68 in a concentration of 0.6 mg./ml. of blood was added to the prime solution. The total perfusion time was 2 hours. The results of these studies indicated that the effect of Pluronic F-68 on the level of plasma hemoglobin was inconsistent. A lower concentration of plasma hemoglobin was noted, but the difference was not statistically significant.

Miyauchi *et al.* (1966) and Hymes *et al.* (1968) reported experimental studies in which they compared the effect of various agents in the prime solutions with Pluronic F-68. All of the data indicated that the

administration of Pluronic F-68 after the end of persufion markedly improved capillary circulation and caused an immediate increase both in venous return and arterial pressure. The most significant difference between Pluronic and non-Pluronic groups was noted in plasma hemoglobin levels. Miyauchi also demonstrated that fat embolism and fibrin deposition on the filter of the oxygenator were greatly reduced by use of Pluronic F-68.

Wells *et al.* (1968) performed studies *in vitro* to evaluate the effects of polymethylsiloxane as a defoaming agent. They showed that it greatly reduced the resistance of red blood cells to lysis by mechanical trauma. The addition of the surfactant agent (Pluronic F-68) prevented the hemolytic process without interfering with the action of the anti-foam material. Similar studies of plasma showed the reduction of hemolytic effects of the Pluronic F-68 was to some extent mediated by the plasma.

In 1970 the effects of Pluronic F-68 were evaluated in 103 patients during cardiopulmonary bypass. The patients were divided into control and treatment groups. The final concentration of Pluronic in the pump prime was approximately 1 mg./ml. The results indicated that Pluronic F-68 significantly protected the red blood cells from hemolysis at all times during perfusion up to 90 minutes. After that time the effect diminished gradually. Serum potassium levels were significantly lower in the Pluronic group, possibly reflecting the decrease in hemolysis (Danielson *et al.*, 1970).

During the past few years, vitamin E and Pluronic F-68 have been employed in intracardiac surgery by Tamiya and his associates (1970), who noted their marked suppressive effects on hemolysis. They stated that vitamin E, when directly added to the pump perfusion, was

ineffective. However, daily intramuscular administration prior to surgery reduced the hemolysis significantly. Various doses of Pluronic F-68 were employed during perfusion in human subjects. These included concentrations of 0.6 mg./ml., 1.0 mg./ml. and 2.0 mg./ml. of the circulating blood volume. Hemolysis was reduced to about 80% of that observed in the control in the 0.6 mg./ml. group. It was reduced even further in the 1.0 mg./ml. and 2.0 mg./ml. groups. Postoperative laboratory studies generally revealed advantages in the Pluronic group, with the exception that higher blood urea nitrogen levels were observed in some members of the 2.0 mg./ml. group. Therefore, it was recommended that the level of Pluronic F-68 not exceed 1.0 mg./ml. of circulating blood volume during cardiopulmonary bypass.

Blood Viscosity. Increased blood viscosity during bypass reflects increased microaggregation, alteration in the physical properties of erythrocytes, increased fibrinogen, and hemoconcentration (Replogle *et al.*, 1967). Experimental studies by Grover *et al.* (1969) indicated that the intravenous injection of Pluronic F-68 prior to bypass lowered blood viscosity. Further reduction in viscosity occurred due to the hemodilution produced by the solution used to prime the bypass apparatus and persisted during the first hour of perfusion with no change in hematocrit values.

Platelet Aggregation and Adhesiveness. Platelet aggregation is an important causative factor in the development of microembolism which commonly occurs during extracorporeal circulation. Grover *et al.* (1969) gave Pluronic F-68 by slow intravenous infusion at the rate of 25 mg./kg./hour to open heart surgery patients. Platelet adhesiveness was reduced from 53% prior to intravenous administration to 15% ten minutes afterward.

Grover concluded, therefore, that Pluronic F-68 should decrease platelet aggregation during perfusion.

Tamiya *et al.* (1970) indicated that this agent is also effective in preventing a reduction in the platelet count during perfusion.

Splenic Vascular Perfusion. Pluronic F-68 has been shown to decrease vascular resistance in a series of 10 porcine spleens perfused with human blood. The reduction in vascular resistance during heterologous perfusion is comparable to that noted during homologous perfusion (Moore, 1968).

Experimentally Induced Amniotic Fluid Embolism. The effect of intra-arterial human amniotic fluid embolism on the mesenteric microcirculation of the rabbit has been studied. Amniotic cellular debris results in an aggregation of cellular material that may obstruct the microcirculation and cause a drop in blood pressure. Infusion of Pluronic F-68 prior to human amniotic fluid embolism prevented impairment of the microcirculation and shock (Hymes *et al.*, 1970).

Cardiac Assist Devices. Pluronic F-68, chemically coupled with heparin and epoxy, is one of the most promising anti-thrombic agents which has been developed. This compound has been used to coat surfaces in circulatory-assist devices. The mechanical characteristics of this compound show the advantages of extreme durability and compatibility with saline. The chemically incorporated heparin epoxy polymers of this material have been shown to be nonhemolytic, harmless to platelets and blood cells, to have no effect on plasma proteins, and to have only a small effect on plasma enzymes. Most significant was the fact that the heparin was not readily depleted from these polymers. Attempts at leaching it were ineffective (Salyer *et al.*, 1971; Gott *et al.*, 1971).

Hemorrhagic Shock. The treatment of hemorrhagic shock is directed toward restoration of the microcirculation. Pluronic F-68 has been demonstrated to increase the percentage of animals surviving experimentally induced shock syndromes. Restoration of renal function occurred in a significantly higher percentage of these animals than in the untreated control groups. It was reported in these studies that blood surface tension was markedly reduced in the animals treated with Pluronic F-68 (Hyme *et al.*, 1971).

MATERIALS AND METHODS

The experimental studies were divided into 2 categories: (1) evaluation of the effects of Pluronic F-68 on red blood cells *in vitro*, and (2) evaluation of the effects of Pluronic F-68 on red blood cells during cardiopulmonary bypass.

In vitro Studies

Mechanical Fragility of Red Cells. The mechanical fragility of the red cells was measured by an adaptation of the method of Adreason (1964) in which a dilute suspension of red cells is forced under selected conditions through the orifice of a chromatography spray.[1] As with other methods of measuring mechanical fragility, the precise forces leading to destruction of red cells are incompletely understood. Hemolysis is presumed to be the result of turbulent flow at the orifice. Mechanical resistance was evaluated by measuring the percentage of hemolysis which occurred following stress.

Source of Specimens. Fresh blood samples were obtained from 5 dogs in sterile heparinized syringes. Packed cell volumes were determined immediately using the microhematocrit method with centrifugation at approximately 10,000 g for 5 minutes in an International Micro-Capillary Centrifuge.[2] Ten percent suspensions in phosphate-buffered saline were

[1]Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.

[2]International Equipment Co., Needham Hts., Mass., U.S.A.

then made by appropriate dilution. All determinations were made at ambient room temperature, approximately 24 ± 2 C.

Experiment I. Four 1-ml. aliquots of each of the 10% cell suspensions were further diluted to 100 ml. with phosphate-buffered saline solution giving final dilution of 1 in 1000. Pluronic F-68 was added to each of these to yield final concentrations of: (a) 1.0 mg./ml. Pluronic F-68, (b) 3.0 mg./ml. Pluronic F-68, and (c) 5.0 mg./ml. Pluronic F-68. The red blood cells suspended in buffered saline alone served as a control.

The apparatus which was used to determine mechanical fragility is illustrated in Figure 1.

The air line tubing was connected both to a mercury manometer and to the spray tube. This spray tube, made of borosilicate glass, is permanently attached to a molded phenolic plastic screw cap which fits commercial narrow-mouth bottles.

A thumb-controlled air vent is located in the rear of the spray tube. This allows instantaneous control of the spray by variable closure of the vent. The spray tube projects a few millimeters inside the mouth of the separatory funnel which delivers its contents into a test tube.

To provide a measure of the relationship between traumatic hemolysis and air pressure, the latter was adjusted in various experiments to 100, 200, 300, 400 and 500 mm. Hg. The diluted red blood cell suspensions were well mixed before each spraying. Each sample was sprayed into the separatory funnel, and about 5 ml. were collected in the test tube. Triplicate samples were run, and the fraction of the cells lysed was determined from the measured hemoglobin liberated into the supernatant

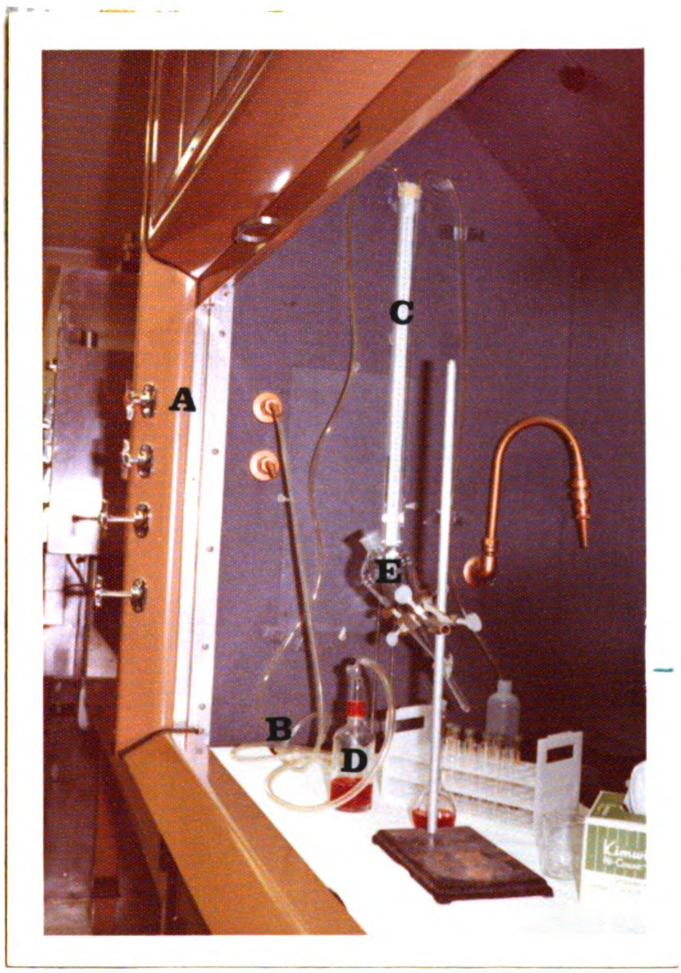

Figure 1. Apparatus for determination of mechanical fragility. (A) air valve; (B) air line tubing to compressed air; (C) mercury manometer; (D) spray apparatus; (E) separatory funnel.

obtained by centrifuging each sample at 2000 rpm for 7 minutes in the International Centrifuge.[1]

Damage produced by mechanical stress to the red blood cells was measured as follows:

(a) The supernatant from the unstressed red blood cell suspension served as a blank.

(b) The test sample was the supernatant from the air-sprayed suspension.

(c) 100% hemolysis was estimated by adding 3 drops of ammonia to a sample of the untreated initial cell suspension and centrifuging as above.

Aliquots of blank, sample, and total hemolysis samples were measured for absorbance at 417 nm. using a Beckman D.B. Spectrophotometer.[2]

Mechanical fragility was calculated according to Shen *et al.* (1944) as follows:

$$\text{Mechanical Fragility} = \frac{100(b-a)}{c-a}\ \%, \text{ where}$$

a = absorbance of blank, b = absorbance of sample, and c = absorbance of the totally hemolyzed sample.

Experiment II. Red blood cells were prepared in 3 ways for determination of mechanical fragility:

(a) Suspended in phosphate-buffered saline solution.

(b) Suspended in 5 mg./ml. of Pluronic F-68 with phosphate-buffered saline solution.

(c) Suspended in 5 mg./ml. of Pluronic F-68 for 3 hours at which time they were washed twice in isotonic saline and resuspended in phosphate-buffered saline.

The red blood cell suspensions were sprayed using an air pressure of 100 mm. Hg.

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[1] International Equipment Co., Needham Hts., Mass., U.S.A.

[2] Beckman Instrument Co., Palo Alto, Calif., U.S.A.

Experiment III. Red blood cells were suspended in plasma for determination of mechanical fragility as follows:

(a) Suspended in plasma.

(b) Suspended in plasma containing 1.0 mg./ml. Pluronic F-68.

(c) Suspended in plasma containing 3.0 mg./ml. Pluronic F-68.

(d) Suspended in plasma containing 5.0 mg./ml. Pluronic F-68.

These plasma suspensions were sprayed at an air pressure of 500 mm. Hg. In Experiments II and III, mechanical fragility was evaluated in the same way as in Experiment I.

Osmotic Fragility of Red Blood Cells. Osmotic fragility of the red blood cells was determined by a modification of the method of Dacie (1938). The resistance of the red blood cells to hemolysis was measured by suspending the cells in decreasing concentrations of phosphate-buffered saline solution and subsequently determining the percentage of hemolysis from the supernatant hemoglobin.

Source of Specimens. Five normal dogs were used as a source of blood. Twenty milliliters of heparinized blood were obtained from each by venipuncture using sterile technique.

Quantities of 5.0, 15.0, and 25.0 mg. of Pluronic F-68 were dissolved, respectively, in 3 tubes, each containing 0.5 ml. of phosphate-buffered saline. A fourth tube of the saline served as a control. Four and one-half milliliters of blood were transferred into each of these 4 tubes to give final concentrations of 0, 1.0, 3.0 and 5.0 mg./ml. These tubes were mixed well by gentle inversion and allowed to stand at room temperature for 30 minutes. The osmotic fragility of each of these samples was determined both immediately and after incubation at 37 C. for 24 hours.

Method. The range of concentration of buffered saline varied from the osmotic equivalents of 0.10% to 1.0% NaCl-PO_4 in 0.05% increments. The concentration of each buffered saline solution was determined by freezing-point depression using an osmometer.[1]

The test was carried out by adding 0.1 ml. of blood to 5 ml. of each buffered saline solution. The tubes were mixed well and incubated at room temperature for 1-1/2 hours. They were subsequently centrifuged at 2,000 rpm for 7 minutes. The absorbance of each supernate was measured at 540 nm. in a Coleman Junior Spectrophotometer.[2] One percent saline was used as a blank. The tube containing 0.1 ml. blood in water represented 100% hemolysis.

The percentage of hemolysis in each tube was calculated as follows:

$$\text{Percent Hemolysis} = \frac{\text{Absorbance of supernate}}{\text{Absorbance of 100\% hemolysis}} \times 100.$$

Percent hemolysis versus concentration of buffered saline was then plotted for each specimen.

In vivo Studies

Source of Specimens. Experimental dogs used for the surgical procedures were generally young mongrels weighing between 20 and 35 kg. They were examined clinically and determined to be in good health.

The dogs were anesthetized with a gas anesthetic mixture of halothane, N_2O, and O_2. A slow intravenous drip of 5% dextrose in water was given by catheter into the cephalic vein, and this route was used also for drug administration. Heparin, 3.5 mg./kg., was used as an

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[1] Advanced Instruments, Inc., Newton Highland, Mass., U.S.A.

[2] Coleman Instruments, Inc., Maywood, Ill., U.S.A.

anticoagulant. Large-size catheters for venous drainage were placed in the superior vena cava and inferior vena cava through the right atrium. The left femoral artery was cannulated for arterial perfusion and the right femoral artery for continuous blood-pressure recordings. Heart action was monitored continuously with a DR8 Recorder.[1] Electrocardiographic tracings were obtained at 15-minute intervals.

The arterial and venous cannulae were connected to the arterial and venous lines from the heart-lung machine. The blood flowed by gravity into a disposable miniprine oxygenator.[2] The roller type Med-Science Electronic[3] was used as a blood pump (Figure 2).

Oxygen was bubbled freely into the oxygenator column of blood at 3 liters O_2/liter blood flow, and CO_2 at 300 cc./minute. The perfusion flow rate was maintained at 80 ml./kg. of body weight/minute. During cardiopulmonary bypass, all lost blood was aspirated by suction and returned to the heart-lung machine in order to minimize blood loss from the system.

In all experiments the priming solution consisted of 1000 ml. of lactated Ringer's solution containing 1 Gm. of Pluronic F-68 and 20 ml. bicarbonate. This was equivalent to 0.35 $\pm$ 0.05 mg./ml. of Pluronic F-68 when the priming volume was added to the circulating blood volume.

Pluronic F-68 was used in 6 of the perfusions while the other 3 procedures served as controls.

Heparainized blood was taken from the arterial catheter to determine blood pH, $_pCO_2$, $_pO_2$, packed cell volume, and hemoglobin concentration,

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[1]Electronics for Medicine, Inc., White Plains, N.Y., U.S.A.

[2]Artificial Organs Division, Travenol Labs, Inc., Morton Grove, Ill., U.S.A.

[3]Med-Science Electronic, St. Louis, Mo., U.S.A.

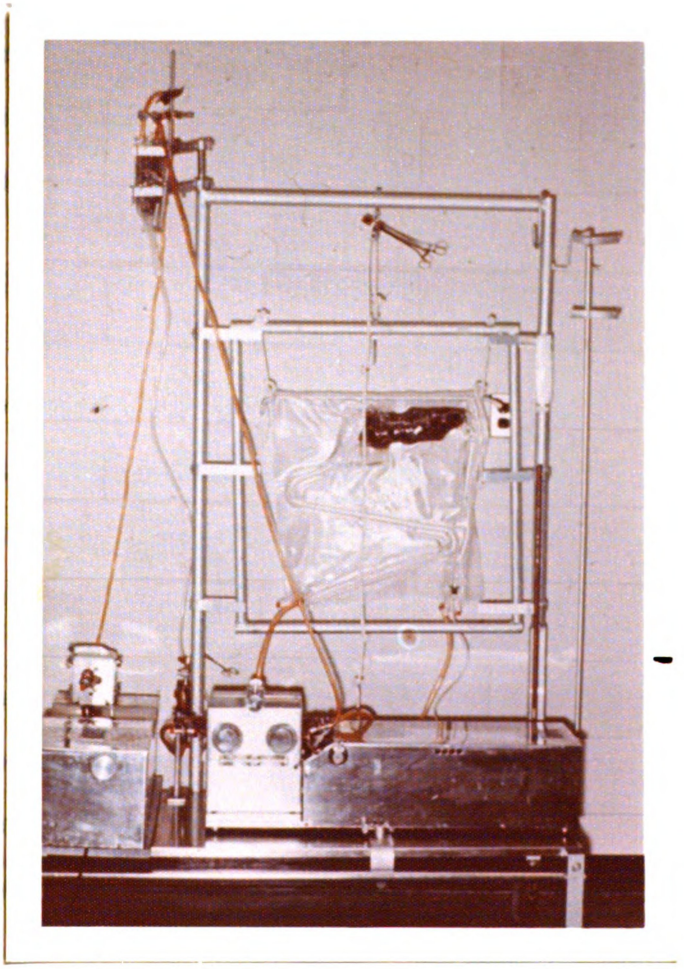

Figure 2. The pump table, front view. Resting on the pump table are the 3 pumps. A disposable oxygenator bag hangs in the frame.

and esophageal temperature was measured at intervals throughout the experimental procedure.

Red Blood Cell Studies. In order to evaluate the effect of Pluronic F-68 on the red blood cells, blood samples were obtained prior to perfusion and during perfusion at 15-minute intervals throughout the bypass for 1 hour. Subsequent samples were obtained at 30-minute intervals.

The following determinations were carried out:

Plasma Hemoglobin. Plasma hemoglobin was determined by Fielding's method (1958). The procedure is based on the use of an Orthotolidine reagent.[1] Colorless orthotolidine acts as an oxygen acceptor and is oxidized to a blue reaction product by the peroxidase activity of hemoglobin.

Method. A quantity of 0.02 ml. plasma was added to 10 ml. of Orthotolidine solution and mixed well. In exactly 8 minutes, the absorbance of the mixture was measured against a distilled water blank at 630 nm. in a Coleman Junior Spectrophotometer. The concentration of plasma hemoglobin was derived from a prepared standard curve.

Calibration Standard Curve. The concentration of hemoglobin in a completely hemolyzed red blood cell mixture was determined by the cyanmethemoglobin method. This mixture was then diluted to appropriate concentrations with distilled water which ranged from 10 to 100 mg./dl. Plasma hemoglobin determination was made on each of the standard dilutions as described above and a standard curve was constructed by plotting absorbance against known hemoglobin concentration.

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[1] Hematest tablet R, manufactured by Ames Company, Inc., Elkhart, Ind., U.S.A.

Osmotic Fragility of Red Blood Cells. The osmotic fragility of red blood cells was determined by a modification of the method of Dacie (1938) as described previously.

Plasma Haptoglobin. Plasma haptoglobin was measured using the method of Owen (1960). This is a simple colorimetric procedure based on the peroxidase activity of haptoglobin-methemoglobin complexes.

Method. This procedure was performed at room temperature (24 to 25 C.). One volume of plasma was diluted with 4 volumes of saline. One milliliter of the diluted plasma was added to 1 ml. of methemoglobin solution for the unknown and 1 ml. to 1 ml. of water for the blank, mixing well. One tenth milliliter of the plasma-methemoglobin mixture was added to 5 ml. guaiacol reagent. For the blank, 0.1 ml. of the plasma-water mixture was added to 5 ml. of guaiacol reagent. One milliliter of freshly made 0.05 M hydrogen peroxide was then added to each tube. The tubes were thoroughly mixed quickly and placed in the dark for color development. This is important as the color fades slowly when exposed to bright daylight. After exactly 8 minutes, the absorbance of the unknown was read against its blank in a Beckman DB Spectrophotometer at a wavelength of 470 nm. These readings should be completed within 4 minutes after the 8-minute reaction time. The concentration of haptoglobin was obtained from a calibration curve and was expressed in terms of bound methemoglobin.

Construction of a Calibration Curve. One milliliter of methemoglobin solution was placed into each of 11 tubes. Pooled normal plasma, free from hemoglobin, was added to each tube, respectively, in increasing amounts of 0.0, 0.1, 0.2...to 1.0 ml. in the last tube. The volume in

each of the 11 tubes was made up to 2.0 ml. with saline. Each sample and a blank prepared with 0.1 ml. NaCl and 5 ml. guaiacol was then submitted to the procedure described above for the unknown. Absorbance was plotted against plasma content for each tube. The inflection point indicates the amount of undiluted pooled plasma which just binds all the methemoglobin present. Thus the reading at this point corresponds to a methemoglobin-binding capacity of 50 mg./dl. This was marked on the abscissa, and the distance between this point and the origin was recalibrated proportionately to give readings of haptoglobin concentration. Samples of test plasma, however, were normally diluted 1 in 5 so that, with these, the reading which corresponds to haptoglobin content should be multiplied by 5.

RESULTS

In vitro Studies

Mechanical Fragility of Red Blood Cells

Experiment I. The results of mechanical fragility tests on Dog A and Dog B using various concentrations of Pluronic F-68 and air pressure are summarized in Figures 3 and 4. In Dog A red blood cell mechanical fragility was related to both the concentration of Pluronic F-68 and air pressure. When plotted on probability paper, the curves relating hemolysis to air pressure for Dog A appeared linear, suggesting that the cumulative hemolysis curve was a simple sigmoid function, and that mechanical fragility was distributed through this population as a normal (Gaussian) distribution. The curves for Dog B were similar except that they tended to plateau at the higher pressures, suggesting that the red blood cell population in that individual was less uniform with respect to mechanical fragility. In the remaining 3 dogs, the curves plotted on probability paper were predominantly linear, but showed a tendency to plateau formation at higher pressures of a lesser degree than with Dog B.

The results of the whole series are recorded in Figure 5. The mechanical fragility regression line was calculated from the percent probit of the data for 5 dogs. This indicated that Pluronic F-68 in phosphate-buffered saline significantly protected the red blood cells.

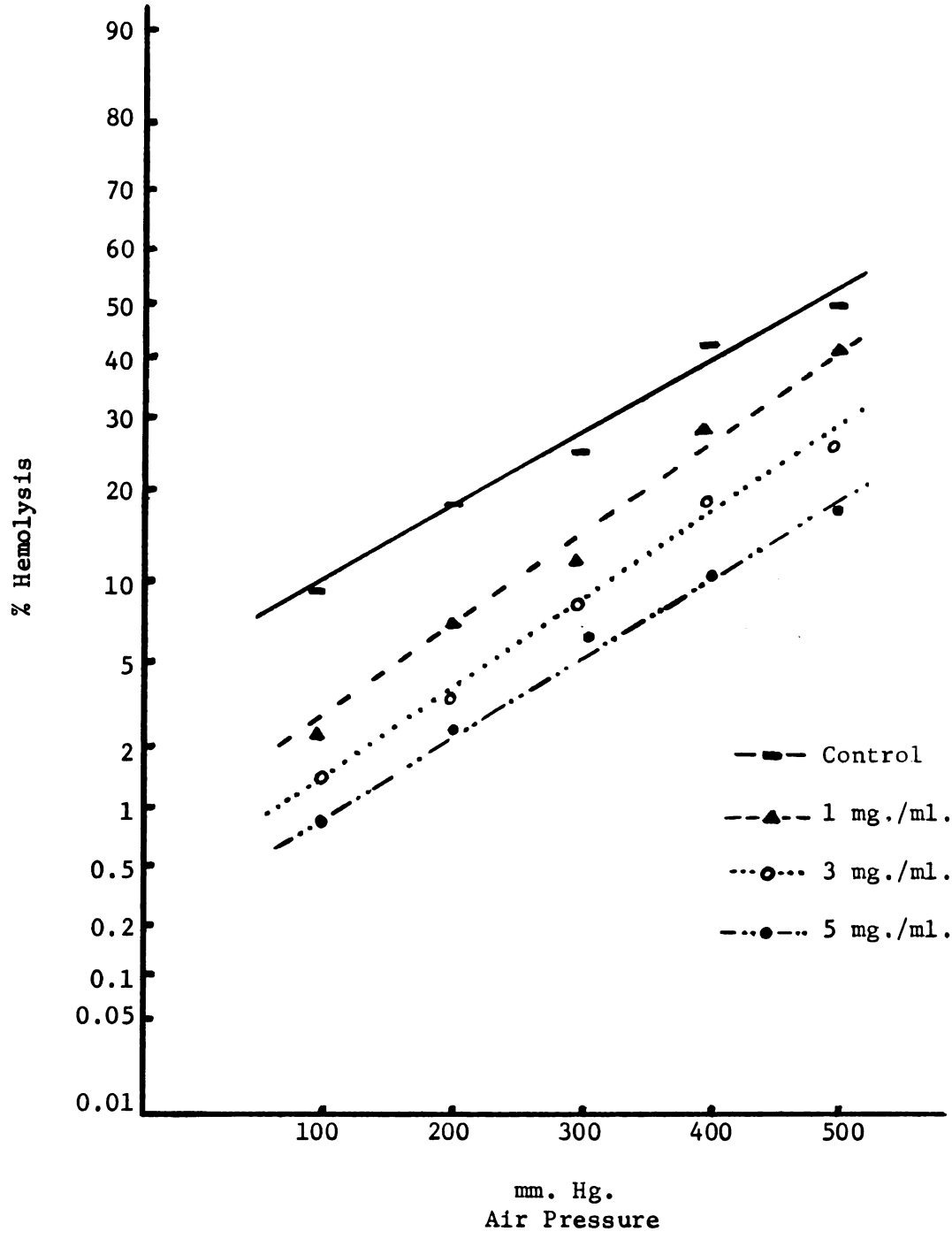

Figure 3. Mechanical fragility (*in vitro*): the effect of varying air pressure and different concentrations of Pluronic F-68 on red blood cells in buffered saline (Dog A).

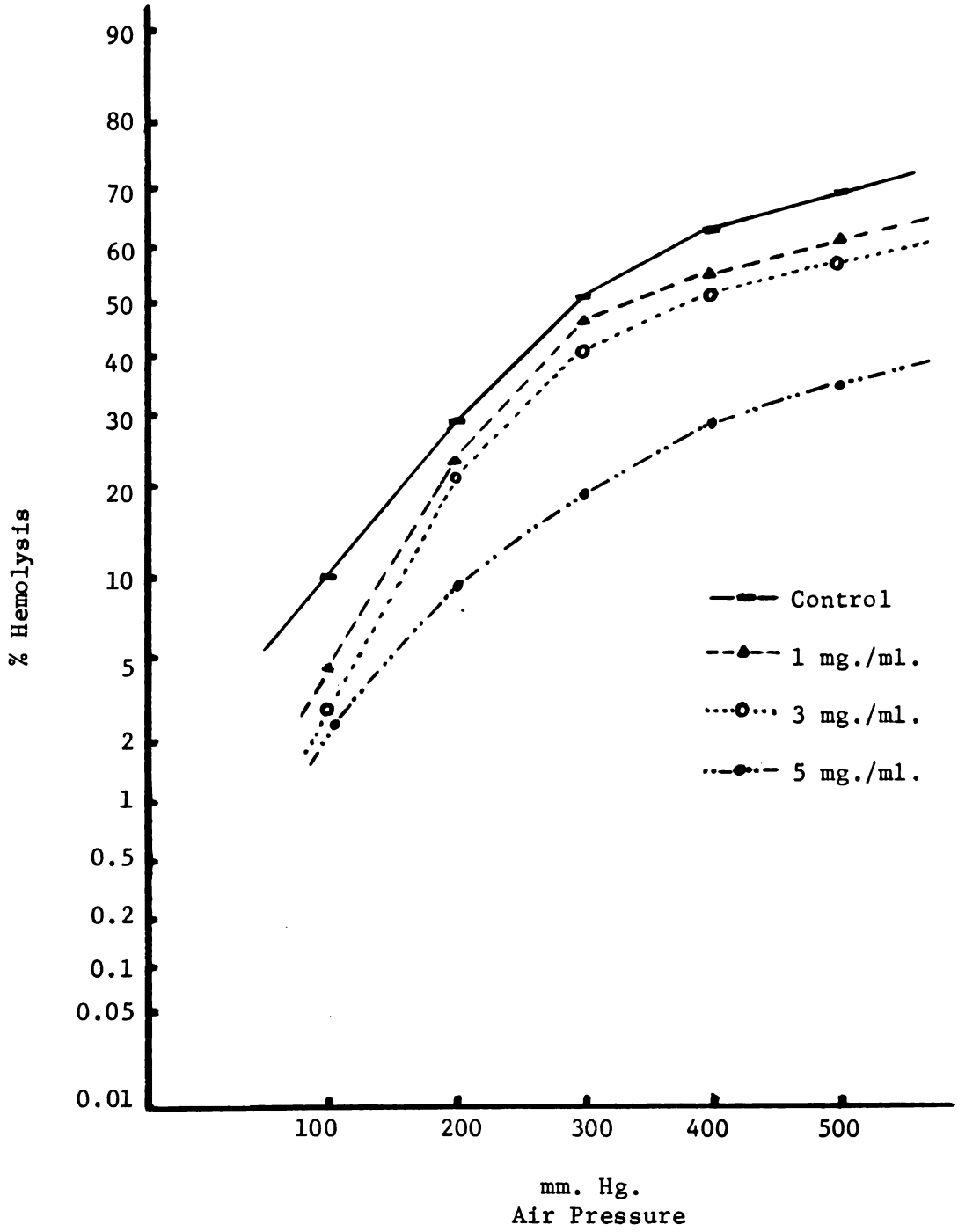

Figure 4. Mechanical fragility (*in vitro*): the effect of varying air pressure and different concentrations of Pluronic F-68 on red blood cells in buffered saline (Dog B).

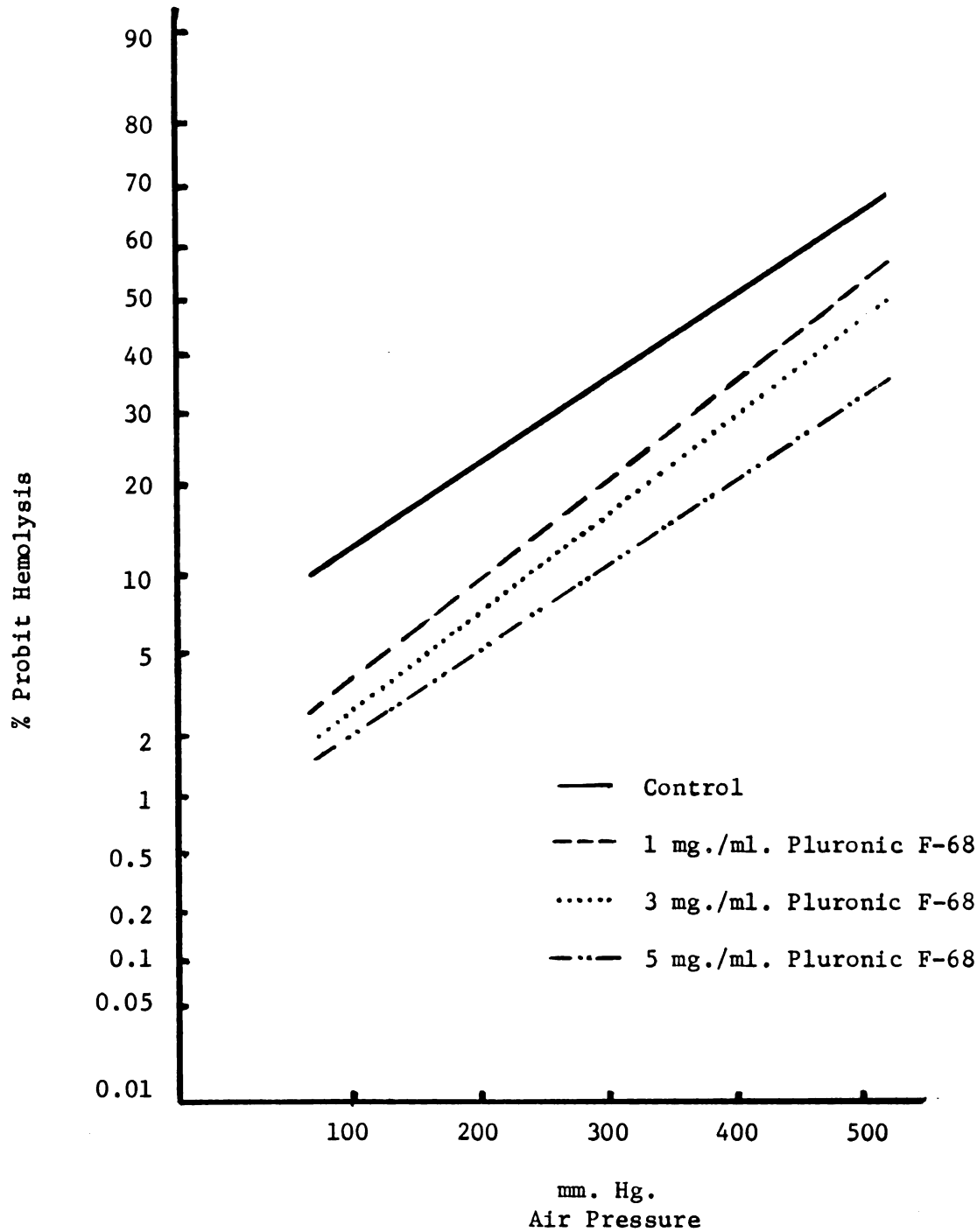

Figure 5. Mechanical fragility (*in vitro*): regression calculated on data from 5 dogs; red blood cells in saline.

The coefficient of correlation of these regression lines ranged from 0.90 to 0.94.

The significance of the effects of different air pressures and concentrations of Pluronic F-68 was calculated by Students' t test: the data showed a significant difference between the untreated and treated red blood cells as indicated in Table 1.

Experiment II. Figure 6 and Table 2 show the effect on mechanical hemolysis of variations in the suspension medium. Red blood cells suspended in Pluronic F-68 in buffered saline were protected to a significant degree. By contrast, red blood cells which were suspended in Pluronic F-68 and buffered saline for 3 hours and then washed with isotonic saline before being subjected to mechanical stress were not significantly protected. Although there was some reduction in hemolysis in the latter cells as compared with controls, the differences were not significant ($P>0.1$).

Experiment III. The results of mechanical fragility testing of red blood cells suspended in plasma are given in Figure 7 and Table 3. A solution of Pluronic F-68 in concentrations of 3.0 mg./ml. or 5.0 mg./ml. of plasma significantly reduced the mechanical fragility of red blood cells as compared with untreated controls (both $P<0.001$). Much less protection was afforded by a concentration of 1.0 mg./ml. of plasma ($P=0.05$).

Osmotic Fragility of Red Blood Cells. The results of *in vitro* osmotic fragility tests are given in Figures 8 and 9. There was no significant difference in the osmotic fragility of cells treated with Pluronic F-68 as compared with controls when tests were performed soon after treatment

Table 1. The effects of different air pressures and concentrations of Pluronic F-68 on the mechanical fragility of red blood cells *in vitro* (mean percent hemolysis, standard deviation and probability for 5 dogs)

Pluronic F-68 Concentration		Air Pressure (mm. Hg.) 100	200	300	400	500
Control	% Hemolysis	11.20	25.76	39.72	55.14	63.10
	S.D.	2.32	7.60	9.88	7.44	7.48
1.0 mg./ml.	% Hemolysis	3.26	12.50	24.52	40.60	49.62
	S.D.	0.84	6.94	13.22	9.69	8.70
	t	7.19	2.88	2.06	2.66	2.63
	P	P<0.001	0.05>P>0.02	0.1>P>0.05	0.05>P>0.02	0.05>P>0.02
3.0 mg./ml.	% Hemolysis	2.72	10.00	21.16	33.44	44.10
	S.D.	0.76	7.36	11.76	11.33	12.96
	t	6.23	3.33	2.71	3.58	4.65
	P	P<.001	0.01>P>0.001	0.5>P>0.02	0.01>P>0.001	0.01>P>0.001
5.0 mg./ml.	% Hemolysis	2.04	6.06	14.96	22.86	30.24
	S.D.	0.68	2.63	4.46	7.88	9.08
	t	8.47	5.48	5.11	6.67	4.74
	P	P<0.001	P<0.001	P<0.001	P<0.001	0.01>P>0.001

Degrees of Freedom = 8.
S.D. = Standard Deviation.
t = as determined by Students' t test.
P = Probability.

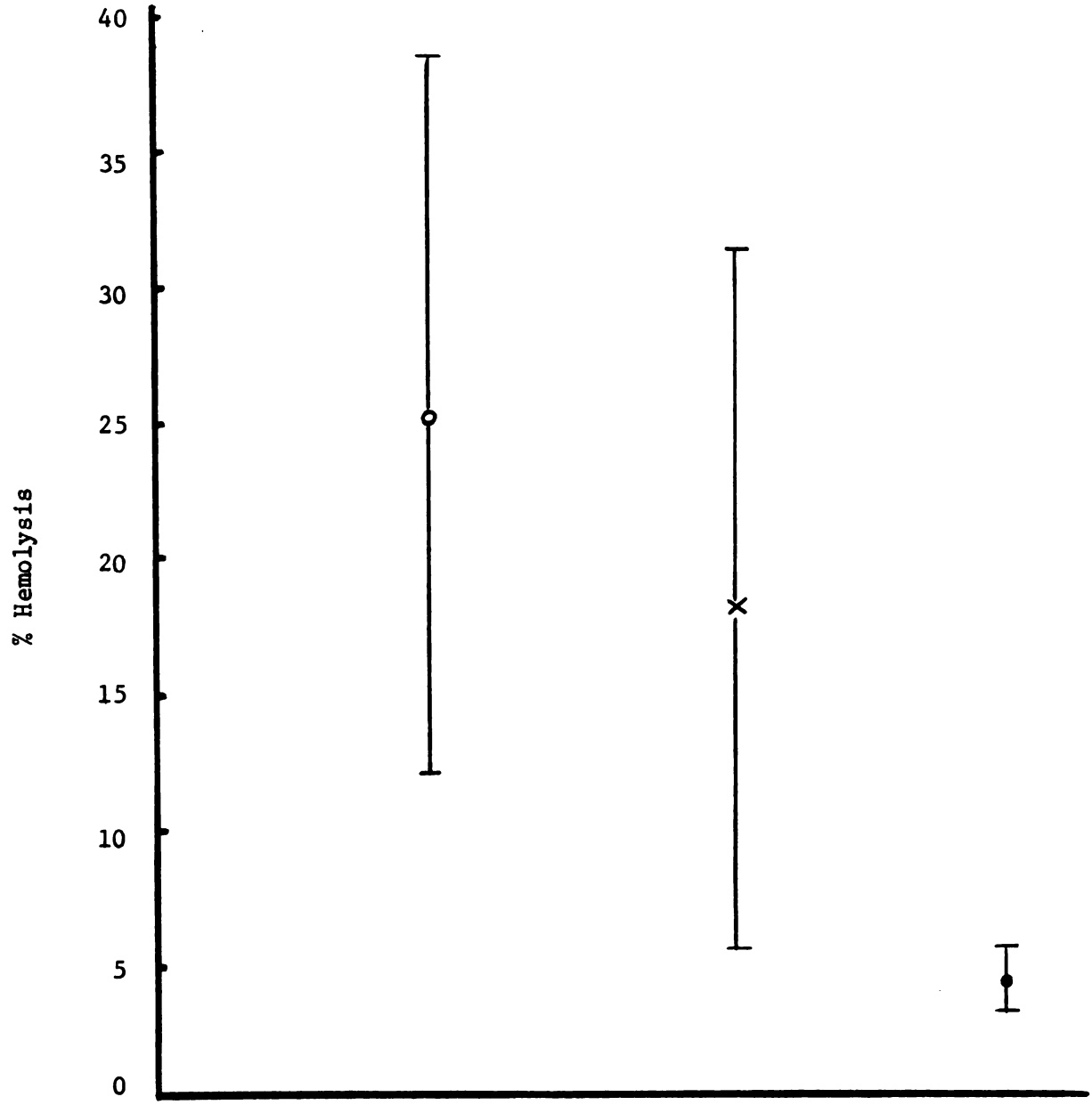

Figure 6. Mechanical fragility (*in vitro*): protective effects of Pluronic F-68 on red blood cells subjected to mechanical stress at 100 mm. Hg. air pressure; summary data from 5 dogs.

Table 2. Red blood cell mechanical fragility *in vitro*: comparison of the effects of Pluronic F-68 on red blood cells subjected to mechanical stress at 100 mm. Hg. air pressure (summary from 5 dogs)

	Control	5.0 mg./ml. F-68 saline wash before test	5.0 mg./ml. F-68 in suspension medium
Mean % Hemolysis	25.28	18.50	4.24
S.D.	13.28	13.01	1.14
t		0.8155	3.5298
d.f.		8	8
P		P>0.1	0.01>P>0.001
Significant		No	Yes

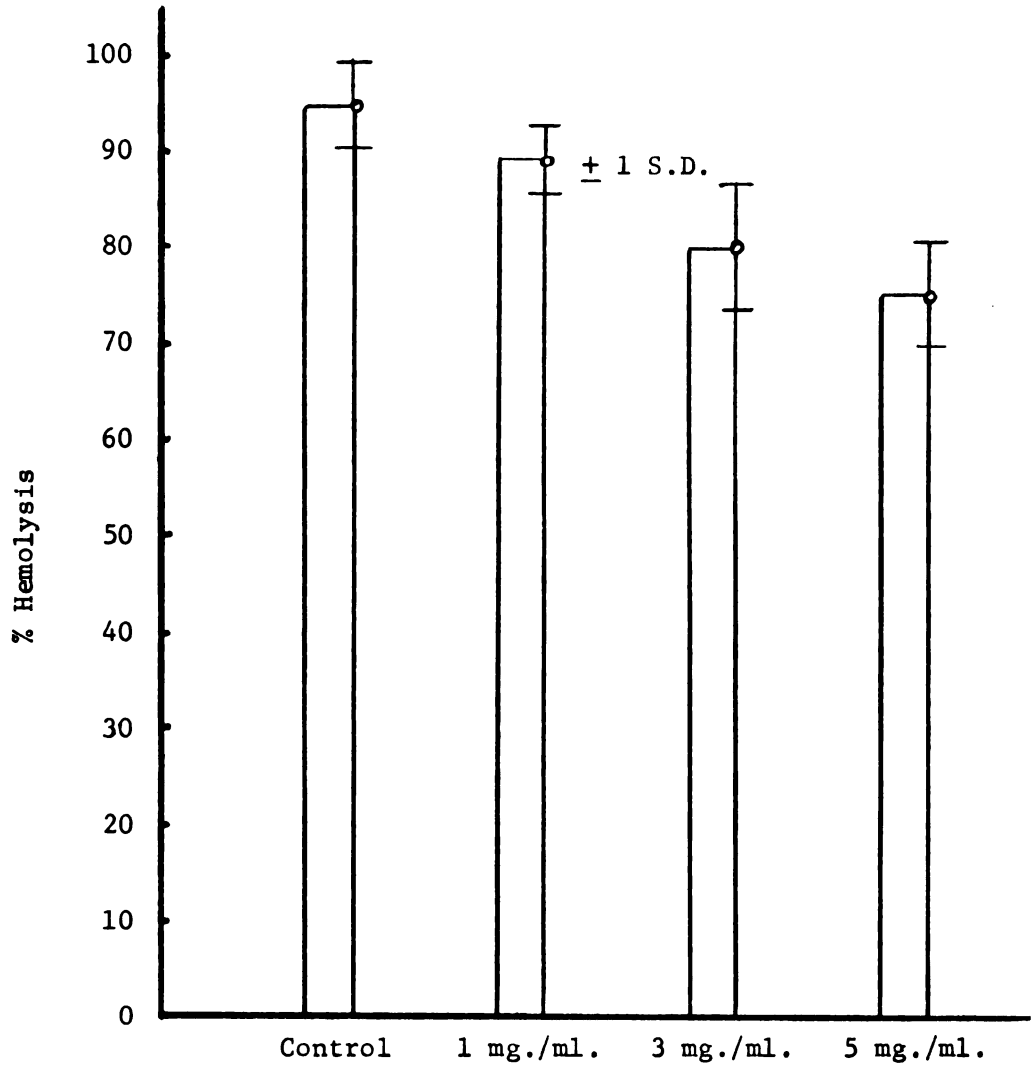

Figure 7. Mechanical fragility (*in vitro*): the effect of varying Pluronic F-68 concentrations on red blood cells in plasma (air pressure = 500 mm. Hg.).

Table 3. Effects of various concentrations of Pluronic F-68 on *in vitro* mechanical fragility of red blood cells in plasma: summary of data from 5 dogs (air pressure = 500 mm. Hg.)

	Control	1.0 mg./ml.	3.0 mg./ml.	5.0 mg./ml.
Mean % Hemolysis	94.82	89.22	79.96	75.08
S.D.	4.32	3.57	6.58	5.33
t		2.308	6.066	6.432
d.f.		8	8	8
P		P=0.05	P<0.001	P<0.001
Significant		Yes	Yes	Yes

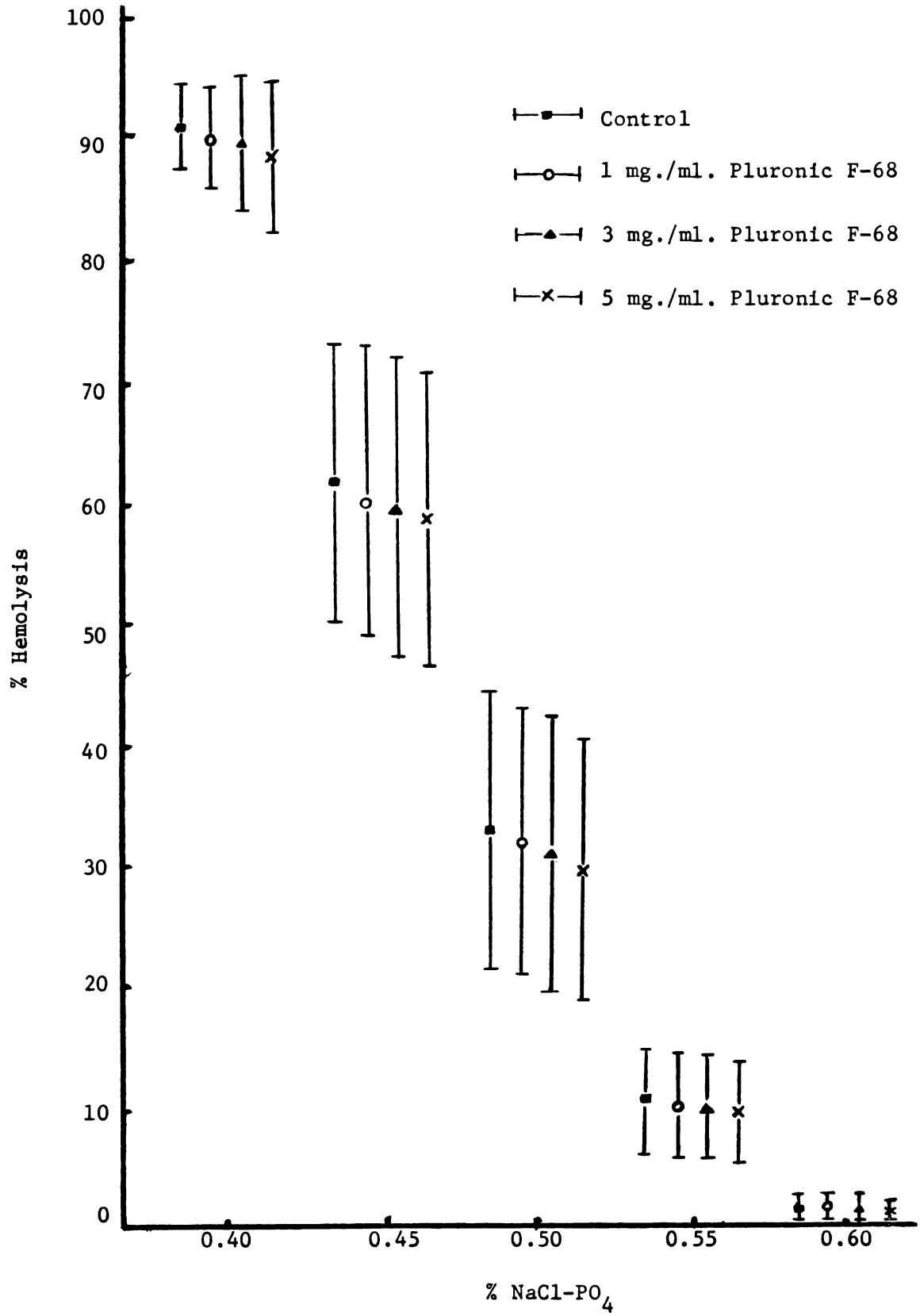

Figure 8. Osmotic fragility (*in vitro*) before incubation: mean ± 1 S.D. in 5 dogs.

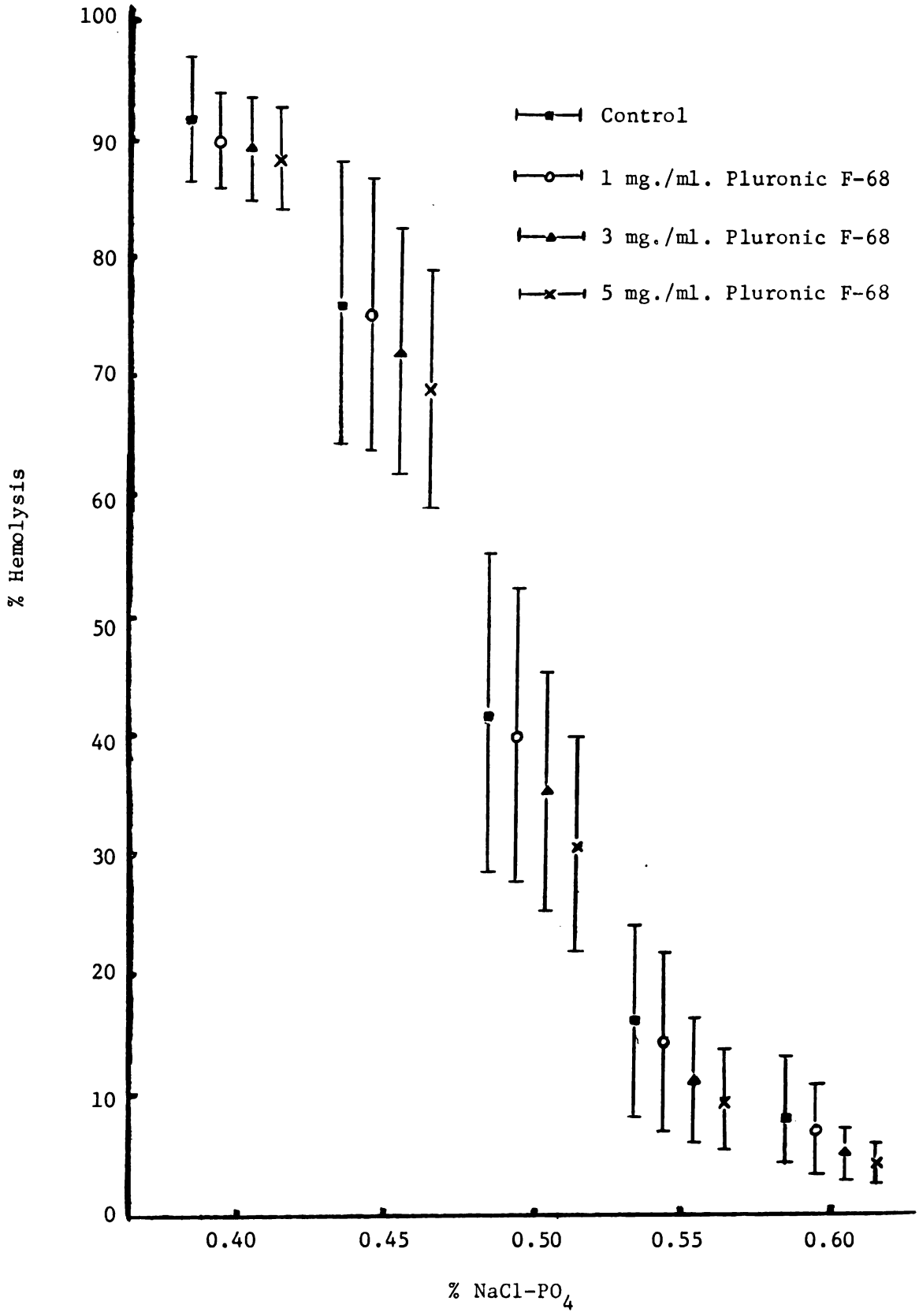

Figure 9. Osmotic fragility (*in vitro*) after 24 hours incubation at 37 C.: mean ± 1 S.D. in 5 dogs.

(P>0.1) (Figure 8). If cells were incubated with Pluronic F-68 for 24 hours at 37 C., osmotic fragility was somewhat reduced, but the differences were not statistically significant (P>0.1) (Figure 9).

In vivo Studies

Red Blood Cell Studies

Plasma Hemoglobin. The average plasma hemoglobin concentrations throughout the period of cardiopulmonary bypass are presented for 9 dogs (Figure 10). There was no significant difference between plasma hemoglobin values in animals treated with Pluronic F-68 and those in untreated controls (Table 4).

Osmotic Fragility of Red Blood Cells. The results of tests of osmotic fragility during cardiopulmonary bypass indicated that Pluronic F-68 had no effect. However, if the blood samples were incubated for 24 hours at 37 C. before testing, Pluronic-treated cells exhibited greater resistance to osmotic hemolysis than did untreated controls (Figures 11 and 12). A comparison of these 2 groups was calculated using Students' t test for 50% hemolysis as demonstrated in Tables 5 and 6.

Plasma Haptoglobin. Data for average plasma haptoglobin concentration during cardiopulmonary bypass are presented for 9 dogs (Figure 13). Plasma haptoglobin concentration fell rapidly during the first 15 minutes as a result of the diluting effect of the priming solution. In untreated controls, with bypass times in excess of 30 minutes, a subsequent fall was found. In Pluronic-treated animals, however, plasma haptoglobin concentrations began to rise after 30 minutes and continued to rise for another 30 minutes. However, as shown in Figure 14, when the data for plasma haptoglobin were corrected for the hemodilution effect of the

($P>0.1$) (Figure 8). If cells were incubated with Pluronic F-68 for 24 hours at 37 C., osmotic fragility was somewhat reduced, but the differences were not statistically significant ($P>0.1$) (Figure 9).

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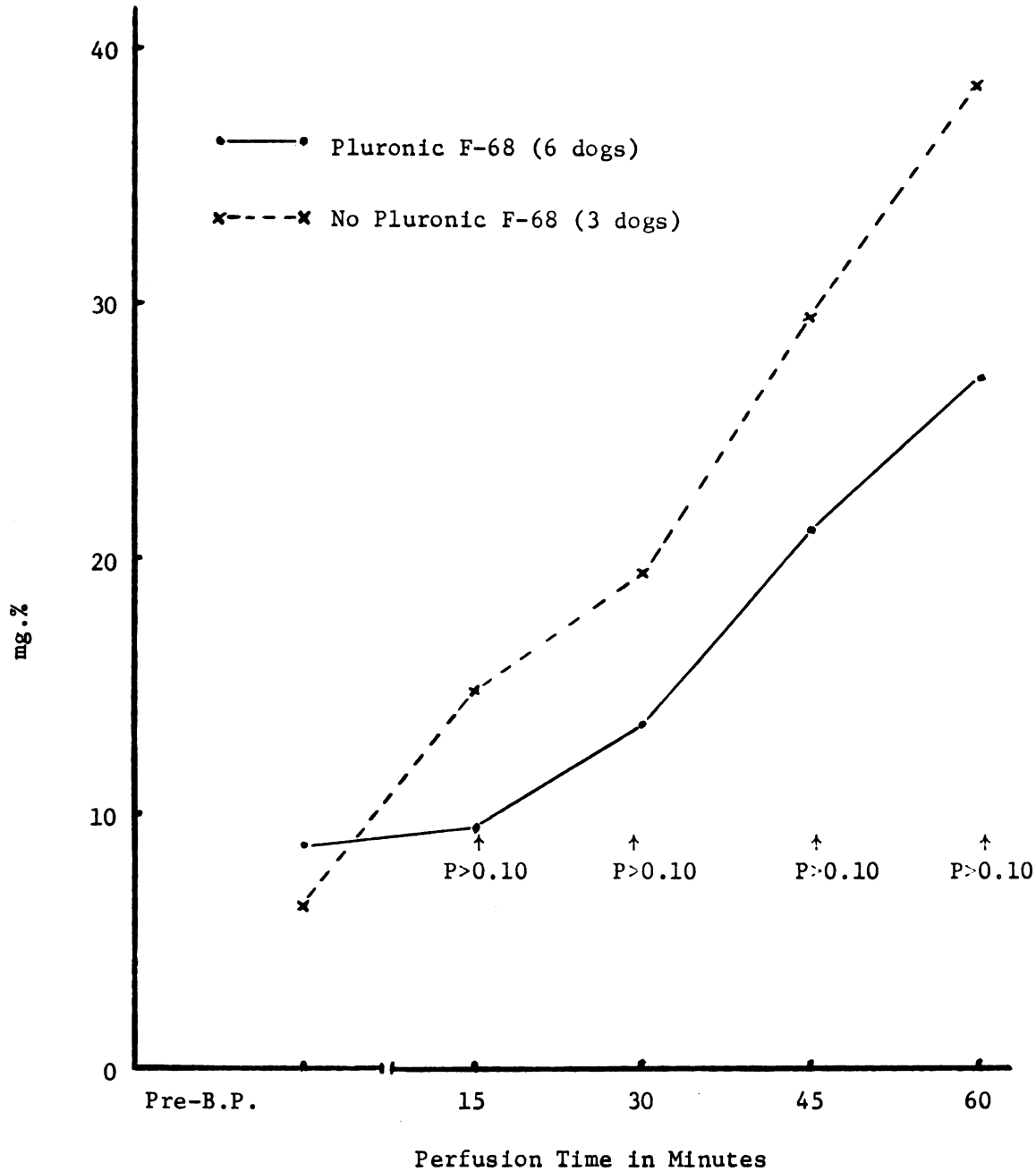

Figure 10. Plasma hemoglobin values in 9 dogs as a function of bypass time (P represents degree of significance of differences of means between 2 groups).

Table 4. Comparison of plasma hemoglobin values during cardiopulmonary bypass with and without Pluronic F-68 in perfusion fluids

	Initial Sample	15 min. B.P.	30 min. B.P.	45 min. B.P.	60 min. B.P.
No Pluronic F-68					
Mean % Hemolysis	6.33	14.83	19.37	29.50	38.77
S.D.	0.29	4.54	4.02	2.50	5.32
Pluronic F-68					
Mean % Hemolysis	8.78	9.47	13.50	21.12	27.20
S.D.	3.89	5.13	7.78	11.16	10.48
Comparison					
t		1.525	1.199	1.243	1.759
d.f.		7	7	7	7
P		P>0.10	P>0.10	P>0.10	P>0.10
Significant		No	No	No	No

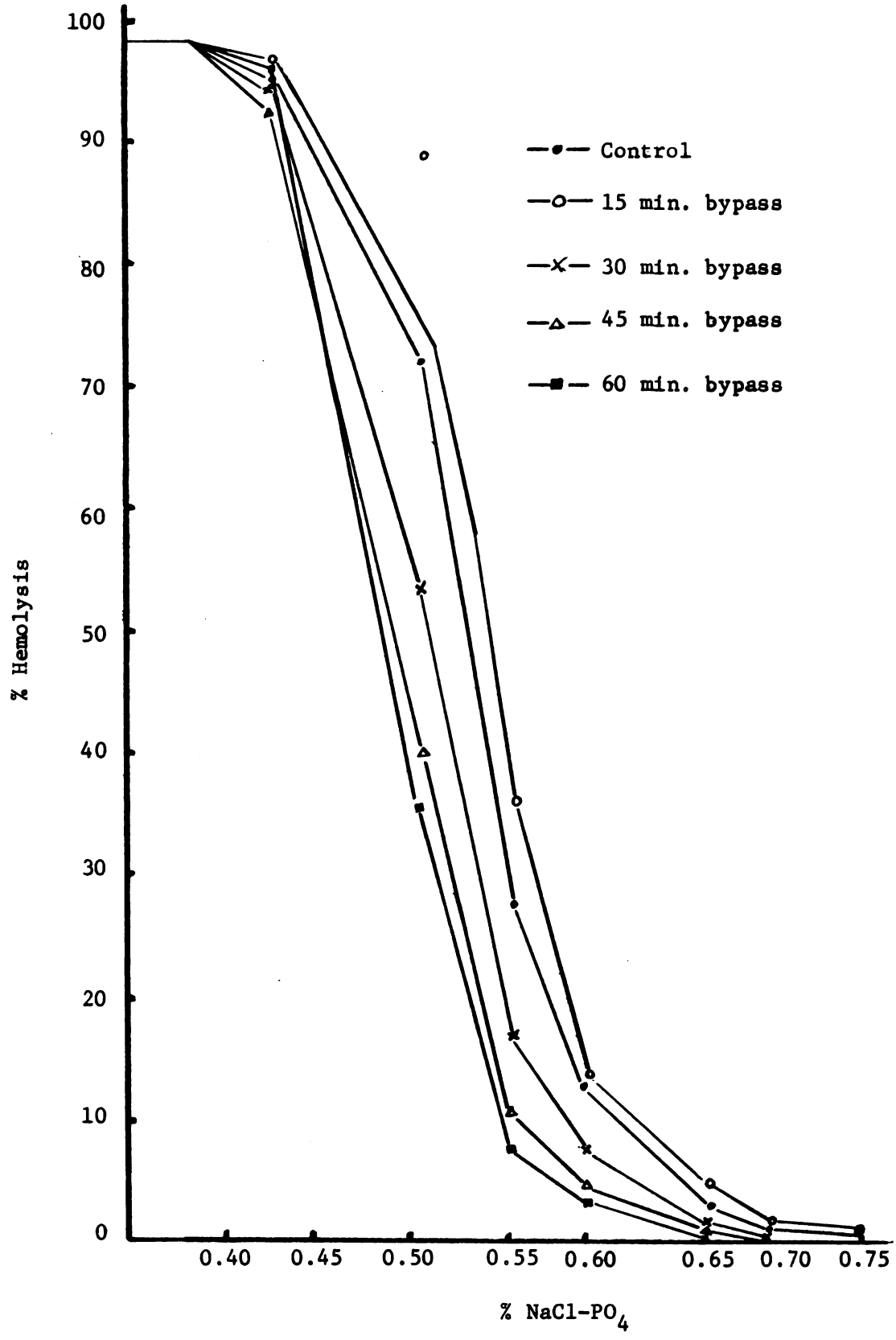

Figure 11. Osmotic fragility (*in vivo*) without Pluronic F-68 after 24 hours incubation at 37 C.

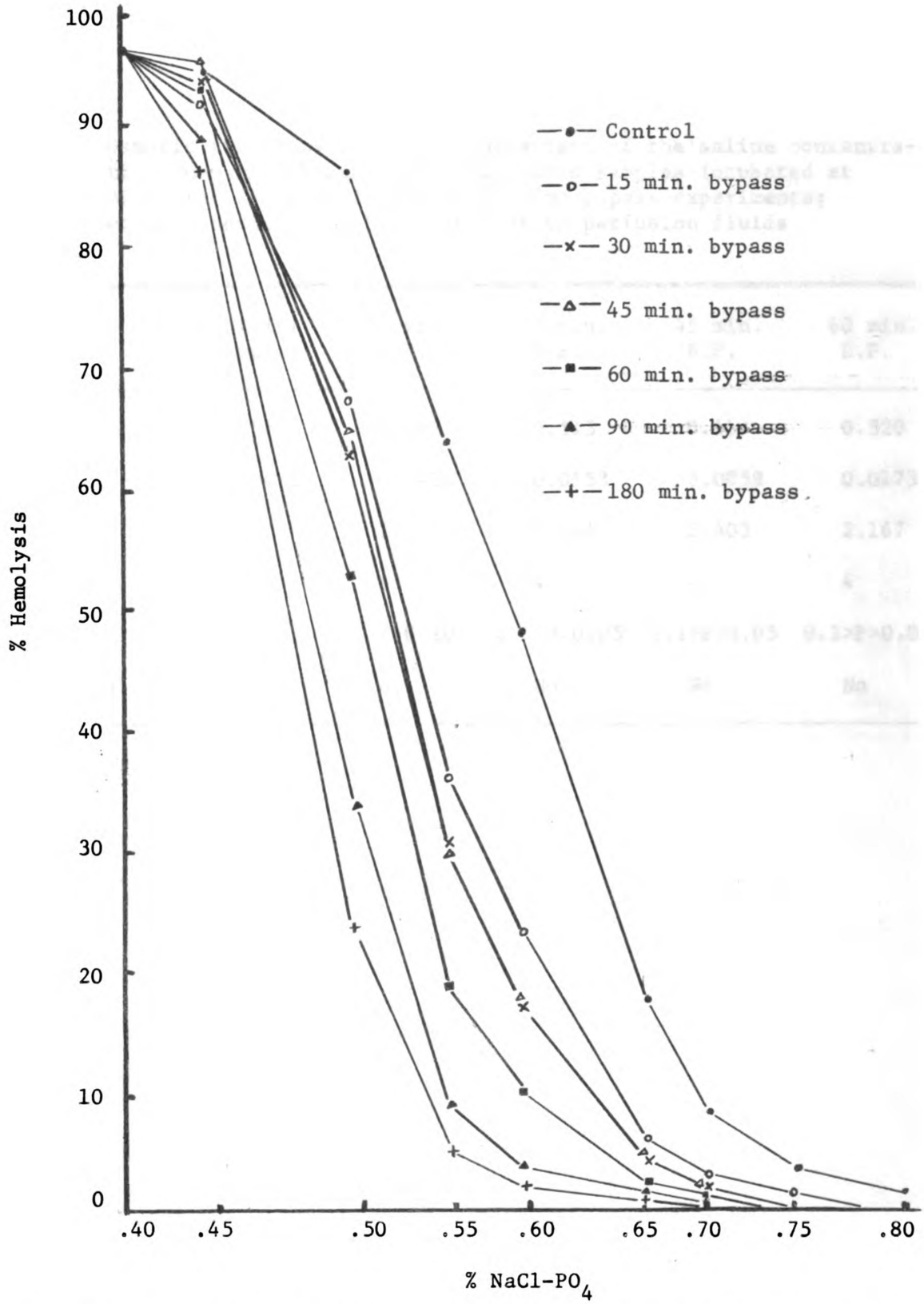

Figure 12. Osmotic fragility (*in vivo*) with Pluronic F-68 after 24 hours incubation at 37 C.

Table 6. Osmotic fragility *in vivo*. Comparison of the saline concentration giving 50% hemolysis in blood samples incubated at 37 C. for 24 hours following *in vivo* bypass experiments; Pluronic F-68 added to perfusion fluids

	Initial Sample	15 min. B.P.	30 min. B.P.	45 min. B.P.	60 min. B.P.
% $NaCl-PO_4$	0.542	0.490	0.475	0.485	0.482
S.D.	0.0402	0.0297	0.0423	0.0281	0.0223
t		2.544	2.809	2.845	3.198
P		0.05>P>0.02	0.02>P>0.01	0.02>P>0.01	0.01>P>0.001
d.f.		10	10	10	10
Significant		Yes	Yes	Yes	Yes

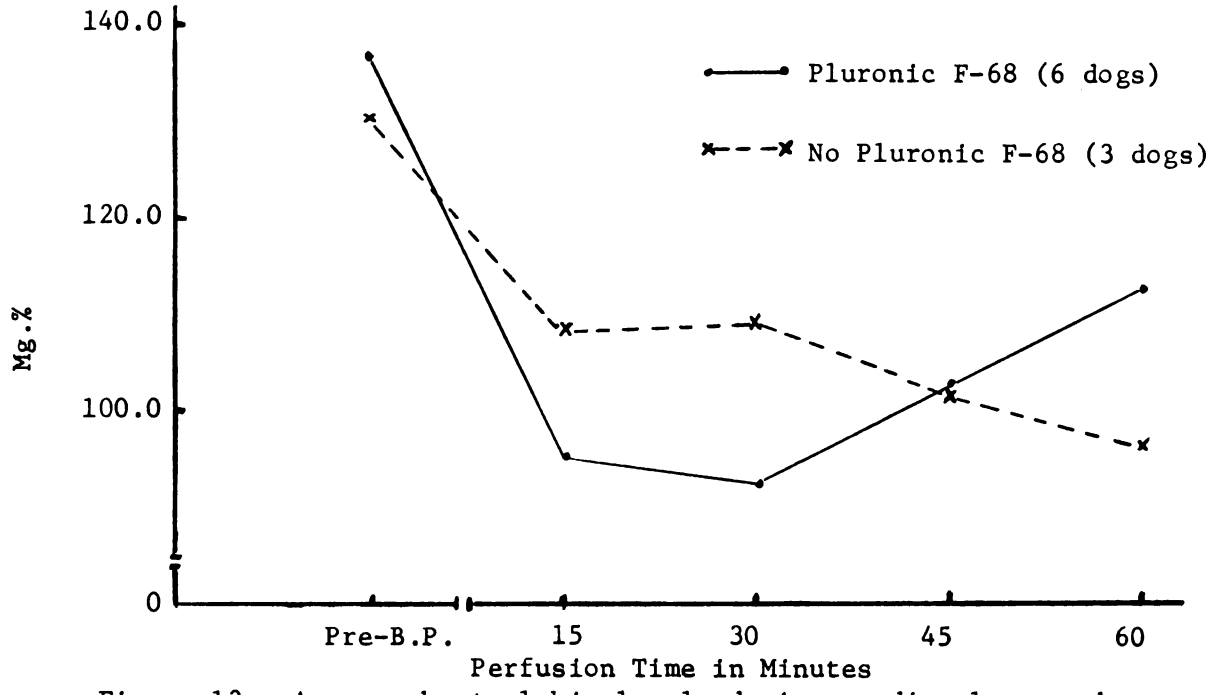

Figure 13. Average haptoglobin levels during cardiopulmonary bypass in 9 dogs.

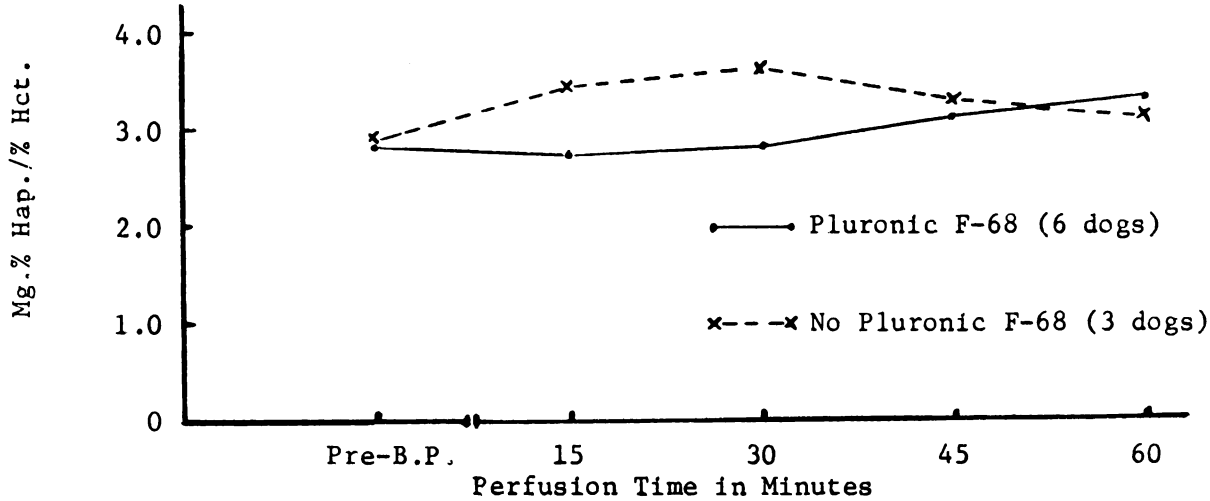

Figure 14. Haptoglobin correction for the effect of hemodilution during cardiopulmonary bypass (haptoglobin level divided by hematocrit concentration).

priming fluids by dividing each value by the hematocrit of the sample, the haptoglobin values showed no significant differences during the first hour. Insufficient data for bypass times in excess of 60 minutes were available for further analysis.

DISCUSSION

The results of these studies lend further support to previously published work suggesting that the nonionic surfactant, Pluronic F-68, reduces the mechanical fragility of erythrocytes (Wells *et al.*, 1968; Tamiya *et al.*, 1970). The data from *in vitro* studies of mechanical fragility indicate that the degree of mechanically-induced hemolysis is diminished by Pluronic F-68, the effect being dependent upon the concentration of the surfactant. This effect is demonstrable both with red blood cells suspended in buffered saline solution and with red blood cells suspended in plasma.

The mechanical fragility of erythrocytes which had been incubated with Pluronic F-68 for 3 hours and then washed was somewhat less than that of untreated cells, but the difference was not statistically significant. This shows that the protective effect with respect to mechanical fragility is dependent upon the surfactant's being present in the surrounding medium and that, if it acts by adsorption to the cell surface, it must be very loosely bound and unable to exert its effect in the absence of significant concentrations in the suspending fluid.

Tests of erythrocyte osmotic fragility were conducted with Pluronic F-68 in the hypotonic media. There was no difference in osmotic fragility between control cells tested in media lacking the surfactant and the treated cells. After 24 hours incubation with Pluronic F-68 at 37 C., the treated red blood cells showed slightly increased resistance to osmotic lysis but the differences were not significant. It therefore

appears that the protective effect of Pluronic F-68 on red blood cell lysis does not arise from changes in the relationship between the surface area of the red blood cell and its osmotically active contents.

Data derived from *in vivo* experiments regarding changes in plasma hemoglobin level are difficult to interpret. The differences in plasma hemoglobin levels between the untreated and treated animals were small and not statistically significant. The series was too small to exclude minor differences, but it seems most probable that the concentrations of Pluronic F-68 *in vivo* (0.35 mg./ml. blood) were too low in these animals to have exerted a significant effect. *In vitro* studies indicated that the inhibition of hemolysis by Pluronic was dose related. In many previous experimental studies, Pluronic F-68 has been employed in considerably higher concentrations. Tamiya *et al.* (1970) suggested that a dose of Pluronic F-68 in a concentration of 1.0 mg./ml. in the circulating blood during perfusion would be effective in preventing hemolysis. The same investigator found that a Pluronic concentration of 2.0 mg./ml. of blood, though more effective in reducing hemolysis, was associated with postoperative elevation of blood urea nitrogen concentration and concluded that this concentration was not to be recommended.

Pluronic F-68 had no significant effect on the osmotic fragility of red blood cells in blood samples collected during cardiopulmonary bypass. However, if the blood was incubated for 24 hours at 37 C. before testing, it was possible to demonstrate a significant increase in osmotic resistance in samples collected later in the bypass period. This effect was noted irrespective of treatment with Pluronic F-68 and in some cases corresponded to an increase in plasma hemoglobin. A possible explanation is that there had been a subtle selective process during the operation

of the bypass, such that those cells which would be more fragile on incubation had been removed from the circulation.

Plasma haptoglobin concentrations were investigated during cardiopulmonary bypass to compare the behavior of free hemoglobin and the hemoglobin-haptoglobin complex with respect to plasma. In general, increased hemolysis is associated with lowering the plasma haptoglobin levels and often its complete disappearance (Brus and Lewis, 1959). In these experiments, Pluronic-treated animals had a higher plasma haptoglobin and lower plasma hemoglobin level as compared to the untreated animals at the end of perfusion, but there were no significant differences between these 2 groups of animals. This is further evidence that the Pluronic F-68 in the concentration used failed to modify *in vivo* hemolysis.

Although the value of Pluronic F-68 during cardiopulmonary bypass is still not conclusively demonstrated, *in vitro* studies indicated marked reduction in mechanical fragility of Pluronic-treated cells.

SUMMARY AND CONCLUSIONS

The Pluronic polyols are ethylene oxide-polypropylene glycol condensation products. Pluronic F-68 is a nonionic polyol surfactant of molecular weight 8,350. This agent has been subjected to extensive toxicologic studies which indicate that it is non-toxic and suggest that it is not metabolized. This surfactant has been used in total body perfusion experimentally and clinically to protect blood cells from trauma. The present investigation is a further study of the effects of Pluronic F-68 both *in vitro* and during cardiopulmonary bypass.

The protective effects of Pluronic F-68 on red blood cells can be summarized as follows:

In vitro Studies

1. Pluronic F-68 in phosphate buffered saline significantly protects red blood cells against mechanical hemolysis.

2. Pluronic F-68 in plasma also significantly increased the resistance of red blood cells to mechanical hemolysis.

3. When erythrocytes were suspended in Pluronic F-68 and then washed with isotonic saline prior to exposure to mechanical stress, a slight reduction in the degree of hemolysis as compared with untreated cells was observed but was not significant ($P>0.1$).

4. Pluronic F-68 had no immediate effects on the osmotic fragility of red blood cells.

5. There was a slight but statistically insignificant ($P>0.1$) reduction in the osmotic fragility of erythrocytes incubated with Pluronic F-68 for 24 hours prior to testing.

In vivo Studies

1. There was no significant difference between the plasma hemoglobin and haptoglobin levels of Pluronic F-68 treated animals with blood concentrations of approximately 0.35 mg./ml. and those of untreated controls during cardiopulmonary bypass.

2. There was no immediate change in the osmotic fragility of erythrocytes of Pluronic F-68 treated animals during cardiopulmonary bypass.

3. If blood was incubated at 37 C. for 24 hours prior to testing for osmotic fragility, the osmotic resistance of samples drawn during the later stages of cardiopulmonary bypass was significantly greater than that of blood collected early in the bypass period whether Pluronic F-68 was used or not. This suggested that there was a selective loss of the potentially more fragile cells during passage through the bypass equipment.

Pluronic F-68 has been widely studied by numerous investigators. It possesses certain properties which make it a uniquely suitable additive to the perfusion system. The use of Pluronic F-68 is a safe and effective means of minimizing the adverse effects of the pump oxygenator on red blood cells during extracorporeal circulation.

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APPENDICES

APPENDIX A

REAGENTS

APPENDIX A

Reagents

Fielding Method for Plasma Hemoglobin Determination

Orthotolidine Reagent. Crush 1 Hematest reagent tablet* and dilute with 25 ml. of distilled water; mix well and allow to stand 30 minutes before filtering. This reagent should be crystal-clear, and stored in the refrigerator.

Modified Owen Method for Plasma Haptoglobin Determination

Guaiacol Reagent. Guaiacol (A.R. 1 Gm./ml.) 3.72 ml. are added in 700 ml. water to which are added 100 ml. of 1 M acetic acid. The pH is adjusted to 4.0 by the addition of 1 N NaOH, using a glass-electrode pH meter. The volume is finally made up to 1 liter.

Hydrogen Peroxide 0.05 M. This is prepared immediately before use by diluting a stock solution with distilled water to a concentration of 0.05 M.

Methemoglobin Solution. To 8 volumes of thrice-washed packed red blood cells are added 3 volumes of water and 1 volume of ether. The mixture is shaken and centrifuged. The hemolysate is pipetted off, and its hemoglobin concentration determined by ordinary clinical

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*Ames Company, Inc., Elkhart, Indiana, U.S.A.

hemoglobinometry (Drabkin's method). It is then diluted until the hemoglobin concentration is 1 Gm./100 ml. To 25 ml. of this solution are added 10 ml. of potassium ferricyanide (100 mg./100 ml.) to convert the hemoglobin to methemoglobin. After 10 minutes the volume is made up to 500 ml. The solution can be stored at 4 C. for several weeks.

APPENDIX B

CALIBRATION OF STANDARD CURVE

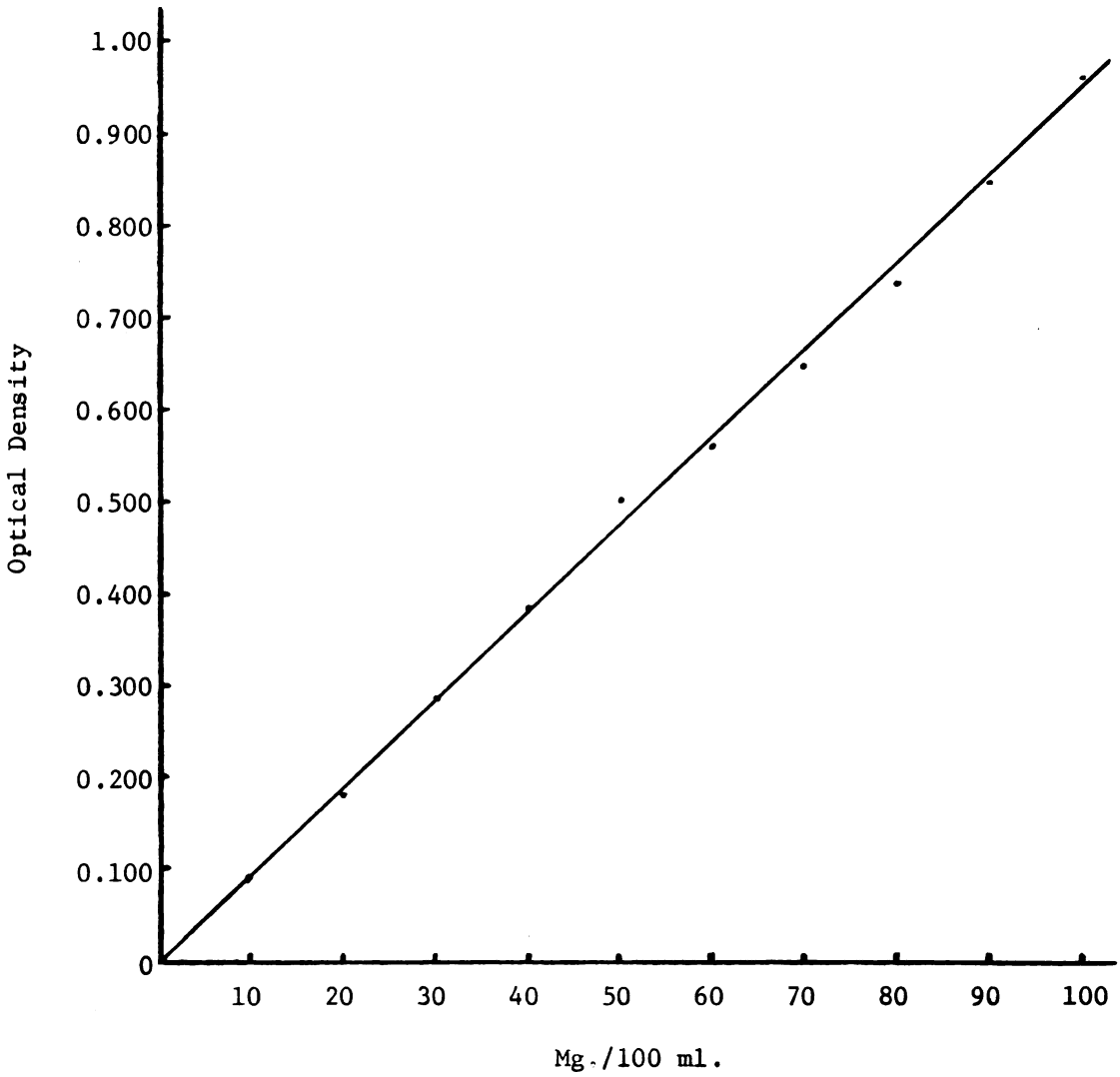

Figure B-1. Plasma hemoglobin standard curve.

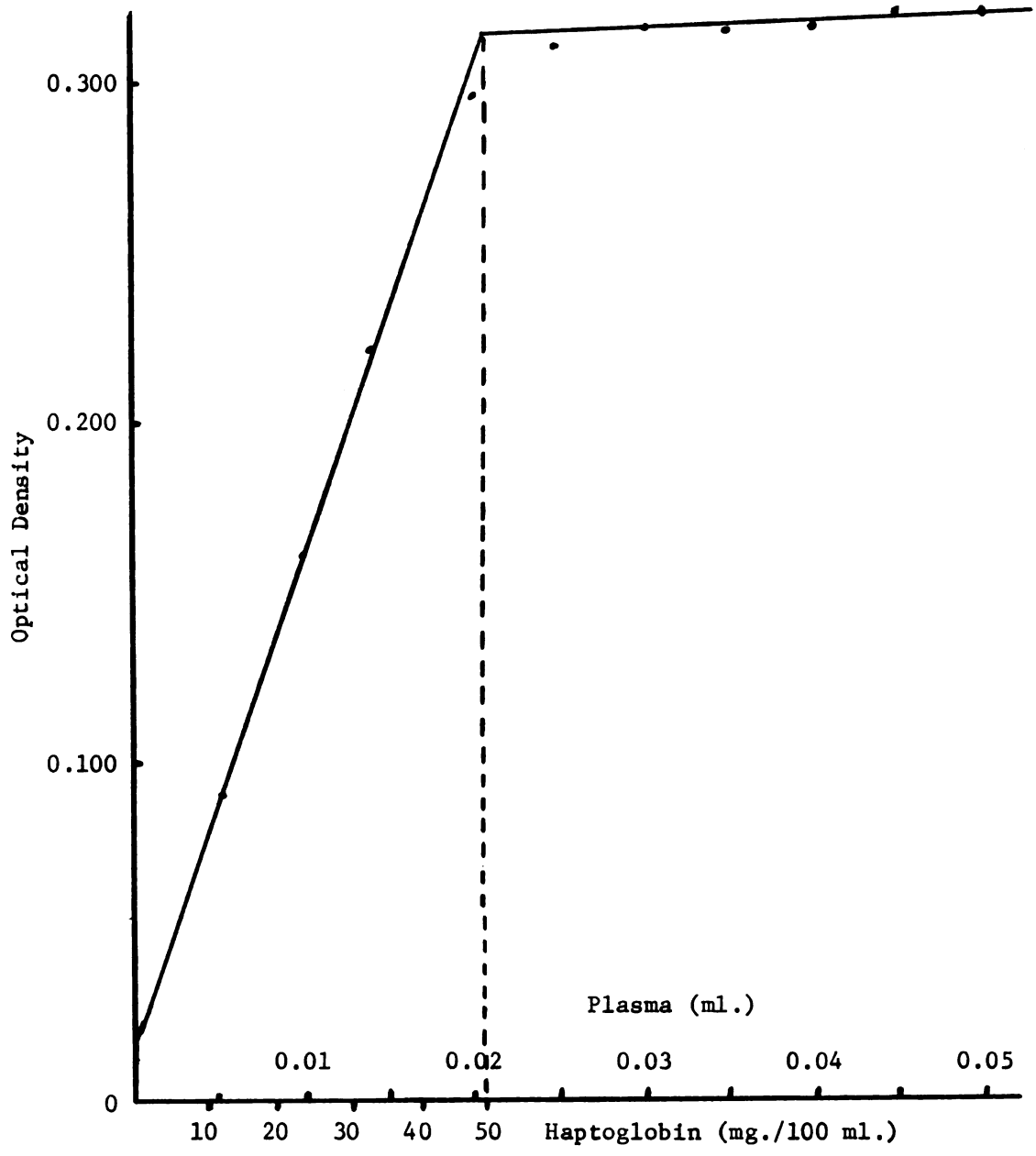

Figure B-2. Calibration curve for estimation of haptoglobin concentration (the abscissa shows at the point of change of gradient the normal pooled plasma contains 50 mg. haptoglobin per 100 ml.).

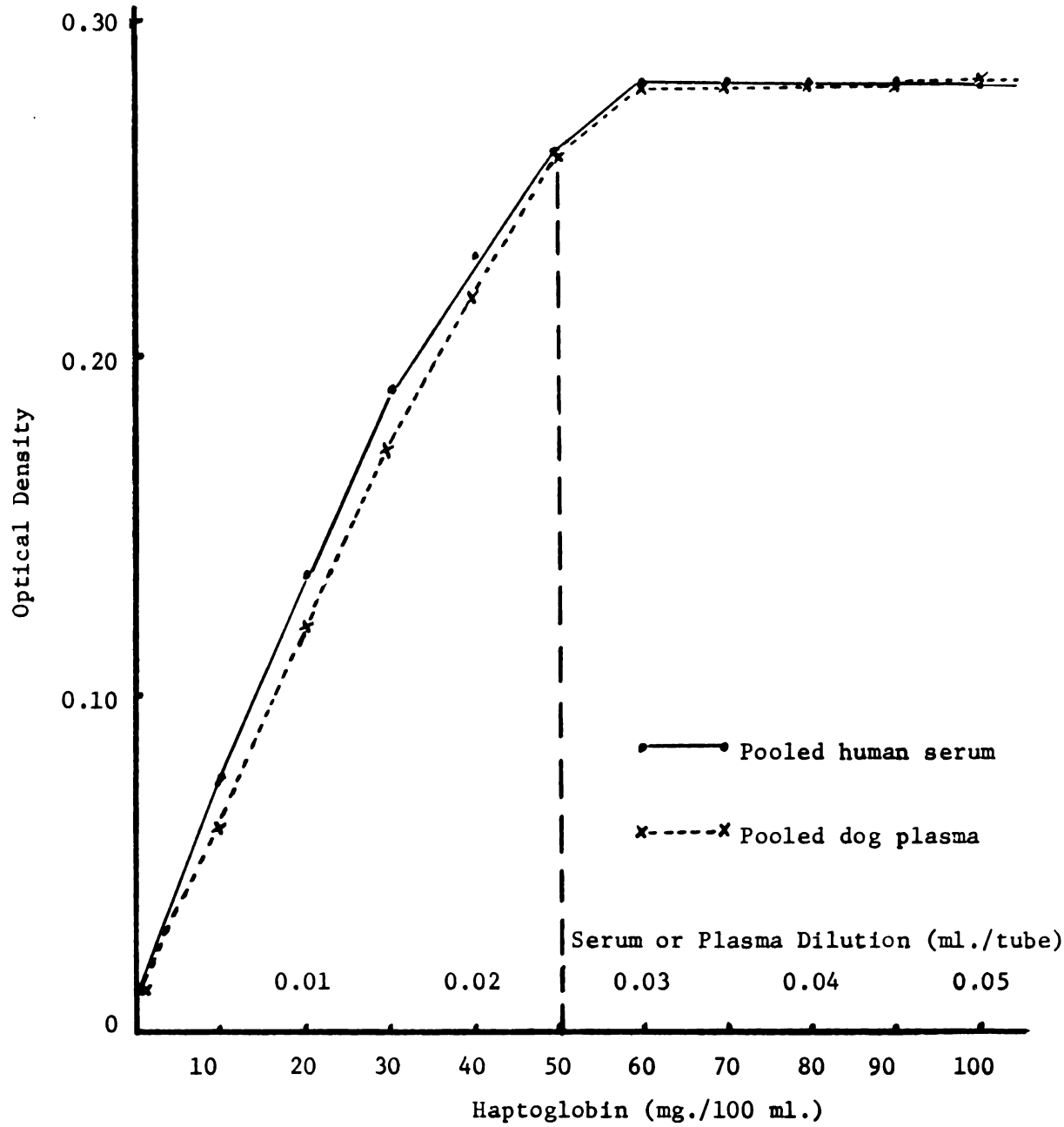

Figure B-3. Haptoglobin calibration curve (comparison human pooled serum and dog pooled plasma).

APPENDIX C

CALCULATION FORMULA

APPENDIX C

Calculation Formula

Comparison of Two Means

To obtain 2 sets of numbers and to test whether the mean of one set is significantly different from the mean of the other set, or whether the 2 sets can be regarded as drawn from 1 population.

Let $X_1, X_2 \ldots X_{n1}$, and $X'_1, X'_2 \ldots X'_{n2}$ be the 2 group samples.

Calculate as following terms

$$\overline{X} = \frac{\Sigma(X)}{n_1}, \qquad \overline{X} = \frac{\Sigma(X')}{n_2}$$

$$\sigma^2 = [\Sigma(X'^2) - \frac{(\Sigma(X'))^2}{n_2} + \Sigma(X_2) - \frac{(\Sigma(X))^2}{n_1}]/(n_1 + n_2 - 2)$$

$$\sigma = \sqrt{\sigma}^{2-}$$

$$t = \frac{\overline{X} - \overline{X}'}{\sigma} \sqrt{\frac{n_1 \times n_2}{n_1 + n_2}}$$

Having found the value of t, this enters the table of t with degree of freedom $(n_1 + n_2 - 2)$. If the value of t exceeds 5% level, this may assure that the populations are different.

Where

$\overline{X}$ = Mean
Σ = Summation
n = Number
σ^2 = Variance
σ = Standard deviation
t = Student t test
$\sqrt{\frac{n_1 \times n_2}{n_1 + n_2}}$ = Standard error of the difference in the mean of the 2 group samples
$(n_1 + n_2 - 2)$ = Degree of freedom