Part I

The Physiological Behavior of Oxypolygelatin

Part II

The Relative Sensitivity of the Mucosal and Peritoneal Surfaces of Guinea Pig Ileum to Histamine, Acetylcholine, and Specific Antigens

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FOREWORD

The retention study presented in this thesis was performed as part of a project on the use of modified gelatin solutions as blood substitutes. The members of the group all worked as a team but had separate duties to perform. Dr. Norman Abrahamsen and Dr. Walter Dandliker were responsible for the physical measurements and the chemical investigations, while the writer was charged with the responsibility for the physiological experiments. Analyses were done by each individual as required, but the main analytical experiments were done by Dr. Frank Lanni, with the aid of the writer.

The presentation of physical measurements and preparative procedures in this paper is used only for purposes of comparison and should not be interpreted as the work of the writer.

The literature review has been accepted for inclusion in a Festschrift issue of the Stanford Medical Bulletin in honor of Professor Thomas Addis.

Summary

The treatment of gelatin with a bifunctional condensing agent, glyoxal, and hydrogen peroxide resulted in the change of its physical state and physiological behaviour as estimated from the results of retention experiments in hemorraged rabbits. It was found that glyoxal treatment enhanced molecular weight and retention while subsequent treatment with hydrogen peroxide tended to decrease these attributes. The Oxypolygelatins were liquid at room temperature and were retained to a greater extent than untreated gelatins of comparable molecular weight.

The presence of Oxypolygelatin in the blood tended to increase the sedimentation rate of the erythrocytes. Experiments performed *in vitro* using systems of washed cells in Oxypolygelatin solutions demonstrated that the sedimentation rate was proportional to the concentration of Oxypolygelatin, dependent on the hydrogen-ion concentration, and varied with the species and age of the cells.

Sedimentation was found to be inhibited by sodium salicylate, the degree of inhibition being dependent on the relative concentrations of salicylate and protein. The inhibitory action of salicylate was found to be dependent on its molecular configuration since spatial changes in the molecule caused a loss of inhibitory activity.

Experiments on the physiology of anaphylactic shock indicated that surviving segments of guinea-pig ileum would react to histamine, acetylcholine and specific antigens only when these substances were presented to the serosal aspect of the gut. Previous exposure of the mucosal surface to these substances did not influence the reactivity of the serosal musculature.

Chapter I

The Effect of Gelatin in Shock Due to Experimental Hemorrhage and Trauma

- A Literature Review -

INTRODUCTION

There is ample evidence in the current literature to indicate that the mechanisms which initiate and sustain shock are extremely complex and involve the breakdown of many physiological and biochemical processes in the organism (1-25). The application of a stress to any one of the functional systems for a sufficient period will disrupt the body economy and eventually produce a cycle of events which leads to an irreversible collapse.

The search for a specific agent or remedial measure to combat shock is complicated by the lack of adequate means of determining when and where the shock cycle has been established. Failure of the circulation (unless shock has been produced by hemorrhage) does not occur in the early stages but only when the syndrome has been well developed; if this point is reached, no single substance is adequate to intercept the processes which sustain shock.

The stage of cardiovascular collapse is characterized by decreased volume flow, lowered blood volume, and, occasionally, by hemoconcentration. On the other hand, the blood pressure often remains at relatively constant

levels for as long as the animal's vasomotor system is patent and can compensate for the reduced blood supply. The impaired volume flow may be due to the failure of the capillary tone and the consequent enlargement of the vascular bed, resulting in a disparity between the capacity of the bed and the quantity of blood available to fill it, or it may be due to an actual loss of fluid and solutes because of an increased permeability of the capillary endothelium.

If the biochemical energy cycles have not yet been depleted (in vital organs such as the heart, liver, muscle, kidney, etc.) or irreversibly "poisoned", the transfusion of blood or plasma will generally restore the blood volume and maintain the flow at levels adequate for survival. The special beneficial effects of plasma reside in the colloidal osmotic effects of the dissolved proteins, of which the most active agent is the serum albumin.

The mechanism by which the circulation is able to maintain an almost constant volume of circulating blood was elucidated by Ernest Starling in 1896. In a paper on the "Absorption of fluid from the tissue spaces" (26), he suggested that a given blood volume was a resultant of two opposing forces: the net driving force of the hydrostatic head tending to force the fluid out of the circulation, and the colloid osmotic pressure of albumin attracting the fluid into the circulation.

Starling was the first to demonstrate that serum proteins could exert a measurable osmotic pressure when separated from a salt solution by a membrane permeable only to the latter. He assumed that there existed a gradient of hydrostatic pressure from the arteriolar to the venous end of the semipermeable capillary wall and that this pressure was somewhat greater than the colloid osmotic pressure at the first site, and lower than the colloid osmotic pressure at the second. According to this hypothesis there should be a transfer of fluid to the extravascular compartment in the first case, where the hydrostatic pressure exceeds the colloid osmotic pressure, and a reabsorption in the second case, where the osmotic pressure is the greater. Any decrease in the hydrostatic head, such as is occasioned by hemorrhage, causes the filtration pressure to fall, and if the drop is below the level of the osmotic pressure hemodilution will result.

Because of the obvious difficulties encountered in obtaining, processing, and preserving whole blood or plasma, investigations were undertaken during the first World War to find adequate substitutes - substances which would have the same colloid osmotic effects as plasma and which would otherwise not interfere with vital processes. The physiological effects of two such materials, gelatin and gum acacia, were studied and on the basis of

the results of Bayliss (27) acacia came into prominent use. It was later discarded because it could not be cleared from the body but tended to be stored in the liver, with consequent hepatic damage manifesting itself in the underproduction of serum proteins.

The undesirable features of gum acacia have been enumerated by Amberson (28). They are as follows: 1) the erythrocyte sedimentation rate is greatly increased; 2) the red blood cells become coated with gum, interfering with the diffusion of oxygen; 3) since gum leaves the circulation rapidly, the osmotic pressure is not maintained for much more than 48 hours; 4) gum is fixed in the tissues and is not digested; 5) retention in the liver results in a diminution of plasma protein output; 6) the material is fairly antigenic.

The first clinical tests of gelatin transfusion were made by Hogan (29), in 1915, who reported that the injection of 15% autoclaved gelatin was well-tolerated by patients in shock, but that occasionally they exhibited slight temperature rises. Since the solutions of gelatin were not liquid at ordinary temperatures it was necessary to autoclave them until they were fluid. As a result of this procedure the protein became severely degraded and the resulting small molecules tended to leave the circulation very rapidly. The seriousness of this condition can be realized by considering the fact that deposition

of particles in the tissue spaces increases the movement of fluid from the circulation to the extravascular compartment and further aggravates shock.

Owing to the development of techniques for the large-scale separation and purification of serum proteins, interest in the use of gelatin and of other non-hematic materials as blood substitutes waned until about 1940, when the exigencies of the war created a demand for an adequate transfusion fluid to supplement the supply of whole blood and for use when whole blood was not available.

A discussion of the value of gelatin in experimental shock is complicated by several factors. First, there seems to be little agreement as to the most desirable method of producing shock or of gauging its severity; second, there are no unique criteria by which the utility of a substitute may be determined since, depending on the time and mode of administration, both plasma and saline may be either effective or valueless. Finally, the preparations employed for transfusion may differ widely in respect to both protein concentration and molecular species.

The results obtained by various investigators will here be evaluated on the following points:

I. Efficacy. There will be discussed the influence of gelatin on those physiological processes which vary directly as a function of the molecular nature of gelatin,

viz., plasma volume, bleeding volume, blood pressure, and retention. II. Toxicity. This topic will treat of the degree of clearance of gelatin from the tissue, its effect on blood clotting, and its possible interference with the normal synthesis of plasma proteins. III. Antigenicity. IV. Pyrogenicity. V. Sedimentation of Erythrocytes.

I. EFFICACY OF GELATIN

The comparison of preparations on the basis of their ability to restore and to maintain blood pressure is a rigorous test, for the blood pressure does not generally show any decline until the stage of irreversible shock (cf. page 1) is reached, when transfusions of plasma or of homologous blood itself are seldom effective.

Ni (30) compared the pressor actions of gum acacia and donkey-skin glue (Ah-Chiao) solutions in their ability to restore and to maintain blood pressure in cats made hypotensive either by hemorrhage or by the injection of 1.5 mg. of histamine. He found that the transfusion of 6% Ah-Chiao solutions (colloid osmotic pressure 128-176) into hemorrhaged cats restored and maintained the blood pressure at pre-hemorrhagic levels even after the procedure had been repeated four times; on the other hand, similar injections of 6% gum acacia failed to maintain the pressure after the second bleeding. Following

the third bleeding the circulation was so depressed that further injection of gum proved useless.

Experiments on hypotension due to histamine shock demonstrated that Ah-Chiao was able to support the blood pressure at levels even higher than normal following four repeated alternate administrations of histamine and Ah-Chiao. Control experiments showed transfusions of saline to be ineffective. It was concluded by Ni that the large particle size of Ah-Chiao was the controlling factor in the maintenance of blood volume and, consequently, of pressure.

Taylor and Waters (31), and Taylor, Moorhouse, and Stonyer (32) found that 7% solutions of isinglass, a gelatin prepared by the acid extraction of fish-sounds, restored and maintained the blood pressure and volume (as judged by hematocrit) of hemorrhaged dogs, bled to permanent hypotensive levels of 30 mm. Hg, but were unable to observe this recovery when the blood pressure was reduced by traumatic shock resulting from the pulping of the thigh muscles or from the application of tourniquets to the hind quarters. They claimed that the failure of isinglass to restore the blood pressure was due to its lack of retention in the vascular system, since in their experiment hemoconcentration was not observed and since transfusion of whole blood into a control series, similarly traumatized, was likewise inadequate in maintaining the circulation and in supporting life.

However, it must be pointed out that in a previous paper (33) Waters had shown transfused isinglass to be cleared rapidly from the circulation via the kidneys. Urine samples taken 4.5 hours after transfusion gelled on cooling and were shown to contain 11.5% isinglass.

Comparative studies on the retention of isinglass and autoclaved gelatin were made by Waters (op. cit.) by determining the amount of non-plasma N in dogs subjected to 50% hemorrhage and subsequent transfusion with the respective materials. Blood samples taken at given intervals in both groups showed that there was more non-plasma N in the case of the gelatin-treated animals than in the ones transfused with isinglass and that gelatin N was still detectable three days after transfusion, while no isinglass could be found after 9.5 hours.

Waters' method of determining retention, however, was inaccurate. Inasmuch as he did not determine plasma volume, and thus was unable to know the total amount of circulating protein, he had to base his calculations entirely on the percent of gelatin. Since hemodilution occurs after hemorrhage, determinations of gelatin concentration are without real value in estimating the amount of injected protein remaining. Judging from the rise in hematocrit in the instance of the isinglass-treated dogs and from the maintenance of low hematocrits in the gelatin group, it is probable that his conclusions are only qualitatively correct.

Another source of error lies in his method of analysis. The gelatin determination carried out by Waters were based on the differential precipitation of plasma protein by trichloroacetic acid. Total protein N was determined by Kjeldahl analysis of tungstic acid precipitates while plasma protein N was estimated by similar analyses of trichloroacetic acid precipitates, the difference in the two values being considered due to gelatin-N. Because co-precipitation of gelatin with the serum proteins resulted when plasma samples were treated with trichloroacetic acid, this method was shown to be unreliable (Lanni, Feigen, and LeRosen (34); Knoefel and Lehmann(35)) and subject to as much as 100% error in the presence of low concentrations of gelatin.

The relative efficacies of glucose-saline, defibrinated blood, and autoclaved gelatin in restoring and maintaining carotid blood pressure in etherized dogs subjected to a standard massive hemorrhage were studied by Gordon, Hoge, and Lawson (36). The three preparations were transfused in volumes equal to that of withdrawn blood, and the percentage restoration of carotid arterial pressure, the maintenance of arterial pressure up to two hours following a transfusion, and the ability of the dogs to withstand a second hemorrhage were used as criteria in gauging the effects of the injected materials.

All three of these solutions produced almost complete recovery of blood pressure. The restitution of pressure by gelatin was generally still complete at the end of the first hour, and 86% complete at the end of the second hour, whereas defibrinated blood showed complete restoration at that time. In contrast to this, dogs treated with glucose-saline solutions showed a drop to 62% of the post-transfusion pressure within thirty minutes. The animals treated with gelatin suffered no greater fall in pressure during the second hemorrhage than did those which were given defibrinated blood, while dogs injected with glucose-saline were less able to tolerate a second hemorrhage than animals of the other two groups. The average restoration of the blood volume at the time of the second hemorrhage, compared to that of control, was 49% in the instance of the glucose-saline group and 86% for the group transfused with gelatin.

The ratio of bleeding volumes before and after the administration of a substitute was found by Lawson and Rehm (37) to be useful in judging the relative merits of various fluids given in replacement of blood lost by hemorrhage in barbitalized dogs. The justification for this method of evaluation is based on the assumption that those preparations which yield the larger bleeding volumes, when given to subjects who continue to lose fluid, might reasonably be expected to support life longer than materials which give the smaller volumes.

The first bleeding volumes (taken by controlled hemorrhage) represented the amount of blood which had to be removed to lower the arterial pressure to 60 mm. Hg, it having been determined by previous control experiments that an additional small hemorrhage beyond this point would be fatal.

A comparison of the ratios of the first to the second bleeding volumes failed to reveal any difference in effectiveness between plasma or serum (H_2/H_1 - 68-73%) but showed that both were slightly more effective than autoclaved "isomolar" gelatin solutions (H_2/H_1 - 61%). The injection of saline after hemorrhage showed the second bleeding volume to be restored to 38%, whereas if no treatment were given the second, fatal, hemorrhage amounted to only 19% of the control bleeding. It was concluded that the bleeding volume is a function of the amount and type of colloid used in the replacement fluid.

Using a similar technique, these authors recently showed a certain preparation of gelatin (P-20) to be more effective than plasma or serum in restoring the bleeding volume four hours after a previous controlled hemorrhage (38). Barbitalized dogs pre-treated by intravenous injections of saline (to equalize the hydration states) were almost completely exsanguinated by means of controlled bleeding and transfused with various replacement fluids. Comparisons of the fatal four-hour hemorrhage

volumes showed the order of relative utility of the fluids in reversing the effects of the first hemorrhage to be the following: 1) whole blood and 4% gelatin; 2) plasma and serum; 3) 3.45% gelatin L-80; 4) 2.8% gelatin B-20610-51; 5) 0.9% NaCl. The gelatin solutions were approximately isomolar and had about the same osmotic pressure in vitro as dog plasma.

Little and Wells (39) studied the relative losses of plasma and intravenously administered 6% gelatin from the intestinal capillaries of the dog after trauma was applied to an exteriorized loop of the ileum. Following irritation of the gut, the loop was suspended in a beaker containing warmed saline solution and the transudate was collected at regular intervals. Analyses of the blood and transudate for gelatin and plasma protein revealed that the intestinal capillaries were injured sufficiently to permit the partial or complete passage of serum proteins but to allow only 35-60% of the gelatin to escape.

Parkins and co-workers (40) found that transfusions of gelatin into anesthetized dogs shocked by immersion into hot water (72° C.) compensated for the loss of plasma from the blood-stream and prevented hemoconcentration to the same extent as did the administration of plasma in a separate series of animals. Although seven of the eight dogs treated with gelatin succumbed after a progressive decline in blood pressure, the average

survival time showed a 100% increase over that of saline controls. Under identical conditions only one out of four of the plasma-treated dogs died.

The effectiveness of gelatin, plasma, and saline in combatting post-hemorrhagic circulatory failure was compared by three different bleeding techniques (40). Immediate injection of gelatin or saline preparations into dogs subjected to a massive rapid hemorrhage resulted in the restoration and maintenance (3-24 hrs.) of their blood pressures and in subsequent hemodilution. Although the percent recovery for both groups at the 3- and the 24-hour periods was the same, gelatin showed a greater restitution fifteen minutes after injection than did saline.

A controlled triple-hemorrhage procedure resulted in a more critical method of evaluating gelatin. Nembutalized dogs were bled and transfused with equal volumes of gelatin or saline, three times at hourly intervals. Untreated dogs died within a few hours following the third hemorrhage. In spite of the fact that restoration of plasma volume and blood pressure was not marked after transfusion of saline, compensation did occur and two of five dogs made a gradual recovery. The gelatin-treated dogs were definitely more resistant to repeated bleeding. The blood pressure drop was only slightly greater after the second hemorrhage than following the first; and in

every instance the full amount of blood (30 ml./kg.) could be removed during the last bleeding without the blood pressure's declining to dangerous levels. Three of the dogs treated with gelatin showed a normal blood pressure after the last hemorrhage, and recovered. Homologous plasma injections did not prove more efficacious under similar conditions of testing.

The homeostatic adjustments of the cardiovascular system to hemorrhage and the efficiency of fluid replacement are greatly influenced by the speed of the bleeding and by the length of persistence of the low blood pressure. When the bleeding is protracted and the blood pressure is maintained at critical values, the reaction to replacement of the plasma volume is lessened and the efficacy of the infused material diminished. In spite of the fact that hemodilution and increase in blood pressure may still occur upon infusion of substitutes after a protracted hemorrhage, the mechanisms determining the peripheral resistance might not be adequately restored and circulation will fail. Under such conditions saline transfusions are seldom of value while similar treatment with plasma is often effective.

Parkins et al. (40) also compared the effectiveness of gelatin with that of saline and plasma in dogs subjected to prolonged hemorrhage and sustained hypotension produced by bleeding the animals in three stages

over a period of thirty to forty minutes and maintaining a pressure of 30-40 mm. Hg for an additional thirty minutes by intermittent bleeding. This experiment gave the following results: 1) all of the untreated dogs died within two and one-half hours; 2) three-fifths of the saline treated animals died in two to six hours, the survivors recovering in spite of a poorly maintained blood pressure; 3) all of the gelatin- and plasma-treated dogs lived. There was no difference in the blood pressure recovery after gelatin or plasma infusion; in both cases it was restored to prehemorrhage levels. Hemodilution after gelatin exceeded that after plasma, presumably because of the fact that 6% gelatin preparation employed in this study had a greater colloid osmotic pressure value than plasma.

The superiority of intermittent over single massive transfusions of plasma, gelatin, or saline was demonstrated by Swingle and his associates (41, 42, 43) in experiments on anesthetized dogs rendered traumatic by one of the following methods: 1) chronic application of tourniquets; 2) the application of 750 pounds of pressure by means of an hydraulic press; 3) muscle-pulping with a raw-hide mallet; and 4) gun-shot. The results of the three papers are presented in some detail because they are important for the subsequent discussion.

A. Tourniquet Shock

Shock was produced by releasing tourniquets which had been applied to both hind legs for a period of five hours (41). The symptoms shown by control animals were a marked swelling of the legs, hemoconcentration (71%), and a great reduction of plasma volume. The injection of a dose of plasma amounting to 25 ml./kg. ameliorated the hemoconcentration but in most cases failed to prevent shock, whereas the administration of gelatin or plasma, given intermittently in five equal portions, prevented all signs of shock and insured the survival of the injured animals.

B. Muscle-Pulping

The hind legs of anesthetized dogs were traumatized by the delivery of 400-800 blows with a raw-hide mallet to the external aspects of both thighs (41). As the result of this treatment the legs were numbed and severely bruised, but the skin was unbroken and the bones unfractured. Trauma was continued until the mean arterial pressure fell to 60-70 mm. Hg. In consequence of such injury, the plasma volume fell but the cell volume remained unaltered.

Although shock following muscle-pulping was not greatly responsive to transfusions, the intermittent injection of plasma prevented shock and aided survival of

77% of the animals while the single injection of plasma prevented shock in only 20% of the dogs. Fifty percent of the dogs treated with gelatin or saline (isotonic or hypertonic), irrespective of the method of their administration, survived, indicating that the effect of these two materials on this type of shock was due to factors other than colloid osmotic pressure.

C. Duncan-Blalock Shock

Dogs, deeply anesthetized with morphine and Nembutal, were traumatized for seven hours by the application of 750 pounds of pressure to the right hind leg with an hydraulic press (42). The average survival period of untreated dogs upon release from compression was eleven hours. The blood pressure declined slowly for several hours but fell precipitously immediately preceding death. The progressive reduction in blood pressure was accompanied by hemoconcentration, decrease in plasma volume, and swelling of the limb resulting from the increased transudation of plasma. Most of the decline in plasma volume was due to plasma loss into the area of injury.

Immediate transfusion of a massive dose of gelatin (40 ml./kg.) did not prevent death. It was poorly retained due to the capillary damage. Intermittent gelatin transfusions, given over an eight-hour period, prevented shock in 73% of the dogs but similar injections of saline

prevented death in only 35% of the cases. Heparinized homologous plasma proved to be more effective than gelatin solutions.

D. Shock Due to Gun-Shot Wounds

Three shots, containing an average of 375 lead pellets, were fired into each of the thigh muscles of a deeply anesthetized dog from a distance of seven feet (43). A certain amount of bleeding resulted, was accompanied by noticeable swelling of the leg first injured. Ninety percent of the control animals succumbed to this type of trauma.

The blood pressure declined to low values immediately after injury. Hemoconcentration was not marked but the plasma volume was lowered by 50%, indicating loss of blood. This was supported by necropsy findings, which showed the presence of large amounts of extravasated blood collected around the ruptured vessels.

Transfusions of plasma given in five divided doses prevented shock in most of the animals whereas a single massive infusion did not. A great deal of the infused plasma was lost into the injured area. Intermittent infusions of gelatin and of isotonic saline were both efficacious in preventing shock, with gelatin slightly the more effective. A single large injection of saline was without value.

An interpretation of the greater efficacy of small intermittent infusions over single injections can be made in the light of the mechanisms proposed by Starling (op. cit.). Thus, in the case of the former, the volume given at each injection is not sufficiently large to increase the pressure appreciably (driving force) so that less plasma will be driven from the vascular compartment into the injured tissues, while in the latter, on the other hand, the arterial pressure is raised and the transfused material leaks out rapidly.

The uncertainty surrounding the evaluation of the comparative efficacy of gelatin as a substitute is typically illustrated in the previously discussed results of Swingle et al. This difficulty derives from the qualitative differences of conditions under which the tests were performed. Thus, although gelatin appears to be as effective as plasma in shock resulting from chronic eschemia (limb tourniquets, Duncan-Blalock press), it is only as adequate as saline when trauma is due to contusion of the thigh muscles.

The comparative efficiency is also varied with the method of administration. The intermittent infusions of plasma or its substitutes, when given in small amounts over a period of hours, are more effective in preventing shock than a single massive infusion.

Some of the paradoxical results obtained in gauging the relative efficiency of gelatin become clearer when one

considers the nature and the results of the various shock-inducing procedures. The experimental shock states in which the action of substitutes was compared by Swingle et al. resulted from either one or both of two methods, contusion and compression. Compression shock results from tissue changes after a prolonged ischemia and is characterized by loss of plasma, alone, into the site of trauma. Contusion shock succeeds upon actaul damage to the muscle substance and involves the loss of whole blood.

The blood volume is reduced in both cases but hemoconcentration results only in the former; since cells are not lost in this instance, the shock lends itself more readily to treatment by plasma substitutes. A further complication which accompanies contusion shock is the severe damage to the nervous system. This has many undesirable influences, not the least of which is the reduction of vasomotor tones causing the extensive accumulation of blood in "silent areas" such as the splanchnic region.

On the basis of the effects just mentioned an analysis of the factors involved in the four types of experiment made by Swingle will explain the success or failure which attended the use of gelatin in each instance. The results are summarized in the appended table.

Summary of Initiating and Sustaining Factors in Shock
Resulting from Various Forms of Trauma

Experiment	Type	Initiating Factor(s)	Sustaining Factor(s)	Pathological Findings	Action of Gelatin
Limb tourniquet	Compression	Ischemia without muscle damage	Increased capillary permeability	Plasma loss	Good
Duncan-Blalock press	Duncan-Blalock press	Ischemia; some damage to muscle substance	Increased capillary permeability	Plasma loss	Poor
Muscle-pulping	Contusion	Damage to muscle substance; hemorrhage; nervous factor. No ischemia	Capillary atony; blood loss	Reduced blood volume and stagnation	Good in intermittent doses
Gun-shot wounds	Contusion	Severe muscle injury; blood loss. No ischemia	Chronic blood loss	Hemorrhage into area of injury	Fair in intermittent doses

II. TOXICITY

There are two orders of toxic reactions, the explicit and the implicit, encountered in subjects transfused with gelatin solutions. One of these is due either to infection by various spore-forming organisms (such as anthrax or tetanus) which are not entirely removed during the process of extraction of the hog- or calf-skin, or to the intoxication with by-products (e.g., histamine); the other is implicit in the macromolecular nature of gelatin and leads to the suppression of plasma protein synthesis by damaging the liver and the reticulo-endothelial system through its failure to be digested or excreted.

The extraction of unwashed hides in acid solution, used in the earlier processes of manufacturing gelatin, frequently resulted in the carry-over of viable anthrax spores (31) into the preparation. Eventual heat-treatment served only to rupture the spores and to liberate the organisms. Modern methods of gelatin production which utilize bones or tendons as sources of the protein have completely removed the danger of potential infection as a bar to the transfusion of gelatin. Indeed, from the standpoint of sterility gelatin solutions present a better choice than whole blood! Gelatin is a poorer growth medium than the latter and, whereas gelatin can be autoclaved to insure freedom from contamination, whole blood

is generally assumed to be sterile by having been collected and stored under aseptic conditions. One is often prone to overlook the fact that blood for transfusion is subjected to comparatively few tests, e.g., blood-typing and complement fixation. The presence of such organisms as P. vivax, trypanosoma, or leishmania and the occurrence of viruses often go disregarded. A follow-up study made by Brightman and Korns (44) on 605 patients who had been transfused with pooled plasma revealed that such therapy carried a significant "risk of a serious and possibly fatal complication" due to the fairly high incidence of post-transfusion homologous serum jaundice. The causative agent of such icterus was unknown but the possibility of its viral origin has not been ruled out.

Taylor and Waters (31) chose isinglass as their source of gelatin in order to obviate the possibility of transferring tetanus or anthrax by transfusion. Although the use of this material reduced the danger of infection it did not eliminate toxic reactions. The starting material, when dissolved in water, liberated "globules of oily matter which tended to collect on the surface and was in great part responsible for a disagreeable fishy odor." Even after the fatty impurities had been removed the odor persisted and the material, upon injection into hemorrhaged dogs, tended to aggravate the hypotension.

Subsequent work of Waters (33) and Taylor, Moorhouse, and Stonyer (32) failed to reveal any untoward effects of

isinglass or gelatin on the clotting mechanism of transfused dogs; furthermore, they were able to show that the rate of regeneration of plasma proteins in hemorrhaged dogs was uninfluenced by isinglass. Histological examination of the livers, kidneys, and spleens of chronically isinglass-treated rabbits showed these organs to be normal and gave no evidence of storage.

Knoefel and Lehmann(35) attributed the death of one of their dogs to be due to a transfusion with a high molecular-weight gelatin fraction (M.W. 69,000), obtained by alcohol precipitation. Twenty minutes after injection, the animal suffered respiratory stridor and a fall in blood pressure; death occurred fifteen minutes later. Necropsy showed diffuse bronchopneumonia; atelectasis was marked and a number of alveoli and bronchioles containing fluid were observed.

Excepting this case, the transfusion of various gelatin fractions was well-tolerated by the other dogs. No reduction in total circulating plasma protein followed the injection of gelatin and none of the gelatin fractions reduced the amount of hemoglobin available.

Gordon, Hoge, and Lawson (36) found no evidence of immediate toxicity due to the presence of depressor substances when Knox calf-skin gelatin was injected into hemorrhaged dogs in doses up to 90 ml./kg. Transfusion of these solutions into man were uneventful. Post-mortem

examinations of the test animals produced no evidence of thrombosis, hemolysis, embolism, or capillary damage.

Lawson and Rehm (op. cit.) found no toxic sequelae following the transfusion of 6% gelatin (Knox P-20) into dehydrated, hemorrhaged dogs. In these cases blood replenishment during the final hemorrhage was as great as in animals treated with whole blood. Higher concentrations of gelatin produced no significant cardiovascular damage.

Parkins et al. (op. cit.) observed that dogs tolerated repeated administrations of large volumes of gelatin without any serious toxic reactions specific to gelatin. Unfavorable effects, when present, were reversible and were frequently produced by the injection of comparable amounts of saline or plasma. Measurements of urea clearance showed the kidneys to be unimpaired after single or repeated infusions of gelatin. Liver function, as judged by the glucose tolerance and prothrombin time methods, was normal; bromsulphalein tests showed an increased retention of the dye after plasma as well as gelatin transfusions.

The intravenous administration of gelatin following a rapid massive hemorrhage did not interfere with the replacement of plasma proteins, restitution occurring at about the same rate as the disappearance of gelatin from the circulation. Chronic tissue storage was not observed in any of the gelatin-treated dogs; tissue changes which

did occur were reversible and only slightly more obvious than those which followed transfusion of an equivalent volume of plasma.

Robscheitt-Robbins, Miller, and Whipple (45) studied the effects of chronic gelatin administration on the ability of doubly-depleted dogs (anemic and hypoproteinemic) to produce hemoglobin and plasma proteins. The intravenous injection of gelatin into the experimental animals gave no immediate toxic response and occasionally contributed to the synthesis of new hemoglobin and plasma protein. The results of concurrent administration of amino acid mixtures or casein digests offer definite proof of utilization of the amino acids to form blood proteins. The site of the toxic action of gelatin was not established since the histological findings were negative but the depression of blood-protein synthesis seemed to implicate the liver. Experiments on the oral administration of gelatin showed the material to be utilized to form needed plasma proteins and hemoglobin--the absent amino acids being presumably supplied from the body's protein stores or from tissue protein catabolism.

III. ANTIGENICITY

None of the investigations, in which an especial effort was made to detect hypersensitivity due to calf-skin or ossein gelatin (36, 37, 40, 45) proved gelatin

to be antigenic. On the other hand, isinglass was found capable of producing mild hypersensitivity when injected after precipitation with alum (31). Mild anaphylactic reactions were observed in isinglass-treated etherized dogs when they were re-injected with this material within twelve days. When the interval was extended to three or four weeks such reactions were absent.

Taylor and Waters (31) attributed the antigenicity of this material to the presence of fish protein contaminating the isinglass, and later Waters (33) showed that isinglass solutions could be rendered non-antigenic by heat treatment.

IV. PYROGENICITY

Febrile reactions following transfusion with gelatin were observed by investigators who used isinglass (31, 32, 33) and some of the earlier gelatin preparations. Pyrogenicity was likewise observed by Parkins et al. (40) in solutions of saline as well as of gelatin, but the temperature rises were not serious.

Pyrogenicity is not a property unique to gelatin but can occur in any solution carelessly prepared or incautiously handled before transfusion. Once present, pyrogens are difficult to remove since they are generally considered to be heat stable.

Taylor, Moorhouse, and Stonyer (op. cit.) claim to have reduced the pyrogenicity of their isinglass solutions by autoclaving at twenty pounds' pressure for fifteen minutes and by more efficient filtration. Campbell and Cherkin (46) eliminated reactions from pyrogenic solutions by treating them with suitable amounts of hydrogen peroxide.

V. SEDIMENTATION OF ERYTHROCYTES

The increase in erythrocyte sedimentation rate due to the presence of gelatin or of other long-chain molecules in the blood has been a universal observation (27, 32, 40). This decreased suspension stability has been attributed to the great tendency of these substances to induce rouleaux formation among the red cells. Although no definite pathology has yet been ascribed to this phenomenon it is conceivable that oxygen carriage may be hampered and the danger of thrombus formation (47) increased. A fuller treatment of the mechanism of red cell sedimentation in gelatin is reserved for a subsequent chapter.

Chapter II

The Retention of Gelatin and Oxypolygelatin in the Circulation of Hemorrhaged Rabbits

Chapter II

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Chapter II

The Retention of Gelatin and Oxypolygelatin in the Circulation of Hemorrhaged Rabbits

I. INTRODUCTION

Protein solutions have been extensively used in the prophylaxis and management of clinical shock. The restoration of fluid to the circulation and the prevention of its further loss are both dependent on the colloid osmotic pressure exerted by the injected material. Providing that no fundamental changes have occurred in the biochemical energy cycles of the body to render the shock irreversible, gelatin solutions may often be efficacious in treating certain types of experimental shock produced by the acute loss of blood or by the progressive loss of plasma.

Gelatin is easily available and can be rendered sterile by autoclaving; it is non-toxic, as evidenced by its failure to be stored in the tissues or to interfere with the normal action of various organs; and it can readily be eliminated through the kidneys. The injection of large quantities of gelatin has shown the substance to be well-tolerated and entirely devoid of antigenic action; furthermore, it engenders no chronic pathology when administered to dehydrated animals; and it does not depress the normal synthesis of hemoglobin or plasma proteins.

The chief objection to the use of gelatin for transfusion is a practical one: in reasonable concentrations at room temperature the material is a gel and requires the use of special warming apparatus for its successful administration. It is possible to circumvent such gelling by extensive autoclaving but such a procedure introduces a further objection in that the resulting product becomes severely degraded and therefore is rapidly lost from the circulation. (48)

One of the necessary conditions for the effectiveness of a blood substitute is that it persist in the circulation; that is to say, the material must remain in the blood stream long enough to tide the injured animal over a crisis to the condition in which its own homeostatic mechanisms can once again become effective.

Since undegraded gelatin solutions are well-retained, the aim of our general program was to find a suitable chemical means by which their gelling proclivity could be decreased without the sacrifice of retention. In a separate report (49) there have been presented the details of manufacture and the physico-chemical properties of a material, Oxypolygelatin (OPG), which fulfills these requirements.

The purpose of this report is to describe the retention assay, to indicate the influence of each reagent on the retention of the final product, and, finally, to present the results obtained on testing several pilot

plant lots of gelatin and Oxypolygelatin designed for clinical experiments.

II. EXPERIMENTAL

The preparation of Oxypolygelatin consists of two basic processes: one, the treatment of gelatin with glyoxal, which results in the formation of a viscid condensate, the "polygel", and, two, the oxidation of the polygel with hydrogen peroxide at autoclave temperatures to produce the liquid Oxypolygelatin.

The selection of the reagent ratios used in each step was based on the following considerations: the amount of glyoxal used in the condensation reaction should permit the formation of large aggregates without causing the material to gel in the first step of the reaction, consequently to interfere with the peroxide treatment, and, secondly, the amount of peroxide had to have a lower limit imposed by the quantity of this material required for the destruction of pyrogens (46, 50). The upper limit was to be set by the requirement that the product should be well retained in the circulation.

Two questions had to be answered before the final reagent ratios were chosen; a) What is the relative influence of the reagents on the molecular weight? and b) How does the retention of the final product vary with this function? In a separate publication by the members of the blood substitutes group (49) changes in various

physical properties of gelatin as a function of glyoxal and hydrogen peroxide have been considered. In this report an attempt will be made to show how such reagent variations influenced the retention values and to what extent these were correlated with the physical findings.

The large scale preparations were, in the main, designed to reveal the reproducibility of the basic process and to elucidate the changes in retention which could occur if other variables were imposed. The retention of several untreated gelatins was also studied for comparison. To interpret the variations in retention results obtained in assaying the pilot plant batches a brief description of the preparations and a table (Table I) summarizing their physical properties is included in the following section.

A. Materials

1. Oxypolygelatins

Although the exact details of manufacture of the Oxypolygelatins varied somewhat from lot to lot, the main process can be summarized as follows.

Removal of Calcium.- Early experiments indicated that decalcification reduced the tendency of both the treated and the untreated gelatin to form gels. In order to obviate such gelling it was necessary either to start with a commercial gelatin containing negligible amounts of Ca^{++} or to remove Ca^{++} from the parent material by passing the warmed 7.5% solution of gelatin through a

Table I. Physical, Chemical, and Physiological Properties of Oxypolygelatins and Gelatins

Preparation	Parent Material	Remarks- Heating and Treatment Conditions		Reagent Ratio g. Glyoxal / g. Gelatin	Reagent Ratio g. H_2O_2 / g. Gelatin	Condition at Room Temperature	% Protein	Neutralization Equivalent ml. 0.1 N NaOH per Liter
Oxypolygelatins		Poly.	Oxy.					
91	Knox C-1	20'-121°C	20'-121°C	0.025	0.031	Liquid	5.47	105.0
111	"	20'-121°C + $NaHCO_3$	20'-121°C	"	"	"	4.75	60.0
113	"	20'-121°C	20'-121°C	"	"	"	4.70	122.5
116	"	"	"	"	"	"	4.65	151.5
117	"	"	"	"	"	"	5.35	102.5
118	"	"	"	"	0.027	"	5.54	98.0
11,115AY	"	"	"	0.014	0.026	"	4.89	-- *
11,381Y	"	30'-90°C	60'-90°C + 20'-121°C	0.017	0.025	"	5.25	--
12,676AY	Wilson 50,980	59'-90°C	150'-70°C + 20'-121°C	0.010	0.010	"	5.08	--
Knox I-3	Knox	20'-121°C	20'-121°C	0.025	0.030	"	6.54	--
Knox II-3	"	"	"	0.0125	0.015	"	6.55	--
Knox II-5	"	"	"	"	0.0225	"	6.55	--
Gelatins								
Knox C-1	Knox C-1	40°C-30'				Gel	5.26	--
78P	"	20'-121°C, Decalcified				"	6.48	--
Knox P-11-20	Knox	"				"	6.00	--
Wilson A-I	Wilson 50,980	50'-95°C				Liquid	4.50	--
Upjohn-B20610-50						"	5.70	--

* not tested

Table I. (cont'd.)

Preparation	pH	Relative Viscosity pH 7.3 at 37° C	Osmotic Pressure (mm. H_2O for 1% soln.) pH 7.3	Osmotic Pressure (mm. H_2O for 1% soln.) pH 4.5	Calculated Mean Mol. Wt. at pH 7.3 37° C, 1% soln.	Retention at 5 hrs. Mean %	Retention at 5 hrs. σ_{Mean}	Retention at 24 hrs. %
Oxypolygelatins								
91	5.65	1.23	99	97	26.460	41.8	±1.58	13.2
111	6.67	1.23	136	108	19.260	27.75	±2.34	0
113	5.32	1.32	108	104	24.250	33.85	±2.60	0
116	5.12	1.22	96	94	27.290	63.00	±6.32	15
117	5.63	1.28	113	92	23.190	33.40	±1.73	6.7
118	5.63	1.32	100	72	26.200	37.50	±6.67	14.8
11,115AY	5.23	1.20	122		21.480	35.20	±1.63	12.8
11,381Y	5.62	1.35	98		26.730	39.00	±4.05	9.76
12,676AY	5.29	1.26	90	87	29.110	37.60	±3.26	15.2
Knox I-3		1.23		107	24.480	47.63	±6.38	23.3
Knox II-3		1.25		112	23.390	39.60	±2.59	25.7
Knox II-5		1.21		109	24.030	23.90	±4.42	6.8
Gelatins								
Knox C-1		1.40	62		42.250	47.65	±8.47	12.66
78P	7.30	1.43	78		33.160	29.80	±4.36	14.83
Knox P-11-20	7.20	1.38	80	80	32.750	37.66	±1.35	9.00
Wilson A-I	7.15	1.33	62		42.250	34.65	±4.66	0
Upjohn B20610-50	6.90	1.22	127	120	20.620	8.80	±4.49	11.4

column containing an ion-exchange resin (Zeo-Karb-H). The solution was considered to be decalcified when the addition of 5 drops of 1.5 M $K_2C_2O_4$ formed no precipitate with a 5-ml. portion of gelatin. Once the decalcification was complete, the solution was adjusted to 5% protein on the basis of an analysis for nitrogen (34). At this point the pH of the solution was 6.8-6.9.

Treatment with Glyoxal.- The 5% decalcified gelatin solution was brought to a temperature of 95° C. in a glass-lined steam-jacketed tank. Glyoxal (usually 0.025 g. glyoxal/g. gelatin) was stirred in and the temperature was then maintained at 95-100° C. for 30 minutes. The material became very dark at this stage and would form an unworkable resin if the heating were allowed to continue without performing the following step of oxidation.

Treatment of the "Polygel" with H_2O_2.- Sodium chloride, 9.0 g./liter, was first added to the polygelatin solution, after which the pH was adjusted to 7.3-7.4 by the addition of about 2.5-3.0 ml. 5 N NaOH per liter of gelatin. Thirty percent H_2O_2 (du Pont Technical grade) was next added (0.0166 g./g. gelatin) and the heating was continued until the polygel was completely liquid; the material was then filtered, packed in transfusion bottles, and autoclaved for 20 minutes at 121° C.

The resulting yellow or light-brown solutions were sterile and non-pyrogenic. Freedom from pyrogenicity was achieved by the use of pyrogen-free distilled water and

the employment of sufficient hydrogen peroxide in the process of manufacture to destroy pyrogens (46).

Oxypolygelatins thus prepared were liquid at room temperature and had a pH, of ca. 5.6. In spite of the low pH, the preparations could be safely injected without previous neutralization, because of the low titratable acidity of Oxypolygelatin. Some of the properties of the various batches are presented in Table I.*

2. The Untreated Gelatins

Knox C-1 Gelatin.- This material was a special dried calcium gelatinate supplied by the Knox Gelatine Protein Products, Inc., through the courtesy of Dr. D. Tourtelotte who also supplied preparations OPG I-3, II-3 and II-5. A 5% solution of the gelatin required warming to 35° C. before injection. This particular lot of gelatin was used as the starting material in the preparation of most of the Oxypolygelatins listed in Table I.

Knox Gelatin 78-P (C-1, decalcified and autoclaved).- The first step in the manufacture of Oxypolygelatin, as has been indicated previously, was the removal of calcium by treating gelatin solutions with Zeo-Karb-H. It was desired to determine whether the removal of Ca^{++} had any effect on retention. The 5% solution of 78-P was a gel at room temperature.

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*The Oxypolygelatins prepared by ourselves were manufactured either in our own laboratory or in the plant at Don Baxter & Co. We wish to express our gratitude to Mr. Arthur Cherkin at this organization for his interest and aid rendered in the course of this work.

Knox Gelatin P-11-20.- The P-11-20 preparation has been extensively tested by other laboratories and was supplied especially for transfusion. It is a Knox gelatin similar to C-I, which has been autoclaved for twenty minutes at 121° C. Like 78-P, the 5% solution of P-11-20 gelled at room temperature.

Upjohn Gelatin B20610-50.- The Upjohn preparation was a so-called degraded gelatin which had been autoclaved for 180 minutes until a 5% solution no longer gelled at room temperature. The solution was supplied us by the Upjohn Company, through the courtesy of Dr. John F. Norton.

Wilson Gelatin AI.- The parent material, Wilson Lot No. 50,980, was kindly supplied by the Wilson Company through the courtesy of Dr. David Klein. The AI preparation was made by heating Wilson 50,980 at 95° C. for 50 minutes to yield a liquid material at room temperature.

B. Experiments on the Variation of Reagent Ratios

The independent influences of glyoxal and peroxide on the retention of the final product were examined by testing a system of preparations in which one of the substances was constant while the other was present in variable amounts.

Experiments designed to test the influence of glyoxal and peroxide on retention and osmotic pressure were set up in the following manner: Wilson gelatin 50,980 was dissolved in sufficient water to yield a 5% solution. This solution was then separated into four batches, each

lot being treated with a different ratio of glyoxal per gram of gelatin (0, 0.005, 0.01, 0.02), and heated at 95° C. for 20 minutes. After heating, each batch was subdivided into five aliquots which were respectively treated with different amounts of hydrogen peroxide, ranging from 0 to 0.0666 g. H_2O_2/g. gelatin. The reaction was completed by autoclaving the solutions at 121° C. for 20 minutes.

Peroxide ratios in excess of 0.0166 g./g. gelatin produced too great an increase in the osmotic pressure of the resulting material to warrant physiological testing, while the higher glyoxal ratios (particularly under conditions of low peroxide concentration) yielded resinous and often completely insoluble preparations. Thus the selection of materials for detailed physiological characterization was limited to the range of reagent ratios which would not only produce fluid preparations but which would give measurable retention values. The glyoxal ratio giving fluid products with every concentration of H_2O_2 was 0.01 g./g. gelatin, while the peroxide concentration yielding fluid preparations for this ratio of glyoxal was 0.0166 g./g. gelatin.

C. Methods

1. Hemorrhage, Transfusion, and Sampling

The necessity for bleeding the animals prior to the administration of the plasma substitutes was established from results of preliminary experiments made in this laboratory (50) and from concurring reports of other in-

vestigators (51), which showed that gelatin and other substances are removed faster from a normal animal than from one previously subjected to hemorrhage.

Rabbits undifferentiated as to sex or breed and ranging in weight from 2.5 to 5.0 kg. were used for these studies (see Figure 1). An initial volume of blood amounting approximately to one-third of the total blood volume (20 ml./kg.)* was withdrawn by cardiac puncture into a large syringe containing 2-3 ml. of 10% sodium citrate to prevent coagulation. The plasma was separated by centrifugation and preserved for future analytical operations. Immediately after the hemorrhage an equal volume of 5% substitute was injected via the marginal ear vein, after which the animal was freed from constraint and was placed in a cage which contained neither food nor water.

Blood samples for the estimation of plasma volume and gelatin concentration were obtained 5 and 24 hours after the transfusion. The animal was anesthetized with ether, the operative field was shaved, and an incision was made in the region of Scarpa's triangle immediately above the maximum pulsation of the femoral artery. By means of blunt dissection the femoral artery and vein were exposed for a distance of about 2 centimeters distal to the level of their emergence from the peritoneum and

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*This assumption does not entail too great an error if the animal's weight is between 2.5 and 4.0 kg. (see Figure 1).

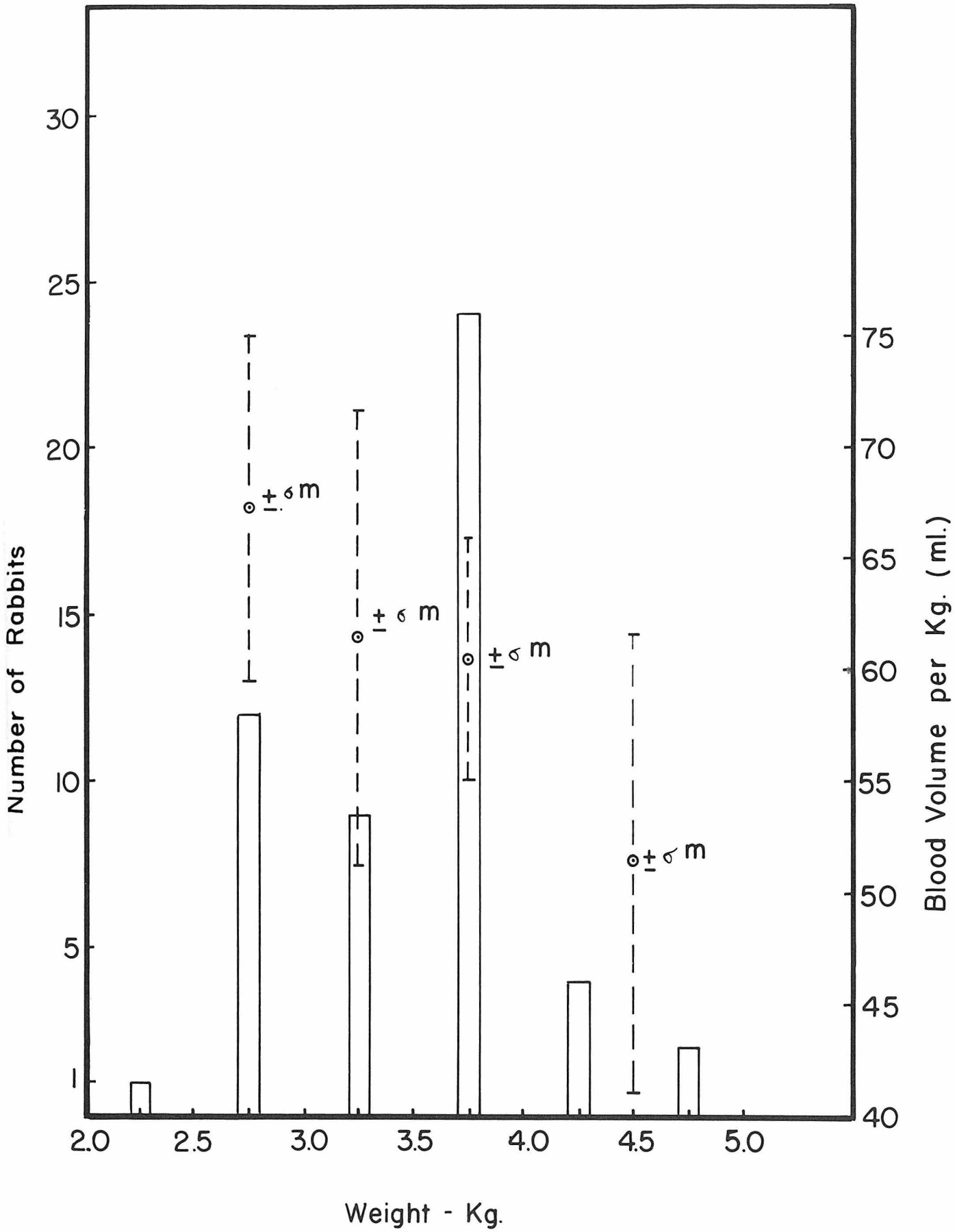

30
25
20
15
10
5
1
Number of Rabbits
75
70
65
60
55
50
45
40
Blood Volume per Kg. (ml.)
± σ m
± σ m
± σ m
± σ m
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Weight - Kg.

Figure 1

slings were placed around the vessels to facilitate their manipulation. A quantity of 8 ml. of blood was then drawn from the femoral artery into a calibrated 10-ml. syringe containing 2 ml. of a mixed oxalate anticoagulant (51). The syringe was rocked and the contents were delivered into a 15-ml. graduated centrifuge tube. Five milliliters of Evans' Blue (T-1824), containing 0.2 mg. of dye per ml., was next injected into the femoral vein, and 15 minutes later, when complete mixing of the dye had been insured, another 8-ml. sample of arterial blood was taken in the usual manner. This sample was used for the determination of hematocrit and of the concentrations of dye and gelatin. At the end of the sampling operations the femoral artery was ligated distally to the profunda, the incision was sutured, and the animal was returned to its cage.

2. Determination of Cell Volume

Hematocrit values were determined directly in the 15-ml. centrifuge tubes by reading the volumes of packed cells after centrifuging the blood samples for 45 minutes at 2,000 r.p.m. in an International clinical centrifuge. Suitable corrections were made for the dilution of the plasma by the anticoagulant solution by means of the following expression:

$$H = \frac{\text{Level of cells}}{\text{Level of fluid -- vol. of anticoagulant}}$$

3. Plasma and Blood Volume

The retention values were obtained from the expression

$$\frac{\text{Total weight of circulating gelatin}}{\text{Weight of gelatin injected}} \times 100$$

and hence the determination of plasma volume was required for the conversion of the percent gelatin in the plasma to the total circulating gelatin expressed in grams. The need for a precise estimate of the plasma volume at the time of sampling follows from the unstable condition of the circulation succeeding hemorrhage. Although it is possible to extrapolate values for plasma volume from a previous dye injection when the circulation is at equilibrium, by assuming a constant rate of dye loss (51, 52), it is erroneous to make such estimates when the animal has been subjected to extensive hemorrhage and has been transfused with a foreign protein of considerable osmotic activity.

Plasma volume was estimated on aliquots of the supernatants obtained in the hematocrit determinations. Optical densities of pre-dye and post-dye samples at 620 mμ were determined with a Beckman photoelectric spectrophotometer equipped with Corex cells. After subtracting blank readings and correcting for dilution with anticoagulant, the post-dye values were referred to a corrected calibration curve (Figure 3) from which plasma volume could be obtained directly. Hemolysis rarely occurred; when present, a correction could be applied from readings taken at 540 mμ. The blood volume was calculated from the plasma volume and hematocrit by the expression

$$BV = \frac{PV}{100 - \text{hematocrit}}$$

A calibration curve relating optical density to concentration of dye was established by measuring the optical densities of 5-ml. aliquots of two plasma samples and one sample of distilled water to which had been added various volumes (0.02-0.20 ml.) of T-1824 solution containing 0.2 mg. of dye per ml. After correction for the appropriate blanks, the plot was linear with respect to the volume of dye added, all the points lying on the same line. The dilution for each point was calculated $\left(\frac{\text{vol. serum}}{\text{vol. dye added}}\right)$ and was multiplied by volume of dye used in the injections, and a curve was plotted relating plasma volume to optical density (see Table II, Figure 3). This curve was corrected for dye disappearance occurring within 15 minutes by means of the average rate of dye loss estimated from time-dilution studies made on three rabbits.

The determination of individual disappearance rates was made in the following manner: fasted animals were anesthetized with 50 mg. of Nembutal and prepared surgically according to the usual method. Five milliliters of the standard dye solution was injected into the femoral vein and samples (8 ml.) were taken into calibrated syringes at regular intervals 15 minutes after the injection and continued until death of the animal. After centrifuging, the optical densities of the plasma samples were determined at 620 mμ by the usual spectrophotometric method with correction for blank (pre-dye value). The corrected values were plotted and the linear portions of the curves were then projected to zero time (see Table II, Figure 2).

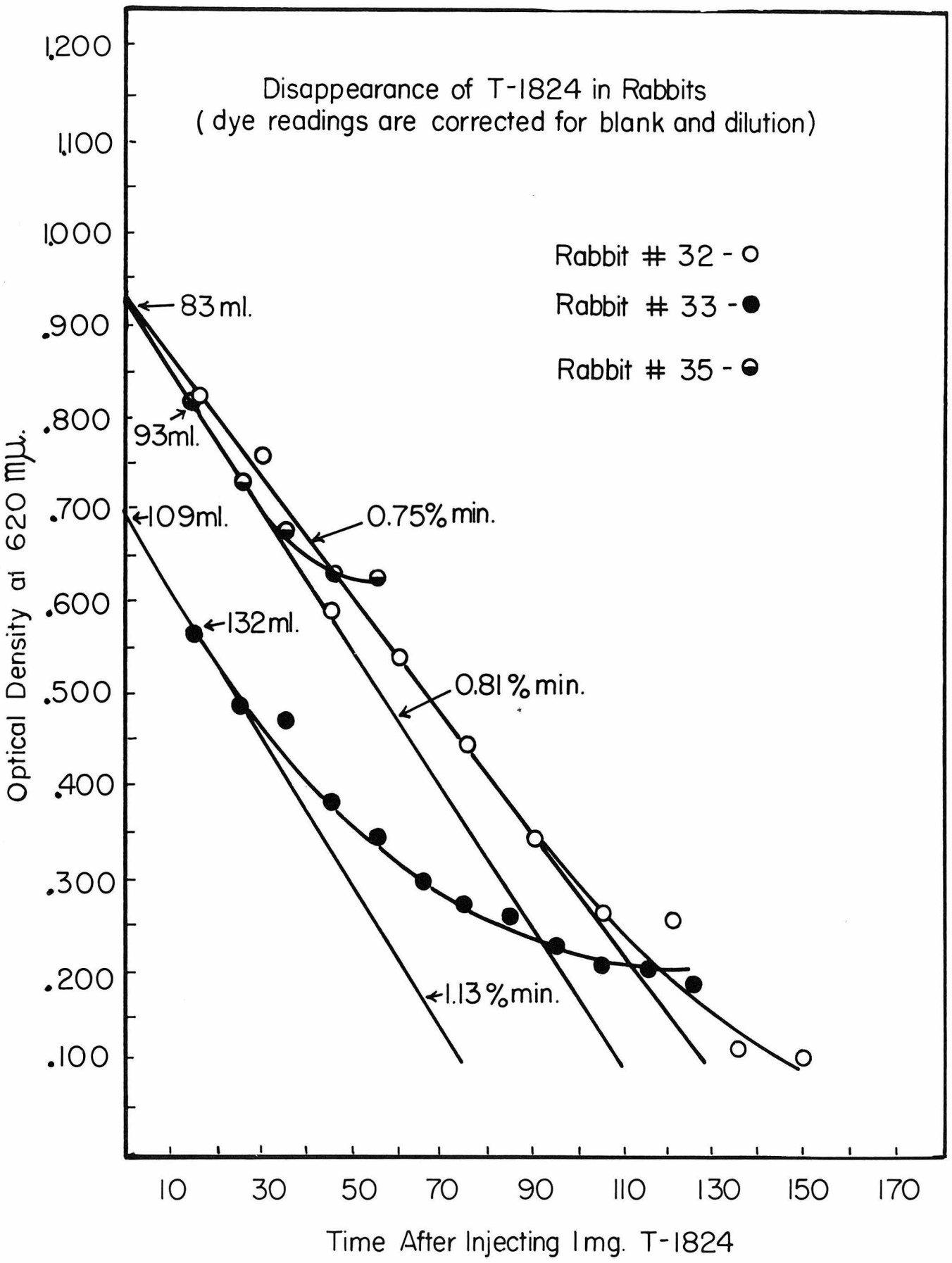

Disappearance of T-1824 in Rabbits
(dye readings are corrected for blank and dilution)
Rabbit # 32 - O
Rabbit # 33 - ●
Rabbit # 35 - ◒
83 ml.
93ml.
109ml.
132ml.
0.75% min.
0.81 % min.
1.13 % min.
Optical Density at 620 mμ.
1.200
1.100
1.000
.900
.800
.700
.600
.500
.400
.300
.200
.100
10
30
50
70
90
110
130
150
170
Time After Injecting 1mg. T-1824

Figure 2

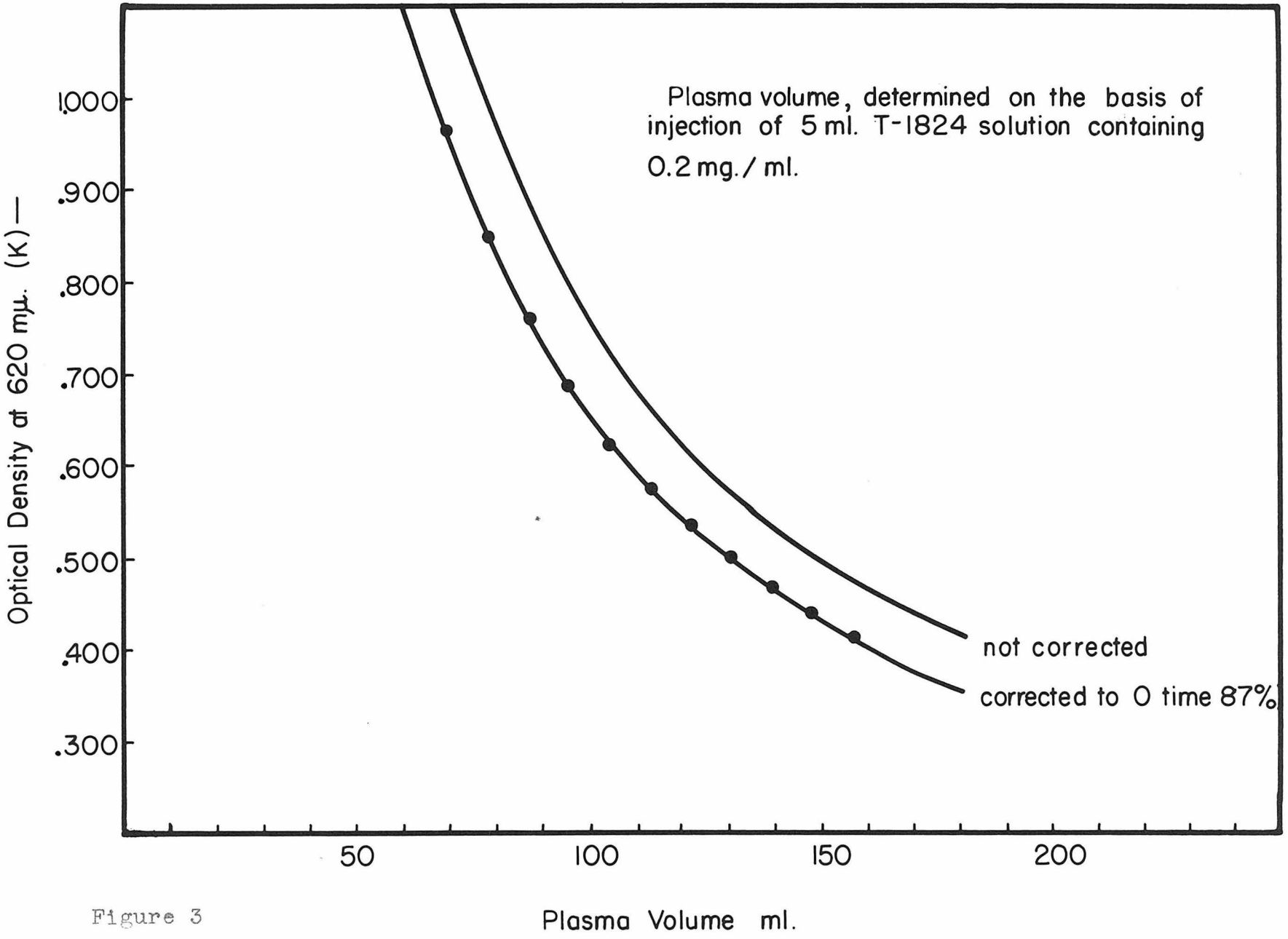

Plasma volume, determined on the basis of injection of 5 ml. T-1824 solution containing 0.2 mg./ ml.
not corrected
corrected to 0 time 87%
Optical Density at 620 mμ. (K) —
1.000
.900
.800
.700
.600
.500
.400
.300
50
100
150
200
Plasma Volume ml.

Figure 3

Table II

The Disappearance of T-1824 from the Circulation of Normal Rabbits

	Readings Corrected for Blank and Dilution		
Rabbit No.	32	33	35
Rabbit Weight	2.7 kg.	3.6 kg.	3 kg.
Time. Min. after injection of 5 ml. T-1824 solution			
0			
5			
10			
15	824	566	819
20			
25		486	730
30	759		
35		472	678
40			
45	594	385	631
50			
55		346	628
60	540		
65		299	
70			
75	447	281	
80			
85		261	
90	346		
95		232	
100			
105	267	209	
110			
115		206	
120	258		
125		193	
130			
135	119		
140			
145			
150	108		
Disappearance Rate, % per min. (Calculated from graph)	0.75% per min.	1.13% per min.	0.81% per min.

Since deviations from linearity generally did not occur until several samples had been withdrawn, it is very likely that such deviations resulted from hemodilution due to a decline in the arterial pressure. This is borne out by the fact that the steepest departure from linearity occurred in Rabbits 33 and 35 in which the sampling was most rapid (10-min. intervals), while the curve of Rabbit 32 (sampled at 15-min. intervals) was linear for a considerably longer period. Death occurred earlier in the rapidly-sampled rabbits.

The disappearance rates calculated according to the foregoing method were 0.75%, 1.13%, 0.81% /min. for Rabbits 32, 33, and 35, respectively, and the mean used in making the correction for zero time in the optical density vs. plasma volume curve was calculated as 0.89% /min. Thus 15 minutes after injection the true plasma volumes were only 86.7% of the apparent readings.

A Comment on the Measurement of Plasma Volume by Dye-dilution

The measurement of plasma volume by dye-dilution is based on the uniform distribution of the injected dye to all regions of the intravascular compartment and on the tendency of the injected material to be lost very slowly from the circulation. The blue dye T-1824 has been generally employed for the determination of plasma volume because of its slow rate of clearance (5-10% /hr.) and because its spectral characterisitics are such that it can be

easily distinguished from the plasma constituents even when a fair degree of hemolysis is present in the plasma samples. The persistence of T-1824 in the blood stream is enhanced by its property of uniting with serum albumin (53), which greatly restricts its tendency to diffuse across the capillary walls. The mechanism by which the diffused dye is removed from the body is not well established, but it is thought by some authors (51) to be mainly phagocytic action and hepatic excretion.

Serious errors in the estimation of plasma volume by the dye-dilution method can result if blood samples are taken before the injected dye has become distributed throughout the peripheral vascular beds. Such errors are unidirectional (often involving under-estimates of 20%) but not regular, since they tend to be influenced by the circulation rate, the blood flow to the "silent areas," and the vasomotor adjustments which a given animal might make in response to the experimental situation. Mixing has been found to be essentially complete after 15 minutes and the blood volume can be estimated if the rate of dye disappearance is known, by extrapolating the curve to zero time.

Since it is the <u>concentration</u> rather than the <u>total</u> amount of dye which is being determined, sampling procedures requisite for the construction of the curve should be without influence on rate of dye loss if serious hemodilution does not occur to produce a spurious increment in rate.

Inasmuch as both water and protein are removed at the time of sampling and the blood pressure (net driving force) remains unchanged due to the compensatory vasomotor adjustments, little hemodilution is expected to take place until an appreciable proportion of the total blood volume has been removed by sampling. It is very likely, therefore, that the slope of the time-concentration curve does not differ greatly from that of the dye-disappearance curve in the early period of sampling. In the present investigation it was not of interest to follow changes in fluid shifts; therefore the volumes at two definite times--5 and 25 hours--were determined by independent injections of dye at the stated intervals and the need of correcting the curve for sampling and, consequently, for hemodilution was obviated.

4. Gelatin Analysis

The concentration of gelatin in the oxalated post-dye plasma samples was determined by the previously mentioned colorimetric micromethod (34), which is based on the difference in the capacities of plasma proteins and gelatin to produce color with the Folin-Ciocalteu phenol reagent. The color developed is due to the action of the reagent on the aromatic amino acid residues which are abundantly present in the plasma proteins but are found only to a limited extent in gelatin.

The determination of gelatin in this investigation involved the separate analyses of a tungstic acid precipitate (representing total proteins) by two independent

methods: a) the Kjeldahl-Nessler, which depends on the nitrogen content of the precipitate and b) the colorimetric method of Pressman (54) based on the detection of aromatic residues by the Folin-Ciocalteu reagent. Because plasma proteins and gelatin are similar in their nitrogen content but different in their content of aromatic residues, it was possible to establish a curve relating the aromatic residue content of a mixed precipitate to the percentage of the total protein as gelatin.

Calibration experiments made on a series of mixtures, variable in their plasma protein/gelatin ratios but constant in respect to the concentration of total protein, revealed that a plot of specific Folin color (i.e., the color developed per milligram of protein) against the composition of the precipitate expressed as percent gelatin was a straight line passing through the values for pure plasma protein and for pure gelatin. Such linear relationships, demonstrated in several calibration experiments, made it possible to refer the specific Folin color of a post-transfusion plasma sample presumed to contain gelatin to a composition curve established from the analytically determined specific Folin color values of a pre-transfusion plasma sample and a sample of the gelatin administered in the particular experiment.

The determination of specific Folin color for the pre-hemorrhage plasma of every animal rather than the use of a value for pooled plasma was made in order to minimize

the contingency that the specific Folin color of an individual animal might be a statistical deviate from normal.

The reliability of this method has been demonstrated by Lanni (5), who extended these investigations and found the constants of his empirical linear equation relating color production in mixtures of differing plasma protein-gelatin ratios at a given total protein concentration to be invariant for pooled plasma and for a series of thirteen randomly-chosen samples. Moreover, he showed that these constants were independent of the source of rabbit plasma protein and the kind of gelatin or Oxypolygelatin used.

The method, presented in terms of a typical calibration experiment, is found in the subsequent extract from the article (34).

The Determination of Gelatin in the Presence of Plasma Proteins

Frank Lanni, George A. Feigen and Arthur L. Le Rosen [1]

From the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California [2]

Experimental

Method

Citrated rabbit plasma and a solution of decalcified Knox gelatin were adjusted to protein concentrations of 0.926% and 0.940%, respectively, by dilution with 0.85% sodium chloride solution, on the basis of preliminary estimation of nitrogen. These reference solutions were mixed volumetrically to provide suitable solutions ranging from 0 to 1 mg. gelatin per mg. protein. In addition, the two reference solutions and a solution prepared by mixing these in equal parts were each diluted with an equal volume of saline. Each of the solutions was then treated as follows:

Preparation of the tungstic acid precipitate: Pipet 1.00 ml. (in quadruplicate for the reference solutions, in triplicate for the others) into a graduated 15-ml. centrifuge tube. Add from a 10-ml. buret 0.25 ml. of 0.67 *N* H_2SO_4 and mix by rotating. Add, also from a 10-ml. buret, 0.25 ml. of 10% sodium tungstate and mix by rotating, and flipping. if necessary. Allow to stand at least 15 minutes at room temperature. Centrifuge for 15 minutes at approximately 2000 r.p.m. Decant supernatant and allow tube to drain.

Solution of the precipitate: To the precipitate add from a buret 1.0 ml. of 1.00 *N* NaOH and dissolve the precipitate with the aid of gentle heating over a low flame. (Precipitates containing more than about 40% gelatin dissolve with difficulty; therefore, they are warmed and allowed to stand several times to complete the process. Complete solution is indicated when no *schlieren*, arising from gradients in the refractive index, appear when a tube which has been allowed to stand for several minutes is gently tapped.) Bring to a volume of 10.0 ml. with distilled water. Cover and mix thoroughly.

Determination of total protein: Pipet 1.00 ml. of the solution of the precipitate into a digestion tube calibrated at 35 and 50 ml. Add from a buret 1.0 ml. of concentrated sulfuric acid which has been diluted with an equal volume of distilled water. Digest to dense white fumes over a Meker burner in a hood. (For this purpose we have found it convenient to utilize a circular copper rack supported by a tripod. Each rack can be made to hold 8 or 10 digestion tubes and one analyst can readily manage more than 40 digestions at one time.) Remove from the flame and allow to cool for 30 seconds. Add one drop of a good grade of nitrogen-free 30% H_2O_2 directly to the digest and heat again to dense fumes. Remove from the flame and allow to cool for 15 minutes.

Bring the volume to about 30 ml., but not over 34 ml., with distilled water. Add from a buret 1.0 ml. of 5.0 *N* NaOH and swirl. Add immediately 15.0 ml. of Nessler reagent from a large buret or other suitable volumetric container and swirl. Bring the volume to 50.0 ml. with distilled water and mix by bringing a clean dry footed glass rod up and down several times in the solution. After 15 minutes read the color intensity with a suitable colorimetric instrument. (For this purpose we have found a Klett-Summerson photoelectric colorimeter satisfactory.)

Subtract from this reading the reading given by a *blank* in which the protein is omitted. A *standard* may be prepared by adding to a blank, before or after digestion, 1.00 ml. of a standard solution of ammonium sulfate containing 0.100 mg. nitrogen per ml. (It has been our practice to include one or more blanks with each set of 8 or 10 analyses and to include sufficient standards to establish the standard value adequately.)

The amount of protein in the analyzed aliquot is obtained from the relation

$$\text{mg. protein} = \frac{\text{unknown reading}}{\text{standard reading}} \times 0.1 \times 6.25$$

The use of the customary conversion factor 6.25 for both plasma protein and gelatin introduces no uncertainties into the determination of the proportional amount of gelatin retained at a given time.

Determination of the phenol color: With the same pipet used in the total protein determination, pipet 1.00 ml. of the solution of the tungstic acid precipitate into a calibrated 15-ml. centrifuge tube. From this point the analysis is conducted exactly as described by Pressman (4) except that the volume is brought only to 6.0 ml. after the addition of alkali and is adjusted to 7.5 ml. after digestion and cooling. The color intensity is recorded in the arbitrary units of the colorimeter scale.

The advantages of this method are found in that a) no fractionation of gelatin and plasma proteins is required, obviating errors due to co-precipitation characteristic of the differential precipitation with trichloroacetic acid as used by Waters (op. cit.); b) the analytical procedures are minimized by the formation and analysis of a single precipitate; c) errors due to volumetric apparatus up to the point of the differential analyses of the precipitate affect only its quantity but not its composition, since the delivery of aliquots can be made with the same pipette.

The present method of gelatin analysis is subject to the limitation imposed by the fact that the intensity of the color production per unit weight of a given protein is not constant because the relation between phenol color and weight of protein is not linear (54) but is described, rather, by a curve which rises sharply from the origin to 200 gamma, the slope becoming constant after that point. Since determinations of gelatin in this investigation were all made in the region of the curve far beyond the 200-gamma limit, and since they were done within narrow limits of total protein concentration, this objection is not serious, in view of the large errors inherent in the retention determination themselves.

5. Determination of Retention

Knowing the plasma volume and the gelatin concentration the total gelatin in the circulating plasma may now be obtained. The determination of the total circulating

gelatin in subject to errors in addition to those involved in the analysis. These are introduced in the measurement of the volume of the blood withdrawn from the animal, the volume of the anticoagulant fluid admixed, and the total volume of plasma.

The determination of the retention of a gelatin, defined as the percentage of gelatin injected which is present in the circulation at a given time, is subject to an additional error which is associated with the measurement of the quantity of gelatin administered. It is therefore possible for the final retention values to be in error by about 10% (see section on statistical evaluation).

Example of Retention Calculation

For purposes of describing the retention calculations, a typical protocol is herewith presented.

Rabbit 400 weighing 3.0 kg. was bled of 57 ml. by cardiac puncture. Following the hemorrhage 60 ml. of 4.93% Oxypolygelatin was administered via the marginal ear vein. Samples were taken 5 hours later under the surgical conditions previously described.

Plasma and Blood Volume.- The pre- and post-dye blood samples were centrifuged, the cell volumes (26/100 and 21/100, respectively) were recorded, and the supernates were decanted. The plasma dilution factors, obtained from the expression $f = \frac{\text{ml. supernate}}{\text{ml. supernate - ml. oxalate}}$ were found to be 1.37 and 1.34, while the corresponding

hematocrit values were 32.5% and 26.3%.

The supernates were poured off and their optical densities at 620 mμ were determined spectrophotometrically. The values observed were 66 and 390 for the pre- and post-dye plasma samples. Multiplying each value by its appropriate dilution factor and subtracting the products yielded a difference of 429 mμ . This value was referred to the calibration curve, extrapolated to zero time, and was shown to correspond to a plasma volume of 151 ml. The blood volume calculated from plasma volume and hematocrit was estimated as 205 ml.

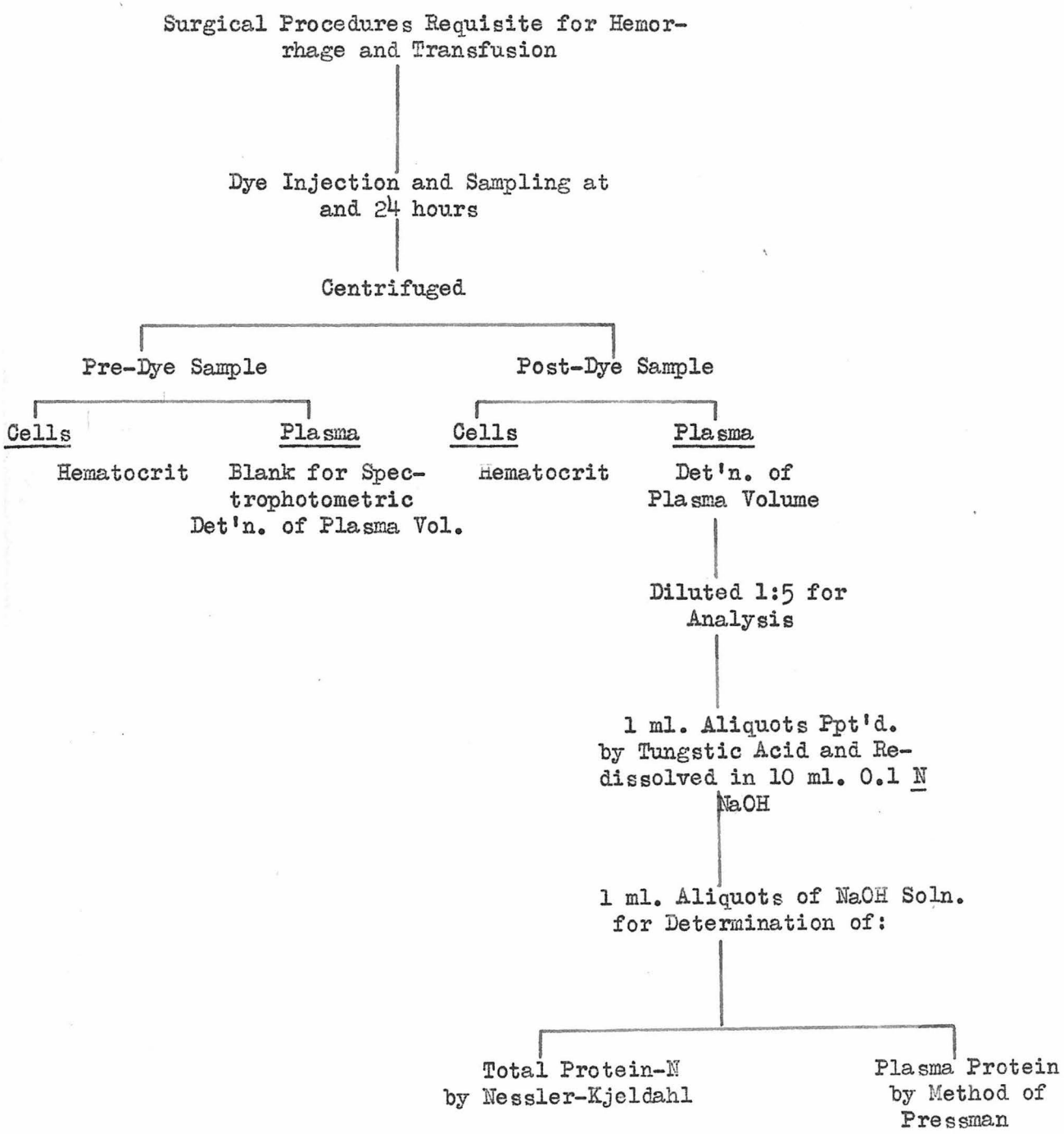
Determination of Gelatin in the Five-Hour Plasma Sample.- The supernate of the dye-containing sample was diluted five-fold and 1 ml.-aliquots were taken for the estimation of total protein and plasma protein by the Kjeldahl-Nessler and Folin-Ciocalteu micromethods described earlier. The Nessler analysis showed the sample to contain 0.76 mg. protein (N X 6.25) for which the corresponding corrected Folin reading was 303, yielding a specific Folin color value of 399 per mg. protein. Folin values similarly obtained were 79 for the Oxypolygelatin and 458 for the gelatin-free plasma proteins, the latter being obtained from the initial (zero time) hemorrhage samples. The ratio of gelatin to total protein was found graphically to be 0.16. The weight of circulating total protein, obtained by multiplying the amount of Nessler total protein-N by the dilution factors (1.34 and 5) and the plasma volume

(151), was found to be 7.70 g.

The amount of gelatin present at 5 hours (7.70 X 0.16) was 1.23 g. The percent retention was obtained on dividing this value by the grams of gelatin injected (4.93% X 60 = 2.96 g.) and found to be 42%.

The procedures necessary for the retention studies are schematically summarized by the flow sheet presented below.

Flow Sheet of Retention Determination



D. Results

1. The Influence of Reagent Variation of Oxypolygelatin.-

a. The effects of varying the glyoxal concentration on retention and osmotic pressure.- Four lots of 5% Wilson gelatin (50,980) were treated with 0, 0.005, 0.01, and 0.02 g. glyoxal/g. gelatin, respectively. The solutions were heated to 95° C. after the pH was adjusted to 7.0 with a predetermined quantity of 5 N NaOH. Heating was continued for 50 minutes, and NaCl (in quantities sufficient to render the final solution isotonic) and 0.0166 g. H_2O_2/g. gelatin were then added. All samples were autoclaved for 20 minutes at 121° C. and the resulting preparations then were subjected to the usual physiological and physico-chemical tests. The results of increasing glyoxal concentration on the osmotic pressure and retention are presented in Table III and illustrated by Figure 4.

When a constant amount of H_2O_2 was added to polygels of increasing glyoxal concentration there appeared, first of all, an increase in the 5-hour retention values without a corresponding rise in the molecular weight. For instance, as the amount of glyoxal was increased from 0 to 0.005 g./g. gelatin the retention showed a two-fold increase whereas the molecular weight exhibited a slight drop. As the ratio of glyoxal was increased to twice the previous level (0.01 g./g. gelatin), the 5-hour retention

Table III. Effect of Increasing the Amount of Glyoxal per Gram of Gelatin on the Retention and Osmotic Pressure of Oxypolygelatins at Constant Values of Hydrogen Peroxide (0.0166 g./g. Gelatin)

Grams Glyoxal per Gram Gelatin	% Retention at 5 hrs.	24 hrs.	Mean 5-Hr. % Retention	Osmotic Pressure[1] (mm. H_2O)	Relative[1] Viscosity	Molecular Weight
0	15 26	0 0	20	89	1.25	29,500
0.005	33 44	0 0	38	101	1.25	26,000
0.010	42 38	17 0	40	92	1.28	28,600
0.020	50 82	39 --	60	67	1.47	39,200

[1] = 1% solutions at pH 7.3.

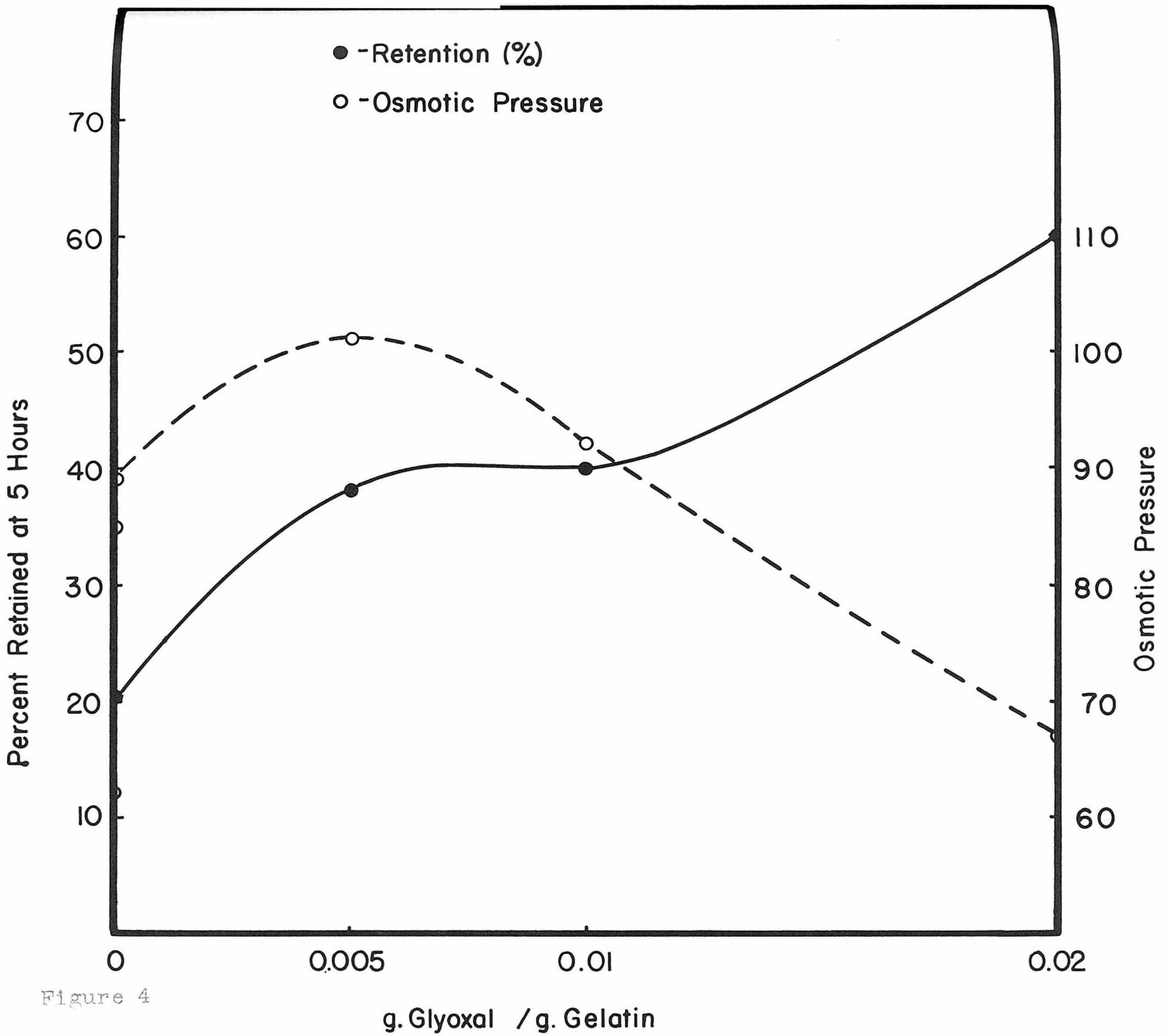

● -Retention (%)
o -Osmotic Pressure
Percent Retained at 5 Hours
Osmotic Pressure
70
60
50
40
30
20
10
110
100
90
80
70
60
0
0.005
0.01
0.02
g. Glyoxal / g. Gelatin

Figure 4

remained almost unchanged but there was a slight increase in the molecular weight, and an indication that the 24-hour retention was rising. A further increase of the glyoxal concentration now markedly increased the molecular weight and affected the 5- and particularly the 24-hour retention values in the same direction.

To summarize the relative effects of increasing the glyoxal concentration on the molecular weight and retention under the present conditions, it can be stated that the effects of such treatment are more evident on the retention than on the molecular weight of the Oxypolygelatins.

b. <u>The effects of varying the hydrogen peroxide ratio on the retention and osmotic pressure</u>.- Aliquots of the polygel made with 0.01 g. glyoxal were treated with H_2O_2 in the following amounts per gram gelatin, 0, .0033, 0.0166, 0.0333, 0.0666, and autoclaved in the usual way. Treatment of these polygels with increasing amounts of H_2O_2 resulted in a progressive reduction in the molecular weight of the respective Oxypolygelatins so produced, this decrement being covariant with the concentration of hydrogen peroxide employed. The effect of peroxide treatment on the retention is equivocal. By reference to Table IV it is noticed that in spite of the progressive fall in molecular weight values from 46,000 to 13,800 the retention

Table IV. Effect of Increasing the Amount of Hydrogen Peroxide per Gram of Gelatin on the Retention and Osmotic Pressure of Oxypolygelatins at Constant Values of Glyoxal (0.01 g./g. Gelatin)

Grams Peroxide per Gram Gelatin	% Retention at 5 hrs.	24 hrs.	Mean 5-Hr. % Retention	Osmotic Pressure[1] (mm. H_2O)	Relative[1] Viscosity	Molecular Weight
0	33 44	24 D	38.5	57	1.39	45,964
0.0033	57 68	28 54	62.5	52	1.41	50,385
0.0166	42 38	17 D	40	92	1.28	28,478
0.0333	29 22	2 0	25.5	136	1.19	19,265
0.0666	5 5 10	D D 4	6.69	190	1.15	13,790

D = died; [1] = 1% solutions at pH 7.3.

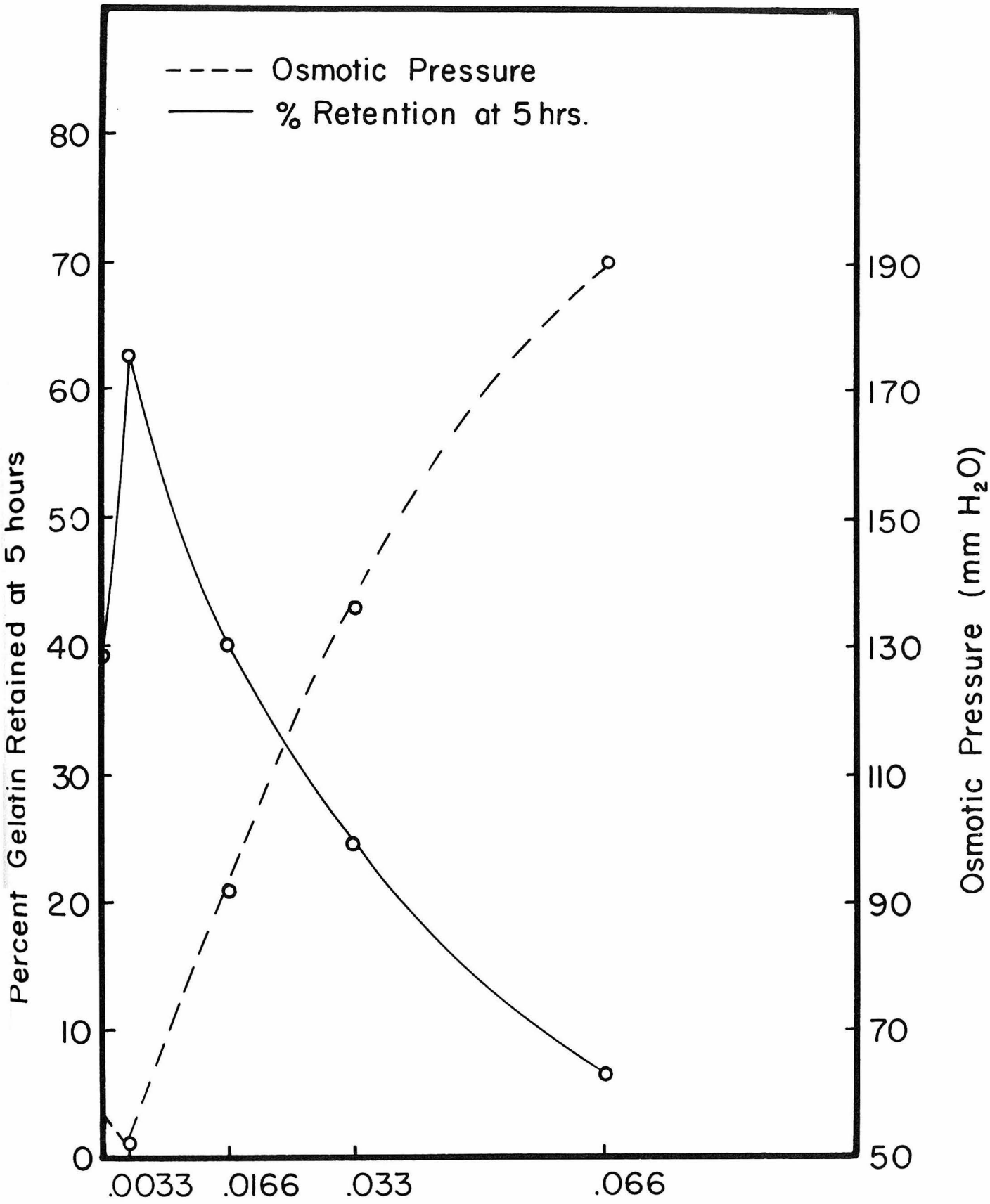

Osmotic Pressure
% Retention at 5 hrs.
Percent Gelatin Retained at 5 hours
Osmotic Pressure (mm H_2O)
0
10
20
30
40
50
60
70
80
50
70
90
110
130
150
170
190
.0033
.0166
.033
.066
g H_2O_2 /g Gelatin

Figure 5

rose from 38.5% to 62.5% with only a small increase in molecular weight, returning to 40% when the molecular weight reached 28,500, and then progressively declined to about 7% when twenty times as much H_2O_2 was added and the molecular weight became 13,800.

c. Combined effects of reagents.- Figure 4 portrays the combined effects of peroxide and glyoxal on the 5-hour retention and molecular weight values of the Oxypolygelatins. An examination of the graph relating molecular weight to retention for the constant glyoxal and the constant peroxide systems reveals the following:

1. Glyoxal in the amount of 0.01 g./g. gelatin increased the molecular weight and the retention of the untreated material (retention 35.5%, M.W. 42,000) to a slight extent (retention 38.5%, M.W. 46,000) while 0.0166 g. peroxide/g. gelatin in a glyoxal-free system decreased the molecular weight and the retention of the untreated gelatin to a great degree (retention 20%, M.W. 29,500).

2. The presence of 0.0033 g. peroxide/g. gelatin increased the molecular weight of the 0.01 polygel only slightly (46,000 to 50,385) while affecting the retention markedly (38% to 62%). The presence of 0.005 g. glyoxal/g. gelatin (as compared to zero glyoxal) resulted in a decrease of the molecular weight (29,500 to 26,000) and in the increase of the amount retained (20% to 38%) when

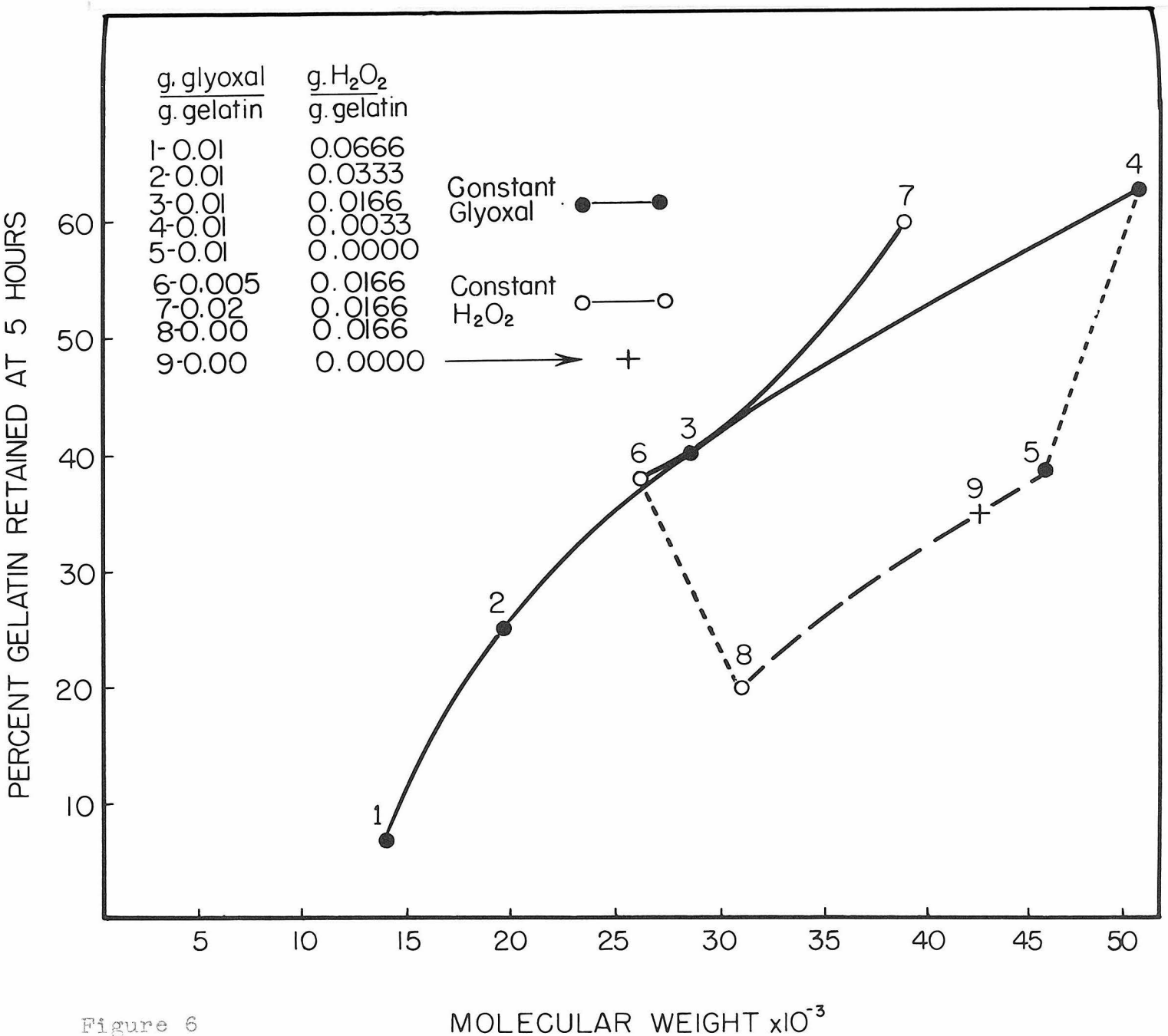

g. glyoxal / g. gelatin
g. H_2O_2 / g. gelatin
1- 0.01 0.0666
2- 0.01 0.0333
3- 0.01 0.0166
4- 0.01 0.0033
5- 0.01 0.0000
6- 0.005 0.0166
7- 0.02 0.0166
8- 0.00 0.0166
9- 0.00 0.0000
Constant Glyoxal
Constant H_2O_2
PERCENT GELATIN RETAINED AT 5 HOURS
MOLECULAR WEIGHT x10^{-3}
10
20
30
40
50
60
5
10
15
20
25
30
35
40
45
50

Figure 6

both gelatins were finally treated with 0.0166 g. peroxide/g. gelatin.

3. A comparison of the relative degree of the molecular weight-retention correlation of the constant glyoxal Oxypolygelatins (glyoxal ratio - 0.01; peroxide ratio - 0.0033 to 0.0666) with that of the constant peroxide family (glyoxal ratio - 0.005-0.02; peroxide - 0.0166) shows that within the conditions of the experiment changes in the glyoxal concentration produce a greater rate of change in retention with respect to molecular weight than do variations in peroxide concentration. An increase of the peroxide concentration (at constant glyoxal values) yields first a paradoxical increase in retention, with small amount of H_2O_2, and then a parallel decrease in the retention and molecular weight.

2. A Comparative Study of the Retention-Molecular Weight Dependence of Gelatins and Oxypolygelatins Manufactured on the Pilot Plant Scale.-

Table V gives the detailed results of the 5- and 24-hour retention studies on a group of 59 hemorrhaged rabbits made on 5 gelatins and 12 Oxypolygelatins. The means and standard errors* of the 5-hour retention values

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* $\sqrt{\frac{Sx^2}{n-1}} \times \frac{1}{\sqrt{n}}$ Fisher, R. A., Statistical Methods for Research Workers, Oliver and Boyd, London, 1936.

Table 5

Summary of Plasma Protein, Gelatin, Plasma Volume and Hematocrit Values Obtained at Five and Twenty-four Hours After Injection of Substitute Materials into Hemorrhaged Rabbits

Preparation	Type	Conc. %	Animal	Ml. Gel. Injected	Gm. Gel. Injected	Plasma Protein % 5 hr.	Plasma Protein % 24 hr.	Gelatin % 5 hr.	Gelatin % 24 hr.	Total Protein % 5 hr.	Total Protein % 24 hr.	Gm. Plasma Prot. 5 hr.	Gm. Plasma Prot. 24 hr.
91	OPG	4.82	A	43	2.08	3.82	4.65	.912	.244	4.73	4.89	3.32	4.23
			B	46	2.22	4.42	5.04	.958	.333	5.38	5.37	4.42	4.79
111	OPG	4.23	A	40	1.69	3.54	4.80	.51	0	4.05	4.80	2.83	3.89
			B	47	1.99	3.79	4.70	.40	0	4.19	4.70	3.94	4.84
			C	41	1.73	4.15	5.24	.655	.107	4.81	5.35	3.28	4.13
			D	46	1.95	4.03	4.83	.656	0	4.69	4.83	3.47	4.15
113	OPG	4.28	A	37	1.58	4.28	5.76	0.54	0	4.82	5.76	3.30	4.27
			B	48	2.05	3.91	4.95	.75	0	4.66	4.95	3.99	5.40
			C	42	1.80	4.81	5.33	.62	0	4.80	5.33	3.64	4.59
			D	42	1.80	3.92	4.98	.74	0	4.66	4.98	3.18	4.63
116	OPG	4.08	A	39	1.59	3.92	4.86	1.22	.192	5.14	5.05	3.37	4.18
			B	47	1.92	3.59	5.02	1.06	.266	4.65	5.29	4.67	4.87
			C	41	1.67	4.29	5.16	0.89	.318	5.18	5.48	3.73	4.28
			D	55	2.24	3.59	4.74	1.07	.324	4.66	5.06	3.84	5.59
117	OPG	4.74	A	75	3.55	4.16		1.09		5.25		4.20	
			B	55	2.61	3.47	4.09	.782	.216	4.25	4.31	3.85	4.38
			C	40	1.90	3.65	4.53	.508	.21	4.16	4.74	3.72	4.08
			D	39	1.85	3.70	4.32	.70	0	4.40	4.32	3.36	3.59
118	OPG	4.77	A	46	2.20	4.11	4.71	.514	.096	4.62	4.81	4.76	5.70
			B	46	2.20	3.92	4.63	.762	.168	4.68	4.80	3.68	4.21
			C	55	2.62	3.68	4.42	1.10	.64	4.78	5.06	4.79	4.90
			D	46	2.20	3.74	5.01	.564	.41	4.30	5.42	4.15	4.51
11,115	OPG	4.06	A	50	2.03	4.05	3.75	0.57	0.20	4.62	3.95	5.55	5.68
			B	49	1.99	5.12	5.81	0.70	0.31	5.82	6.12	5.55	6.89
			C	50	2.03	3.98	4.48	0.50	0.00	4.48	4.48	4.99	5.65
			D	50	2.03	4.21	4.88	0.55	0.18	4.76	5.06	5.40	9.80
11381Y	OPG	5.25	A	80	4.20	3.88	4.43	1.27	0.69	5.12	5.13	5.68	6.32
			B	65	3.41	4.47	5.45	1.02	0.91	5.49	6.36	6.35	7.65
			C	75	3.94	4.28	4.53	0.75	0.39	5.02	4.92	7.02	8.08
12676AY	OPG	4.73	A	80	3.78	4.82	5.70	1.21	0.43	6.03	6.13	6.42	7.70
		"	B	80	3.78	4.25	5.81	0.87	0.44	5.12	6.25	6.66	7.36
		5.08	C	90	4.57	5.26	--	0.59	--	5.85	--	12.61	--
		"	D	50	2.54	4.79	--	0.78	--	5.57	--	5.17	--
		"	E	100	5.08	4.79	7.40	1.56	0.56	6.35	7.95	7.46	10.72
Knox I-3	OPG	5.43	A	70	3.80	3.62	4.33	0.74	0.204	4.36	4.52	6.52	5.50
			B	75	4.07	3.70	3.20	1.33	0.785	5.02	5.40	5.92	4.62
			C	70	3.80	3.82	4.69	1.60	1.14	5.42	5.83	5.00	5.54

Preparation	Gm. Gelatin		Gm. Total Protein		Plasma Vol. ml.		Hematocrit		Blood Vol. ml.		% Gel. Retained	
	5 hr.	24 hr.	5 hr.	24 hr.	5 hr.	24 hr.	5 hr.	24 hr.	5 hr.	24 hr.	5 hr.	24 hr.
91	.793	.222	4.11	4.45	87	91	31.6	25.7	127	122	40.6	11.4
	.96	.316	5.38	5.10	100	95	42.5	35.5	174	147	43	15
111	.41	0	3.24	3.89	80	81	30.4	23.7	115	106	26	0
	.42	0	4.36	4.84	104	103	31.1	26.3	143	140	22.2	0
	.52	.10	3.80	4.23	79	79	33.7	27.3	119	108	31.8	5.2
	.56	0	4.03	4.15	86	86	32.1	26.9	126	118	35.8	0
113	.42	0	3.71	4.27	77	74	32.1	32.7	113	110	28.4	0
	.76	0	4.75	5.40	102	109	28.2	22.8	142	141	39.5	0
	.54	0	4.18	4.59	87	86	36.4	32.1	137	127	31.7	0
	.60	0	3.78	4.63	81	93	30.8	22.1	117	119	35.8	0
116	1.05	.165	4.42	4.34	86	86	24.1	20.3	113	108	71	11
	1.38	.258	6.05	5.13	130	97	21.5	23.1	166	126	76	14
	.775	.264	4.51	4.55	87	83	26.3	20.9	118	105	50	17
	1.14	.382	4.99	5.97	107	118	25.3	17.7	143	143	53.6	18
117	1.10		5.30		101		25.3		135		32.9	
	.868	.231	4.71	4.61	111	107	25.0	21.5	148	136	35.1	10
	.52	.19	4.25	4.26	102	90	19.2	18.4	126	110	28.9	10.7
	.64	0	4.00	3.59	91	83	29.1	22.7	128	107	36.7	0
118	.596	.116	5.36	5.82	116	122	34.2	31.7	176	177	28.3	5.5
	.72	.15	4.40	4.36	94	91	26.6	22.8	128	118	34.6	7.4
	1.43	.71	6.21	5.62	130	111	27.5	20.3	179	139	57.1	28.3
	.625	.37	4.77	4.88	111	90	25.0	24.7	148	119	30	17.9
11,115	0.78	0.30	6.33	5.96	137	151	22.6	17	177	182	38	15
	0.76	0.37	6.29	7.22	108	118	35.3	28.9	167	166	38.2	18.6
	0.63	0.00	5.60	5.64	125	126	24.2	18.2	165	154	31	0
	0.71	0.36	6.09	10.12	128	200	29.6	23.6	182	262	34.8	17.8
11381Y	1.86	0.99	7.48	7.34	146	143	23.2	19.2	190	177	44.5	23.8
	1.43	1.27	7.80	8.90	142	140	27.6	26.7	196	191	41.9	37.2
	1.23	0.70	8.23	8.76	164	178	21.9	16.1	210	212	31.3	17.7
12676AY	1.61	0.58	8.02	8.28	133	135	35.2	31.2	205	196	42.5	15.4
	1.37	0.55	8.04	7.81	157	125	27.0	25.2	215	167	36.2	14.5
	1.42	--	14.04	--	240	--	29.3	--	339	--	31.0	--
	0.842	--	6.02	--	108	--	29.0	--	152	--	33.1	--
	2.43	0.81	9.91	11.53	156	145	27.5	24.9	215	193	47.7	15.9
Knox I-3	1.34	0.259	7.85	5.74	180	127	26.0	21.2	243	161	35.3	6.83
	2.13	1.13	8.03	7.78	160	144	32.8	25.0	238	192	52.5	27.80
	2.10	1.34	7.10	6.88	131	118	32.2	27.2	193	162	55.2	35.40

Preparation	Type	Conc. %	Animal	Ml. Gel. Injected	Gm. Gel. Injected	Plasma Protein % 5 hr.	Plasma Protein % 24 hr.	Gelatin % 5 hr.	Gelatin % 24 hr.	Total Protein % 5 hr.	Total Protein % 24 hr.	Gm. Plasma Prot. 5 hr.	Gm. Plasma Prot. 24 hr.
Knox II-3	OPG	5.72	A	77	4.40	3.80	--	1.40	--	5.20	--	5.12	--
			B	70	4.00	4.09	4.86	1.08	0.86	5.17	5.72	6.18	7.63
			C	80	4.60	3.58	4.79	1.39	0.53	4.97	5.32	4.50	6.48
			D	75	4.30	3.87	4.98	1.09	0.95	4.96	5.93	5.80	6.29
Knox II-5	OPG	5.50	A	60	3.30	3.85	4.48	0.680	0.24	4.53	4.72	3.85	5.00
			B	55	3.03	3.60	4.24	0.40	0.11	4.00	4.35	4.75	6.42
			C	60	3.30	3.81	5.07	0.96	0.13	4.77	5.20	4.94	8.60
			D	56	3.08	3.56	--	0.61	--	4.17	--	3.84	--
Knox (C-1)	Un-auto-claved	5.28	A	70	3.69	4.12	4.79	.96	.23	5.08	5.02	5.52	7.14
		5.25	A-2	46	2.42	4.59	5.65	.86	0	5.45	5.65	4.77	5.82
		5.25	B-2	41	2.15	4.07	5.25	.906	.25	4.98	5.50	3.50	4.78
		5.28	C	72	3.80	3.98	5.62	1.62	.417	5.60	6.04	6.53	8.26
78P	Decal-cified Knox Autocl.	6.48	A	55	3.56	3.49	4.80	.762	.434	4.25	5.23	3.94	5.04
			C	45	2.92	3.18	4.45	.933	.445	4.11	4.89	3.12	3.47
			D	49	3.17	3.87	5.06	.844	.576	4.71	5.64	4.26	4.75
Knox P 11-20	Auto-claved	6.10	A	42	2.56	3.36	4.30	.90	.24	4.26	4.54	3.53	4.26
			B	43	2.62	4.00	4.66	.76	.29	4.76	4.95	4.65	4.80
			C	39	2.38	3.97	4.90	.79	.10	4.76	5.00	4.17	5.00
Upjohn B20610-50	Auto-claved	5.24	A	50	2.62	4.48	5.97	.274	0	4.75	5.97	4.94	6.15
			B	40	2.10	4.92	5.56	0	0	4.92	5.56	4.77	5.45
			D	39	2.04	4.35	5.06	.318	0	4.67	5.06	3.79	4.15
AI Exp't. 131	Un-auto-claved Wilson	4.50	A	68	3.05	4.40	5.40	0.840	0.35	5.24	5.75	6.30	5.35
			B	70	3.15	3.73	--	0.62	--	4.35	--	5.62	--

Preparation	Gm. Gelatin		Gm. Total Protein		Plasma Vol. ml.		Hematocrit		Blood Vol. ml.		% Gel. Retained	
	5 hr.	24 hr.	5 hr.	24 hr.	5 hr.	24 hr.	5 hr.	24 hr.	5 hr.	24 hr.	5 hr.	24 hr.
	1.89	—	7.02	—	135	—	20.2	—	169	—	43.1	—
Knox	1.64	1.34	7.81	8.98	151	157	29.5	27.7	214	217	41.0	33.8
II-3	1.74	0.72	6.21	7.18	125	135	28.2	24.2	174	178	38.0	15.5
	1.62	1.20	7.39	7.47	149	126	30.7	28.9	215	177	37.6	27.9
	0.68	0.27	4.53	5.29	100	112	29.1	36.8	141	177	20.6	8.20
Knox	0.53	0.17	5.28	6.61	132	152	21.9	17.9	169	185	17.5	5.45
II-5	1.23	0.22	6.15	8.84	129	170	36.8	32.9	204	253	37.4	6.71
	0.65	—	4.50	—	108	—	22.9	—	140	—	21.0	—
	1.29	.35	6.80	7.52	134	149	30.3	31.3	192	217	ca .40	10
Knox (C-1)	.985	0	5.66	5.82	104	105	32.7	32.5	154	156	38.8	0
	.78	.23	4.28	5.01	86	91	28.8	29.0	121	128	38.8	11.4
	2.66	.613	9.18	8.88	164	147	29.6	31.2	230	214	73	16.7
	.86	.46	4.80	5.49	113	105	23.1	22.1	147	135	25.4	13.5
78P	.92	.35	4.03	3.82	98	78	29.9	24.7	140	104	33.2	12.8
	.928	.54	5.18	5.30	110	94	27.3	27.6	151	130	30.8	18.2
	.94	.24	4.48	4.50	105	99	18.5	16.3	129	118	39	10
Knox	.88	.30	5.53	5.10	116	103	31.3	31.6	169	151	35	12
P 11-20	.83	.10	5.00	5.10	105	102	25.0	22.5	140	132	39	5
	.30	0	5.23	6.15	110	103	28.5	30.0	154	147	12	0
Upjohn	0	0	4.77	5.45	97	98	38.9	34.6	159	150	0	0
B20610-50	.277	0	4.07	4.15	87	82	32.5	24.7	129	109	14.4	0
	1.20	0.35	7.49	5.69	143	99	32.6	29.8	212	141	39.4	11.4
AI Exp't. 131	0.94	—	6.57	—	151	—	32.3	—	223	—	29.9	—

for all the preparations are plotted against their respective molecular weights in Figure 7. A summary of the percent post-hemorrhagic restoration of total circulating plasma protein, plasma volume, and blood volume is found in Table VIII.

a. Physical properties.-

(1) Molecular weight.- The calculated molecular weights** of the Oxypolygelatin group were lower and more restricted than those of the untreated gelatins. A comparison of the two types of material with respect to molecular weight reveals that the values ranged from 19,260 to 29,110 with a mean value of 34,210.

(2) Gelling tendency.- All of the Oxypolygelatins were liquid at room temperature in 5% solution, whereas most of the untreated preparations of similar strength were firm gels.

(3) Hydrogen-ion concentration.- The range in pH was the same for both types of material, the values lying between 4.5 and 6.5. Although the apparent values were quite low, the titratable acidity (measured for some of the Oxypolygelatins - Table I) was likewise low and

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** OP = 2.62 X 10^5 mm. H_2O/mole at T = 310° K

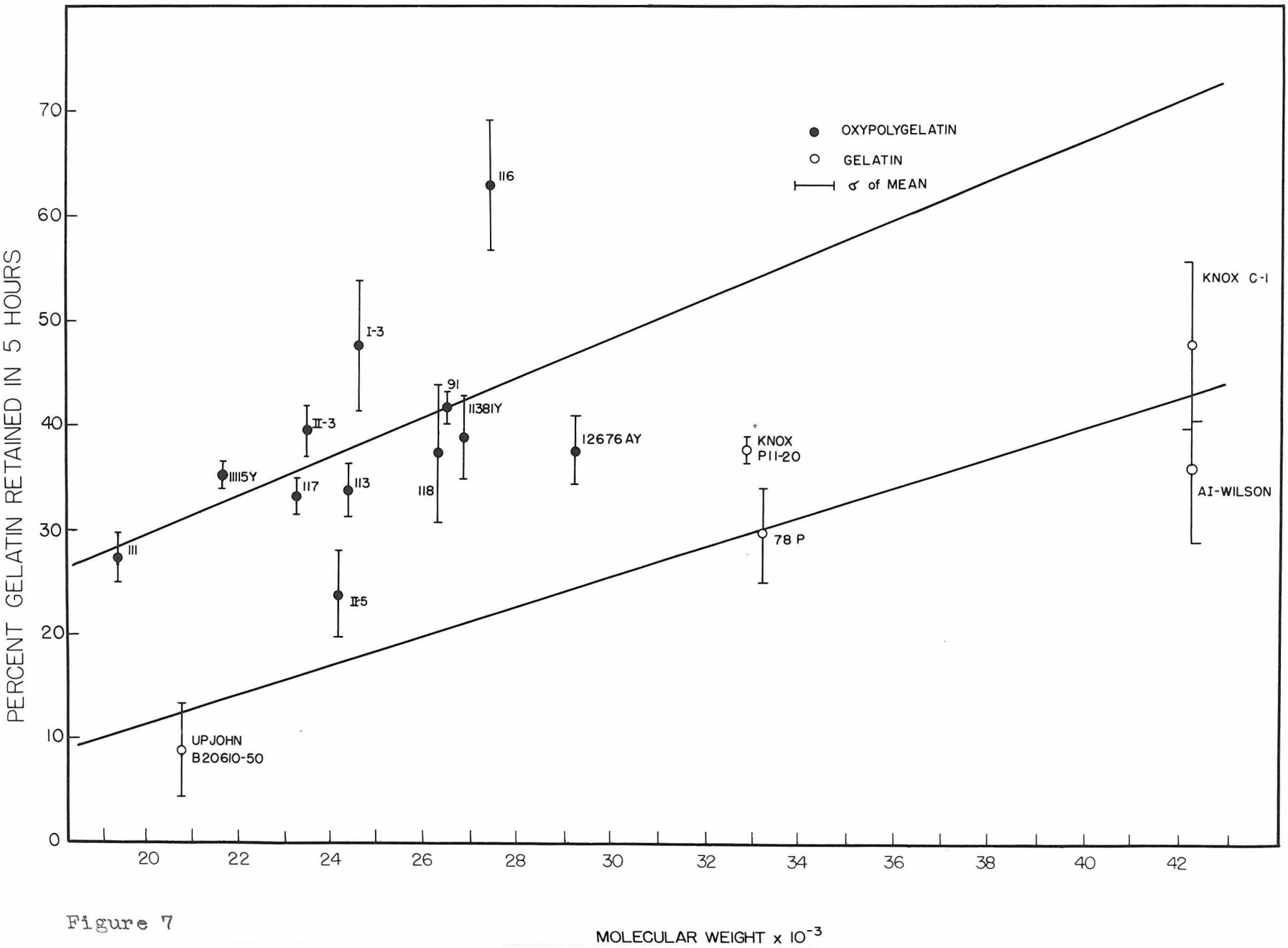
OXYPOLYGELATIN
GELATIN
σ of MEAN
PERCENT GELATIN RETAINED IN 5 HOURS
MOLECULAR WEIGHT x 10⁻³
0
10
20
30
40
50
60
70
20
22
24
26
28
30
32
34
36
38
40
42
116
I-3
91
II381Y
II-3
II115Y
117
113
118
12676AY
KNOX P11-20
KNOX C-1
AI-WILSON
111
II-5
78 P
UPJOHN B20610-50

Figure 7

intravenous transfusions of large quantities of the material into normal mice ($\frac{10 \text{ ml.}}{100 \text{ g.}}$) and hemorrhaged rabbits (60 ml./kg.) were tolerated and did not result in respiratory embarrassment.

b. Physiological effects.-

(1) Retention.-

(a) Five hours.- The series of mean 5-hour retention values of the Oxypolygelatin group was found to be higher than the corresponding series of values for the gelatins employed in this investigation. The Oxypolygelatin mean retention values ranged from 23.5% (II-5) to 63.5% (116) with an average of 38.5% and a standard deviation of ± 6.85% (i.e., retention units). The gelatin values were found to vary between 8% (Upjohn B-20610-50) and 47% (Knox C-I) with an average value of 31.71% and a standard deviation, ± 11.96%. Since there was no reason to believe that the higher standard deviation of the Oxypolygelatins was due to causes other than random sampling (as shown by the non-significant F-test in each case - see Table VI), another analysis of variance was performed, this time on the entire sample of 17 preparations involving 59 rabbits, from which a new estimate of the standard deviation, ± 8.95%, was obtained. This value was taken to represent the precision of a single retention determination derived under the conditions of this experiment.

Table 6

Separate Analyses of Variance of Five-Hour Retention Data Among Gelatins and Oxypolygelatins

Oxypolygelatin Source of Variation	Degrees of Freedom	Sum of Squares	Estimate of Population Variance (Mean Square)	Experimental Error
Individuals within groups	33	2038	61.75	±6.85
Among means	11	1067.77	97.07	
Total	44	3105.77		

$$F = \frac{97.07}{61.75} = 1.53; \quad P = > .05$$

Gelatin Individuals within groups	10	1427	142.7	±11.96
Among means	4	827.24	206.81	

$$F = \frac{206.81}{142.7} = 1.46; \quad P = > .05$$

Table 6 (cont'd.)

Pooled Analysis of Variance of Five-Hour Retention Data

Source of Variation	Degrees of Freedom	Sum of Squares	Estimate of Population Variance (Mean Square)	Experimental Error
Individuals within groups	43	3465	80.58*	±8.95
Group means	16	2106.25	131.64	
Total	59	5571.25	94.25	±9.7

Test of Significance

$$F = \frac{131.64}{80.58} = 1.63$$

P (for df 16 and 42) = > .05 (not significant)

1% 2.46 - 2.44

5% 1.89 - 1.88

* Experimental error = ±8.95

A regression (least squares) line was fitted to each series of mean retention values (Figure 7) and the standard error of estimate for each regression line as the standard error of each regression coefficient was determined. The regression line for Oxypolygelatin is given by

$$E_{OPG} = 38.35 + \frac{150.53}{80.26} (X - 24.66)$$

and for gelatin

$$E_{G} = 31.71 + \frac{456.46}{317.21} (X - 34.21) .$$

The regression coefficients and their respective standard errors were found to be 1.44 $\pm$ 0.419 for the gelatins and 1.88 $\pm$ 1.00 for the Oxypolygelatins. By the employment of the t-test (Snedecor, Chapter 3) with n - 2 degrees of freedom, where t is equal to the quotient of the regression coefficient and its standard error, it is seen that the regression coefficient for gelatin is significant, whereas the regression coefficient for the Oxypolygelatins is not. The former fell in the 2 to 5% probability level and the latter in the 5 to 10% category, 5% being conventionally taken as the limit for significance. The standard errors of estimate for the regression lines were found to be $\pm$ 7.46 for gelatins and $\pm$ 8.95 for Oxypolygelatins. An analysis of covariance made according to the method of Snedecor showed that these two regression lines were not

statistically distinct. Although their standard errors are not significantly different, it is still of interest to inquire into the possible reasons for the observed differences existing among individual preparations.

Table VII presents a summary of the covariance analysis of mean retention and molecular weight data of the two groups. Since the mean rather than individual retention values are considered, the precision of each point is correspondingly increased.

Table VII. Regression and Correlation Data Between Retention and Molecular Weight of Gelatins and Oxypolygelatins

Type of Preparation	Sample Size	Sums of Squares and Products			Correlation Coefficient(1)	Regression Coefficient(2)	Errors of Estimate			
		Sx^2	Sxy	Sy^2	r	$b = \frac{Sxy}{Sx^2}$	Sum of Squares (3)	Degrees of Freedom (n-2)	Standard Error of Estimate $s_{y.x} = (3)/n-2$	Standard Error of Regression Coefficient $s_b = \frac{s_{y.x}}{Sx^2}$
Gelatin	5	317.21	456.46	826.65	0.89	1.44	170	3	±7.46	0.419
Oxypolygelatin	12	80.26	150.33		0.511	1.88	801	10	±8.95	1.00

Type of Preparation	Significance $t = b/s_b$	P
Gelatin	3.45	0.02-.05
Oxypolygelatin	1.88	0.05-.10

Test of Significance Between Regression Coefficients				
Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Average Within Groups	16	984		
Deviation from Individual Groups	15	971	64.73	
Between Regression Coefficients	1	13		4.98

(1) $r = Sxy/\sqrt{Sx^2 . Sy^2}$; (2) $b = Sxy/Sx^2$; (3) Sum of squares $= Sy^2 - (Sxy)^2/Sx^2$; S = sum of terms

Statistical Formulae Employed*

Regression Equation:

$$E = \bar{y} + \frac{Sxy}{Sx^2}(X - \bar{x})$$

E is estimated value of Y for a given value of X

$\bar{y}$ = mean 5-hour retention value

$\bar{x}$ = mean molecular weight value X 10^{-3}

Sxy = algebraic sum of products of deviates from $\bar{x}$ and $\bar{y}$

Sx^2 = sum of squared deviations from $\bar{x}$

$\frac{Sxy}{Sx^2}$ = regression coefficient (slope)

$\frac{Sxy}{\sqrt{Sx^2 . Sy^2}}$ = correlation coefficient, r

$\sqrt{\frac{Sy^2 - (Sxy)^2/Sx^2}{n - 2}}$ = $s_{y.x}$ standard error of estimate of regression line

$\frac{s_{y.x}}{\sqrt{Sx^2}}$ = s_b standard error of regression coefficient

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* From Snedecor, G. W., Statistical Methods, Iowa State College Press, Ames, Iowa, 1940.

(b) Retention at twenty-four hours.- The preparations, with few exceptions, did not show appreciable retention values at the 24-hour period and little correlation between the molecular weight and 24-hour retention was evident. In general, preparations which exhibited high 5-hour retentions showed the higher 24-hour values. The latter values manifested greater deviations from the respective lot means than did the 5-hour values, such variations being due presumably to the larger relative error encountered in the analytical determination of small amounts of protein characteristically present at that time.

(2) Protein and fluid shifts.- Because of the suggestion by some authors (45) that the administration of large quantities of gelatin might exert a toxic reaction which would be reflected by inability of the animal to restore serum proteins lost through hemorrhage, it was of interest to compare the two groups of material in respect to the percentage of the 5-hour total protein, blood volume, and plasma volume which each permitted to be restored at the 24-hour level.

Since basal protein levels were not determined in this investigation, the interest being centered only on the values at the time of sampling, comparisons with zero time are not available, but the differences between the 5- and 24-hour values are indicative of repletion (see Table VIII).

Table VIII

Comparison of Percent Maintenance of Plasma Protein, Plasma Volume, and Blood Volume Between Five and Twenty-Four Hours After Hemorrhage and Transfusion

Preparation	Plasma Protein, %	Plasma Volume	Blood Volume
	Gelatins		
Upjohn	116	96.27	92
Knox C-1	129.2	102.0	103
78P	116.8	89.5	84
Knox P-11-20	114.7	93.8	92
Wilson A-I			
Mean (group)	117.62	93.8	92
	Oxypolygelatins		
116	122	96.5	90
113	134	104	98.2
111	126	100	94.5
91	117	100	90.5
117	110	94	88
118	112	92.2	87.7
11,115	130	119	109
11,381Y	115	103	97.6
12676AY	125	91	88
Knox I-3	111	83.7	77
Knox II-3	117	97.7	94.4
Knox II-5	146	119.5	128
Mean (group)	122	85	95

The mean plasma protein levels at 24 hours without exception showed an increase over the values obtained at 5 hours; these values ranged from 110% for Oxypolygelatin 117 to 146% for Oxypolygelatin Knox II-5, no significant differences being observed between the group average gains of the gelatins and Oxypolygelatins.

III. DISCUSSION

During the early part of the war, when the present study had its inception, no positive criteria for gauging the effectiveness of a blood substitute were available. Janeway (48) suggested the following conditions for the utility of such a material: stability, high osmotic activity, low viscosity, non-antigenicity, non-toxicity, and adequate retention. Since extensively autoclaved gelatin possessed most of these qualifications but was not well retained, the last criterion was employed in comparing the several preparations.

Underlying the choice of retention as a measure useful in distinguishing differences among the preparations were two fundamental questions: Is this a valid test for estimating the usefulness of a blood substitute? What is the degree of reliability of this test?

In view of the diverse opinions concerning the efficacy of transfused substitutes presented in the literature review, the validity of retention or of any other

single test as a measure of the utility of gelatin is a moot question. However, certain conditions of experimental trauma,* in which the animals have a chance of surviving if transfused with an adequate colloid, but of dying if such a material is inadequately retained, the retention test offers a measure of the probable efficiency of the transfusion fluid and may be used as a guide in the comparison of various preparations.

The retention of any substance given in transfusion is the resultant of many processes which tend to clear the material from the circulation, among which are digestion, phagocytic action, and filtration. Hyman (56) demonstrated that the rate of filtration of gelatin, pectin, and other colloids through the capillaries of a perfused frog was inversely but not linearly dependent on the osmotic pressure, providing the perfusions were executed at an invariant head. He accounted for the departure from linearity as being attributable to the fact that gelatin was a polydisperse material containing a spectrum of particle sizes, ranging from 10,000 to 90,000, of which a large portion had dimensions small enough to permit escape across the capillaries into the tissue fluid. He found,

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* where shock is due mainly to plasma loss and capillary atony and providing chronic blood loss or irreversible tissue damage have not occurred

moreover, that as the ratio of small particles was increased by heat degradation the rate of edema formation was accelerated.

The loss of gelatin from the circulation of the intact rabbit observed in the present investigation can be assumed to occur mainly by filtration. This conclusion is supported by the following facts: a) the retention of gelatin is markedly decreased if large volumes of substitute are injected into an un-bled animal, with the result that the increased volume significantly raises the blood pressure (i.e., the driving force) and this accelerates the disappearance rate (50, 51); b) injected gelatin can be almost quantitatively recovered from the blood and the urine several hours after its administration, the average particle size of the urinary gelatin being considerably smaller than that of the material remaining in the circulation (35, 49).

The discussion of retention results obtained in the present investigation will be made from the standpoint of the degree of influence exerted by each of the several treatments designed to modify the spatial configuration or the molecular weight of gelatin on the physical filtration of this material across the capillary walls. The covariation of retention and molecular weight is implicit to the validity of this point of view. The reliability of the retention determinations has been treated in a previous section on statistical analysis.

Since gelatin molecules are considered to be long polypeptide chains, with an equatorial diameter estimated to be 18 Å.*, the probability of their escape through the glomerular capillaries, the pores of which possess an effective diameter of about 30 Å., is very high. This escaping tendency can be physically modified in two ways: 1) by an increase in the particle size, or 2) by a change in the spatial configuration of the molecule. An increase in the particle size should be reflected by the decrease of osmotic pressure per unit weight and an increase in percent retention, while a change in spatial configuration without corresponding increase in mass could be indicated only by a change in retention without a coincident change in the osmotic pressure.

In evaluating the difference in the retention-molecular weight dependence found between the two groups of preparations it is necessary to consider the fact that the in vitro osmotic pressure of gelatin, because of the polydisperse nature of its molecules, can yield only a statistical average of the molecular weight. In consequence of the polydispersity, it follows that iso-osmotic gelatin solutions (i.e., furnishing the same number of particles per unit weight of protein) can vary in their distribution of particle masses. Since the osmotic pressure measurements are frequently conducted with the employment of membranes relatively impermeable to the smallest units, differences in

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* Cohn, E. J., Proc. Amer. Phil. Soc., 88, 159 (1944).

size distributions among iso-osmotic gelatin solutions are not apparent. However, if a series of graded membranes of differing pore size be employed, variations in distribution will be reflected by differences in the residual amounts of protein remaining in the bags and in the relative decrements of their osmotic pressure values.

The implications of the preceding discussion on the evaluation of differences observed between the two retention-molecular weight regressions can be readily appreciated if one considers the fact that the systematic and glomerular capillary walls are normally permeable to gelatin and that differences in particle size distributions of *in vitro* iso-osmotic gelatins will be reflected in a greater retention of those gelatins which possess either the larger particles or the greater number of particles above a critical size. Evidence that such *in vivo* fractionation occurs was obtained by measuring the osmotic pressure and determining the cataphoretic migration of an Oxypolygelatin isolated from the blood and urine of a rabbit 2 hours after its injection. The results, presented in an appendix to this chapter, showed the osmotic pressure of the urinary gelatin to be much greater than that of the gelatin injected or that which was isolated from the circulation. Cataphoresis patterns indicated that the urinary gelatin was the least homogeneous of the three.

The effects of chemical treatment may result in the increase of osmotic pressure without a change in retention if the larger molecules are merely broken into smaller units, each being above the critical size for loss from either the circulation or the artificial membrane. If the rupture is uneven and a number of small particles is formed which are capable of being lost from the circulation but not from the physical osmometer, the increased in vitro osmotic pressure will be reflected in a decreased retention.

A. The Influence of Reagent Variation on the Retention of Oxypolygelatin

1. Variation of Glyoxal in the Presence of Constant Hydrogen Peroxide.-

The means by which glyoxal increased the retention is probably not simply a formation of large aggregates since under constant peroxide concentration the addition of a small amount of glyoxal results in a great increase in retention without a corresponding change in the molecular weight, while the addition of a larger amount increases the molecular weight slightly without affecting the retention. A still greater increase in the glyoxal concentration increases both the molecular weight and retention although the effect on retention is the more marked.

Since it was impossible to study the variations of glyoxal concentration in the absence of H_2O_2 statements

regarding its mode of action cannot be made with certainty. It is very likely, however, that the combined action of glyoxal and H_2O_2 results first in a changed spatial configuration, as evidenced by the great increase in retention without a change in molecular weight noted in the case of the 0.005 polygel, and then in aggregation as the proportion of glyoxal to peroxide is increased. This view is supported by physical evidence which reveals an increase in the molecular weight and viscosity values.

Glyoxal might augment the retention without affecting the molecular weight of gelatin by causing the formation of large aggregates which are subsequently broken by the peroxide treatment in such a way that the total number of molecules (as estimated in the physical osmometer) remains the same as before any treatment but with the difference that some of the smaller units have exceeded the critical size for loss from the circulation.

2. The Variation of Hydrogen Peroxide in the Presence of Constant Glyoxal.-

Inasmuch as the treatment of the .01 polygel with 0.0033 g. H_2O_2/g. gelatin increases the molecular weight of the resulting Oxypolygelatin only slightly, while influencing the retention value (particularly that at 24 hours) to a marked extent, it can be presumed that such

quantities of peroxide exert a facilitating effect on the reaction between glyoxal and gelatin and increase the retention by a change in spatial configuration. This view is supported by the observation that increasing the peroxide ratio to 0.0166 g./g. gelatin decreases both the molecular weight and viscosity without affecting the retention of the polygel, suggesting that a change in shape has occurred as the result of the breakdown of large polygels into smaller particles which are still too large to be lost rapidly from the circulation. Further increments in H_2O_2 decrease retention along with the molecular weight and relative viscosity. Thus, the effect of peroxide on the polygels is probably the resultant of two competitive processes, one favoring the condensation reaction and the other causing the degradation of the polygel.

B. The Variability of the Gelatins and Oxypolygelatins Manufactured in Pilot Plant Lots

The preceding discussion has served to indicate the manner in which changes in the physico-chemical and physiological properties of Oxypolygelatins were induced by varying the ratio of either of the reagents with respect to gelatin. The results show that the retention of Oxypolygelatins is highly dependent on their molecular weight but that such a dependence differs in degree from that of untreated gelatin, Oxypolygelatin, or polygelatin. An

inspection of Figure 6 reveals that Oxypolygelatins of comparable molecular weights have higher retention values than either of the corresponding intermediates or the parent material.

Having examined the relative effects of the two reagents, glyoxal and H_2O_2, on the retention of the final product, an analysis of the factors which produced differences in retention among the individual Oxypolygelatins manufactured on a large scale can be undertaken. In the preparation of these materials another variable, the degree of heating, was introduced; the effects of this condition will be considered in the discussion of the retention values of the untreated gelatins.

Although the regression line plotted for the untreated gelatins represents the dependence of retention on molecular weight with a relatively high degree of fidelity, this is not true of the line plotted for the Oxypolygelatin group. Since the experimental error of the Oxypolygelatin group is not significantly different from that of gelatin, the greater scatter of the Oxypolygelatins is probably less the result of errors in retention measurement than of variations in the conditions under which these preparations were manufactured (see Table I) which involved the simultaneous alteration of more than one variable. This is borne out by the fact that there is a high degree of correlation between the mean retention and molecular weight values of

Oxypolygelatins in which only one condition, the H_2O_2 ratio, was varied (Figure 6). Since there is no a priori reason to assume that the precision of the individual determinations should have been different in the two experiments, the low correlation of retention and molecular weight among the Oxypolygelatins made in the pilot plant might be attributed to qualitative differences in the preparations.

It is tempting to speculate that such qualitative differences are produced by a) the variation of reagent ratios, and b) the duration of autoclaving and that these differences are possibly reflected in the existence of a family of curves (in which two of the conditions are held constant while a third is altered) of varying degrees of steepness, each relating retention to molecular weight but at different rates. For example, provided that the heating conditions are constant, it is not unreasonable to postulate the existence of a number of curves for different constant glyoxal ratios in which the variation in molecular weight is produced by changes in the quantity of added peroxide.

A case in point is the difference among preparations I-3, II-5, and II-3. These Oxypolygelatins were all made under the same heating conditions but with different ratios of glyoxal and peroxide. Thus preparation II-3, made with 0.125 g. glyoxal and 0.015 g. H_2O_2/g. gelatin has a higher retention but a slightly lower molecular weight than II-5

which was prepared with the same amount of glyoxal but with 1.5 times the amount of peroxide; on the other hand, Oxypolygelatin I-3, which was made with exactly twice the amount of each reagent used in the preparation of II-3, has a higher mean retention than either II-3 or II-5 but almost the same molecular weight as the latter.

A further illustration is found in the case of preparations 117 and 118, which were made with the same glyoxal ratio, but with a slightly lower peroxide ratio in the case of 118. This resulted in a lower molecular weight value for 117 without a noticeable change in retention. Preparation 11,115 was made with about one-half the glyoxal ratio used in 118 but with the same quantity of peroxide, resulting in a much lower molecular weight in the former case with a slight but insignificant increase in 5-hour retention.

The notable exception to the trend is Oxypolygelatin 116, which was made under the same conditions as 117 but has both a higher molecular weight and 5-hour retention. Such variations are undoubtedly due to technical errors involved in making large scale pilot plant preparations.

Time of Autoclaving.- The influence of heating on the molecular weight and retention is best exemplified by the regression of untreated gelatins. In this instance, the decline in molecular weight from a value of 42,000 for the unautoclaved Knox or Wilson gelatin, to 20,600 for the

autoclaved (180 minutes) Upjohn preparation, is followed by a linear reduction in retention. The deviations in percent retention observed among gelatins possessing the same molecular weight (i.e., between Knox C-I and Wilson A-I at 42,000, and between Knox P-11-20 and Knox 78P at about 33,000) lie within the range of their respective standard errors.

The magnitude of the heating effect on the chemically treated gelatins may be appreciated by comparing the parent material, Knox C-I, which was merely dissolved in warm physiological saline with Oxypolygelatin and gelatin preparations subjected to the same degree of heating. Thus, preparations 91, 113, 116, and 117, which were treated with 0.025 g. of glyoxal and 0.031 g. of H_2O_2 per gram of gelatin and subsequently autoclaved for 20 minutes at 121° C., have lower molecular weight values than C-I without significant differences in retention. It is noticed that the molecular weight drops from 42,000 for C-I to 27,000 for 116 and 23,000 for 117 whereas the retention values (except in the case of 116, which is considerably higher than the rest of the Oxypolygelatins) fall within one or two standard errors of the C-I value. Heating, therefore, produces a greater change in the molecular weight of the Oxypolygelatins than of the gelatins, without materially altering their retention values. Inasmuch as variations in heat treatment alone do not greatly affect the

retention values of the Oxypolygelatins, when the proportions of reagents are constant, the variability of retention values observed within this group must then be ascribed to reagent factors.

C. Analysis of the Factors Responsible for the Difference in Molecular Weight-Retention Dependence of Gelatins and Oxypolygelatins

Knoefel and Lehmann(35) studied the retention of gelatin fractions (of varying molecular weight) which were prepared by the alcoholic fractionation of bovine long-bone gelatin. Presumably the variation of the molecular weight of such fractions was due not to a progressive hydrolytic cleavage of the gelatin molecules but to differences among molecular species of which the unfractionated gelatin was composed. The results of their physiological tests showed retention to be associated with, but not proportional to, the molecular weight of the particular fraction studied. They reported that gelatins of average molecular weight of 25,000 or less showed only a very slight tendency to remain in the circulation while preparations with molecular weights in excess of 50,000 showed little further increase in the retention, the steepest change observed lying between 29,000 and 36,000.

These conclusions are confirmed by the results of studies on untreated gelatins made in the present report. Providing that allowances are made for the difference in the time of sampling, the values obtained by Knoefel and Lehmann at comparable values of molecular weight appear to lie within the experimental error of the regression line plotted for the heat-degraded gelatins of the present study, the greater absolute molecular weight values observed by these workers being attributable to a process of selection by the alcohol fractionation.

Since the fractionation procedures employed by Knoefel and Lehmann did not result in severe structural molecular changes and since the dependence of retention on molecular weight was similar to that of the present investigation in which the variation of molecular weight was produced by heat degradation rather than selection, the difference in such a covariation observed in the case of the Oxypolygelatins implies the action of degrading agents on two <u>independent structural types</u> of starting material. Physico-chemical evidence of the existence of such a qualitative difference is found in the enhancement of molecular weight and viscosity of the glyoxal-treated gelatin over the untreated materials when both are autoclaved for the same length of time; in the color change and resinous consistency of the polygels; and in differences in molecular weight (see Figure 6) which attend the treatment of gelatin and polygelatin with H_2O_2.

The effect of heat also displays differences in the two types of material: the molecular weight and retention of untreated gelatin shows a progressive decline as the time of heating is prolonged, whereas the molecular weight of the polygel is originally increased by heating and then declines as heating is subsequently continued. This is undoubtedly due to the effect of heat on two competing processes, one favoring condensation and the other degradation. The condensation reaction is enhanced by the presence of low concentrations of H_2O_2 while the higher concentrations of H_2O_2 favor degradation of the polygels. Finally, an important difference is found in the fact that Oxypolygelatins no longer form gels at room temperature as do the less severely heat-treated gelatins of comparable molecular weight and protein concentration.

IV. CONCLUSIONS

A. The Influence of the Respective Reagents on Retention of Oxypolygelatin

The retention of Oxypolygelatins was shown to depend on the amounts and proportions of the reagents glyoxal and hydrogen peroxide used in their manufacture.

1. The effect of increasing the ratio of glyoxal under constant peroxide is to increase the retention, molecular weight, and viscosity of the Oxypolygelatin.

2. Treatment of a polygel with increasing amounts of H_2O_2 decreases these attributes. A paradoxical increase in retention without a corresponding decline in molecular weight is noted in the case of a 0.01 g./g. polygel treated with 0.0166 g. H_2O_2/g. gelatin.

3. The effect of increments in the heating period is to produce a concomitant decline in molecular weight, retention, and viscosity in untreated gelatins; this effect is not so definite in the Oxypolygelatins since increments in heat during the first stage of the reaction often favor the formation of an intractable, resinous polygel.

B. Correlation Between Retention and Molecular Weight

Oxypolygelatins prepared under constant ratios of glyoxal but variable ratios of peroxide show a high

correlation between retention and molecular weight. This relationship is not so evident if the peroxide is kept constant but the glyoxal varied, since the range of molecular weight values observed was more limited in this case.

Gelatins in which the variation of molecular weight was achieved by prolonged exposure to heat demonstrate a high correlation (0.89) between molecular weight and retention. The regression coefficient is 1.44 and its standard error is $\pm$ 0.419. The standard error of estimate of the least squares line is $\pm$ 7.46%. For Oxypolygelatin manufactured in the pilot plant these values are: regression coefficient = 1.88 $\pm$ 1.00; standard error of estimate of least squares line = $\pm$ 8.95%, and correlation coefficient = 0.511. The regression for the untreated gelatins was shown to be significant (P = 0.02-0.05), while it was shown to be not significant in the case of the Oxypolygelatins (0.05-0.10. The low correlation among the Oxypolygelatins is attributed to variations in manufacture rather than to experimental error.

C. Effects on Protein and Fluid Shifts

Neither gelatin nor Oxypolygelatin interfered with the restoration of plasma proteins, red cells, or fluid which had been lost after the initial hemorrhage.

D. Physical Properties

All the Oxypolygelatins studied were liquid at room temperature and, in general, had lower molecular weights than gelatins. The pH values of both types of material were low but because of the small titratable acidity, these substances could be safely injected in large quantities without attendant respiratory embarrassment.

V. SUMMARY

This report has described a new retention assay of known reliability by means of which it was possible to establish a difference between the physiological behavior of untreated gelatin and gelatin subjected to the action of glyoxal and hydrogen peroxide. The method possesses the following features: 1) the tests are performed on rabbits previously subjected to a standard degree of hemorrhage permitting the injection of large volumes of protein solutions without imposing artifacts which might otherwise result due to excessive increments in blood volume; 2) the determination of gelatin in plasma samples can be made with a high degree of precision; 3) the weight of circulating gelatin, rather than the percent present, was obtained by determining the plasma volume at the time of sampling.

The retention of both the Oxypolygelatin and untreated gelatin preparations was found to be dependent on their molecular weight as estimated by osmotic pressure measurements in physical equipment, although such a dependence was not clearly demonstrated by those Oxypolygelatins which were made by pilot plant methods. The low correlation between molecular weight and retention noted in the latter case was attributed to changes in reagent ratios and to uncontrolled factors present in large scale manufacture, since Oxypolygelatins produced under laboratory conditions, in which molecular weight changes were achieved by the alteration of only one variable, showed the correlation to obtain. Under such conditions increments in glyoxal tended to increase the molecular weight and retention while the progressive addition of hydrogen peroxide caused a decrease in both attributes.

The following differences in relation between the retention and physical properties of gelatin and Oxypolygelatin were noted.

1. All Oxypolygelatins were liquid at room temperature, whereas most of the gelatins were solid and, in general, had higher molecular weights.

2. Equal degrees of heating produced greater decrements in the retention values of the gelatins than of the chemically-treated preparations.

3. The retention of Oxypolygelatins at comparable values of molecular weight was higher than that of gelatins.

4. By varying the conditions of preparation it was possible to influence the retention of the Oxypolygelatins without significantly affecting their mean molecular weight.

5. The greatest degree of retention was exhibited by an Oxypolygelatin with a molecular weight considerably below that of the parent material.

These facts suggest that a qualitative difference exists between the two types of material.

An estimate of the precision involved in a single determination for this was made by means of a statistical analysis of variance on retention tests of 17 preparations involving 59 hemorrhaged rabbits, and shown to be around ± 10% under the conditions of this experiment.

Appendix to Chapter II

The purpose of this appendix is to present supplementary information concerning the effect of Oxypolygelatin on the dynamics of the circulation and to amplify the discussion of the *in vivo* fractionation of Oxypolygelatin to which reference has been made in Chapter I.

I. THE MAINTENANCE OF BLOOD PRESSURE FOLLOWING PLASMAPHERESIS AND TRANSFUSION WITH OXYPOLYGELATIN

Two sets of experiments, made with use of somewhat different techniques, were carried out to study the relative efficacy of Oxypolygelatin and autoclaved gelatin in maintaining adequate blood pressure of rabbits during the plasmapheresis.

A. Auscultatory Blood Pressure Measurement

The rabbit was placed on a board in a supine position and a clinical blood pressure cuff was adjusted around its abdomen, between the lower costal margin and the iliac crests. The cuff was inflated and the blood pressure was determined by auscultation of the abdominal aorta distal to the cuff and proximal to its bifurcation into the common iliacs. One-third of the estimated blood volume was then withdrawn by cardiac puncture and an

equivalent amount of Oxypolygelatin or autoclaved gelatin was injected intravenously. The results obtained are shown in Figure 1. It is seen that injection of Oxypolygelatin 91 produced no essential change in the systolic pressure and only a slight decrease in the diastolic pressure during a period of 6 hours. Injection of decalcified autoclaved Gelatin 97 resulted in a rapid drop in both systolic and diastolic pressure soon after the transfusion, and death of the animal in 4 hours.(see Figures 1 and 2). Subsequent investigations of Feigen and van Harreveld* showed the validity of this method of measuring the blood pressure to be questionable since the ipso facto constriction of the aorta often produces severe pressor responses. However, the fact that the pressure was maintained in the animal transfused with Oxypolygelatin, but was decreased in the rabbit subjected to the same procedures following the administration of a hydrolyzed gelatin, suggests that the circulatory efficacy of the two materials in post-hemorrhagic trauma was different.

B. Direct Measurement of Blood Pressure

Rabbits were prepared for direct measurement of blood pressure by anesthetization with Nembutal (15 mg./kg.) and ether. Incisions were made to expose the femoral artery

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* Feigen, G. A. and A. van Harreveld, "On the Nature of the Pressor Response Due to Aortic Constriction" (In preparation)

Auscultatory Blood Pressure of Hemorrhaged Rabbits
Following Administration of Gelatin

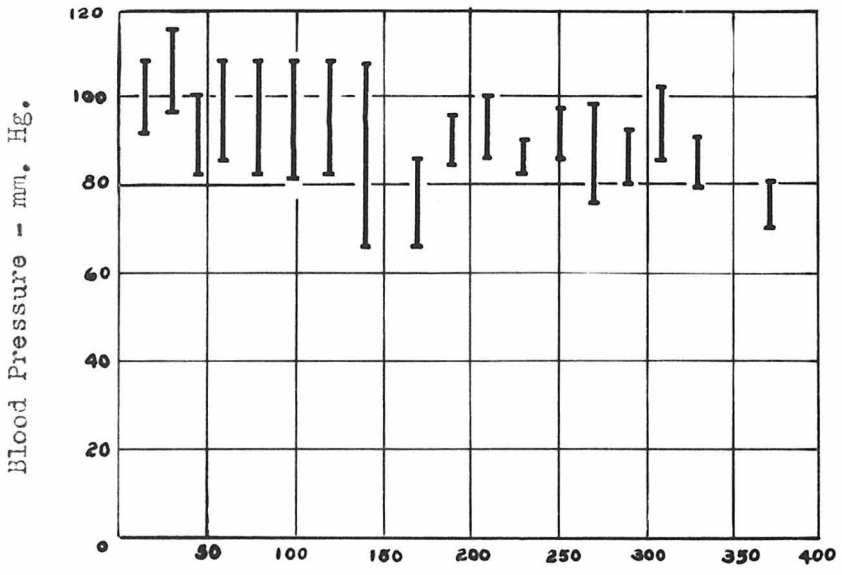

Fig 1 Rabbit transfused with oxypolygelatin No. 91 (Survived)

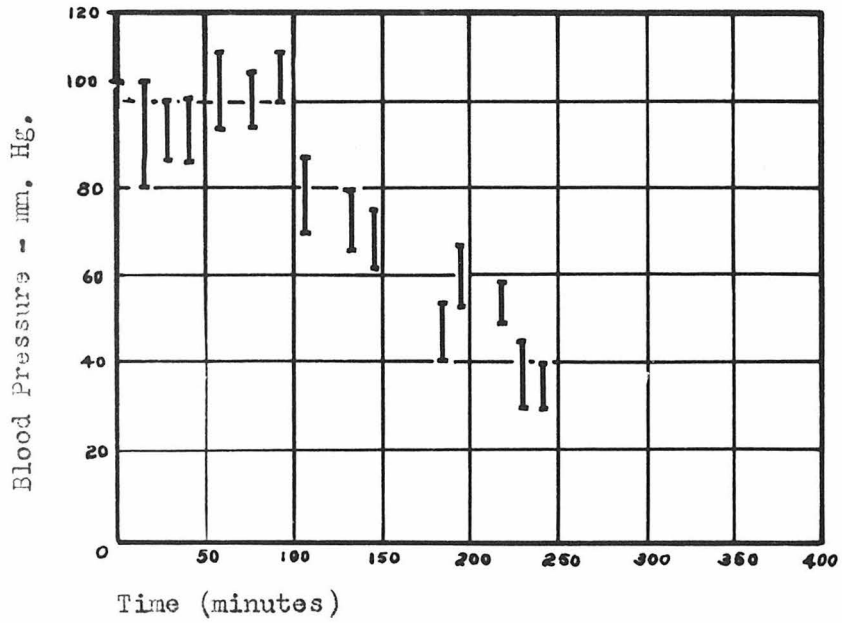

Fig 2 Rabbit transfused with hydrolyzed gelatin No. 97 (died in 240 min.)

and vein on each side. The rabbit was then treated with enough heparin to render its blood incoagulable for 5 hours, 5 mg./kg. usually being sufficient. A cannula was inserted in the femoral artery and blood pressure tracings were made on a kymograph by the usual manometric method, with either 10% sodium citrate or 5% Fastusol (BPA Pink) as anti-coagulant in the manometer circuit. The rabbit was bled from the femoral artery once an hour to the amount of 20 ml. of blood/kg. body weight. The hemorrhage was followed by replacement with plasma substitute equal in volume to the blood withdrawn. Five hemorrhages and injections were usually carried out, washed erythrocytes being added during the third and later injections. The results are given in Table I, and some of the blood pressure records are reproduced in Figures 3, 4, and 5. It is to be seen that replacement of the blood removed by an equivalent amount of Oxypolygelatin or of Oxypolygelatin with added erythrocytes restores the blood pressure adequately, and that, in general, the rabbits survive 4, 5, or 6 hemorrhages and injections. On the other hand, decalcified autoclaved Gelatin 97 was not effective under these conditions.

II. EXPERIMENTS ON FLUID BALANCE AND SURVIVAL

Experiments designed to assay the efficiency with which Oxypolygelatin and related materials would maintain

Table I Maintenance of Blood Pressure in Rabbits Following Plasmapheresis and Transfusion with Oxypolygelatin and Gelatin

Preparation	Rabbit	1st Hemorrhage			2nd Hemorrhage			3rd Hemorrhage			4th Hemorrhage			5th Hemorrhage			6th Hemorrhage		
		A*	B**	% recovery	A	B	% recovery	A	B	% recovery	A	B	% recovery	A	B	% recovery	A	B	% recovery
Oxypolygelatin 113	44E	70	70	100	90	75	83.5	68	+										
	44H	72	90	129	90	76	84.5	84	96	115	80	84	105	76	74	97.5			
	44I	84	94	127	92	84	91.5	84	98	117	88	85	96.5	94	96	102	84	78	93
	44G	84	90	107	80	76	95	70	70	100	70	70	100						
	44F	64	53	83	44	38	86.5	30	46	153	34	12							
Oxypolygelatin 111	44J	104	92	88.5	82	65	79.4	60	64	107	64	60	94	56	44	78.5			
	44M	104	66	63.5	66	52	79	40	+										
	44L	110	100	91	90	80	89	80	90	112	90	80	89	70	70	100			
	44K	100	+																
Oxypolygelatin 116	44O	76	76	100	63	60	95	+											
	44P	110	92	83.5	90	70	77.6	94	80	85	86	76	88.4	80	78	97.5			
	44R	134	116	86.5	122	104	85.2	90	110	122	96	84	87.5	66	45	68.3			
	44Q	104	104	100	84	84	100	84	80	95	80	100	125						
	44N	86	112	130	90	86	95.5	82	82	100	86	86	100						
Autoclaved Gelatin 97	44S	80	20	+															

* Blood pressure immediately preceding hemorrhage

** Blood pressure 30 minutes after hemorrhage

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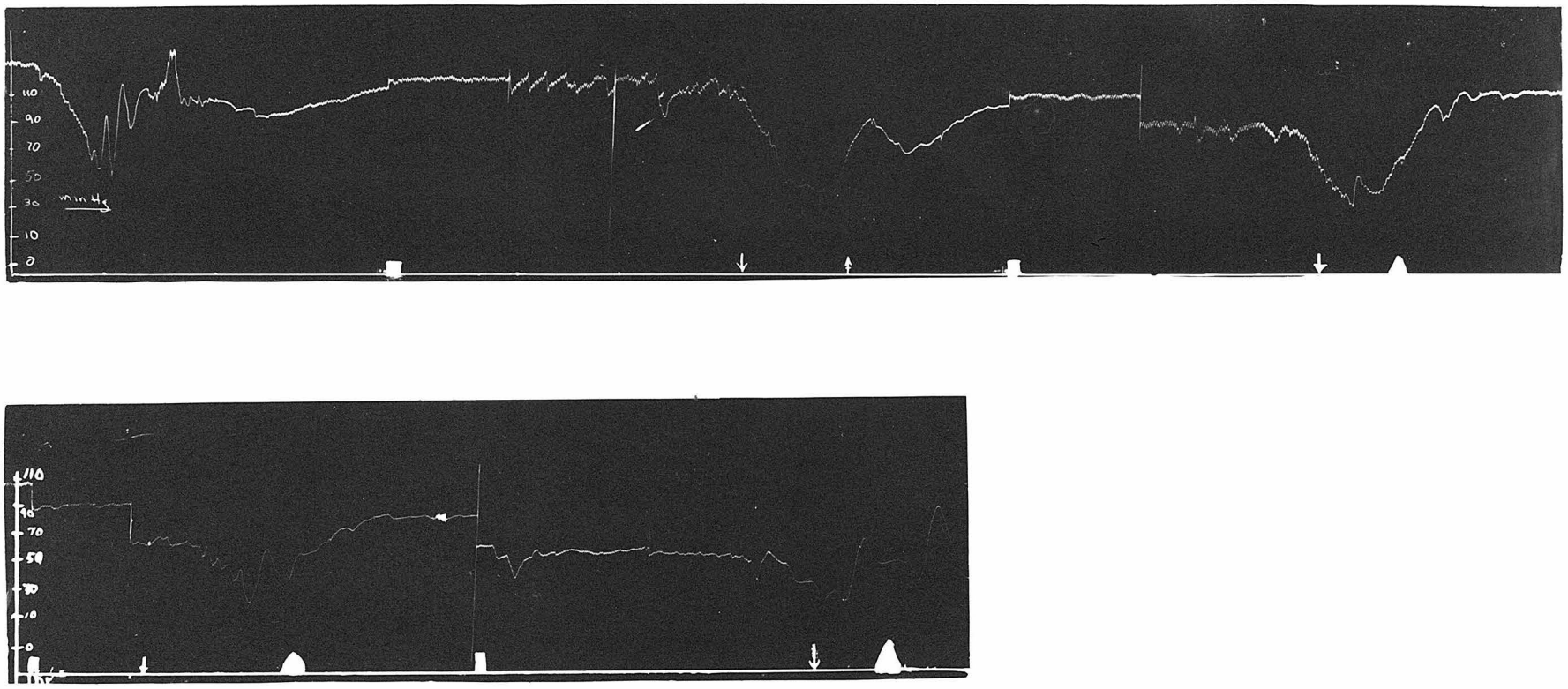


Figure 3 Kymograph record of continuous blood pressure measurement on Rabbit 44-R treated with OPG 116, (↓) indicates removal of 40 cc of blood, (↑) administration of 40 ml OPG, (▲) the administration of 40 ml OPG 116 + red cells, and (■) the passage of one hour.

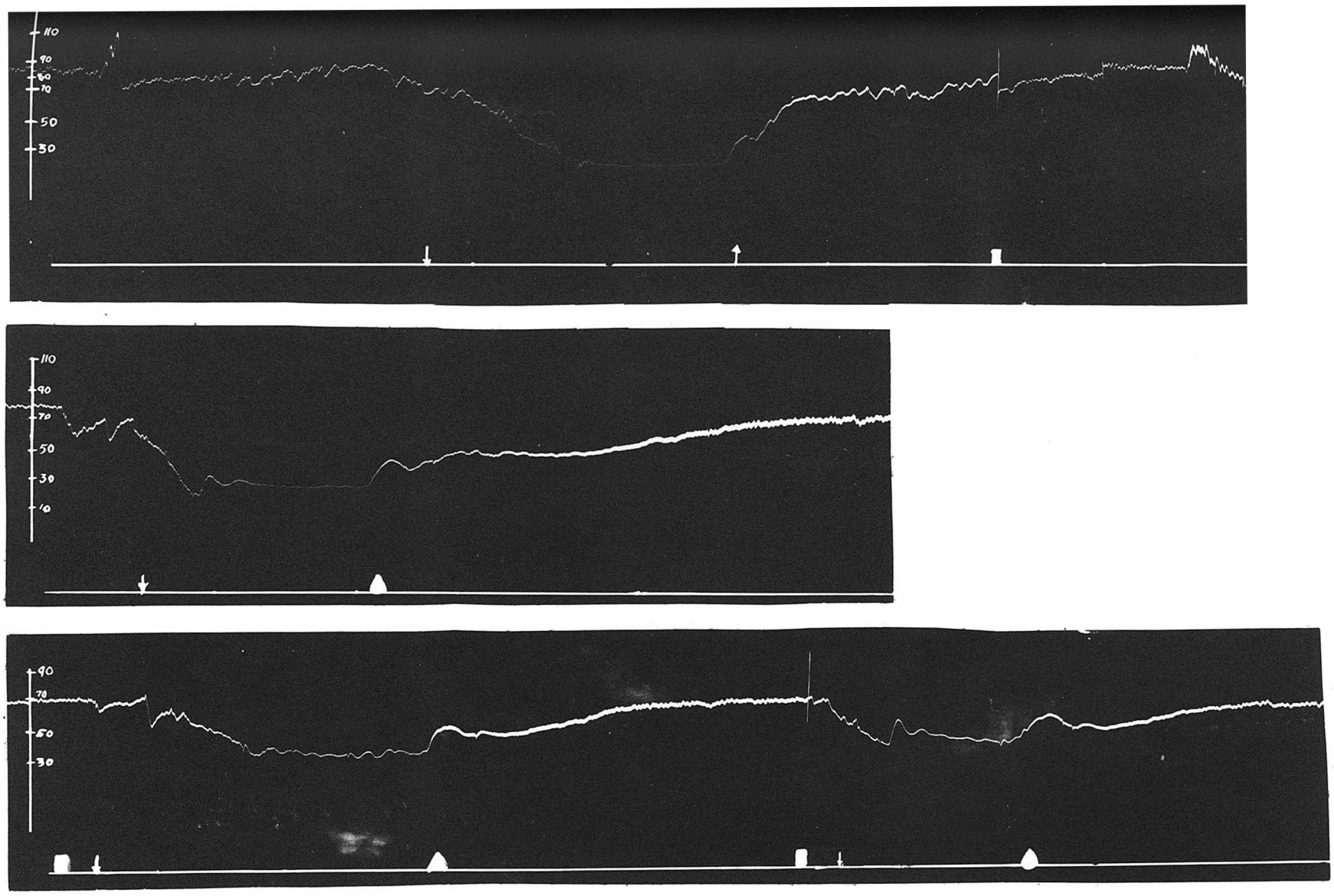


Figure 4 Kymograph record of continuous blood pressure measurement on Rabbit 44-G treated with OPG 113, (⬇) indicates removal of 40 cc of blood, (⬆) administration of 40 ml OPG, (▲) the administration of 40 ml OPG 113 + red cells, and (■) the passage of one hour.

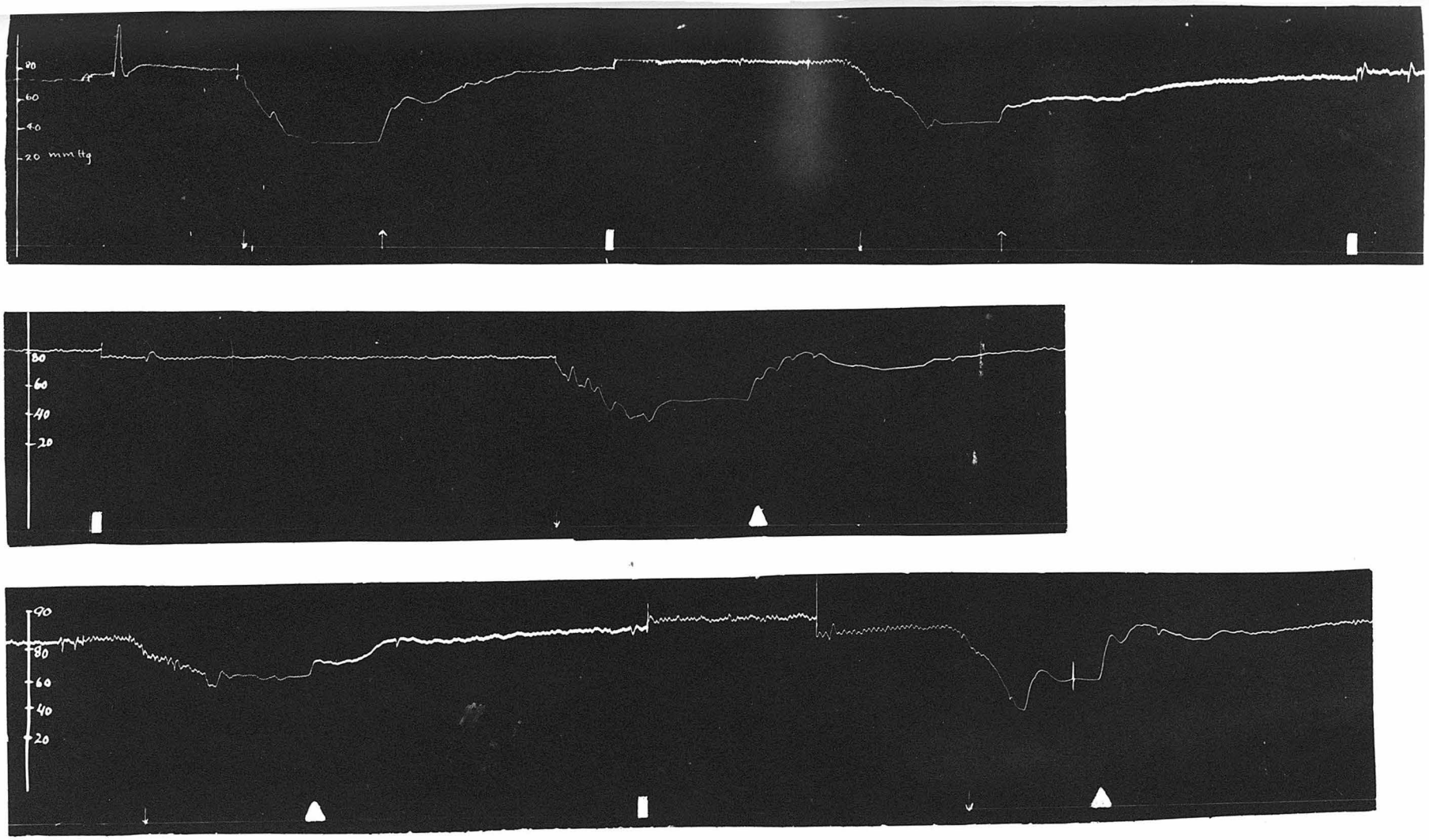
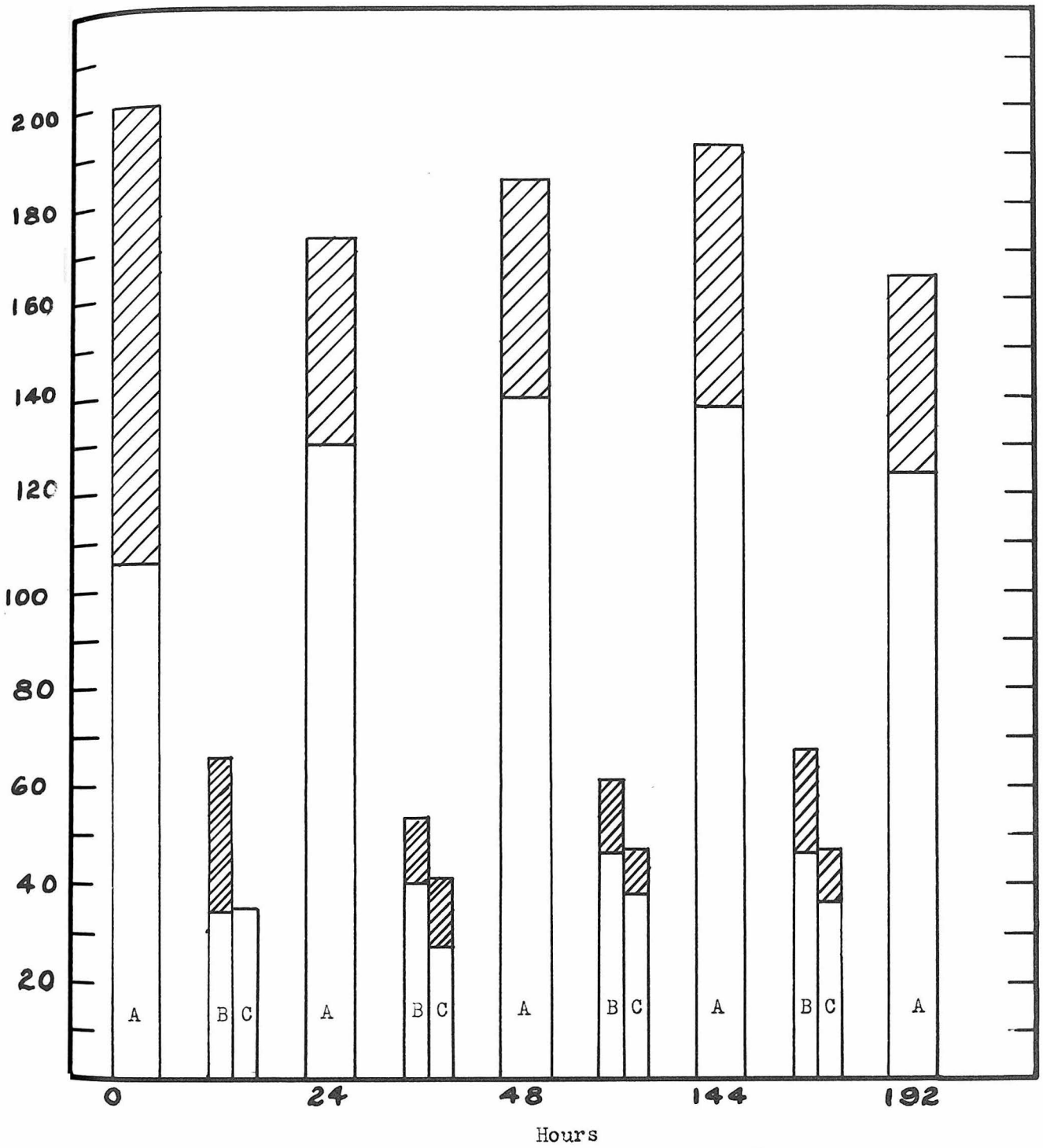


Figure 5 Kymograph record of continuous blood pressure measurement on Rabbit 44-H treated with OPG 113, (↓) indicates removal of 40 cc of blood, (↑) administration of 40 ml OPG, (▲) the administration of 40 ml OPG 113 + red cells, and (▮) the passage of one hour.

an adequate circulating volume, under conditions of repeated hemorrhage were performed on 86 rabbits. For information regarding possible changes which might occur in the plasma proteins, cataphoresis studies were made in selected cases. The findings of the fluid balance studies are the subject of a report in preparation but for illustrative purposes three protocols will be presented.

Rabbits were subjected to the usual procedures of hemorrhage, transfusion, and sampling under the surgical conditions previously described. Immediately after the hemorrhage an amount of Oxypolygelatin, gelatin, or saline roughly equivalent to one-third of the estimated plasma volume was injected. Prolonged experiments required the re-injection of saline-washed erythrocytes which had been withdrawn at a previous bleeding and were refrigerated. Periodically, as the animals' condition would warrant, the hemorrhage and transfusion procedures were repeated and continued until death of the animal.

Rabbit 13 showed the longest survival period and after 4 massive hemorrhages and transfusions maintained an adequate plasma volume but showed decreased capacity to restore cells and death ensued during the course of the fifth hemorrhage, made 192 hours after the beginning of the experiment (Figure 6). Cataphoresis patterns, made for this case by Dr. Stanley Swingle and kindly interpreted by him and Professor Dan H. Campbell, suggested "that either



Legend

CROSS-HATCH - Cells
CLEAR - Plasma
A - Blood Volume
B - Vol. of blood drawn by hemorrhage
C - Substitute + cells injected.

Figure 6 The effect of transfusion with OPG 91 on the blood and plasma volume of Rabbit 13.

Oxypolygelatin had become heterogeneous after injection or had formed complexes with the remaining serum proteins" (49). Since the in vitro mixing of Oxypolygelatin and plasma does not give patterns identical with those found intravenous injection, part of the complexity is due to the increase in plasma proteins, aminly fibrinogen and globulin, a condition characteristically observed after hemorrhage.

The results of experiments on Rabbits 16 and 17 are illustrated in the fluid balance protocol presented in Figure 8. After a relatively small loss of blood and subsequent saline injection the animal showed a restoration of plasma volume but had either lost cells or the cells had sedimented out since the hematocrit value was decreased. After about 4 hours the animals' condition began to decline and the animal was lost in the course of the dye dilution test. Rabbit 17 survived two hemorrhages and showed a restoration of plasma volume at 24 hours although the hematocrit was low. The injection of cells along with the saline after the second hemorrhage failed to prolong survival.

It is of interest to note here the changes in the rabbits which resulted from the prolonged transfusion with modified gelatin preparations. The animals lost weight due to insufficient intake of food in consequence of the anorexia which succeeded upon hemorrhage, no effort having been made to provide a diet more nutrient than the stock diet in use. An increased tendency to infection was noted in

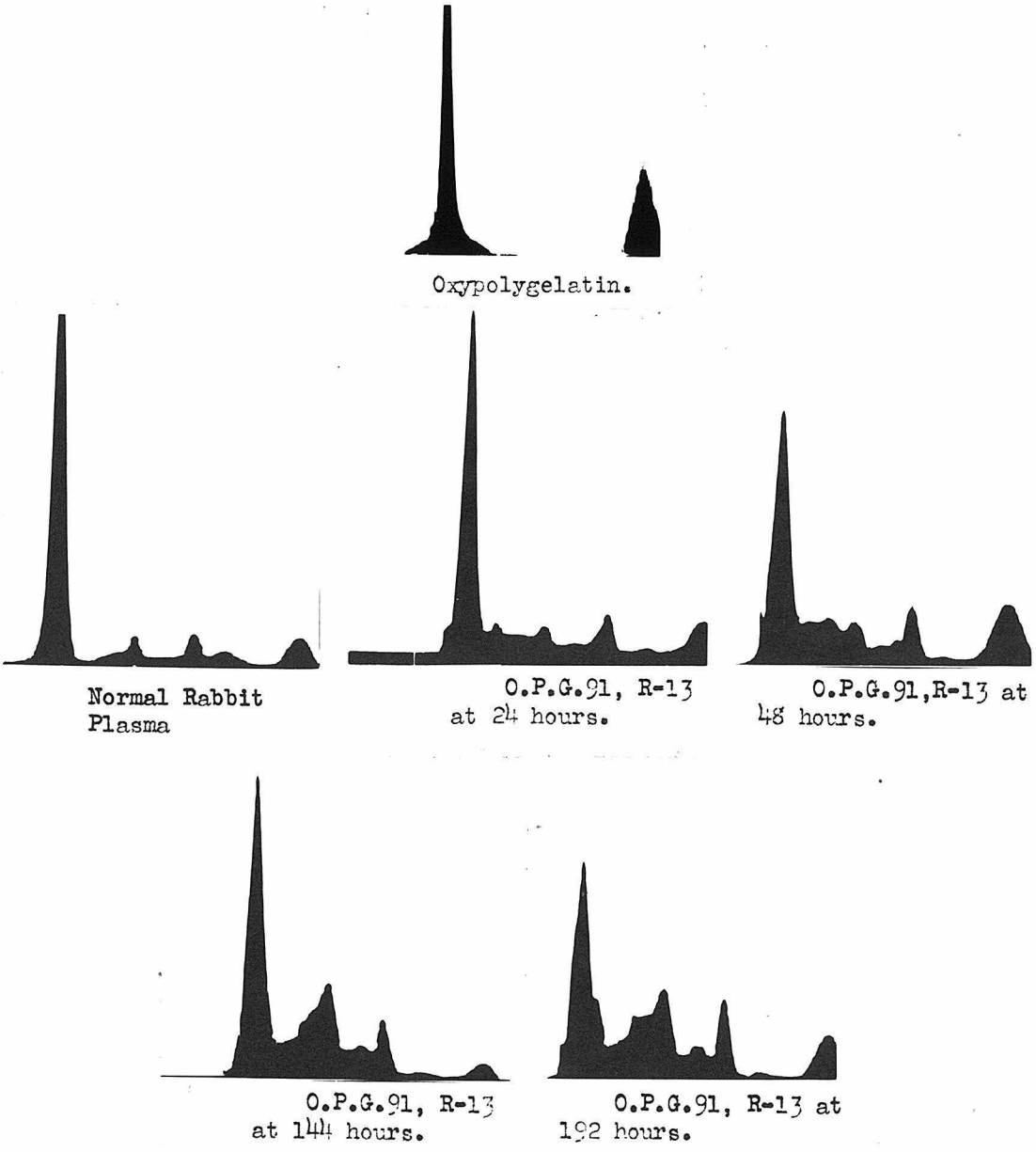
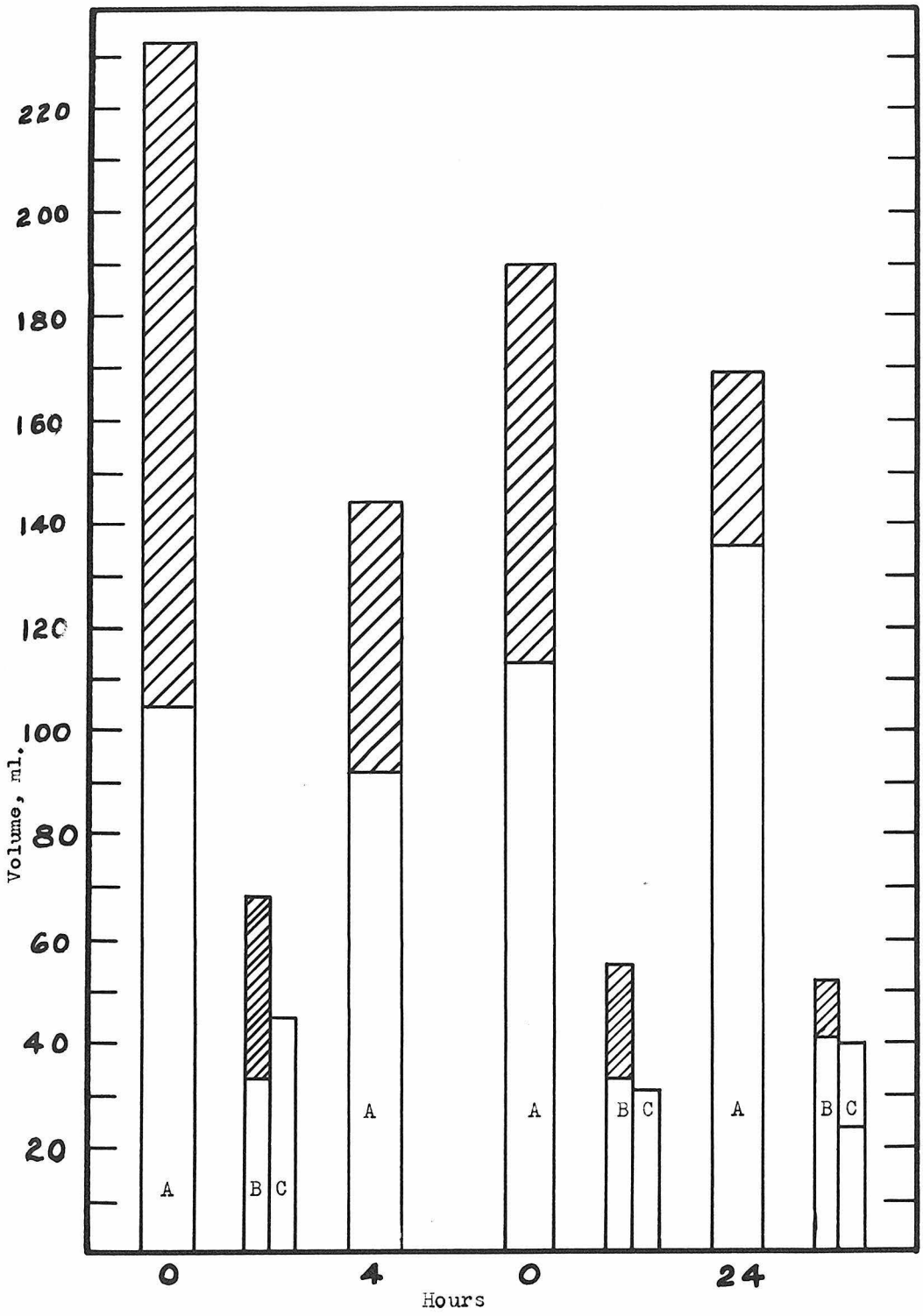


Figure 7. Changes in the cataphoretic patterns of plasma samples taken from Rabbit 13 after several massive hemorrhages and transfusions with Oxypolygelatin 91. The determinations were kindly made by Dr. Stanley M. Swingle. The current strength used was 20.5 ma for about 100 min.



Rabbit 16

Rabbit 17

Figure 8

The effect of transfusion with saline on the blood and plasma volume of Rabbits 16 and 17.

Legend

CROSS-HATCH - Cells
CLEAR - Plasma
A - Blood Volume
B - Vol. of blood drawn by hemorrhage
C - Substitute + cells injected.

most animals subjected to this type of experiment; this finding is related to that of Whipple and his collaborators,* who observed that on depletion of the serum proteins of a dog by plasmapheresis the state of persistent hypoproteinemia induced a lowered resistance to infection and to certain toxic materials.

III. IN VIVO FRACTIONATION OF OXYPOLYGELATIN 91 AFTER ITS ADMINISTRATION TO A HEMORRHAGED RABBIT

A rabbit weighing 1.8 kg. was anesthetized with 70 mg. of Nembutal and prepared for hemorrhage and transfusion according to the customary surgical procedures. After the withdrawal of about 60 ml. of blood, 54 ml. of Oxypolygelatin 91 were infused into the femoral vein. A retention catheter was passed into the bladder and the urine formed was continuously collected. Five hours later the animal was bled from the right femoral artery until 67 ml. of blood had been received and the animal went into tonic spasms. Saline was therefore continuously instilled via the femoral vein in an effort to maintain cardiac action thus to insure an adequate flow during the blood collection. The saline perfusion was maintained, and after 120 ml. had been thus administered the perfusate became entirely clear and the collection was stopped. The bladder contents were delivered into the catheter by squeezing the abdomen and last traces of urine were removed by washing with saline.

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* Madden, S. C., R. R. Woods, F. W. Shull, and G. H. Whipple, J. Exp. Med., 79, 607-24 (1944).

The blood and urine samples were centrifuged at 4500 r.p.m. and the respective supernatants were treated with equal volumes of trichloroacetic acid after which they were stored in the cold. The plasma proteins precipitated out whereas no precipitate in the urine was noted, either in the urine or in a sample of Oxypolygelatin 91 similarly treated with trichloroacetic acid. The solutions were next dialyzed against frequent changes of distilled water for two days and then dried by lyophil. The osmotic pressures of 1% solutions of untreated Oxypolygelatin 91, TCA-treated 91 and of the plasma and urine gelatin were measured and it was found that the mean molecular weight of the material retained in the circulation was higher than that of the material found in the urine.

Table II

Sample	Osmotic ($mm. H_2O$) Pressure (1% soln.)	Calculated Mean Molecular Weight
OPG 91 (untreated)	99	26,500
OPG 91(TCA treated)	86	29,300
Plasma 91	60	42,000
Urine 91	185	13,600

Cataphoretic studies were kindly performed by Dr. Stanley M. Swingle who concluded that both the plasma and the urinary gelatin appeared homogeneous with respect to charge. The cataphoresis patterns are presented in Figure 9.

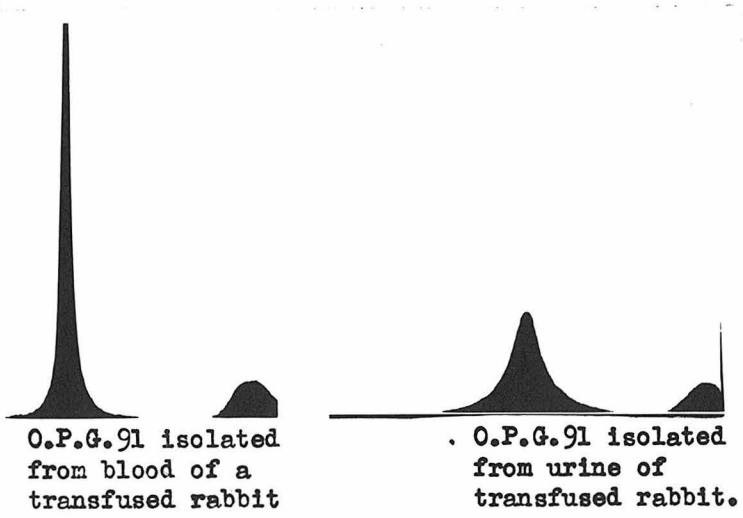

Figure 9. Cataphoresis patterns of Oxypolygelatin 91 isolated from the blood and urine of a rabbit 5 hours after its administration. Current 15 ma for 133 min. (cf. Figure 7).

BIBLIOGRAPHY

1. Beatty, C. H., The Effect of Hemorrhage on the Lactate/Pyruvate Ratio and the Arterial Venous Differences in Glucose and Lactate, Amer. J. Physiol., 143, 579 (1945).

2. Beatty, C. H., The Ability of the Liver to Change Blood Glucose and Lactate Concentrations Following Severe Hemorrhage, Amer. J. Physiol., 144, 233 (1945).

3. Behrmann, V. G., V. Schelling, and F. W. Hartman, Blood Histamine Levels in Experimental Burns, Amer. J. Physiol., 145, 483 (1946).

4. Bollman, J. L. and E. V. Flock, Changes in Phosphate of Muscle During Tourniquet Shock, Amer. J. Physiol., 142, 290 (1944).

5. Bollman, J. L. and E. V. Flock, Phosphate Turnover in Muscle During Shock, Amer. J. Physiol., 144, 437 (1945).

6. Darrow, D. C. and F. L. Engel, Liver Water and Electrolytes in Hemorrhagic Shcok, Amer. J. Physiol., 145, 32 (1945).

7. Engel, F. L. and M. C. Engel, Urea Synthesis from Amino Acids During Hemorrhagic Shock in the Nephrectomized Rat, Amer. J. Physiol., 147, 165 (1946).

8. Eversole, W. J., W. Kleinberg, R. R. Overman, J. W. Remington, and W. W. Swingle, The Nervous Factor in Shock Induced by Muscle Trauma in Normal Dogs, Amer. J. Physiol., 140, 490 (1944).

9. Hamilton, A. S. and D. A. Collins, Renal Hemorrhage Mechanism in Hemorrhage and Shock, Amer. J. Physiol., 144, 661 (1945).

10. Harkins, H. N. and C. N. H. Long, Metabolic Changes in Shock After Burns, Amer. J. Physiol., 136, 275 (1942).

11. Huidobro, F. and E. Braun-Menendez, Amer. J. Physiol., 137, 47 (1942).

12. Kline, D. L., The Effect of Hemorrhage on the Plasma Amino N of the Dog, Amer. J. Physiol., 146, 654 (1946).

13. LePage, G. A., Biological Energy Transformations During Shock as Shown by Tissue Analysis, Amer. J. Physiol., 146, 267 (1946).

14. McShan, W. H., V. R. Potter, A. Goldman, E. G, Shipley, and R. K. Meyer, Biological Energy Transformations During Shock as Shown by Blood Chemistry, Amer. J. Physiol., 145, 93 (1945).

15. Meyer, R. K., W. H. McShan, A. Goldman, and E. G. Shipley, Biochemical Changes in the Blood of Rats After Reapplication of Limb Tourniquets, Amer. J. Physiol., 147, 66 (1946).

16. Mylon, E., C. W. Cashman, and M. C. Winternitz, Studies on the Mechanism Involved in Shock and its Therapy, Amer. J. Physiol., 144, 494 (1945).

17. Mylon, E. and M. C. Winternitz, Factors Concerned with the Induction of Tourniquet Shock, Amer. J. Physiol., 144, 494 (1945).

18. Mylon, E., and M. C. Winternitz, The Differentiation of Potassium from Other Agents Associated with the Toxic Effects of Tourniquet Shock, Amer. J. Physiol., 146, 254 (1946).

19. Mylon, E., M. C. Winternitz, R. Katzenstein, and G. J. de Suto-Nagy, Studies on Mechanisms Involved in Shock, Amer. J. Physiol., 137, 280 (1942).

20. Phillips, R. A., V. P. Dole, P. B. Hamilton, K. Emerson, Jr., R. M. Archibald, and D. D. Van Slyke, Effects of Acute Hemorrhagic and Traumatic Shock on Renal Function of Dogs, Amer. J. Physiol., 145, 314 (1946).

21. Rapport, D., R. Guild, and A. Canzanelli, The Transmission by Crossed Circulation of a Shock-Producing Factor, Amer. J. Physiol., 143, 440 (1945).

22. Russel, J. A. and C. N. H. Long, Amino Nitrogen in Liver and Muscle of Rats in Shock After Hemorrhage, Amer. J. Physiol., 147, 175 (1946).

23. Wilhelmi, A. E., J. A. Russel, F. L. Engel, and C. N. H. Long, The Effects of Hepatic Anoxia on the Respiration of Liver Slices In Vitro, Amer. J. Physiol., 144, 669 (1945).

24. Wilhelmi, A. E., J. A. Russel, M. G. Engel, and C. N. H. Long, Some Aspects of the Nitrogen Metabolism of Liver Tissue from Rats in Hemorrhagic Shock, Amer. J. Physiol., 144, 674 (1945).

25. Wilhelmi, A. E., J. A. Russel, C. N. H. Long, and M. G. Engel, The Effects of Anoxia and of Hemorrhage Upon the Metabolism of the Cerebral Cortex of the Rat, Amer. J. Physiol., 144, 683 (1945).

26. Starling, E. H., On the Absorption of Fluids from the Connective Tissue Spaces, J. Physiol., 19, 312 (1896).

27. Bayliss, W. M., Methods of Raising a Low Arterial Pressure, Proc. Roy. Soc., 89B, 380 (1917).

28. Amberson, W. R., Blood Substitutes, Biol. Revs., 12, 48 (1937).

29. Hogan, J. J., The Intravenous Use of Colloidal (Gelatin) Solutions in Shock, J. Am. Med. Assoc., 64, 721 (1915).

30. Ni, T. G., The Use of Ah-Chiao (Donkey-Skin Glue) for Circulatory Failure Encountered in Severe Hemorrhage and Shock, Chinese J. Physiol., 10, 125 (1936).

31. Taylor, N. B. and E. T. Waters, Isinglass as a Transfusion Fluid in Hemorrhage, Canadian Med. Assoc. J., 44, 547 (1941).

32. Taylor, N. B., M. S. Moorhouse, and A. J. Stonyer, The Use of Isinglass as a Blood Substitute in Hemorrhage and Shock, Canadian Med. Assoc. J., 49, 251 (1943).

33. Waters, E. T., Comparison of Gelatin and Isinglass as Blood Substitutes, Canadian Med. Assoc. J., 45, 395 (1941).

34. Lanni, F., G. A. Feigen, and A. L. LeRosen, The Determination of Gelatin in the Presence of Plasma Proteins, Arch. Biochem., 8, 251 (1945).

35. Knoefel, P. K. and G. Lehmann, Behavior in the Body of Some Fractions of Gelatin, J. Pharmacol. Exp. Therap., 83, 185 (1945).

36. Gordon, H., L. Hoge, and H. Lawson, Gelatin as a Substitute for Blood After Experimental Hemorrhage, Amer. J. Med. Sci., 204, 4 (1942).

37. Lawson, H. and W. S. Rehm, The Relative Value of Various Fluids in Replacement of Blood Lost by Hemorrhage with Special Reference to the Value of Gelatin Solutions, Amer. J. Physiol., 140, 431 (1943).

38. Lawson, H. and W. S. Rehm, The Efficacy of Gelatin and Other Cell-Free Fluids in Reversing the Effects of Nearly Complete Exsanguination, Amer. J. Physiol., 133, 217 (1945).

39. Little, J. M. and H. S. Wells, Capillary Permeability to Intravenously Administered Gelatine, Amer. J. Physiol., 138, 495 (1943).

40. Parkins, W. M., C. E. Koop, C. Riegel, H. M. Vars, and J. S. Lockwood, Gelatin as a Plasma Substitute: With Particular Reference to Experimental Hemorrhage and Burn Shock, Ann. Surg., 118, 193 (1943).

41. Kleinberg, W., J. W. Remington, W. J. Eversole, R. R. Overman, and W. W. Swingle, The Effectiveness of Plasma Gelatin and Saline Transfusions in Preventing Shock Induced by Leg Muscle Trauma and Tourniquets, Amer. J. Physiol., 140, 197 (1943).

42. Swingle, W. W., W. Kleinberg, and H. W. Hays, A Study of Gelatin and Saline as Plasma Substitutes, Amer. J. Physiol., 141, 329 (1944).

43. Swingle, W. W. and W. Kleinberg, Plasma, Gelatin, and Saline in Experimental Wound Shock, Amer. J. Physiol., 141, 329 (1944).

44. Brightman, I. J. and R. F. Korns, Homologous Serum Jaundice in Recipients of Pooled Plasma, J. Am. Med. Assoc., 133, 268 (1947).

45. Robscheit-Robbins, F. S., L. L. Miller, and G. H. Whipple, Gelatin, its Usefulness and Toxicity, J. Exp. Med., 80, 145 (1944).

46. Campbell, D. H. and A. Cherkin, The Destruction of Pyrogens by Hydrogen Peroxide, Science, 102, 535 (1945).

47. Knisely, M. H., T. S. Eliot, and E. H. Bloch, Sludged Blood in Traumatic Shock. I. Microscopic Observations of the Precipitation and Agglutination of Blood, etc., Arch. Surg., 51, 220 (1945).

48. Janeway, C. A., In Mudd, S. and W. Thalheimer, Blood Substitutes and Blood Transfusion, Charles C Thomas, Springfield, Illinois and Baltimore, Maryland (1942).

49. Campbell, D. H., J. B. Koepfli, L. Pauling, N. S. Abrahamsen, A. L. LeRosen, F. Lanni, W. B. Dandliker, and G. A. Feigen, The Condensation and Oxidation of Gelatin with Glyoxal and Hydrogen Peroxide for the Preparation of a Plasma Substitute (Oxypolygelatin), In preparation.

50. Pauling, L., Responsible Investigator, OEMcmr-153, Chemically Modified Proteins as Blood Substitutes (1946).

51. Price, P. B. and W. P. Longmire, The Use of T-1824 in Plasma Volume Determinations, Bull. Johns Hopkins Hosp., LXXI, 51 (1942).

52. Mather, K., R. G. Bowler, A. C. Crooke, and C. J. O. R. Morris, The Precision of Plasma Volume Determinations Made by the Evans Blue Method, Brit. J. Exp. Path., XXXVIII, 12 (1947).

53. Gregersen, M. I. and J. G. Gibson, Conditions Affecting the Absorption Spectra of Vital Dyes in Plasma, Amer. J. Physiol., 120, 494 (1937).

54. Pressman, D., The Determination of Microquantities of Certain Proteins, Ind. Eng. Chem., Anal. Ed., 15, 357 (1943).

55. Lanni, F., Ph.D. Thesis. II. The Determination of Gelatin in the Presence of Plasma Proteins, California Institute of Technology (1946).

56. Hyman, C., Ph.D. Thesis. The Rate of Filtration from the Vascular System as Related to Changes in the Driving Force Under Various Conditions, New York University (1944).

Chapter III

The Effects of Oxypolygelatin and Sodium Salicylate on the Sedimentation of Erythrocytes *in vitro*

Chapter III

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I. INTRODUCTION

Fåhraeus (1) proved that the sedimentation characteristics of a given blood sample were dependent mainly on the plasma by demonstrating that cells derived from a slowly sedimenting blood could be made to sink rapidly when they were added to the plasma of a rapidly sedimenting sample. He ascribed the decreased suspension stability of the blood observed in pregnancy and in many clinical conditions to be dependent on the plasma proteins; thus, increases in fibrinogen or relative increments in the globulin albumin ratio which tended to occur in the course of a disease were found to be correlated with the increased sinking velocity of the cells. The effect of fibrinogen and globulin in increasing the sedimentation rate was found to be due to the heightened tendency of the cells to aggregate into rouleaux, the size and frequency of such aggregates being dependent on the concentration of these proteins. The suggestion that sedimentation rate is a measure of plasma fibrinogen has been recently made by Ham and Curtis (2) who observed a linear correlation between sedimentation rate and the amount of fibrinogen added to defibrinated blood.

Knowledge concerning the mechanism of cell aggregation is scant; it has been suggested by Ponder (3) to

be due to the increased "stickiness" of the cell surface, induced by alternations in the physico-chemical state of the plasma protein. The possibility that increased sedimentation is the result of protein adsorption to the red cell surface was doubted by Monaghan and White (4) since they failed to observe changes in the electrophoretic mobility of cells by the introduction of proteins which caused sedimentation to occur.

The rate of erythrocyte sedimentation is markedly accelerated in the presence of a number of substances such as methyl cellulose, pectin, gum acacia, and gelatin, and recent interest in the problem of erythocyte suspension stability has paralleled the increased use of these materials as blood substitutes. Another source of interest in the problem has arisen from the observations relative to the inhibiting action of salicylates on sedimentation.

The present investigation was undertaken as part of the program on blood substitute in an attempt to determine the nature of the conditions under which sedimentation of red cells occurred in Oxypolygelatin and the subsequent orienting experiments to be presented here were made with the idea of sufficiently familiarizing ourselves with the conditions under which sedimentation in Oxypolygelatin occurs to be able to propose a problem suitable for future proof.

II. PURPOSE

The purpose of this investigation is to present evidence that sedimentation is based on some type of inter-

action of cells and Oxypolygelatin based on considerations of the difference in the sedimentation behavior of cells in saline and Oxypolygelatin, the difference in behavior of cells from different donors, the effect of concentration of Oxypolygelatin on the sedimentation rate of cells, the influence of different fractions of Oxypolygelatin on the sedimentation rate, and the influence of chemical structure on inhibition of sedimentation. Included also are several studies in which an attempt has been made to learn something of the site of action of the inhibitor, sodium salicylate, by efforts to determine the quantitative aspects of its action with respect to changes in protein concentration, its influence on the physical and chemical properties of the protein, and its action on the erythrocytes.

III. EXPERIMENTAL

A. The Influence of Oxypolygelatin on the Sedimentation Rate of Erythrocytes

1. Materials

a. Erythrocytes.- Human as well as rabbit erythrocytes were employed in these experiments.

Rabbit Cells.- Blood was obtained from 4-6 kg. rabbits by means of cardiac puncture. Generally 50-70-ml. samples were drawn into a large syringe containing 5 ml. of an oxalate anticoagulant solution. The cells were separated by centrifuging in an International clinical model apparatus, equipped with either an angle or short-arm head, for a period of 30 minutes at 2000 r.p.m. The supernatant plasma was then removed and the cells poured into an Erlenmeyer flask containing sufficient isotonic saline to make a final 10% suspension of the cells. The cells were swirled for 2 minutes in this vessel and then transferred into 50-ml. plastic centrifuge cups. The cell suspensions were centrifuged under the conditions previously described, the washing and centrifuging being continued for at least six, and for as many more times as were necessary to free the supernatant saline from traces of hemoglobin or serum protein.

Human Cells.- Human cells were made available to us by the Hyland Laboratories. Blood was received under aseptic conditions into transfusion units containing citrate as the anticoagulant and the cells and plasma were separated

by centrifugation. These cells were obtained within 24 hours of their withdrawal from the subjects and sufficient quantities were processed for one complete series of tests. Since it was found that the cell residues contained variable amounts of fibrin, it was necessary to remove the clots by passing the cells through a Baxter "Filter-drip" equipped with a 200-mesh screen. One such passage was, in general, sufficient to remove all clots.

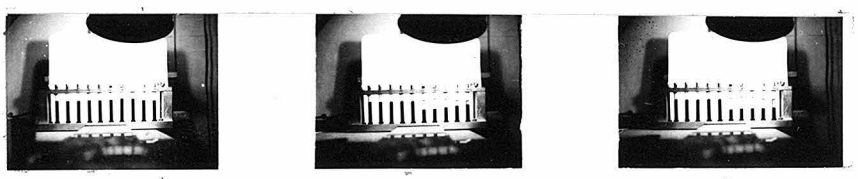
Following their filtration, the cells were washed once in 9 volumes of 1% sodium citrate and 7 times in isotonic saline; the same volumes, speeds, and times being involved for human cells as were used for rabbit cells.

b. Oxypolygelatin.- The stock solutions used in these investigations contained 5% Oxypolygelatin; although the pH of the Oxypolygelatin solutions was in the region of 5.5 the addition of washed cells was sufficient to adjust the suspension to pH 7.2.

2. Method.-

One ml. of 5% Oxypolygelatin (or a suitable dilution) was delivered into each of the ten 6 X 80 mm. calipered tubes which were held rigidly in a specially constructed rack.* Packed cells (0.5 ml. per tube) were next added

* Figure 1. A typical experiment arrangement is illustrated in the series of photographs presented below.



Zero time 10 min. 20 min.

Tubes 1-5 (l. to r.) - 5.75% OPG 0.6% salicylate
Tubes 6-10 " " " - " " no salicylate

from a 2-ml. syringe fitted with a 20-gauge needle. Care was taken not to injure the cells by sudden changes of pressure, and to this end the delivery from the syringe was made by "screwing" the plunger into the barrel.

The tubes were stoppered with corks, and their contents mixed for 10 minutes by rotating the rack through 180°, 10 times per minute. At the end of the agitation period the rack was placed on a stand to which, at a given distance, was fastened a camera. The amount of sedimentation of a given series was recorded simultaneously by making exposures at 5-minute intervals over a period of 1 hour.

The processed negatives were projected on the screen of a microfilm reader and the sedimentation values of each tube for every period was recorded. The readings (distance from top of the meniscus to layer of packed cells) were expressed directly in millimeters, since both the exposure and projection distances were equal.

In some of the preliminary experiments it was noted that variations in sedimentation rates were attributable to traces of acid remaining in the tubes after cleaning, hence the following procedure was employed in subsequent preparation of tubes: immediately after use the tubes were rinsed clean with distilled water; they were then swabbed out with an applicator on the end of which was fixed a

a piece of cotton moistened with a dilute Aerosol solution. Following treatment with Aerosol, the tubes were rinsed with 15 changes of distilled water and finally allowed to drain until dry.

a. Comparative influence of saline, Oxypolygelatin, and serum proteins on the sedimentation rate of erythrocytes.

(1.) Saline.- The sedimentation of rabbit erythrocytes in saline was found to be exceedingly slow and dependent inversely on the proportion of cells to supernatant solution. The results of an experiment in which the cell volume was varied from 6.77% to 35.7% showed the fastest sedimentation to occur in the tube which had the smallest proportion of cells (See Table 1 and Figure 2.)

(2.) Serum Proteins.* Fåhraeus (op. cit.) reported that both fibrinogen and globulin accelerated the sedimentation rate of human erythrocytes while albumin had no effect. The sedimentation rate of human cells was measured in globulin and albumin solutions made up to concentrations of 5%, 3.75%, 2.5%, and 1.25% protein. The sedimentation rate of cells in all systems was, in absolute terms, very slow. However, at 10 hours definite differences were noted, both between albumin and globulin, on the one hand, and between the higher and the lower concentrations of globulin, on the other.

* The albumin and globulin samples used in the experiments were obtained from the Department of Physical Chemistry, Harvard University, through the kindness of Dr. E. J. Cohn.

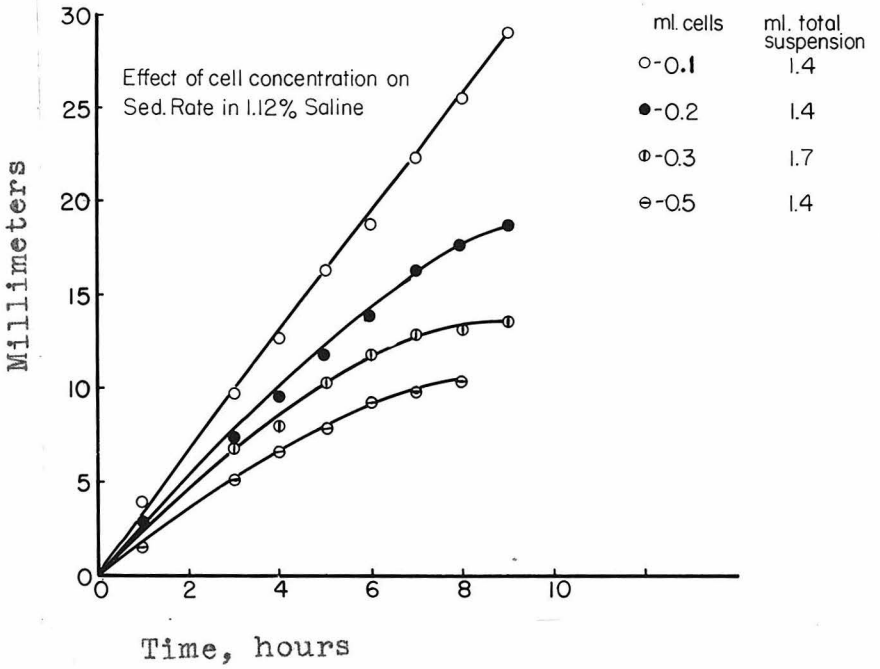

Effect of cell concentration on
Sed. Rate in 1.12% Saline
ml. cells
ml. total suspension
○-0.1
1.4
●-0.2
1.4
⊕-0.3
1.7
⊖-0.5
1.4
Millimeters
Time, hours
0
5
10
15
20
25
30
0
2
4
6
8
10

Fig. 2

The cell levels for any concentration of albumin did not differ significantly from those in saline nor from the 1.25% and 2.5% solutions of globulin. The cell levels in the 3.75% and 5.0% globulin solutions were found to be much lower.

Table I. Sedimentation of Cells in 1.12% NaCl

Ml. Cells	Final Ml. Suspension	% Cells in Suspension	Sed. Rate Mm./Hour
0.1	1.4	6.77	3.25
0.2	1.4	14.3	2.40
0.3	1.7	17.65	2.05
0.5	1.4	35.7	1.50

Table II

	Cell Levels* (Mm.) at 10 Hours				
% Protein	5%	3.75%	2.50%	1.25%	0.9%
Albumin	3.5	3.5	3.0	4	--
Globulin	10	8	4	4	--
Saline	--	--	--	--	3.5

* Distance from top of meniscus to layer of packed cells.

(3) Oxypolygelatin.- One-half-ml. portions of packed rabbit cells were added to sedimentation tubes containing Oxypolygelatin 116 solutions of varying protein concentration, each buffered to pH 7.2 with 0.145 M phosphate buffer. Figure 3 shows the sedimentation rate of such systems to be symbatic** with protein concentration. Systems containing 3% and 4% Oxypolygelatin 116 sedimented the most rapidly, their curves coinciding at all points.

** This term implies variance in the same direction but not proportionally.

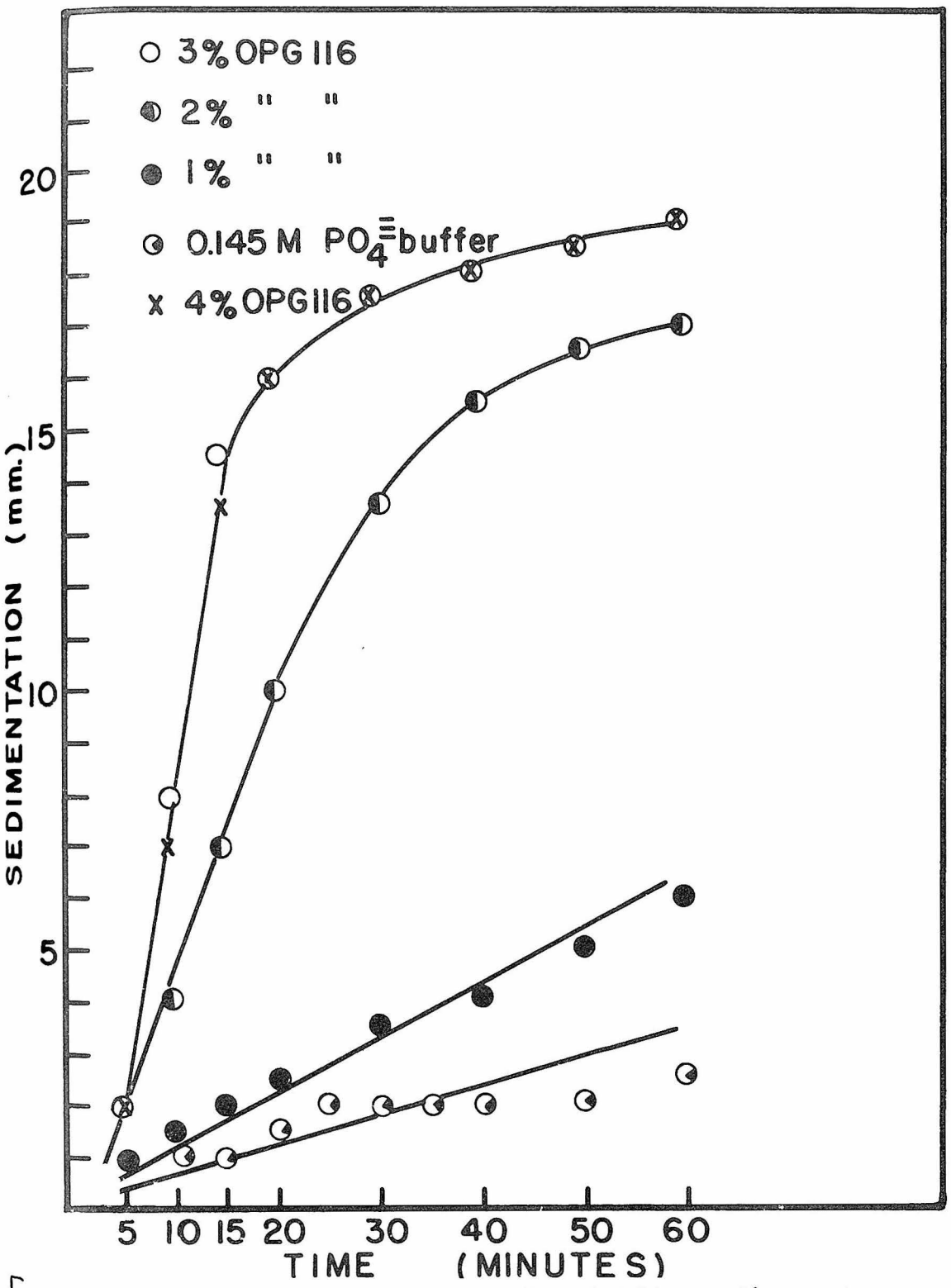
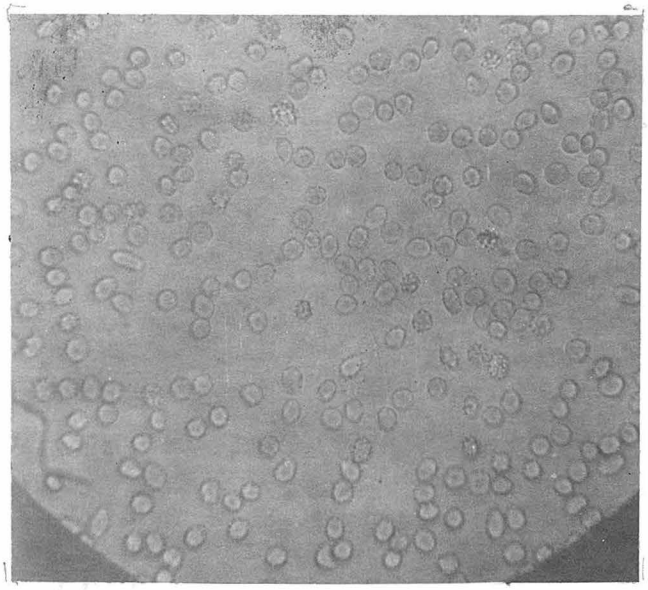


Fig 3 Sedimentation rate of rabbit erythrocytes in various concentrations of OPG116 (0.145 M $PO_4^{\equiv}$ buffer pH 7.2)

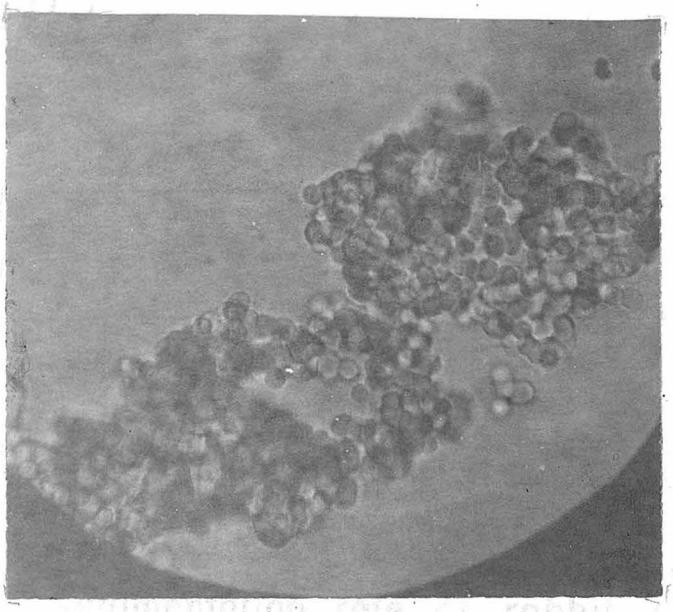
The system containing 2% Oxypolygelatin was the next in rapidity, while the tube containing 1% Oxypolygelatin was quite slow in settling, differing only slightly from the condition in which buffer alone was present.

Photomicrographs taken of all cell samples 30 minutes after their exposure to 1.1% saline and 5% Oxypolygelatin 12676AY, respectively, revealed that the cells in gelatin formed a large aggregates, whereas the cells in saline solution remained individuated. (Figure 4.) This sort of aggregation is responsible for the increased in vitro sedimentation rate of cells in a gelatin medium.

b. The effect of incubation and resuspension of erythrocytes on their sedimentation rate in Oxypolygelatin.- It has been suggested in the preceding section that the accelerated sedimentation rate of red cells in gelatin solutions might be attributed to their increased tendency to form aggregates. The intrinsic strength of such aggregates was tested by resuspending the system represented in Figure 3 after it had been incubated in Oxypolygelatin at room temperature for 3 hours. The results after such resuspension by agitation are indicated in Figure 5. The only difference noted between these two figures is the very slight change in the order of sedimentation rate of the 3% and 4% categories; Figure 3 represents their values as coincident, whereas Figure 5 (after 3 hours' incubation) shows the 3% curve to be slightly steeper in slope. Since



Rabbit Erythrocytes after 30'
in 1.12% NaCl solution



Rabbit Erythrocytes after 30'

in 5% OPG 12676AY

Fig. 4

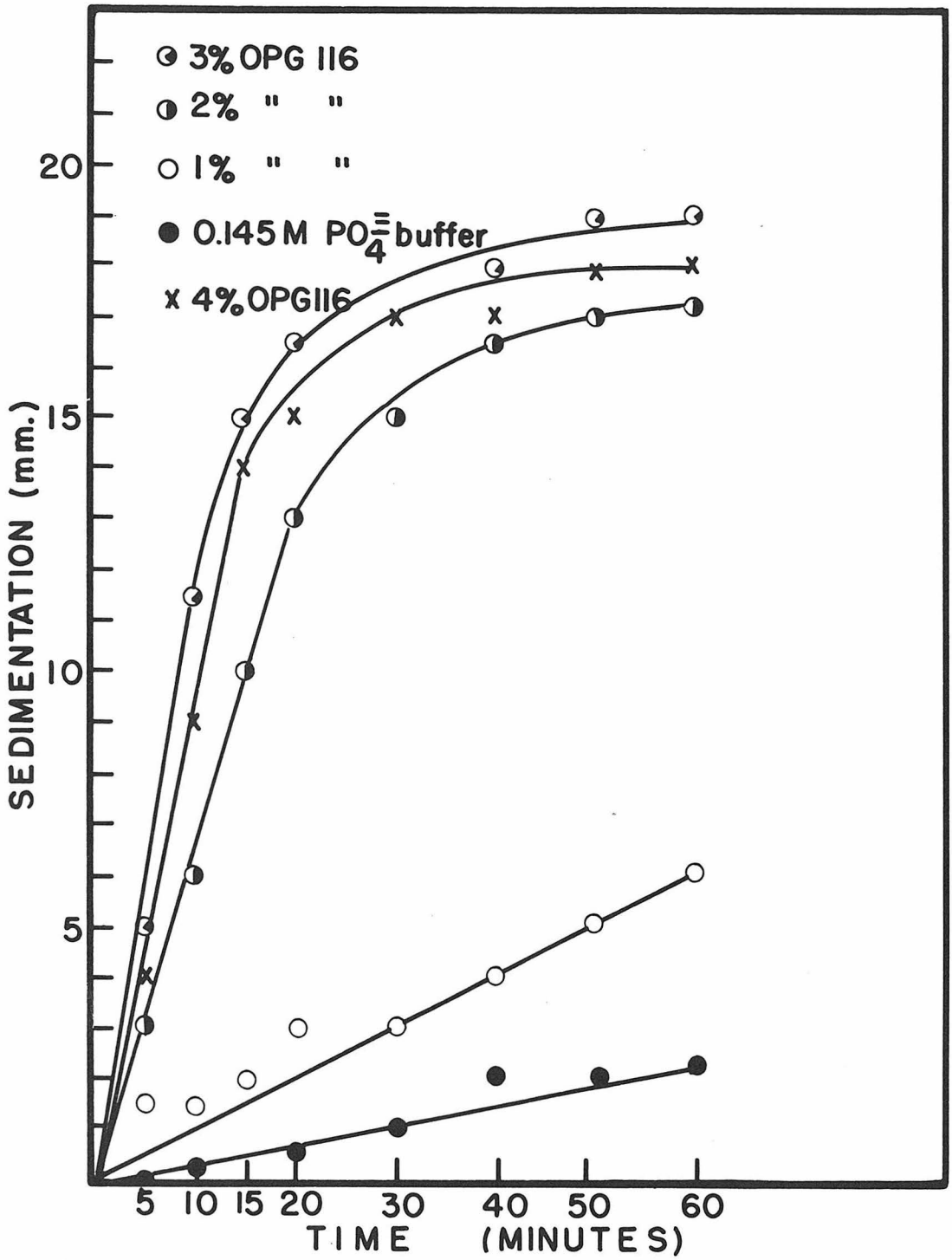


Fig 5 Effect of incubation and resuspension on the sedimentation rate of rabbit erythrocytes in various concentrations of OPG 116 in 0.145 M $PO_4^{\equiv}$ buffer.

these values were read visually, the slight difference in time of measurement might account for the variation in otherwise identical results.

An alternate explanation might consider the possible fact that the rouleaux formed by 4% protein do not dissociate with quite such readiness as those formed by the lower concentrations.

c. The influence of the source and age of erythrocytes on their sedimentation rate in Oxypolygelatin.- Preliminary experiments indicated that human and rabbit erythrocytes would settle in Oxypolygelatin under the conditions of this experiment whereas samples of cells of the cat, sheep, and ox were not susceptible to its influence. This peculiarity might strengthen the hypothesis of the existence of some specialized areas on the cell surface, present in the human and rabbit cells but absent in the cells of other species tested, which possess means of interacting with Oxypolygelatin.

For the sake of experimental uniformity, and because the preparation and washing of a given quantity of cells was rather time-consuming, it was customary to process enough erythrocytes from a given donor to suffice for several experiments. Experience had shown that a greater reproducibility of sedimentation could be achieved if, after washing, the cells were incubated for about 18 hours in the refrigerator before their use. An experiment designed to test the reproducibility of the sedimentation behavior of cells after storage was made on rabbit cells incubated for several days under the previously described conditions. One-half-milliliter portions of cells were delivered into each of 10 tubes containing 1 ml. of Oxypolygelatin 12676AY and the sedimentation recorded in the usual way. There was essentially no difference in rate between cells kept for one or two days, although the latter showed a faster rate of fall. (See Figure 6.) Erythrocytes stored for longer periods developed

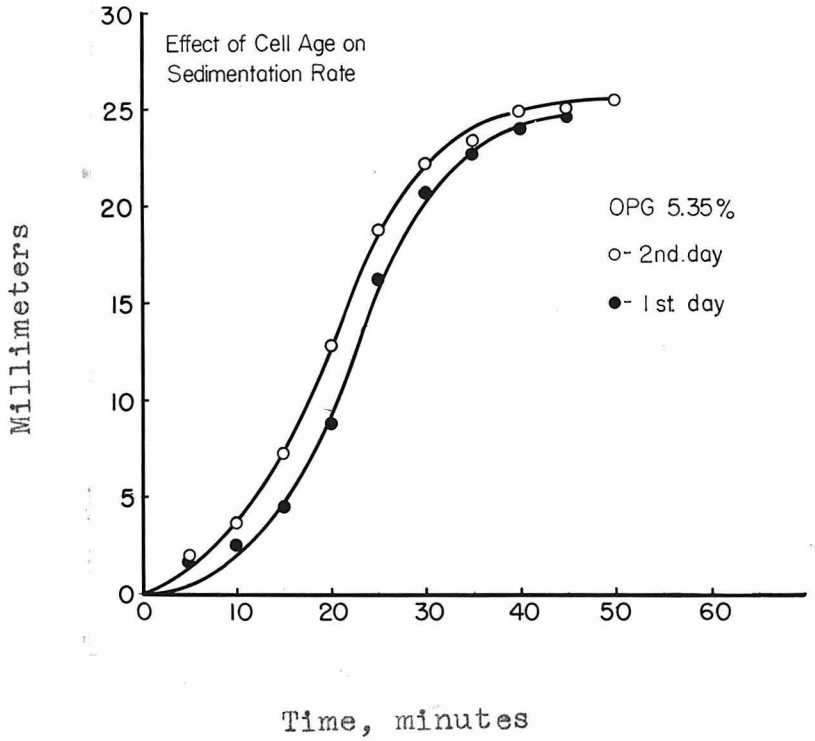

Effect of Cell Age on
Sedimentation Rate
OPG 5.35%
o- 2nd.day
●- 1 st. day
Millimeters
Time, minutes
0
5
10
15
20
25
30
0
10
20
30
40
50
60

Fig. 6

variable degrees of hemolysis which could not be removed by repeated washing and were therefore arbitrarily discarded from the tests.

Although the reproducibility of sedimentation of a given sample of cells was high in a particular experiment of 10 tubes, a considerable amount of variation was observed between samples. This is reflected in a high internal consistency in the following protocol (which involved parallel samples of Oxypolygelatin subjected to exposure and removal of salicylate) and by a great variation observed among mean sedimentation rate curves in which the same Oxypolygelatin sample but different batches of cells were used.

Tubes 1, 2, and 3 contained identical samples of 5% Oxypolygelatin 12676AY while tubes 4 and 5 contained samples of the same bottle of Oxypolygelatin which had been treated with 1 g. of salicylate (as salicylic acid) per 100 ml. and had subsequently (5 days at 37° C. under aseptic conditions) been freed of this compound by dialysis against several changes of salicylate-free Oxypolygelatin.

Table III. Sedimentation in Millimeters

Time, Min.	OPG 12676AY Untreated			OPG 12676AY Dialyzed Until Salicylate-Free	
Tubes	1	2	3	4	5
0	0	0	0	0	0
5	2	2	2	2	2
10	5	5	4.5	4	5
15	13	13	12	12	14
20	22	22	20	20	22
25	25	25	23	23	25
30	27	27	25	25	27

d. The effect of pH on sedimentation rate of cells in various concentrations of Oxypolygelatin.- A system of Oxypolygelatin 116 concentrations similar to the one used in Figure 3 was brought to pH 4.6 with 0.145 M acetate buffer. Figure 7 demonstrates that the rate of red cell sedimentation in the lower concentrations of Oxypolygelatin (1%, 2%, and 3%) was markedly reduced, but still in the same order as at pH 7.2, whereas the sedimentation rate of red cells in 4% Oxypolygelatin was not markedly affected by the increase in H-ion concentration.

The internal consistency of the method was borne out in a similar test involving a different lot of Oxypolygelatin. The sedimentation rate of rabbit cells was determined, in duplicate, at pH 7.08 and pH 6.35, with an Oxypolygelatin concentration of 4% (Oxypolygelatin 113) in each case.

Cells sedimented more rapidly at pH 7.08 than at the lower pH, duplicate values coinciding closely with each other. (Figure 8.)

This experiment demonstrates the action of two competing effects; the influence of hydrogen-ion in inhibiting sedimentation and the action of gelatin in enhancing the rate of fall.

e. The effect of Oxypolygelatin concentration on the sedimentation rate of rabbit erythrocytes.- A detailed study of the influence of protein concentration on the sedimentation rate of rabbit erythrocytes was made in two experiments, one involving variations in the Oxypolygelatin concentration per se and the other possessing an additional variable, the concentration of an inhibitor, sodium

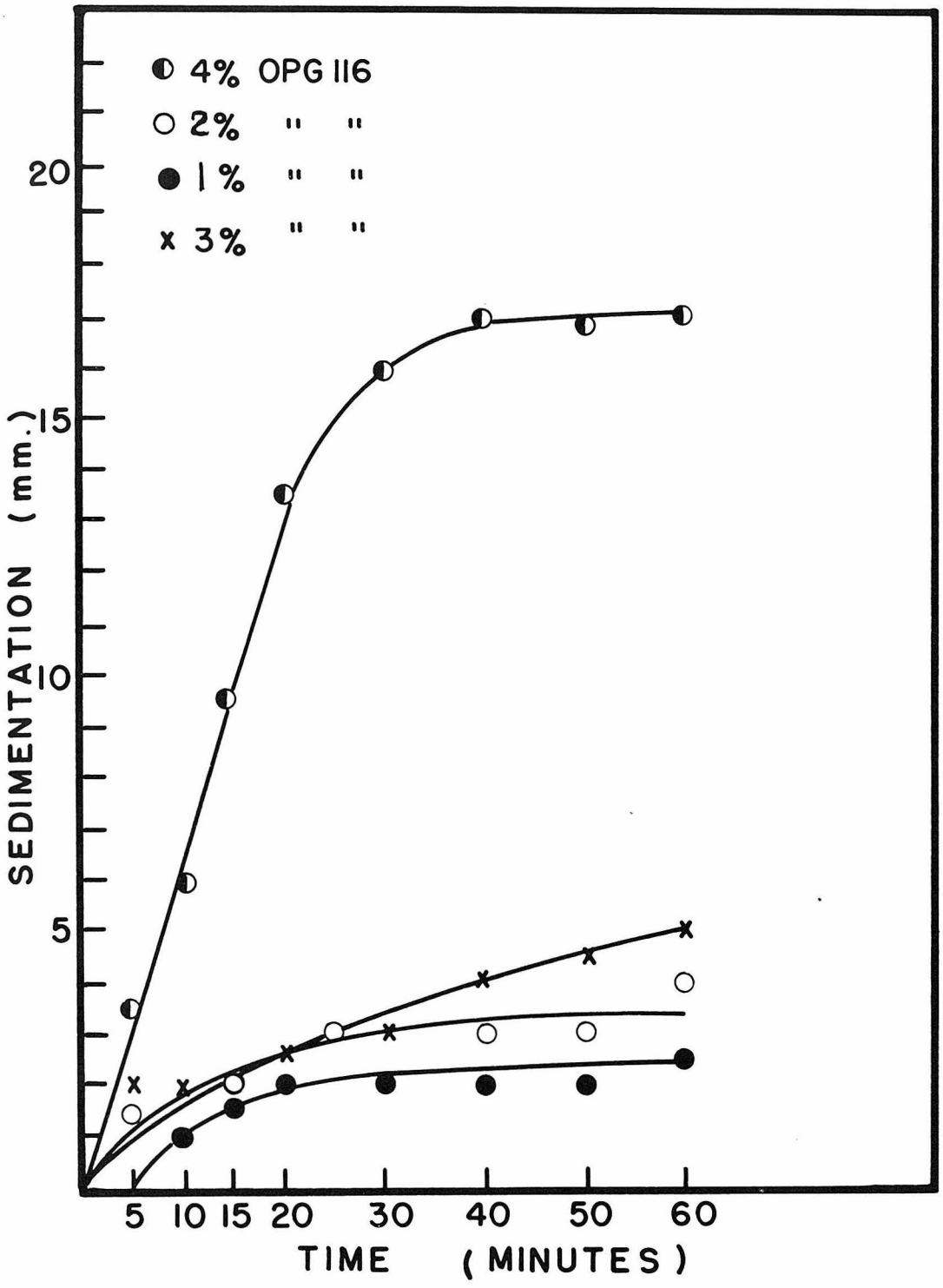


Fig7 Sedimentation rate of rabbit erythrocytes in various concentrations of OPG 116 at pH 4.6 (0.145 M acetate buffer)

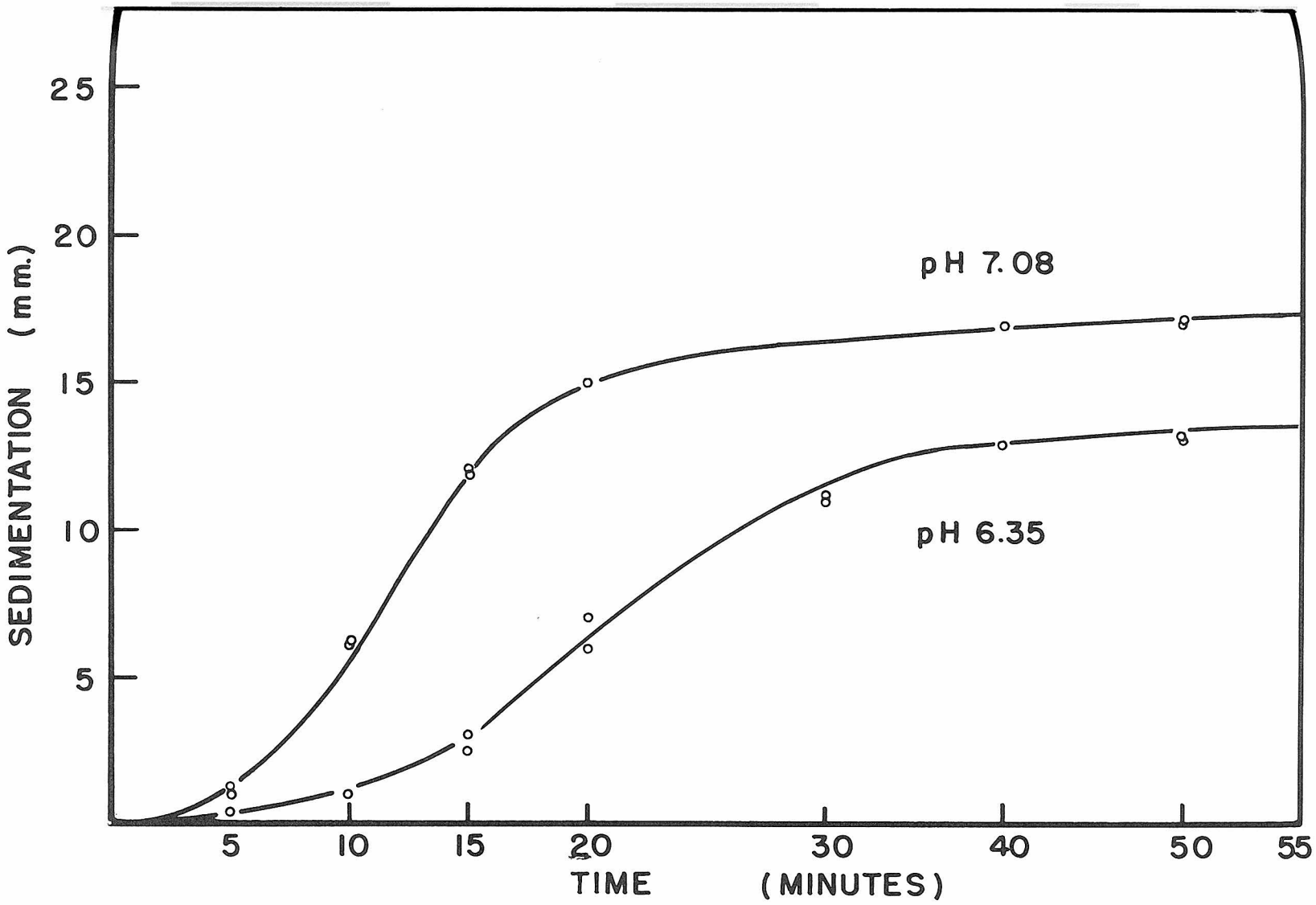


Fig 8 Sedimentation rate of rabbit cells in OPG 113 at pH 7.08 and pH 6.35

salicylate. The control categories of the latter experiment, from which salicylate was absent, but which otherwise contained aliquots of the identical protein solution and cell sample, will be considered jointly with the former in the present discussion.

The usual procedure was followed in these experiments. The sedimentation rates of washed rabbit cells were measured in quadruplicate or quintuplicate for each of the concentrations of Oxypolygelatin 12676AY studied. One replicate test only was performed in most cases except in the instance of the control concentrations 5.75% and 5.35% Oxypolygelatin (for the first and the second experiments, respectively) in which the tests were repeated for the entire array of variations in protein and salicylate concentrations.

An array of Oxypolygelatin solutions containing variable concentrations of protein was made by the suitable dilution of the sterile stock solution. Sufficient quantities of stock solution were taken aseptically, as needed for the separate experiments, diluted with 1.12% saline and analyzed for total N by the Kjeldahl-Nessler colorimetric micromethod described elsewhere. The protein concentrations used in this experiment ranged from 0.56 to 5.75%.

A representative protocol in which the sedimentation of rabbit erythrocytes was simultaneously compared in 5.75% and 0.56% Oxypolygelatin 12676AY is presented below.

Table IV. Sedimentation (millimeters)

Time, Min.	5.75% OPG				0.56% OPG					
Tubes	1	2	3	4	5	6	7	8	9	10
0	0	0	0	0	0	0	0	0	0	0
5	2	1	1	2	0	0	0	0	0	0
10	2	3	4	3	0	0	0	0	0	0
15	4	5	8	7	0	0	0	0	0	0
20	9	10	10	10	0	0	0	0	0	0
25	13	15	15	15	0	0	0	0	0	0
30	18	19	22	18	0	0	0	0	0	0
35	20	21	23	20	0	0	0	0	0	0
40	21	22	24	24	1	0	1	1	1	2
50	23	24	25	23	1	1	1	1	1	1
60	24	25	26	23	1	2	2	2	2	2

Replicate experiments on the effects of protein concentration on the sedimentation rate are presented graphically in Figure 9 (Curves 1-7) and additional evidence can be obtained by an examination of the control curves in the salicylate experiment presented in Figure 15 (Curves 8-30).

Sedimentation rates were estimated from the slopes of lines drawn through the best three or four points of the linear portions of these curves. By plotting the rates thus obtained as a function of the protein concentration (Figure 10), it is possible to draw several conclusions: the sedimentation of erythrocytes in Oxypolygelatin seems to require a threshold concentration of protein. In the present experiment it lies between 2 and 3% Oxypolygelatin; (2) after the threshold is passed small increments in protein concentration rapidly increase sedimentation until a maximum value is reached;

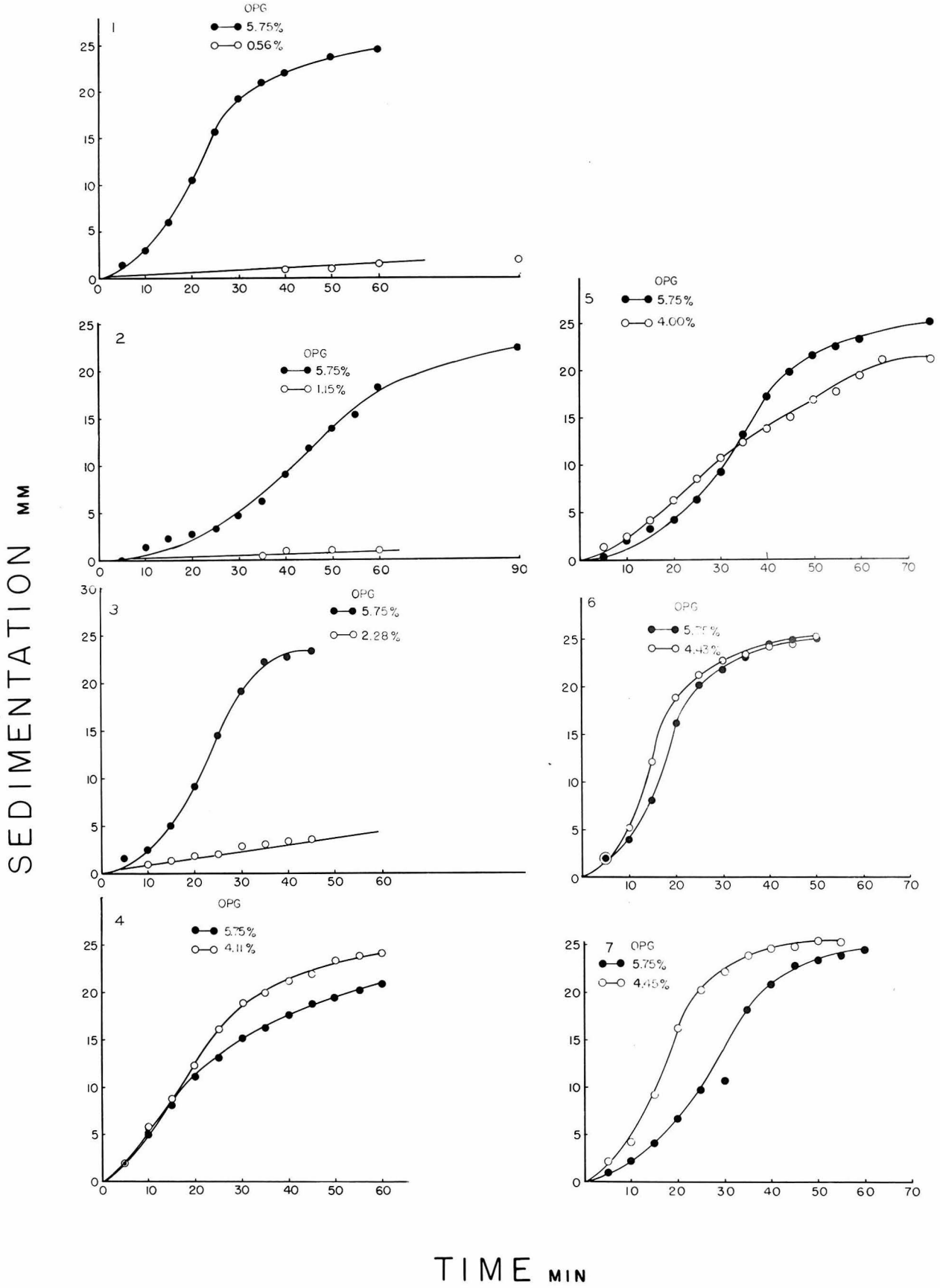

SEDIMENTATION MM
TIME MIN
1
OPG
5.75%
0.56%
2
OPG
5.75%
1.15%
3
OPG
5.75%
2.28%
4
OPG
5.75%
4.11%
5
OPG
5.75%
4.00%
6
OPG
5.75%
4.43%
7
OPG
5.75%
4.65%

Fig 9

(3) further increases in the protein concentration apparently decreases sedimentation. The reason for this is not known but it may be presumed that the increase in viscosity of the medium due to the high protein concentration begins to efface the effects of cell aggregation.

The mean sedimentation rates expressed in millimeters per minute are given for the various protein concentrations.

Table V. The Effects of Varying Oxypolygelatin Concentration on the Sedimentation Rate of Rabbit Erythrocytes

Concentration of OPG 12676AY	Sedimentation Rate (Millimeters/Min.)
0.56	0.03
1.15	0.17
2.28	0.08
3.00	0.68
	0.35
3.45	1.21
3.70	0.95
3.92	1.12
4.11	0.70
4.24	2.12
4.45	1.11
5.35	0.97
5.75	1.31

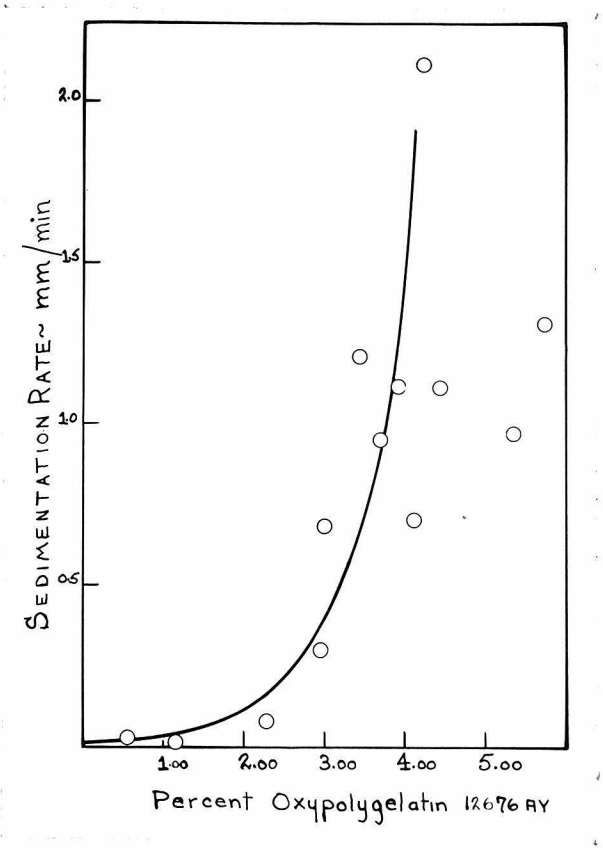

Sedimentation Rate~ mm/min
2.0
1.5
1.0
.5
1.00
2.00
3.00
4.00
5.00
Percent Oxypolygelatin 12676 RY

Figure 10

f. The effect of the molecular milieu on the sedimentation rate.- Fåhraeus, ascribed the decrease in suspension stability of the blood to be dependent on the tendency of the cells to aggregate into rouleaux, the formation of which was, in turn, influenced by the nature of the protein surrounding the cells. Thus relative increments of fibrinogen or globulin in the plasma were shown to accelerate the formation of rouleaux, and consequently to increase the sedimentation rate, whereas such increases in albumin were observed to inhibit this effect. His observations were confirmed and extended by other workers notably Rourke and Ernstene (5) and Ham and Curtis (2) who showed a quantitative relationship to exist between the sedimentation rage of cells and the experimental variation of the fibrinogen content of the plasma.

Knoefel and Lehmann (op. cit.) observed that the presence of various gelatin fractions in the blood of transfused dogs caused an increased sedimentation rate of the erythrocytes in vitro and that this effect was somewhat greater in the fractions of intermediate molecular weight and considerably greater for the fraction possessing the highest value.

Since the sedimentation rate is generally increased after the administration of such diverse substances as methyl cellulose, pectin, gelatin etc., it was of interest to consider whether the rate of sedimentation (i.e.

aggregation) could vary as some physical characteristic, for instance molecular weight of the molecules of the medium.

Using Oxypolygelatin fractions prepared and characterized by Dr. Walter B. Dandliker obtained by the treatment of Oxypolygelatin 11381Y with progressively increasing amounts of ethanol, it was found that the sedimentation rate of human erythrocytes depended to some extent on the molecular weight.

Nine fractions were obtained by adding progressive amounts of alcohol to Oxypolygelatin 11381Y and removing the precipitate formed in each step. These fractions, after being freed of alcohol and dried to a constant weight, were each dissolved insufficient saline to yield 5% solutions, which were employed in the usual fashion for sedimentation studies.

The sedimentation kinetics of the various fraction are presented in Figure 11. An examination of this graph reveals that Fractions 8 and 9 completely inhibited sedimentation. This was to be expected, since the only particles present were of a very small molecular size. Fractions 1 and 2 were moderately effective in accelerating the sedimentation rate of human erythrocytes. The reason for this difference cannot be given with certainty but the available data suggest that it might be due to differences in the physical properties of the particles in the various fractions.

Fractions 3, 4, 5, 6, and 7 all caused marked increase in the rate of sedimentation with a maximum in the care of Fraction 5. Thus, on the basis of sedimentation kinetics, it is possible to distinguish three types of constituent in Oxypolygelatin: a) those characteristic of Fractions 1 and 2; b) those related to Fractions 3, 4, 5, 6, and 7; and c) the degraded or small molecular fragments found in Fractions 8 and 9.

Table VI presents the graphically evaluated sedimentation rates (expressed in arbitrary units) of Fractions 1-9 in terms of the molecular weight calculated by Dandliker.

Table VI

Sedimentation Rate as a Function of Molecular Weight

Fraction	Calculated Molecular Weight	Sedimentation Rate (Slope)
I	39,000	0.542
II	41,000	0.575
III	40,000	1.31
IV	32,000	1.15
V	29,000	1.93
VI	21,000	1.15
VII	16,000	0.68
VIII	13,000	0.02
IX	10,000	0.02
Unfractionated OPG	21,000	3.33

A consideration of the physical properties of the various fractions in relation to their sedimentation behavior fails to reveal the existence of a clear dependence on the molecular weight values although in a rough way sedimentation seems to increase with the molecular

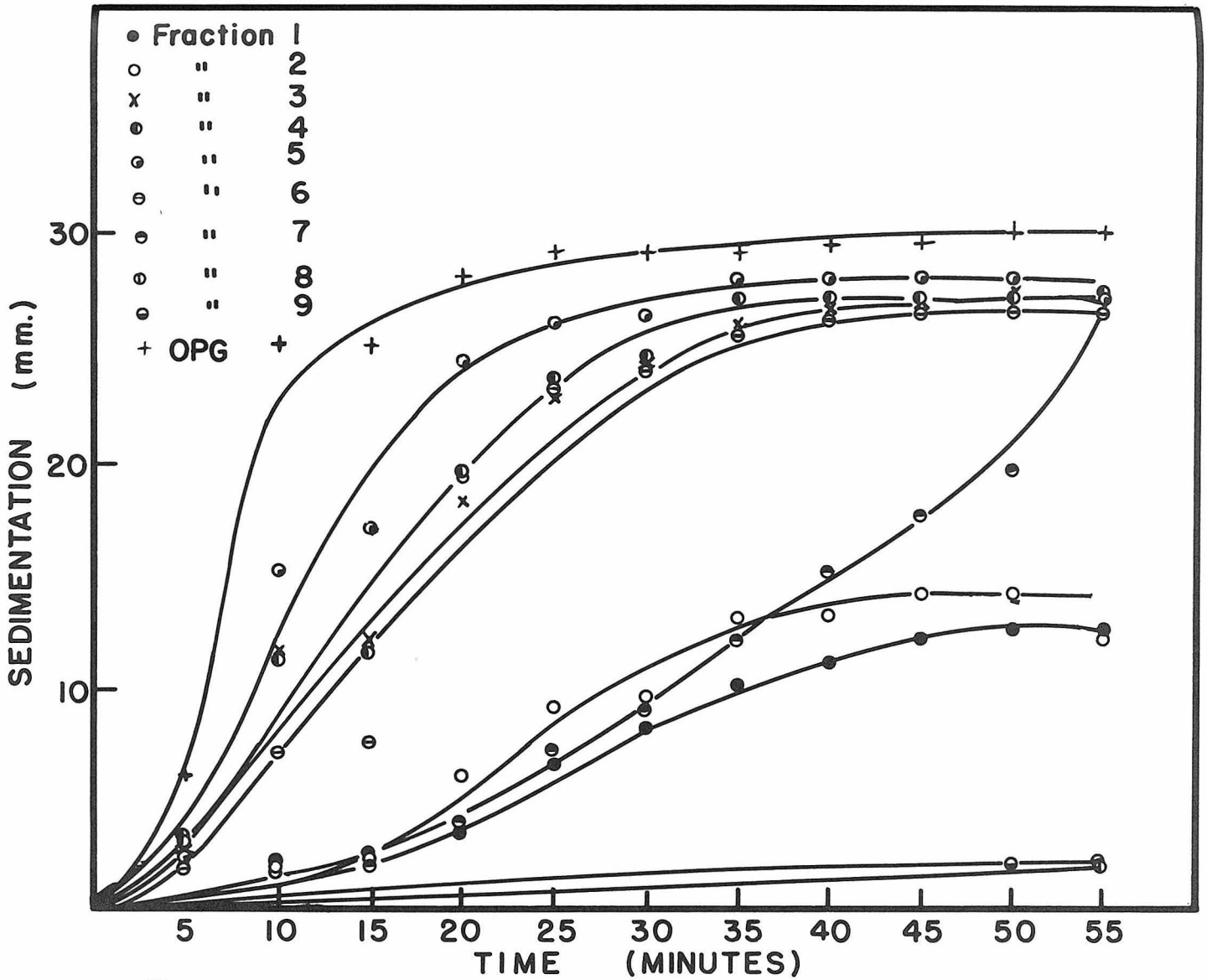


Fig 11 Comporative sedimentation rates of human erythrocytes (O, Rh−) in various 5% fractions of OPG 11381Y

weight.

The failure of sedimentation rates to be correlated with the molecular weights of the fractions might imply the existence of other variables, such as shape or charge which can influence the aggregation of red cells. Thus size and shape may be necessary but insufficient parameters to define or predict sedimentation behavior.

B. The Inhibitory Action of Sodium Salicylate

The slowing of *in vitro* sedimentation by the presence of salicylate was first reported by Bendien, Neuberg and Snapper (7), who observed that the addition of 1 mg. salicylate/ml. blood would significantly retard the sinking velocity. These findings were confirmed by Lichty and Hooker (8) who showed that the presence of 90-120 mg. of salicylate per milliliter of blood would prevent sedimentation. Recent interest in the action of salicylates on sedimentation has resulted from the widespread use of these drugs in the management of rheumatic fever. Since the sedimentation rate is one of the important clinical indices in such cases, the slow clearance of these compounds from the body often produced a spurious evaluation of therapeutic success.

The mechanism of the inhibitory action of salicylate is not known but has generally been attributed to its suppression of the plasma fibrinogen content (Homburger) although this effect has not been clearly demonstrated. Other evidence suggests that the action of salicylates on sedimentation is part of their general effect in influencing chemical reactions of proteins. Thus a depression of antibody production was observed *in vivo* by Swift, (10) who found that administration of salicylates to rabbits reduced the hemolysin titre, and interference with specific precipitation of egg albumin *in vitro* was was reported by Coburn and Kapp (11) who also observed

that salicylate irregularly influenced the non-specific precipitation of egg albumin with sodium tungstate. The recent work of Meyer (12) on the inhibitory influence of sodium salicylate on the spreading factor is further testimony to its generalized biological influence.

Physico-chemical evidence of its action on proteins is furnished by the observations of Chabanier et al. (13) who showed by means of dialysis experiments that serum protein solutions containing salicylate in equal concentration to that found in the external fluid would tend to remove salicylate from the latter site.

The experiments to be subsequently presented in this section are based on the hypothesis that the sedimentation of erythrocytes in protein solutions is due to the interaction of the protein with the surface of the cell and that this interaction depends on the presence of specific conditions* (e.g., reactive sites, or groups) on either the protein or the cell or both. Such a concept finds some support in the results of the experiments presented in the preceding section which have showed that aggregation is dependent on the type of cell, the molecular fraction in the gelatin, and the concentration of the

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* The existence of "key spots" on the cell surface have been suggested by Abramson (15), and Abramson, Furchgott, and Ponder (14).

gelatin present. In this section, our task will be to elucidate the nature of this interaction by blocking sedimentation with an inhibitor and attempting to determine the site and the manner in which such blocking takes place. The site of action of salicylate was investigated by determining its independent effects on the cells and the protein medium, while the nature of its action was tested by experiments designed to show the importance of the particular groups on salicylate molecule in relation to its inhibiting effect.

1. Materials and methods.-

Since the validity of the evidence concerning the site of action of salicylate depended to a great extent on the ability to detect small amounts of salicylate in gelatin or in laked cells a highly reliable analytical method had to be used. The method finally adopted was that of Brodie, Udenfriend, and Coburn (16) which consisted in a colorimetric estimation of salicylate by means of its reaction with ferric nitrate. Since the test is not specific for salicylate it was necessary to extract this substance from acidified gelatin with ethylene dichloride and then to shake out an aliquot of the ethylene dichloride solution with water containing the iron reagent. The details of the method are presented in terms of the following calibration experiment.

A solution containing 1.1600 g. of sodium salicylate /100 ml. H_2O was suitably diluted so that 2 ml. portions

gave 0.05, 0.10, 0.20, 0.30, and 0.50 mg./ml. as salicylic acid. The several solutions were added to 30 ml. of ethylene dichloride in glass-stoppered bottles. After the addition of 0.50 ml. 6 N HCl the mixtures were shaken for 5 minutes on a shaking machine. Following the shaking, the contents of the bottles were transferred to 50 ml. cups and centrifuged for 5 minutes. Twenty milliliter-aliquots were volumetrically transferred into dry 60-ml. glass-stoppered bottles and after the addition of 10 ml. H_2O and 0.25 ml. of the $Fe(NO_3)_3$ reagent, the solutions were stoppered and again subjected to the shaking procedure. After centrifugation, 6 ml.-portions of the purplish-pink aqueous layer were transferred to colorimeter tubes and read on the Klett-Summerson colorimeter through a green (540) filter. A blank run simultaneously produced no color with the iron reagent.

The results of the calibration experiment are presented in Table VII and in Figure 12, which show that the relationship between the weight of salicylate and the iron color is linear and passes through the origin.

Table VII. Calibration Experiment of the Determination of Salicylate by Means of $Fe(NO_3)_3$

Mg. Salicylate Added (as Salicylic Acid)	Klett Reading	Klett Reading Corr. for Blank
0.05	21.5	19.5
0.10	40.0	38.0
0.20	81.5	79.5
0.30	125.0	123.0
0.50	206.0	204.0
0.00	2.0	0.0

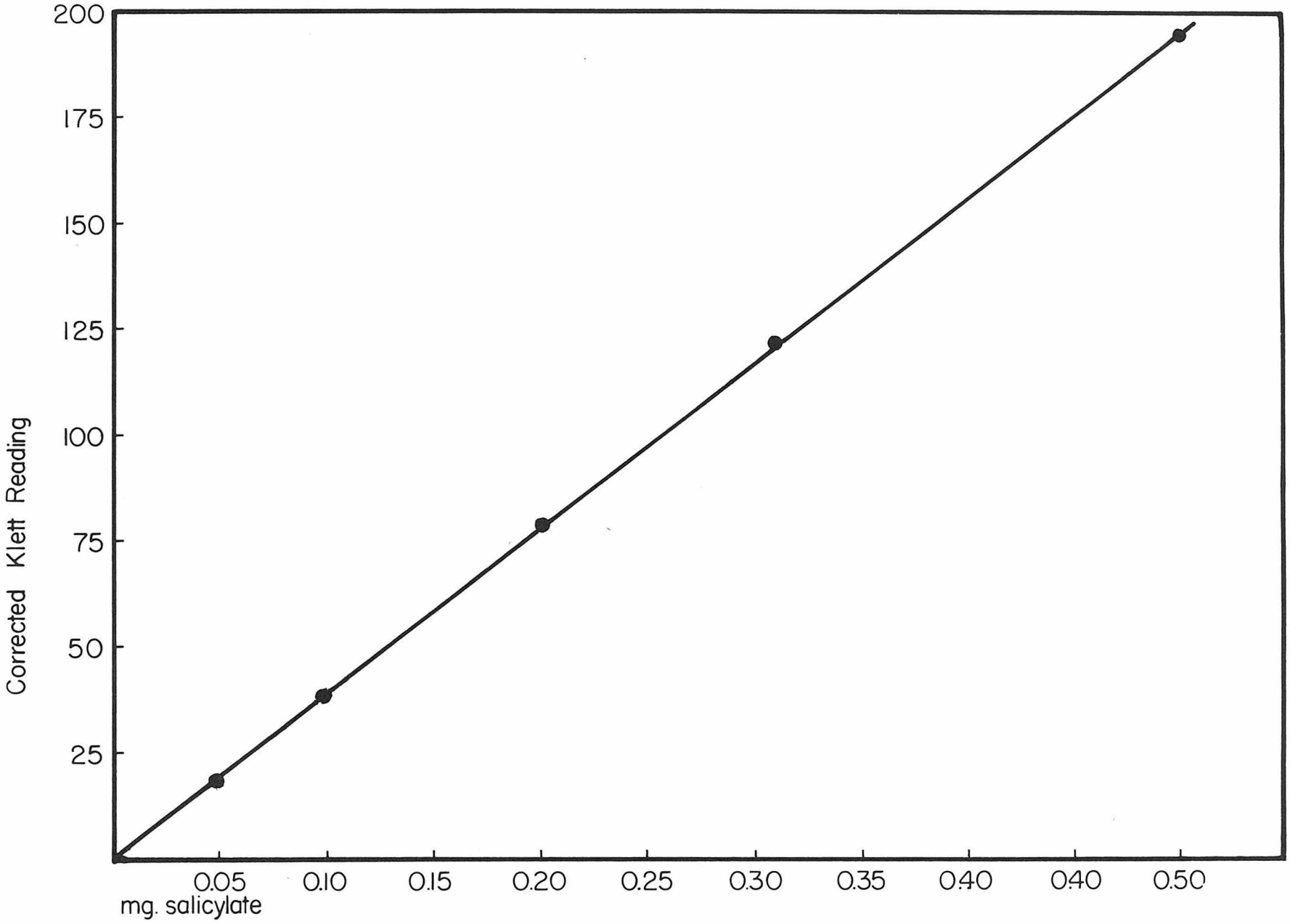


Fig 12

Calibration Curve of Salicylic Acid

An experiment involving greater quantities of salicylate showed the limit of the extraction to be 2 mg. of salicylate estimated as salicylic acid.

To determine whether the presence of gelatin would influence the extraction, experiments involving the recovery of a constant amount of salicylate in the presence of a variable amount of protein, and of variable amounts of salicylate under constant protein concentration were carried out. Table VIII presents the results former case and Table IX the results of the latter.

Table VIII. The Recovery of Salicylate from Variable Concentrations of Oxypolygelatin Containing 0.50 mg. Salicylate/ml. Oxypolygelatin

% OPG in sample	Corr Klett Reading	mg. Recovered	% Recovered
2.50	201	0.52	104
2.25	198	0.51	102
2.00	203	0.524	104.5
1.00	192	0.502	100
0.50	197	0.510	102
	191	0.495	99.2

The recovery of 0.50 mg. of salicylate was uninfluenced by the concentration of Oxypolygelatin in the sample. The mean Klett reading for 0.50 mg. salicylate was 197 and had a standard error of ± 2.13 units.

Table IX. The Recovery of Variable Amounts of Salicylate from 2.5% Oxypolygelatin

Mg. Salicylate in Sample	Corr. Klet Reading	Mg. Salicylate Recovered	% Recovered
0.025	7.5	0.022	88
0.050	20.0	0.050	100
0.10	39.0	0.10	100
0.15	62.0	0.15	100
0.00	0.0	0.00	---

The recovery of even small amounts of salicylate was made with a good degree of precision by this method, the only limit imposed being the lack of sensitivity of the colorimeter under these conditions.

2. Results

a. The general features of salicylate action on red cell suspensions in vitro.-

The addition of salicylate to rapidly sedimenting cell suspensions in 5% Oxypolygelatin inhibit sedimentation by preventing the cells from forming aggregates. This effect is illustrated by a photomicrograph taken half an hour after the addition of solid sodium salicylate to a suspension of washed rabbit erythrocytes in Oxypolygelatin (cf. photomicrograph of cells in gelatin alone).

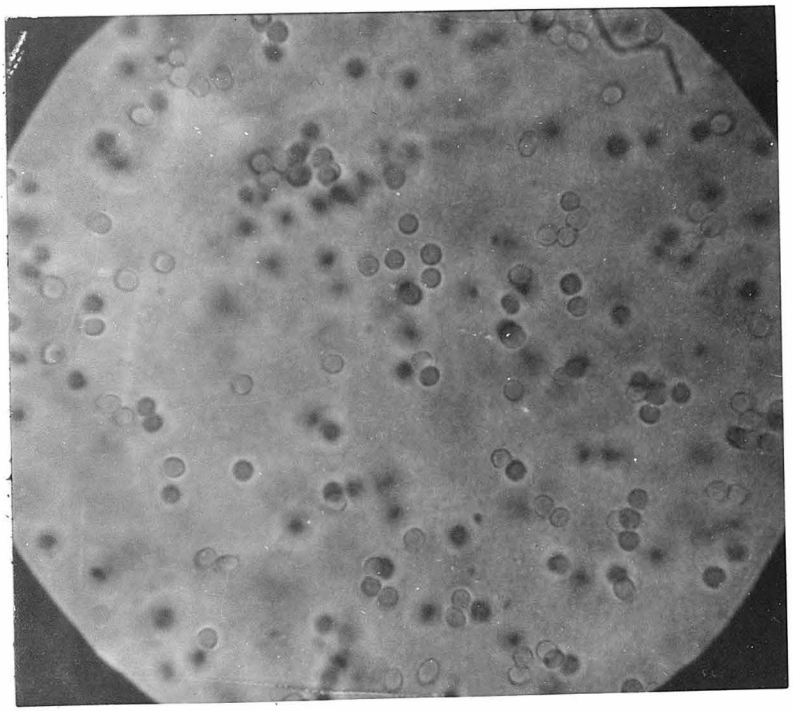


Fig. 13. Rabbit erythrocytes suspended in 5% Oxypolygelatin 12676AY containing 1% salicylate.

The presence of salicylate retards the packing of cells in Oxypolygelatin when such suspensions are centrifuged.

The retarding effect of salicylate was demonstrated by enumerating the cells per cubic millimeter of cell after centrifuging the suspension and removing the supernatant Oxypolygelatin.

One-half-ml. portions of washed rabbit erythrocytes were suspended respectively in 1 ml.-portions of 5% Oxypolygelatin, 5% Oxypolygelatin containing 1% salicylate, 1.12% saline, and 1.12% saline containing 1% salicylate. After an incubation period of 15 minutes at room temperature the tubes were centrifuged for 5 minutes at 2,000 r.p.m., the supernatants were aspirated off, and aliquots of the respective samples of packed cells were taken with hemocytometer pipettes. The results of cell counts made on the various samples are found in Table X.

Table X. Number of Rabbit Erythrocytes per cu. mm. of Suspension Following Centrifugation

Sample	Cells/ cu. mm.
OPG 12676AY 5%	14,510,000
OPG 12676AY 5% & 1% salicylate	2,610,000
NaCl 1.12%	14,130,000
NaCl 1.12% & 1% salicylate	12,750,000

The fact that the presence of salicylate in gelatin retards sedimentation, whereas the presence of salicylate in saline solutions is ineffective, implies that the action of salicylate must be either on the gelatin, or on

some factor peculiar to the situation imposed by the presence of gelatin and cells.

Figure 14 presents the typical results obtained in sedimentation experiments when progressive quantities of salicylate are added to cell suspensions in constant protein concentration. The addition of sodium salicylate in quantities ranging from 1.1 to 9.9 mg. to a series of tubes containing 0.5 ml. washed human cells and 1.0 ml. Oxypolygelatin 12676AY shows that there is a threshold concentration, namely about 4.4 mg., which is just necessary to cause a decrease in rate and an upper limit, between 6.6 and 7.7 mg., which causes complete inhibition.

b. The relationship between protein concentration and the concentration of salicylate required to produce inhibition.- Since it has been observed that the sedimentation rates of erythrocytes varied with the protein concentration and since it was reasonable to assume that salicylate might interfere with the action of gelatin on cells, it was inferred that salicylate reduced the "effective" protein concentration by inactivating a certain amount of gelatin. To test this inference it was necessary to determine whether there was a quantitative relationship between the concentration of protein and the concentration of salicylate required to produce inhibition. Because it was not possible to define an inhibition or an inhibiting dose, since the sigmoid curves of sedimentation rate could be variable in several respects but could be similar in others, the problem was attacked by determining the manner in which

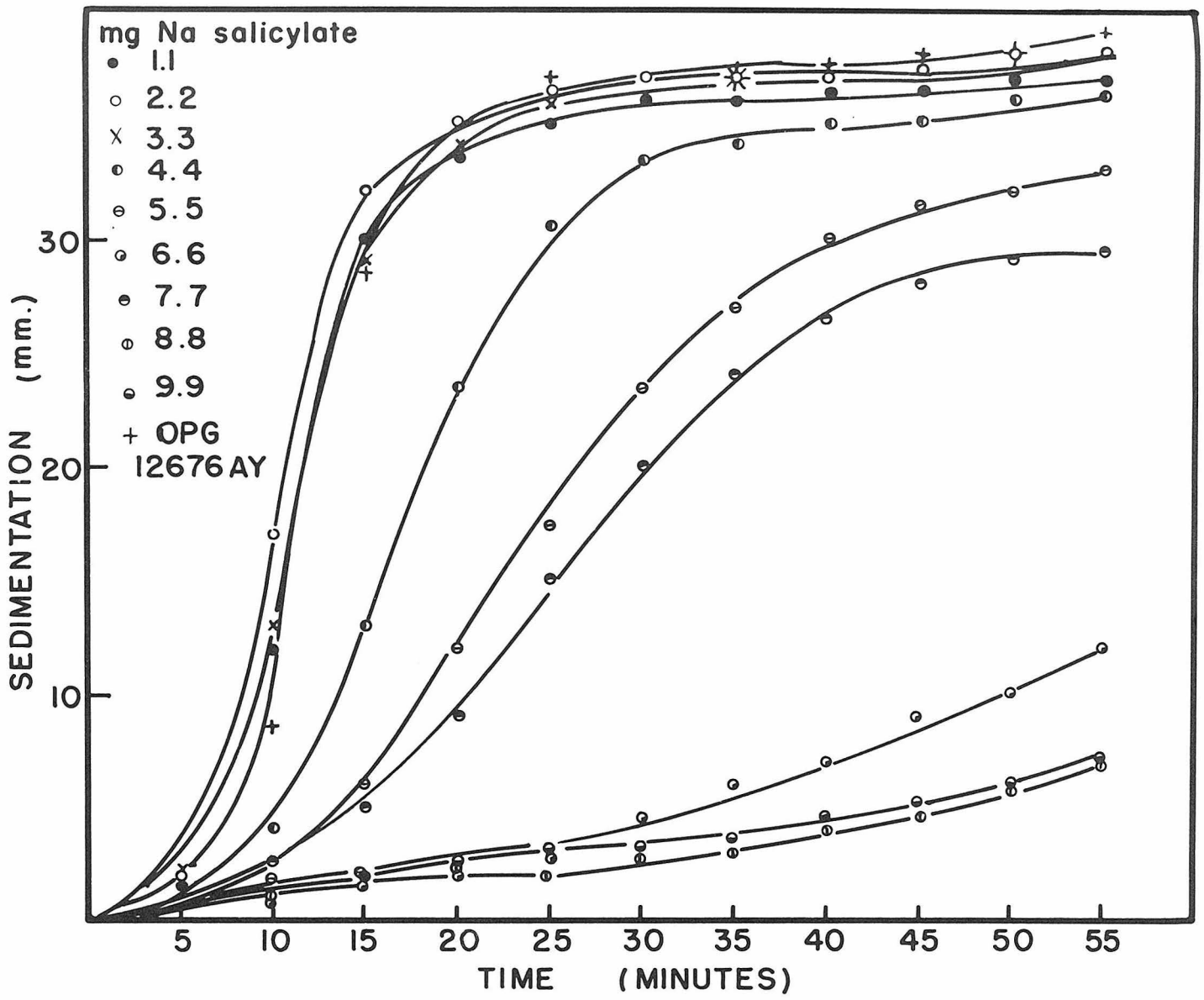


Fig 14 Effect of varying Na salicylate concentration on sedimentation rate of human erythrocytes (O, Rh-) in OPG 12676 AY

salicylate influenced the sedimentation at each value of protein concentration. Since it soon became obvious that at lower protein concentrations there was only a limited range of salicylate activity, "coarse tests" (i.e., orienting experiments involving only one tube per salicylate concentration) were performed to set the limits for the more detailed quintuplicate trials.

Quintuplicate determinations were made for every value of protein and salicylate concentration for each of these tests, a control quintuplicate series from which the salicylate was omitted was simultaneously run. The results of the individual experiments are presented graphically in Figures 15a, 15b, 15c, and 15d (Curves 8--28, inclusive). A visual inspection of the curves brings out the following facts: 1) At the higher concentrations of Oxypolygelatin the range of salicylate concentration which can be employed before inhibition occurs is greater than at the lower protein concentrations. Thus in the case of the 5.35% Oxypolygelatin the inhibiting range of salicylate lay between 0.42 and 0.50 mg. (Curves 8-14 Figure 15a), whereas to produce the same amount of inhibition as the 0.42 value (Curve 12 Figure 15a) only 0.21 mg. salicylate were required in the case of the 3.92% Oxypolygelatin (Curve 23 Figure 15c) in general quantitative comparisons among quintuplicate experiments were only fair for a given protein concentration and relatively poor between different protein concentrations. This was in part due to the variability of the control

values within a given protein concentration and confirms the results of the quotidien variability found in the 0.75% categories of Curves 1-7 (Figure 15a) and lends credence to the suggestion that the variability is resident in differences among the cells; 3) surprisingly enough, the single tube "coarse tests" were more internally consistent than the replicate tests since comparisons of the sedimentation rates under conditions of constant protein but variable salicylate could be made on aliquots of the same sample of cells. Table XI presents the results of comparing six coarse tests of variable protein concentration in terms of the ineffective dose, (moderately inhibiting dose, and limiting, or completely inhibiting dose of salicylate).

Table XI. Comparison of Various Oxypolygelatin Concentrations in Terms of the Ineffective, Moderately Inhibiting, and Limiting Doses of Salicylate

Coarse Test (Curve No.)	%Protein	Ineffective Dose (mg.%)	Moderately Inhibiting Dose (% Salicylate)	Limiting Dose (% Salicylate
15	3.0	0	0.1	0.2
18	3.45	0.1	0.2-0.3	0.4
21	3.70	0.1	0.2	0.3
27	4.24 (1)	0.3	0.5	0.6
26	4.24 (2)	0.1	0.4	0.5-0.6
8	5.35	0.1	0.5	0.6

The differences observed among the various tests suggest, but do not prove, a relationship between Oxypolygelatin and salicylate.

An attempt to compare systems of protein concentration in terms of their respective salicylate dependence by

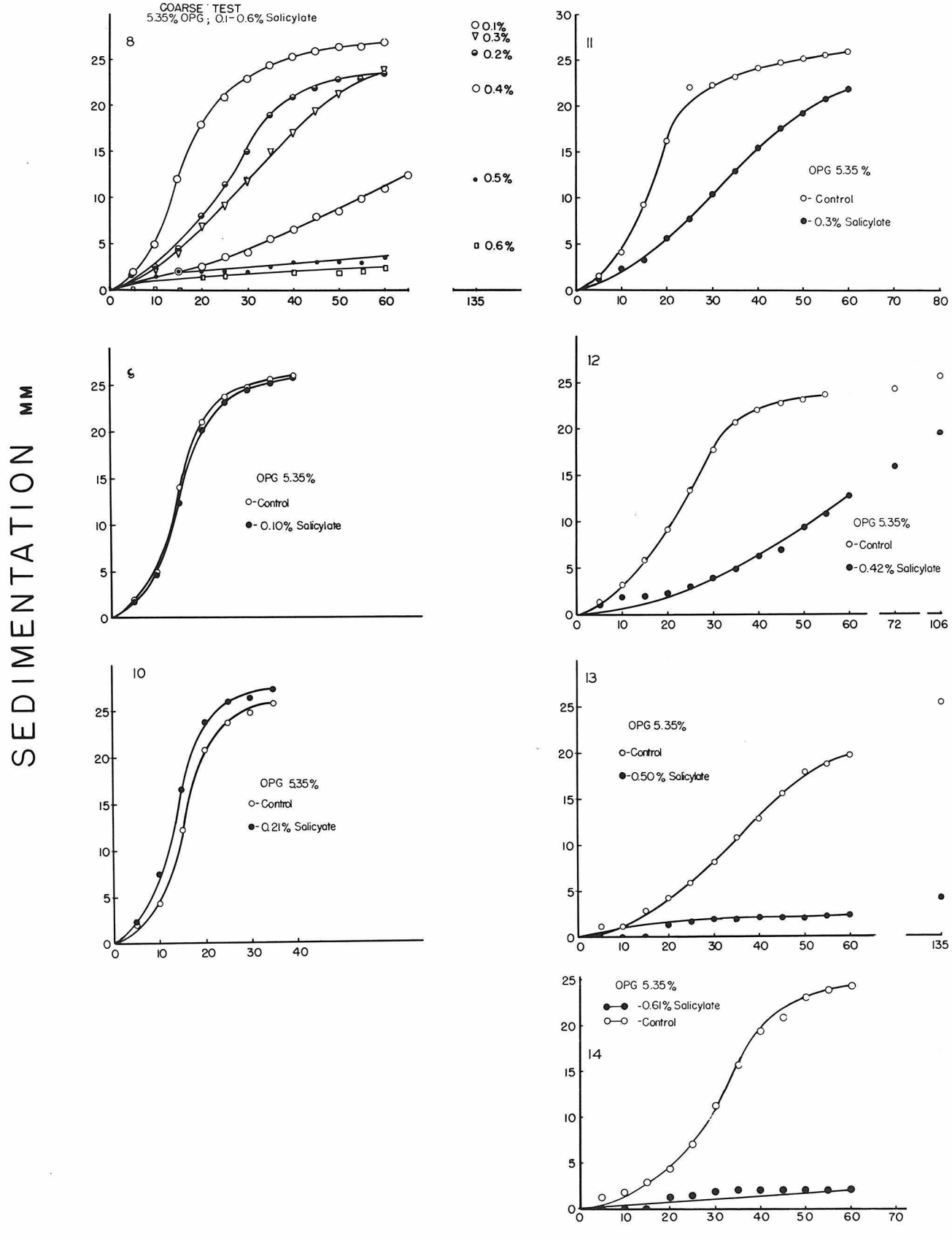
Fig. 15a, b, c, d

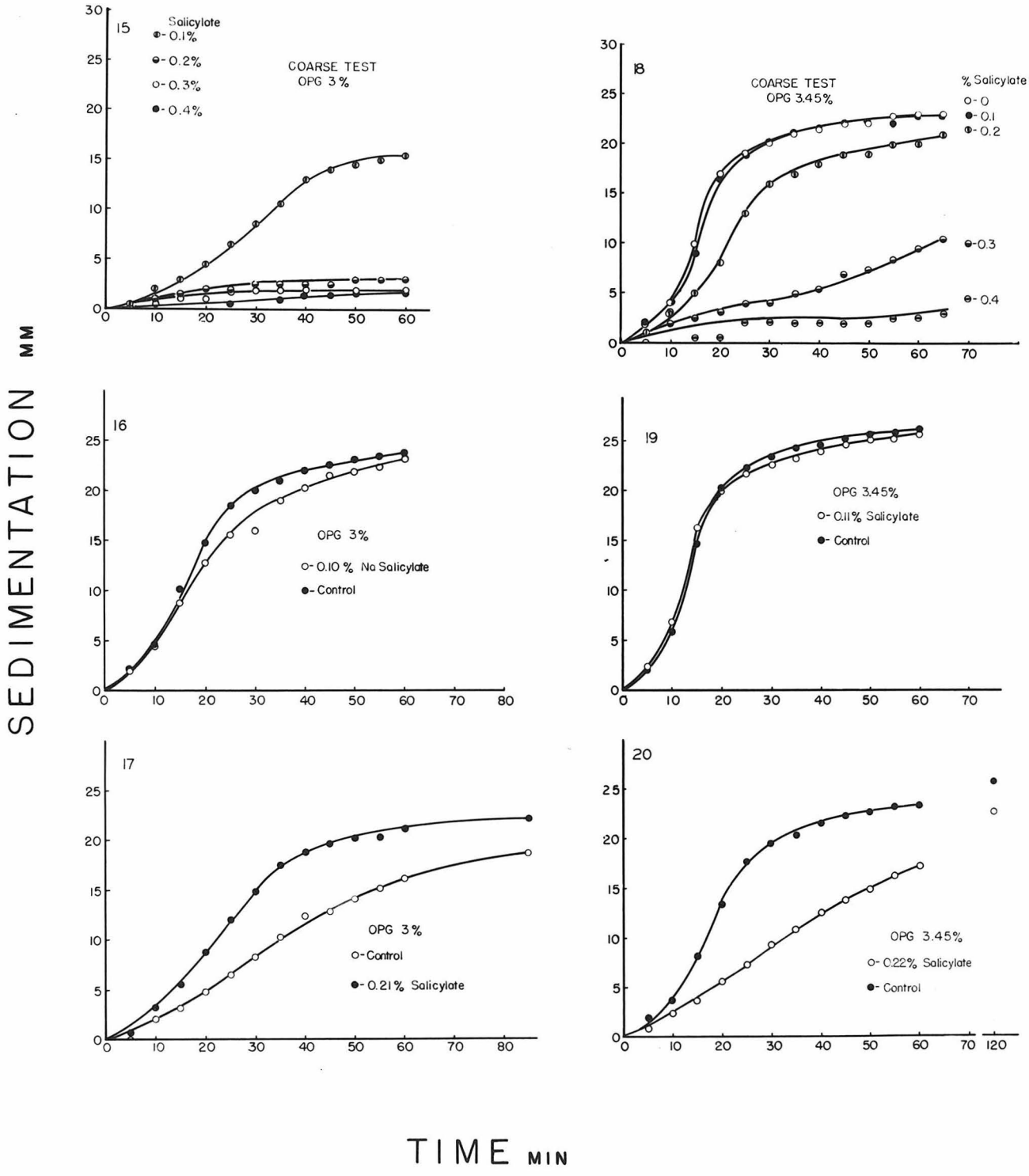
The Relationship Between Protein Concentration and the Concentration of Salicylate Required to Produce Inhibition

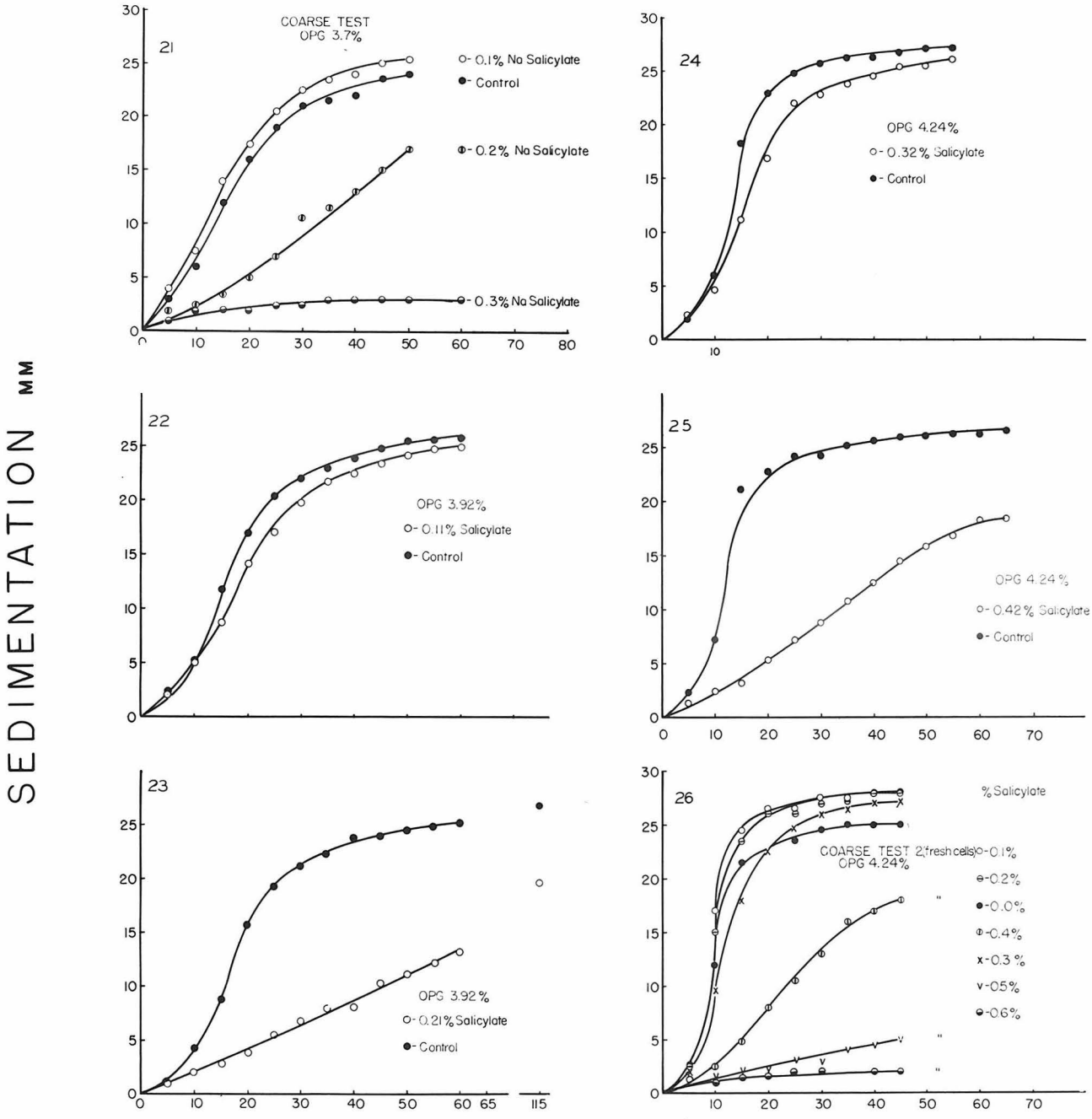
(Curves 1-28)

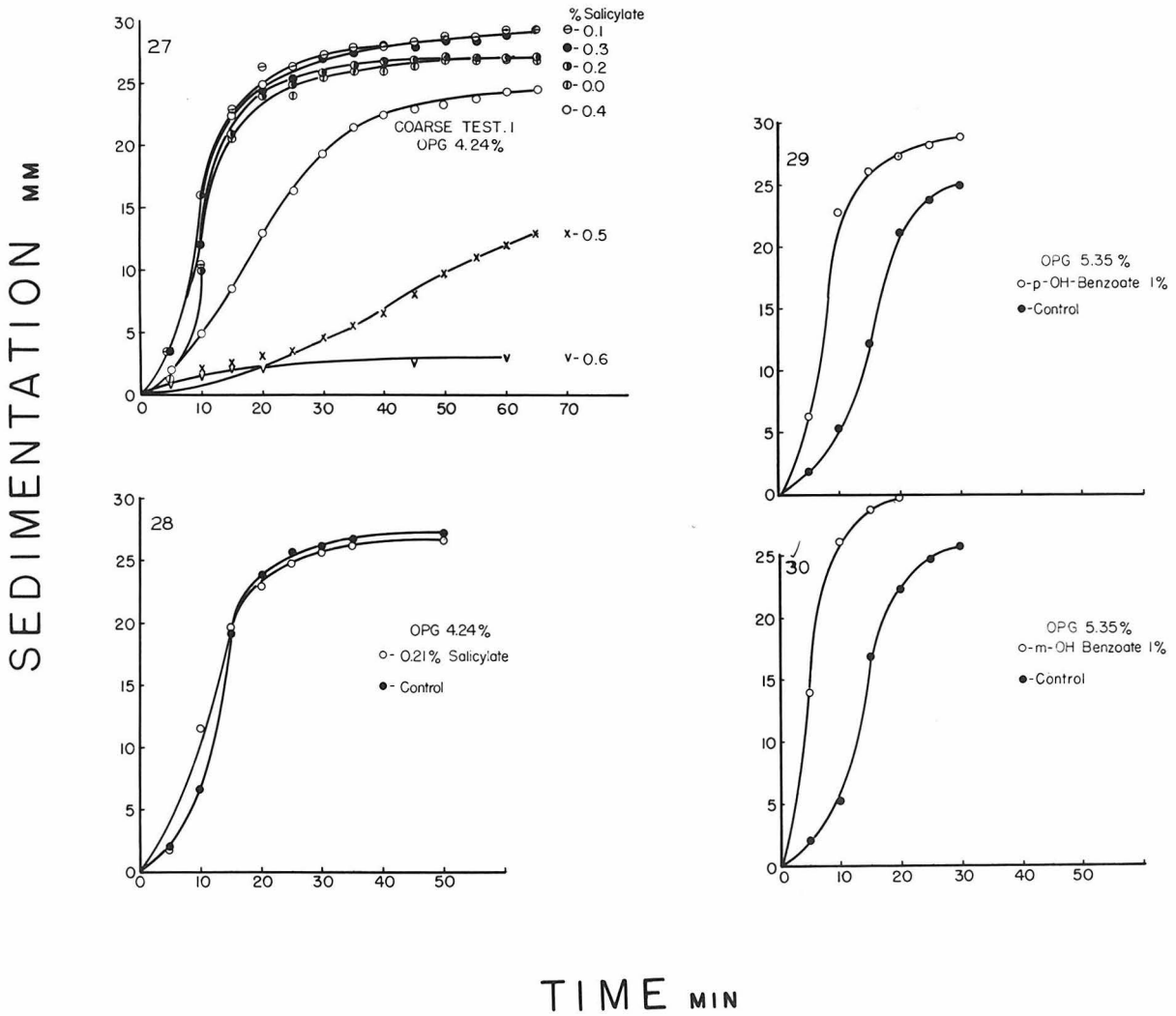
The Effect of Sodium Para-hydroxy Benzoate and Sodium Meta-hydroxy Benzoate on Sedimentation

(Curves 29,30)


COARSE TEST
5.35% OPG ; 0.1-0.6% Salicylate
8
0.1%
0.3%
0.2%
0.4%
0.5%
0.6%
11
OPG 5.35 %
- Control
- 0.3% Salicylate
9
OPG 5.35%
-Control
- 0.10% Salicylate
12
OPG 5.35%
-Control
-0.42% Salicylate
10
OPG 5.35%
-Control
-0.21% Salicyate
13
OPG 5.35%
-Control
-0.50% Salicylate
OPG 5.35%
-0.61% Salicylate
-Control
14
SEDIMENTATION MM
TIME MIN


SEDIMENTATION MM
TIME MIN
15
Salicylate
0-0.1%
0-0.2%
0-0.3%
0-0.4%
COARSE TEST
OPG 3%
18
COARSE TEST
OPG 3.45%
% Salicylate
0-0
0-0.1
0-0.2
0-0.3
0-0.4
16
OPG 3%
0-0.10% Na Salicylate
0-Control
19
OPG 3.45%
0-0.11% Salicylate
0-Control
17
OPG 3%
0-Control
0-0.21% Salicylate
20
OPG 3.45%
0-0.22% Salicylate
0-Control


SEDIMENTATION MM
21
COARSE TEST
OPG 3.7%
o- 0.1% Na Salicylate
●- Control
Φ- 0.2% Na Salicylate
⊖- 0.3% Na Salicylate
24
OPG 4.24%
o- 0.32% Salicylate
●- Control
22
OPG 3.92%
o- 0.11% Salicylate
●- Control
25
OPG 4.24%
o- 0.42% Salicylate
●- Control
23
OPG 3.92%
o- 0.21% Salicylate
●- Control
26
% Salicylate
COARSE TEST 2(fresh cells) o- 0.1%
OPG 4.24%
⊖-0.2%
●-0.0%
Φ-0.4%
x-0.3%
v-0.5%
⊖-0.6%
TIME MIN


SEDIMENTATION MM
27
% Salicylate
⊖- 0.1
●- 0.3
◑- 0.2
◐- 0.0
○- 0.4
COARSE TEST. I
OPG 4.24%
x- 0.5
v- 0.6
28
OPG 4.24%
○- 0.21% Salicylate
●- Control
29
OPG 5.35 %
○-p-OH-Benzoate 1%
●-Control
30
OPG 5.35%
○-m-OH Benzoate 1%
●-Control
TIME MIN

plotting sedimentation rates as a function of salicylate for each protein concentration was generally unsatisfactory, since in most cases there was a great variability in both the control and experimental values and when the curves were idealized it was found that they tended to vary randomly in their slopes. Another factor responsible for the high variability resides in the narrowness of the salicylate range between inhibition and ineffectiveness at the lower protein concentrations.

Table XII presents sedimentation rates of cells at (mm./min.) two protein values as a function of the salicylate concentration. The sedimentation rates were obtained graphically from the linear portions of the quintuplicate curves.

Table XII. Comparison of Sedimentation Rate; Salicylate Dependence in 5.35 and 4.24% Oxypolygelatin

% Protein	% Salicylate	Sedimentation Rate (mm./min.)		
		Control (No Salicylate)	Variable	V/C
5.35	0.10	1.75	1.75	1.00
	0.21	1.73	1.52	0.88
	0.30	1.23	0.50	0.41
	0.42	0.80	0.28	0.35
	0.50	0.45	0.05	0.11
	0.61	0.85	0.03	0.03
4.24	0.21	1.63	1.63	1.00
	0.32	2.25	1.15	0.50
	0.42	2.78	0.38	0.13

Although a fair dependence is found between the percent salicylate and the sedimentation rate the variability of the control values makes the reliability of the curve obtained to be of questionable significance.

3. The Site of Action of Sodium Salicylate.-

The site of the inhibitory action of salicylates on the interaction of erythrocytes and Oxypolygelatin was investigated in a series of experiments designed to reveal its independent effect on gelatin and on the cells. The influence of salicylate on Oxypolygelatin was examined in terms of its stability of union with gelatin by determining whether the removal of salicylate by dialysis would confer any permanent alteration of its sedimentation activity.

a. Studies of the effects of salicylate on Oxypolygelatin,-

(1) The effects of the removal of salicylate by dialysis on the sedimentation behavior of Oxypolygelatin.-

This experiment was carried out to determine whether a) salicylate could be completely dialyzed out of gelatin and b) whether the presence of salicylate for several days at 37° C. would exert a permanent effect on the sedimentation behavior of gelatin.

The dialysis units consisted of short-necked bulbs of 20 ml. capacity which were equipped with side arms through which the external solution could be changed. The cellophane sacs of 2 ml. capacity were attached to serological stoppers and inserted into the necks of the bulbs while other serological stoppers were placed in the side arms. Hypodermic needles covered with sterile cotton and gauze were inserted into each of the stoppers to serve as vents during the autoclaving. The dialysis units

were autoclaved in an assembled condition and after sterilization, 2 ml. of sterile Oxypolygelatin 12676AY containing 1% salicylate was added by means of an hypodermic syringe to the cellophane bag of each of 4 units while 20 ml. of salicylate-free Oxypolygelatin was added through the side arm into the external chamber.

The dialysis units were placed into a 37° C. incubator for a period of 3 days in the course of which the external fluid was changed three times. At the end of the 3-day period the contents of the cellophane sacs were removed and employed for sedimentation studies. The sedimentation studies of this experiment have been presented in tabular form in Table III.

A control consisting of sterile 5% stock solution of Oxypolygelatin from which the test samples originated was kept in the incubator under identical conditions of time and temperature and used as a source of the external solution for the dialysis units. Sedimentation tests were performed on two of the samples which had been dialyzed to freedom from salicylate while the remaining two were pooled and used for analytical investigations to determine the percent of the salicylate remaining after the dialysis. For comparison sedimentation tests were performed also on two samples of the control Oxypolygelatin to which had been added salicylate to a concentration of 1%. The sedimentation test showed rapid and complete sedimentation in the salicylate-free controls and in the samples dialyzed

to freedom from salicylate, while samples containing 1% salicylate showed inhibition.

To determine whether the salicylate was bound to the cells, the cells of one of the tubes containing 1% salicylate in Oxypolygelatin were washed three times with salicylate-free Oxypolygelatin (by repeated suspension and centrifugation) and the washings were pooled and analyzed for salicylate. The analytical results (Table XIII) showed that 92% of the salicylate could be removed by three washings. The cells of this category were resuspended in 1 ml. of salicylate-free Oxypolygelatin and their sedimentation rate measured. Figure 16 shows the sedimentation rates of the washed, resuspended cells and the resuspended cells of the control salicylate-free category. It can be seen that although the sedimentation of both categories was rapid, it was slightly delayed in the case of the washed cells, perhaps because of the residual amounts of salicylate still present.

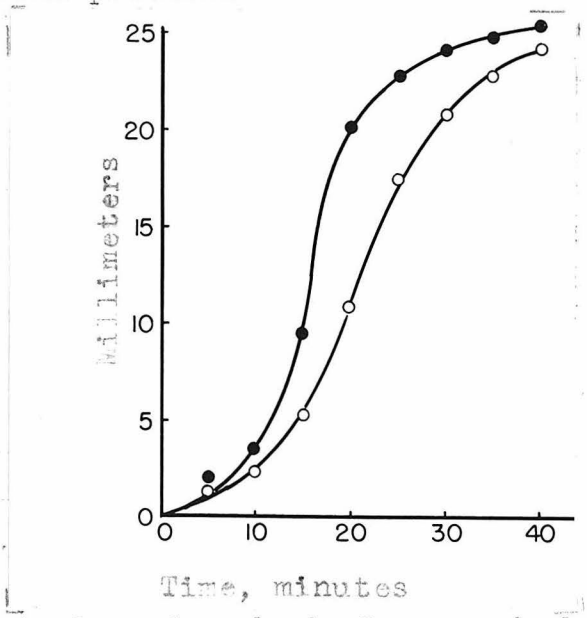


Fig. 16. Control - closed circles; washed 3X in salicylate-free OPG - open circles.

Table XIII

Sample	Corrected Klett Reading	Mg. Salicylate in Sample	Dilution Factor	Mg. Salicylate X dil "f"	% Recovered
OPG with 1% Salicylate	165	0.425	1/25	10.63	100.0
OPG Dialyzed for 3 Days	4	0.02	1/25	0.50	4.71
Control Salicylate-free	1	0	1/25	0	
Washings from Cells	151	0.39	1/25	9.75	92.0

(2) The effects of salicylate on the precipitation of Oxypolygelatin with tungstic acid.-

The results of Coburn and Kapp (11) indicated that the presence of salicylate would inhibit the amount of specific precipitate formed in the reaction between ovalbumin and antiovalbumin; and in the same publication they stated that salicylate tended to influence the precipitation of serum proteins in an irregular manner. It was of interest, therefore, to determine whether salicylate would influence the precipitation of Oxypolygelatin under various conditions of protein and salicylate concentration.

Experiments designed to determine the ratios of total to precipitable nitrogen were performed on two values of protein concentration 2 and 5% using 0, 0.5, and 1.0% salicylate for each category. The protein concentrations were chosen on the basis of the results of a previous experiment designed to indicate the manner in which the ratio total to precipitable N would vary as the function of concentration in the absence of salicylate. Suitable dilutions of Oxypolygelatin 12676AY were made to yield presumptive concentrations of 1, 2, 3, 4, and 5% protein and separate aliquots of each category were taken for the determination of total and precipitable N by the method to be subsequently described. The results are presented in the following table.

Table XIV. The Content of Total and Precipitable Protein on the Basis of Nitrogen for Various Protein Concentrations (Protein = N X 6.25)

% Total Protein	% Precipitable Protein
1.00	1.03
2.00	2.06
3.20	3.09
4.16	3.84
5.49	4.45

The results of the preliminary analysis indicated that the amount of protein precipitated by tungstic a cid at the higher protein concentrations was lower than the values obtained for the total protein. The two values chosen, then, were designed to test differences in salicylate activity in the ranges of incomplete and complete protein precipitation by tungstic acid.

The experiment was designed in the following way: suitable dilutions of an Oxypolygelatin stock solution were made to yield 2% while for the 5% category the undiluted stock solution was employed. The respective solutions were then further subdivided and to aliquots of the 2 and 5% solutions sufficient quantities of sodium salicylate were added to yield 1.0% salicylate (as salicylic acid - 1.1600 g. sodium salicylate per 100.0 ml. Oxypolygelatin). Half-strength, 0.5%, salicylate categories were prepared by diluting aliquots of the 1.0% salicylate solutions at each protein concentration with equal volumes of salicylate-free solutions of Oxypolygelatin. For each protein concentration two salicylate values and one control, containing the same concentration of protein but lacking salicylate, were studied.

For the sake of brevity the analytical details of only one protein concentration category at one salicylate value will be presented, i.e., the experimental results obtained on analyzing for total and for precipitable protein (N X 6.25) in the solution containing 5% Oxypolygelatin and 0.5% mg. of salicylate. For clarity the following code is used.

T = total protein
W = protein precipitated by tungstic acid
X = 1.0% salicylate (as salicylic acid)
5 = 5% protein category
a,b = duplicates

Quadruplicate 1.0-ml. samples of the 5% protein solution containing 10 mg. of salicylate per mg. solution were pipetted into separate 15-ml. centrifuge tubes and the quadruplicates designated, respectively, TX5a, TX5b, WX5a, and WX5b. Samples TX5a and TX5b were then treated by the addition of 0.25 ml. of 10% Na_2WO_4 and 0.25 ml. of 0.67 N H_2SO_4 to precipitate the protein. After precipitation the samples were placed in the cold for one hour and then they were centrifuged for 15 minutes at 1500 r.p.m. The supernatant solutions of the tungstic acid precipitates were decanted off and the samples of all categories treated with 1.0 ml. 5 N NaOH. After the precipitates had been dissolved all of the solutions were diluted with sufficient distilled water to bring the final volumes to 10 ml. Two-milliliter aliquots of each of these categories were further diluted

to 10 ml. in volumetric flasks, duplicate 1.0-ml. portions of these 1:5 dilutions being taken for digestion and analysis by the Kjeldahl-Nessler micromethod. Analyses made on the subdilutions of the total and precipitable N categories gave the results presented in Table XIV.

Table XIV. Analysis of Total and Precipitable Protein (as N X 6.25) in 5% Oxypolygelatin Samples Containing 5 mg. Salicylate/mg. Oxypolygelatin

Overall Dilution	Sample	Corrected Klett Reading	Mg. N in Sample	Mg. Protein (N X 6.25)	Mg. Protein X Dilution Factor	Mode % Protein
1:50	TX5a	355	0.1750	1.092	54.60	5.55%
		360	0.1775	1.109	55.45	
	TX5b	360	0.1775		55.45	
		360	0.1775		55.45	
	WX5a	320	0.1578	0.985	49.25	4.93%
		320	0.1578	0.985	49.25	
	WX5b	340	0.1675	1.047	52.45	
		320	0.1578	0.985	49.25	

(N.B. The use of four significant figures in presenting these results should not be taken to imply that degree of precision of the analyses; they were used merely to facilitate in "rounding off" the final percent protein values.)

The ratio $\frac{\text{Precipitable Protein}}{\text{Total Protein}}$ for 1% salicylate in 5% Oxypolygelatin was found to be 0.89. A summary of the ratios found in two experiments is presented in Table XV.

Table XV. Ratios of Precipitable to Total Protein Under Various Conditions

Protein Category	% Salicylate	Precipitable Protein / Total Protein	
		Experiment 1	Experiment 2
5% OPG	1.0	0.89	0.89
	0.5	0.95	0.90
	0.0	0.85	0.86
2% OPG	1.0	0.97	-- (not done)
	0.5	0.94	0.94
	0.0	0.87	-- (not done)

The difference in the ratios obtained probably cannot be regarded as significant. Although there is an indication in the first experiment that the presence of 0.5% salicylate might increase the amount of protein precipitated this is not borne out by the results of the second experiment. Since only the 0.5% salicylate group in the 2% protein category was repeated, the 10% difference between the control (0) and the 1.0% salicylate group was not confirmed.

Salicylate determinations made on aliquots of some of the samples after tungstic acid precipitation and solution in 0.1 N NaOH showed a salicylate content of 10 to 20% of the original amount addedm whereas corresponding analyses made on the non-precipitated group showed quantitative recovery.

(3) The effect of salicylate on miscellaneous physical properties of Oxypolygelatin.-

The presence in Oxypolygelatin of quantities of salicylate sufficient to affect the sedimentation did not markedly alter the pH or the relative viscosity of the protein under the conditions tested. Viscosity measurements made at 37° C. in an Ostwald viscosimeter showed relative viscosities of 5% Oxypolygelatin 12676AY to be 3.09 and in the presence of 1.0% salicylate to be 3.13. The pH of both solutions was 5.28.

The pH of cell suspensions in Oxypolygelatin solutions of variable salicylate content (0 to 8 mg./ml.) Oxypolygelatin, showed no great differences in pH, the range

being 0.35 pH units.

Table XVI. The Effect of Salicylate Concentration on the pH of Erythrocyte Suspensions in Oxypolygelatin (0.5 ml. Washed Cells plus 1 ml. Oxypolygelatin 12676AY)

Concentration of Salicylate mg./ml. 5% Oxypoly-gelatin 12676AY	pH of Final Suspension
2 mg.	7.40
3 "	7.30
4 "	7.30
5"	7.20
6 "	7.15
7 "	7.15
8 "	7.21
0	7.05

b. The effect of salicylate on the erythrocytes.- Experiments of Smith, Gleason, Stoll and Ogorzalek (17) on the distribution of salicylate between cells and plasma suggested that the cell membrane behaved as though it were freely permeable to salicylate. Calculations based on the assumption that the salicylate found in the erythrocytes was merely dissolved in cell water and was in equilibrium with salicylate in the plasma ultrafiltrate indicated that this was generally true, although in exceptional cases wide variations were observed. The loss of salicylates from the plasma into the cells had been previously suggested by Bendien, Neuberg, and Snapper (op. cit.) and confirmed later by Homburger (op. cit.), who found, in addition, that after the exposure of erythrocytes to plasma samples containing salicylate for some time, the amount of salicylate required to inhibit the sedimentation rate was decreased.

(1) The removal of salicylate from saline solutions as a function of erythrocyte concentration.- The removal of salicylate from saline solutions containing 10 mg. salicylate/ml. saline was found to obey the dilution hypothesis proposed by Smith et al. By increasing the volume of cells added to 1 ml. of saline containing 1% salicylate from 0.1 to 0.9 ml. progressively larger quantities of salicylate were removed from the saline as determined by the analysis of the supernatant. Basing the expected values on the dilution hypothesis, quantitative recovery was obtained. Table XVII summarizes the results of this experiment.

Table XVII. The Uptake of Salicylate from Saline Solutions as a Function of Cell Concentration

Ml. Washed Cells	0.9	0.5	0.3	0.1
Ml. 1% Salicylate	1.0	1.0	1.0	1.0
Ml. total Volume	1.9	1.5	1.3	1.1
Mg. Salicylate Expected by dilution	5.38	6.70	7.70	9.10
Mean mg. Salicylate Found	5.51	6.85	7.75	9.60
% Recovery	103	102	101	106

The experiment was repeated on another series of cell quantities after incubating the cells for 15 minutes or 24 hours prior to removing the supernatant for analysis. The results are presented in Table XVIII.

Table XVIII. The Uptake of Salicylate from Saline Solutions as a Function of Cell Concentrations

Time of Sampling	Ml. Cells	Ml. 0.5% Salicylate in 1.12% Saline	Salicylate Mg. Expected by Dilution	Mg. Found	% Recovery
15 min.	1.0	2.0	3.33	3.20	96.3
24 hrs.	1.0	2.0	3.33	3.20	96.3
15 min.	0.5	2.0	4.0	4.12	103
24 hrs.	0.5	2.0	4.0	4.0	100
15 min.	0.1	2.0	4.8	5.0	104
24 hrs.	0.1	2.0	4.8	5.1	106

(2) The uptake of Salicylate from gelatin by erythrocytes.- Since it was found that cells would take up salicylate quantitatively from saline solutions and since the results of analyses were such as to indicate free passage of salicylate into cells this effect was checked by determining whether the uptake would be different in gelatin solutions. The uptake of salicylate from 2-ml. gelatin solutions containing 1.0, 0.4, and 0.10 ml. packed cells was shown to be quantitative.

To determine whether there was a difference in distribution of salicylate between cells and supernate at 15 minutes and 24 hours, comparative analyses were made on the laked cells and on the supernatants. The experiments were performed as follows: 0.5-ml. quantities of washed cells were added to 6.0-ml. portions of Oxypolygelatin 12676AY containing 1% salicylate. After incubation at room temperature for 15 minutes or 24 hours, tubes were shaken and 1-ml. aliquots of the total suspensions were

removed and delivered into 10-ml. portions of distilled water. After agitation, the laked solutions were centrifuged to remove the stroma and 1-ml. aliquots were taken for analysis. The supernatants were similarly diluted and analyzed.

Salicylate analyses gave the results presented in Table XIX.

Table XIX. Recovery of Salicylate from Supernates and Laked Cells After Exposure to 1% Salicylate

Time of Exposure	Ml. Cells	Ml. Oxypolygelatin 1% Salicylate Solution	Mg. Salicylate Expected by Dilution	Mg. Salicylate Found in	
				Total Suspension	Supernate
15 min.	0.5	6.0	9.25	9.75	10.25
24 hrs.	0.5	6.0	9.25	9.75	9.75

The results of this analysis are not entirely conclusive due to the fact that the values obtained for the total suspensions were higher than expected. However, if this discrepancy can be ascribed to an error in the delivery of the packed cells and the values obtained for the total suspension (which were the same for the 15-minute and the 24-hour categories) assumed to be the expected values, then there is a possibility that at the 15-minute period the salicylate had not yet become distributed.

c. The dependence of inhibition on the molecular configuration.- Homburger (op. cit.) had previously demonstrated that benzoic acid was without effect on the sedimentation of human red cells in plasma. The dependence of inhibitory activity on chemical structure was studied

in this investigation by treating cells suspended in Oxypolygelatin with the sodium salts of para- and meta-hydroxy benzoic acids. The salts were obtained from Dr. David Pressman and used in equimolar concentration to that of salicylate. Inhibition of sedimentation was not noticed when cells were suspended in Oxypolygelatin containing 1% para- or meta-hydroxy benzoate. A slight degree of acceleration as compared to controls was evident in the results presented in Figure 15d (Curves 29 and 30).

Experiments are at present under way to investigate the distribution of these compounds between the cells and gelatin.

IV Summary

The results of experiments on the influence of Oxypolygelatin on the sedimentation rate of saline washed erythrocytes show that sedimentation occurs due to the formation of large sludge-like aggregates. The sedimentation in replicate experiments has been found to vary with the kind of cells used and with the age of the sample. Variations of sedimentation as a function of protein concentration were investigated with the finding that there existed a lower limit beyond which sedimentation would not occur under the conditions of the experiment; this limiting value lies between 2 and 3% Oxypolygelatin. Further increments in protein concentration cause first an acceleration in the sedimentation rate which reaches a maximum at about 4.5% protein and then a slight decline, presumably because of the influence of the viscosity factor.

The inhibition of sedimentation by salicylate was examined in experiments attempting to relate the inhibitory action as a function of the concentration of salicylate and protein. It was found that at the higher protein concentrations a greater salicylate concentration was required to produce an inhibition and the experiments suggest, but do not prove, that there may be a quantitative relationship between the protein and salicylate. The lack of a one to one correspondence between protein and salicylate is due among other factors to the great variability of the sedimentation rates imposed by differences in cell samples used.

The site of action of sodium salicylate was investigated by determining its relative activity on the gelatin and on the cells. It was found that the exposure of Oxypolygelatin to salicylate did not result in permanent changes in its sedimentation behavior as evidenced after almost complete dialysis of the salicylate from the Oxypolygelatin. The effects of salicylate on the amount of protein which could be precipitated at different protein and salicylate concentrations were variable and confirmed the irregularities observed by Coburn and Kapp (op. cit.) in similar precipitation of serum proteins. In concentrations effective for inhibition of sedimentation no effects on either the pH or the viscosity were noted. It was noted that cells would take up salicylate with almost equal facility from Oxypolygelatin as from saline and that this uptake could be predicted on a "dilution" hypothesis by assuming that salicylate distributed itself in the cell water.

The inhibitory action of sodium salicylate was found to be dependent on its structural features (the ortho-hydroxy benzoic acid) since the para- and meta-hydroxy-benzoic acids did not have an inhibitory action in equimolar amounts.

The results of the investigations suggest the following conclusions: cell aggregation is the result of some type of interaction of the cells with the Oxypolygelatin,

and since this does not occur in all cells it must vary with the nature of the cell membrane. To study this type of interaction, the sedimentation technique is too coarse and too susceptible to variations imposed by changes in protein concentration, age of cells, viscosity of the medium, and other technical factors such as slight variations in tube diameters and proportions of cells to supernatant.

BIBLIOGRAPHY

1. Fahraeus, R., Physiol. Rev., 9, 241 (1929).

2. Ham, H. J. and F. C. Curtis, Med., 17, 447 (1937).

3. Ponder, E., J. Exp. Biol., 23, 350 (1947).

4. Monaghan, B. R. and H. L. White, J. Gen. Physiol., 19, 715 (1936).

5. Rourke, M. D. and A. C. Ernstene, J. Clin. Inv., 8, 545 (1930).

6. Knoefel, P. K. and G. Lehmann, J. Pharmacol. Exp. Therap., 83, 185 (1945).

7. Bendien, W. M., J. Neuberg, and I. Snapper, Biochem. Ztschr., 235, 14 (1931).

8. Lichty, J. A., Jr. and S. P. Hooker, Proc. Soc. Exp. Biol. Med., 48, 69 (1941).

9. Homburger, F., Amer. J. Med. Sci., 210, 168 (1945).

10. Swift, H. F., J. Exp. Med., 36, 735 (1922).

11. Coburn, A. F. and E. M. Kapp., J. Exp. Med., 77, 173 (1943).

12. Meyer, K., Physiol. Rev., 27, 335 (1947).

13. Chabanier, H., M. Lebert, and C. Lobbo-Onell, Compt. rend. soc. biol., 88, 178 (1923).

14. Abramson, H. A., R. Furchgott, and E. Ponder, J. Gen. Physiol., 22, 545 (1939).

15. Abramson, H. A., J. Gen. Physiol., 14, 163 (1930).

16. Brodie, B. B., A. Udenfriend, and A. F. Coburn, J. Pharmacol. Exp. Therap., 80, 114 (1944).

17. Smith, P. K., H. L. Gleason, C. G. Stoll, and S. Ogorzalek, J. Pharmacol. Exp. Therap., 87, 237 (1946).

Part II

The Relative Sensitivity of the Mucosal and Peritoneal Surfaces of Guinea Pig Ileum to Histamine, Acetylcholine, and Specific Antigens

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THE RELATIVE SENSITIVITY OF THE MUCOSAL AND PERITONEAL SURFACES OF GUINEA-PIG ILEUM TO HISTAMINE, ACETYLCHOLINE AND SPECIFIC ANTIGENS[1]

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During the investigation by Nicoll and Campbell (1, 2) of *in vitro* anaphylaxis, it was discovered that suitable antigens caused no response of the intestinal strips either when the strips were everted or when antigen was injected into the lumen of the strip. It was apparent, therefore, that in order to set off the mechanism responsible for anaphylactic contraction of intestinal smooth muscle the antigen must come into contact with the peritoneal surface.

These earlier observations have been repeated and extended to include studies dealing with the response to histamine and acetylcholine as well as to antigens of both relatively small and large molecular size.

Preparations of intestinal strips. The studies were made entirely with guinea-pig intestine. No particular selection was made as to sex or to the weights of the animals, which varied from 300 to 500 grams. The animals were usually bled to death by cardiac puncture, since the tissues then seemed to react more normally than when the animal was killed by a blow at the base of the skull. This latter method, which was used in most of the earlier studies, often produced a "refractoriness" in intestinal strips which disappeared only after prolonged exposure to aerated Tyrode's solution or storage in the cold. The abdomen was opened as soon as possible and the lower third of the small intestine was removed and placed in warm balanced salt solution.[2] The large strip was then cut into smaller sections of about 15 cm. to facilitate manipulation and was carefully flushed with warm salt solution. Portions not to be used immediately were covered with salt solution and stored in the refrigerator. Strips for immediate testing were then cut into 25–30 mm. lengths and the ends were tied tightly with silk thread. They were then suspended on a glass support and placed in the 100-ml. muscle bath which has been described by Campbell and McCasland (3).

The following method was used to evert muscle strips: a thin glass rod with a small knob at one end was passed into the intestinal lumen until the bulb emerged. A ligature was then made near the bulb and the free end was pulled over and past the knob until complete eversion was achieved, the entire procedure

[1] This investigation was financially supported by a Rockefeller Foundation fund for immunochemistry research.

[2] The balanced salt solution used was a slight modification of Tyrode's formula and contained per 100 ml. of solution: $MgCl_2 \cdot 6H_2O$ 0.0213 gram, KCl 0.0195 gram, $CaCl_2 \cdot 2H_2O$ 0.0193 gram, $NaHCO_3$ 0.1015 gram, NaCl 0.800 gram, and glucose 0.1000 gram. The final pH at 37°C. was 8.2.

being carried out in a petri dish containing warm salt solution. A suitable portion was then ligated at both ends and placed in the muscle bath for testing. The records clearly show that the failure of such everted preparations to react was not due to the above manipulation, since re-everted strips showed only a slight loss in irritability.

Studies on *in vitro* anaphylaxis were performed on guinea pigs which had been sensitized either to arsanilic-azo-ovalbumin or to histamine-azo-ovalbumin. The former was given intra-abdominally in four injections of 15 mgm. each at 48-hour

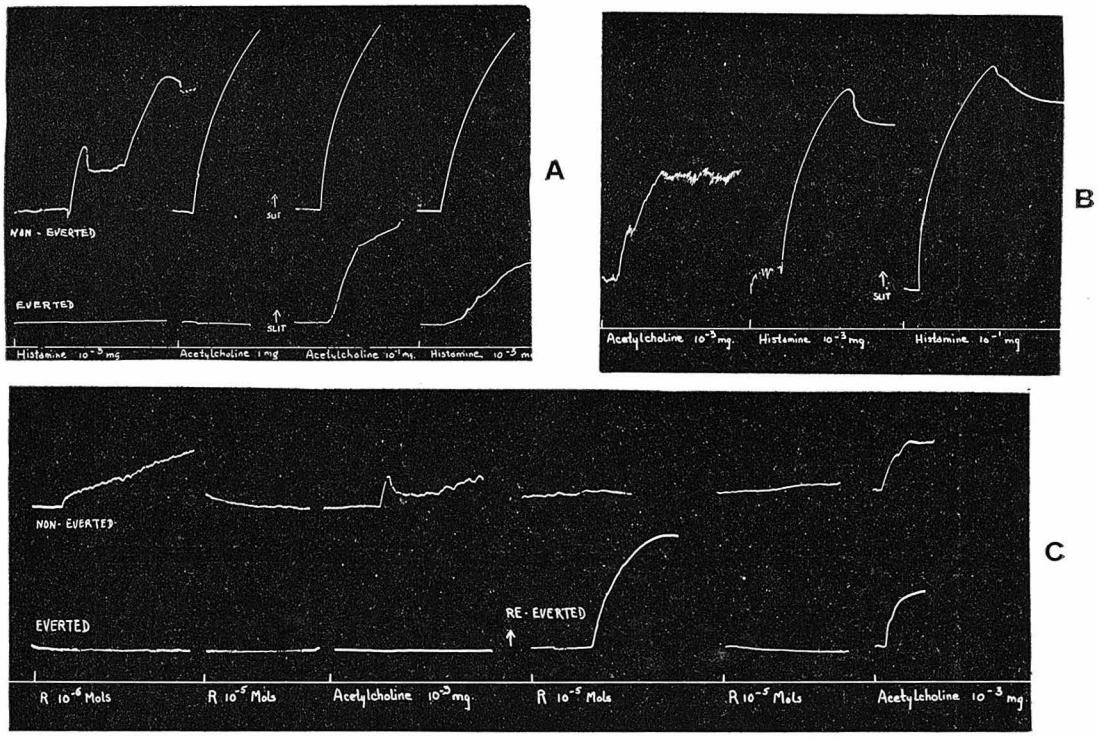


Fig. 1. Kymograph records showing the effects of specific and non-specific chemical agents on everted and non-everted strips of guinea pig ileum.

intervals and the latter in two injections of 25 mgm. each. The animals were used 14 or more days following the final injection.

Results. Typical effects obtained from normal and everted intestinal strips are given in the kymograph records of figures 1 and 2.

Figure 1A. Everted and non-everted strips were first exposed to 10^{-3} mgm. of histamine and then 1 mgm. of acetylcholine in the 100-ml. bath. The non-everted strip responded normally, while the everted preparation showed no response. After a longitudinal slit was made, the everted as well as the non-everted responded to both acetylcholine and histamine.

Figure 1B. This is an additional record to show that a normal non-everted strip apparently reacts normally after being subjected to a longitudinal incision.

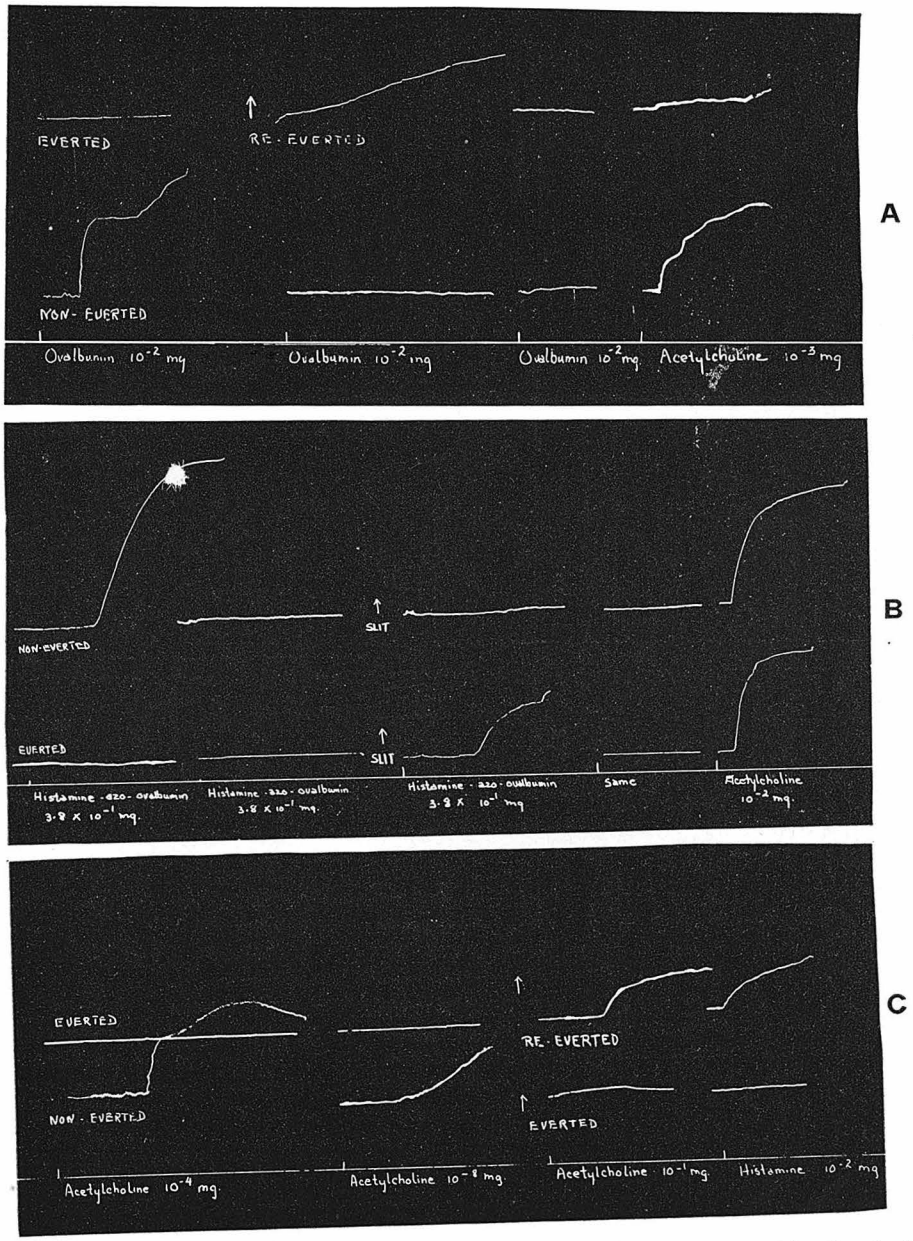
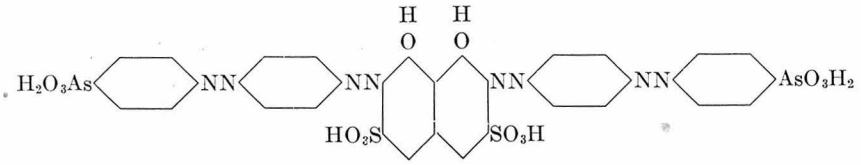


Fig. 2. Kymograph records showing the effects of specific and non-specific chemical agents on everted and non-everted strips of guinea pig ileum.

Figure 1C. These strips were taken from a guinea pig which had been sensitized to arsanilic-azo-ovalbumin. The test antigen was



The addition of 10^{-6} moles of this material produced no responses in the everted strip, but the non-everted strip gave a normal anaphylactic response. After the tissue was washed and allowed to return to its normal state, a second addition of antigen caused no response, indicating that desensitization had taken place. The addition of acetylcholine caused the non-everted strip to contract, proving its irritability. The everted strip was then re-everted, thus exposing the peritoneal surface. When antigen was again added, the re-everted strip gave a typical anaphylactic response and was desensitized, as evidenced by its failure to respond to a second exposure of antigen. Both strips subsequently responded to acetylcholine, proving their viability.

Figure 2A. Intestinal strips from guinea pigs sensitized to histamine-azo-ovalbumin were tested against ovalbumin.[3] Here again the everted strip failed to respond to antigen until re-everted.

Figure 2B. The same type of strips was used here as those of 2A. Histamine-azo-ovalbumin was used as the shocking antigen. Here again the everted strip did not contract until it was slit longitudinally, thus allowing the antigen to come into contact with the peritoneal surface of the intestine.

Figure 2C. Everted and non-everted strips were first tested with acetylcholine. The former contracted, while the latter showed no activity. The strips were then reversed and the initially non-everted muscle was everted while the initially everted strip was re-everted. Responses were then clearly reversed and the initially inactive intestine became responsive upon re-eversion.

Discussion. The foregoing data demonstrate that the smooth muscle of isolated intestinal strips will respond to specific (antigens) or to non-specific (histamine, acetylcholine) chemical stimuli only when the peritoneal surface of the gut comes in contact with these agents. Apparently such chemical substances cannot diffuse through the relatively thick mucosal layer and hence are prevented from reaching the sites responsible for muscle activation. However, it is of interest to note that the muscle response to both large and small molecular substances is extremely rapid when applied to the peritoneal surface, although this surface consists of a serous membrane of appreciable thickness. In fact, the response is so rapid that it would appear as though actual penetration to the muscle layer were not necessary.

King and Robinson (4) reported contraction of *muscularis mucosae* in isolated mucosal strips upon exposure to acetylcholine or histamine. Since we obtained no contraction of everted strips, it must be assumed that either their recording system was more sensitive than ours or that activation was induced by stimulation from the inner mucosal surface rather than from the epithelial surface.

The failure of such stimulants to induce contraction of intestinal muscle when administered orally is due to absorption or detoxification in the mucosal tissue or portal circulation. Of the simple substances, atropine is an exception, but it apparently reaches the intestinal muscles by way of the circulatory system and not by direct diffusion. It is well known that antigenic materials such as ovalbumin can pass through the intestinal mucosa of normal animals. However, it would appear from the foregoing results that the antigen must reach the intesti-

[3] The sensitizing antigen was prepared by coupling the diazonium salt of histamine to ovalbumin. Enough of the original ovalbumin specificity was retained to produce corresponding hypersensitivity. It was of interest to note that there was no evidence of a refractoriness to histamine, which might have been expected.

nal muscle via the circulation if reactions are obtained following ingestion of antigen in a sensitized animal.

SUMMARY

Surviving intestinal strips have been shown to react to specific and non-specific substances only when these agents have access to the peritoneal surface of the gut. The possible reasons for this effect have been discussed.

REFERENCES

(1) NICOLL, P. A. AND D. H. CAMPBELL. J. Immunology **39:** 89, 1940.
(2) NICOLL AND CAMPBELL. Ibid.
(3) CAMPBELL, D. H. AND G. E. MCCASLAND. J. Immunology **49:** 315, 1944.
(4) KING, C. E. AND M. H. ROBINSON. This Journal **143:** 325, 1945.