Studies on the Hemolytic Properties of Protamine

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ABSTRACT The basic protein protamine causes a rapid hemolysis when incubated with the red blood cells of many mammalian species. The age of the cells does not affect the process. Neutralization of the active side groups of the protamine molecule with formalinization demonstrates that a specific degree of charge is necessary for hemolysis, as more than 30 per cent of the guanidine groups must remain unreacted to maintain activity. Unlike the hemolysis induced by the synthetic polypeptides polylysine and polyhomoarginine, protamine hemolysis is temperature-dependent.

Whole lipoprotein material derived from red blood cell membranes inhibits protamine hemolysis to a greater extent than do the membranes themselves, serum, serum protein fractions, or cholesterol. The phosphatide and protein moieties derived from the membranes are quite avid in inhibiting protamine hemolysis. A probable explanation is that intracellular aggregation of these structural elements may cause changes in electrostatic charge and surface tension which result in increased permeability.

The hemolytic and antitumor cell properties of protamine could not be segregated from its animal toxicity. Despite formalinization to a degree which eliminated the former, the compound remained quite toxic to mice and rabbits.

Protamine is a basic protein with a molecular weight of approximately 4000 to 5000 which is separated by salt fraction (1) from its complex with desoxyribonucleic acid in the sperm heads of fish. It is composed of a linear aggregation of amino acids and contains a large percentage of arginine.

It has been previously demonstrated that protamine is capable of inhibiting protein synthesis in ascites tumor cells following its uptake and combination with the cellular nucleic acids (2). Damage to the tumor cell membrane was minimal and the process was temperature-dependent. Protamine is also capable of producing hemolysis *in vitro* and this hemolytic effect may represent a factor in its animal toxicity or another mechanism of cellular damage. In view of the ability of protamine to penetrate cell membranes (2, 3) it appeared possible that its lysis of red blood cells might result from intracellu-

lar activity rather than from the surface activity which might be anticipated from its highly charged structure. Polylysine polypeptides of similar linear structure also cause hemolysis (4). However, analysis of this reaction has indicated that the hemolysis resulted from membrane binding of the polypeptides with no apparent effect resulting from the binding with non-structural cellular components. This reaction is not temperature-dependent.

The following studies were aimed at elucidating the site of interaction of protamine with red blood cells and comparing this reaction with that of the synthetic polypeptides. The conditions necessary for such interaction were also studied. Attempts were made to segregate the hemolytic and animal toxic properties of protamine from its antitumor cell action.

As in the experiments with polypeptides, the site of action of protamine was found to be the cell's stromal material and not an intracellular component. The use of purified fractions of the cell stroma revealed that the major portion of protamine is bound to the lipoprotein material. When this lipoprotein is further fractionated, it yields very active phosphatide and protein fractions, which in free form bind protamine more avidly than the whole lipoprotein. The status of the amino acids' side groups was found to play a key role in protamine's hemolytic ability and also in its antitumor cell properties. Lastly a significant difference was demonstrated between protamine and the polypeptides in that protamine hemolysis was temperature-dependent.

MATERIALS AND METHODS

25 gm. white mice were used throughout the experiments. Blood was obtained by puncture of the orbital plexus *via* the cornu of the eye and was then washed three times with twenty volumes of a high bicarbonate, pH 7.4, balanced salt solution (BSS) (5). Incubation was performed in 5 ml. volumetric flasks at 37°C. in a water bath which rotated the flasks at 88 R.P.M., unless otherwise specified. Hemoglobin concentration was determined by reading its optical density in solution at 540 mμ.

Adult guinea pigs and albino rabbits were bled *via* intracardiac puncture and the blood was treated as described above.

Protamine was obtained from the Biochemical Research Division of the Eli Lilly Company as a relatively pure form of salmine sulfate. The protamine was dissolved in BSS (usually at 5 mg./ml.) and dialyzed against BSS (100:1) for 12 hours in the cold to remove the sulfate. Up to one-half of the original nitrogen content was lost during the procedure. Nitrogen determinations were performed by the standard Kjeldahl method and in most of the experiments the protamine nitrogen concentration was used as the standard measure in expressing concentration. Nitrogen represents roughly one-third of the weight of the free base (1).

Polylysine polypeptide was obtained from the Mann Laboratories, New York City, in the form of a bromide salt. The polypeptide used had an approximate molecular weight of 5000 and was composed entirely of lysine groups.

o-Methyl isourea was obtained from the New Products Development Group of the American Cyanamid Company.

Guanidination was carried out on the polylysine according to the procedure of Klee and Richards (6). Utilizing the Callanan variation of the Sakaguchi colorimetric reaction for arginine (7), the final product appeared to contain 60 to 75 per cent guanidine groups, with a calculated increase in molecular weight of less than 20 per cent. The final product was considered to represent a polypeptide containing 60 to 75 per cent homoarginine groups with the remaining 20 to 45 per cent as unreacted lysine.

Formalinization of the protamine was performed as described by Fraenkel-Conrat (8). Variation in the degree of formalinization was produced by varying the duration of incubation, the pH, and the concentration of the protamine. Under the conditions described (10 per cent formalin, 80°C., and pH 8) 75 per cent formalinization of a 1 per cent protamine solution was produced in 4 days and 30 per cent in 24 hours. The loss of the reactive side groups has been explained as methylolation of the guanidine groups similar to that which can occur with amine groups. When higher concentrations of protamine or protein are used, cross-linkage can occur through these guanidine groups with condensation of the protamine and increase in molecular weight up to three times the normal molecular weight or 12,000 (8).

The purified lipoprotein was prepared by differential ultracentrifugation of human erythrocytes and consists of peptide, free cholesterol, and phospholipid in about equal portions (9). Further fractionation was performed by differential separation in lipid solvents. The resulting phosphatide fraction may be considered as a pure material, while the cholesterol portion is probably contaminated by phosphatide. The protein component appears to be a moderately basic molecule (10).

Characteristics of the Hemolytic Process

When washed red blood cells were incubated with protamine a rapid hemolysis ensued. At a red blood count of 150,000 cells/ml. remarkably small quantities of protamine were found to be capable of producing a significant loss of hemoglobin from each cell. As is shown in Fig. 1, 0.25 μg. of protamine nitrogen/150,000 cells produces 25 per cent hemolysis. The degree of hemolysis is compared with that produced by distilled water, considered as 100 per cent hemolysis for these experiments. At this cell concentration, a tenfold increase in protamine (2.5 μg. of protamine nitrogen) produces a threefold increase in hemolysis, or 75 per cent. 75 per cent hemolysis actually represents the maximal ability of protamine to release hemoglobin from this cell concentration and increases in protamine to concentrations as high as 250 μg. produce no further increase in hemoglobin release. When examined under phase microscopy the cells showed no crenation, clumping, or gross membrane damage and each cell appeared to be equally depleted of hemoglobin.

Speed of Hemoglobin Release

When tested at the minimal protamine concentration which would produce maximal hemolysis of 150,000 cells (1.5 μg. of protamine nitrogen, hereafter referred to as minimal hemolyzing concentration, MHC) and at a 15-fold greater concentration of protamine, hemoglobin was completely released in 10 minutes. The release was somewhat greater at the higher concentration, especially during the first 5 minutes, but was almost equivalent at the end of the 10 minute period.

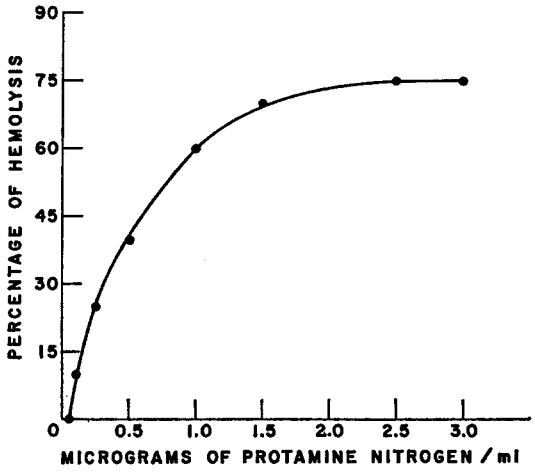

FIGURE 1. The abscissa represents the concentration of protamine nitrogen in micrograms per milliliter; the ordinate, the percentage of hemolysis produced. The red blood cell concentration was 150,000 cells/ml., 3 times washed. The flasks were incubated for 15 minutes at 37°C., pH 7.4, and rotated at 88 R.P.M.

Site of Protamine Action

A membrane preparation was produced from red blood cells by the method of Anderson (11), which was faintly pink but contained no whole cells. The addition of small quantities of this preparation to protamine solutions significantly reduces their hemolytic ability. Clumping and sedimentation of the membranes were noted and centrifugation of these membrane clumps removed protamine from the solutions.

When the lipoprotein material of the red blood cell (9, 13) was added to protamine solutions a similar, though more effective, inhibition of hemolysis was produced. As noted in Fig. 2, 37 per cent inhibition could be produced by a lipoprotein to protamine ratio of 2:1. A ration of 15:1 or 60 μg. of lipoprotein to 4 μg. of protamine was necessary to produce complete inhibition of hemolysis.

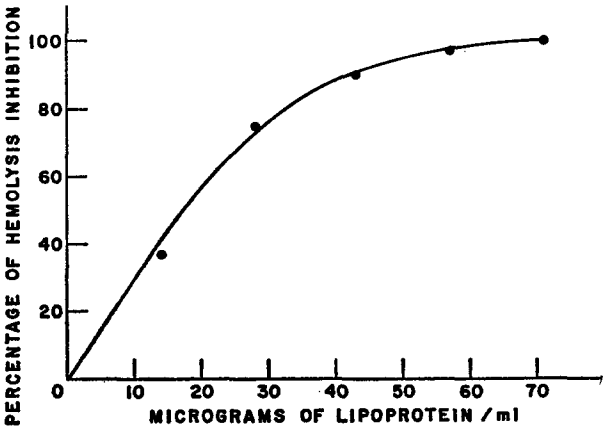

FIGURE 2. The abscissa represents the micrograms of lipoprotein per milliliter; the ordinate, the percentage of inhibition of hemolysis induced. Protamine concentration 4 μg./ml. Red blood cell concentration 150,000 cells/ml., 3 times washed, 37°C. and rotated for 15 minutes.

In order to determine more specifically the site of reaction the stroma was further fractionated (10) and the resulting total lipid, cholesterol, phosphatide, and protein components were added to protamine solutions in equal concentration. As is evident from Fig. 3, the cholesterol fraction was almost inactive as a protamine inhibitor. The activity which was demonstrated was

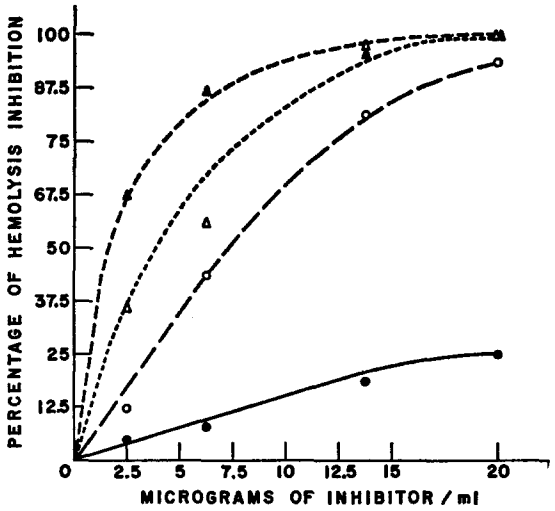

FIGURE 3. The abscissa represents the concentration of inhibitor in micrograms per ml. The ordinate represents the percentage of inhibition of hemolysis induced. Protamine concentration in all experiments was 2.5 μg./ml. Red blood cell concentration 150,000 cells/ml., 3 times washed and incubated in rotating flasks at 37°C. for 15 minutes. ●, cholesterol fraction. ○, total lipid fraction. △, phosphatide fraction. ▲, protein fraction.

most probably the result of phosphatide contamination. On the other hand, the total lipid was moderately and the phosphatide very active as protamine inhibitors. The total lipid fraction was twice as effective as the original lipoprotein, producing total inhibition at a ratio of 7.5:1 while the phosphatide was even more reactive throughout the whole range of concentrations.

A striking feature was the high degree of reactivity of the protein moiety which was the most active component in producing inhibition of hemolysis. At a ratio of protein to protamine of 1:1, 67.5 per cent inhibition of hemolysis was produced.

The doubling of activity of the lipid when freed from its complex with the protein may indicate the release of active binding sites. It was also apparent that both the phosphatide and protein material of red blood cell lipoprotein react with protamine when they are in the form of pure fractions. Despite these findings however, we should only consider the inhibition of protamine as produced by the whole lipoprotein as it is in this form that these substances exist in the membrane.

Factors Affecting the Hemolytic Process

Concentrations of normal mouse serum as low as 0.2 per cent produced 75 per cent inhibition of hemolysis when protamine was used at MHC, but comparable concentrations of serum albumin and globulin (bovine) were less than 30 per cent as effective. Constant results were achieved by washing the cells three times (BSS 1:60). Further washings did not increase the susceptibility of red blood cells and therefore, it appears that the initial concentration of adhering serum was sufficiently reduced by this dilution. No structural alteration can be deduced save perhaps that of loss of adhering, denatured globulins.

This hemolysis is strikingly temperature-dependent. Concentrations of protamine which normally would have produced a 50 per cent hemolysis at 37°C. caused no hemolysis at 0°C. Under similar conditions of cold there was no diminution in the hemolytic activity of the quaternary compound hyamine,[1] polylysine, or of polyhomoarginine.

Changes in the pH of the suspension did not alter the degree of hemolysis. No difference in the reaction was noted when isotonic sodium chloride was substituted for a balanced media (BSS). Glucose was apparently not required nor did excess glucose cause inhibition, and calcium caused only minor inhibition (less than 10 per cent) when raised to twice the concentration of the normal media.

The cells of rabbits and guinea pigs were as sensitive to protamine as were those of mice.

[1] Para (diisobutyl-cresoxyethoxyethyl) dimethylbenzylammonium chloride monohydrate, Rohm and Haas Co., Philadelphia.

In an attempt to delineate the possible effects of the age of the cells, a group of mice was bled repeatedly over a 3 week period and the cells of these mice were compared with those of unprepared mice for protamine susceptibility. The reticulocyte count of the "bled" group was 10 per cent. No differences in protamine sensitivity could be detected at any concentration and it was concluded that no cell-age difference in protamine sensitivity existed such as has been demonstrated in primaquine hemolysis (12).

Formalinization

When a 3 per cent solution of protamine is incubated in 10 per cent formalin, pH 7.4–8 at 80°C. for 4 days, there is an apparent doubling of molecular

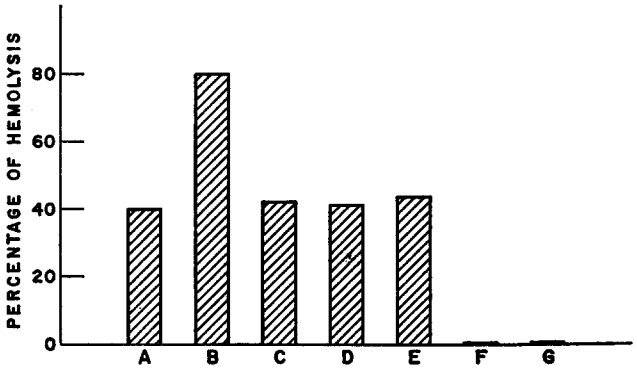

FIGURE 4. The ordinate represents the percentage of hemolysis induced. The red blood cell concentration was 150,000/ml. 3 times washed and incubated at 37°C. for 15 minutes. All protamine preparations (abscissa) were used at a concentration of 0.5 μg. of nitrogen/ml., except G which was 25 μg./ml.

A, untreated protamine.

B, protamine condensate, molecular weight 10,000, with 40 per cent free guanidine groups.

C, protamine with normal molecular weight, 80 per cent free guanidine groups.

D, " " " " " , 60 " " " " " .

E, " " " " " , 50 " " " " " .

F, " " " " " , 30 " " " " " .

G, " " " " " , 30 " " " " " .

weight (as determined by dialysis) and a reduction of 60 per cent in the free guanidine groups. As is shown in Fig. 4 this material is approximately 50 per cent more potent in producing hemolysis (on a weight basis) than untreated protamine. However, this condensate produces an extensive aggregation of the red blood cells, which apparently precedes hemolysis, not noted with untreated protamine.

When a 1 per cent solution of protamine was incubated with formalin, no increase in molecular weight could be detected but a reduction in free guani-

dine groups of 25, 40, and 50 per cent could be produced by varying the incubation periods. As is demonstrated in Fig. 4 no reduction in hemolytic ability could be demonstrated and it was only when a 70 per cent reduction in guanidine groups was produced *via* methylation that a sharp and complete loss of hemolytic activity occurred. In concentrations of this material 15-fold greater than the MHC (25 μg. of protamine nitrogen/ml.) no hemolysis occurred.

This degree of formalinization with a 70 per cent reduction in the reactive guanidine groups produced no significant change in the intraperitoneal toxicity of the treated protamine. On the other hand, its antitumor cell properties were completely eliminated by this reduction in the guanidine groups.

Mechanism of Cellular Alteration

The avidity of the binding of protamine by the structural elements of the red blood cells as well as the rapidity of cell damage indicates that the change brought about by protamine was probably structural in nature. In an attempt to differentiate between the effects which resulted from such alteration and damage which resulted from interference with energy-producing processes, the following experiment was performed. Heparin was added to the cell-protamine suspension at various intervals and one-half of the suspension was immediately plunged into an ice bath while the other half continued to incubate at 37°C. A great excess of heparin was added so that the protamine which remained in solution would be immediately bound. Preincubation of this amount of heparin (1 mg./ml.) with the MHC completely prevented hemolysis. Heparin itself in this concentration had no hemolytic effect and incubation at 0°C. of untreated cells produced no hemolysis.

Despite the addition of heparin at 2, 4, and 6 minutes, hemolysis proceeded undiminished at both temperatures. Final hemolysis reached the expected 65 to 75 per cent in both portions.

These results confirm the observation that protamine is almost instantly bound to the cell structure and produces immediately the cellular alteration necessary for hemolysis. The continued presence of unreacted protamine is not necessary and the cell changes which are so produced are sufficient to permit hemolysis despite the absence of catabolic activity (at 0°C.).

DISCUSSION

Protamine is capable of producing alterations in the red blood cell which result in a release of up to 85 per cent of the cell's hemoglobin. The release is rapid and affects all cells equally.

The basic polypeptides, especially polylysine, are also capable of producing

hemolysis (4). An analysis of the kinetics of this reaction indicates an interaction between the cationic macromolecules and the normally anionic cell surface. A probable explanation is that the spatial alteration which would thus be produced would derange the normal permeability. This type of reactivity has been termed "surface activity." Schulman (14) has described surface activity as a combination of a highly charged compound with the charged molecules of the cell's surfaces, or in some cases, a penetration of the agent into the lipoprotein monolayers of the cell with resultant increase in surface tension and increased permeability.

It might be predicted from protamine's macromolecular structure and cationic nature (1) (dependent upon a large number of free guanidine side groups), that it would demonstrate a considerable degree of surface activity. The experiments demonstrated a clear cut dependence upon the presence of a specific number of unreacted side groups, for although the hemolytic ability was maintained when the guanidine groups were reduced 50 to 60 per cent, it was completely eliminated when they were reduced 70 per cent. The use of this maximally reacted protamine in concentrations such that the total number of free guanidine groups per cell is equal to that of a fully hemolyzing concentration of unreacted protamine causes no cell damage. Thus the loss of hemolyzing ability is not due solely to the depletion of total charge but to a specific charge loss on each protamine molecule probably resulting in a loss of the ability of the molecule to orient itself in a particular manner on the cell's surfaces. A similar loss of antitumor activity at this juncture is probably the result of the loss of protamine's ability to aggregate nucleic acid (2).

The lipoprotein component of the cell membrane demonstrates a high degree of affinity for the protamine molecule. This binding undoubtedly accounts for the inhibition of protamine hemolysis induced by membrane preparations and indicates that the hemolysis of red blood cells most likely follows the fixation of protamine within the lipoprotein stroma. The use of purified fractions also demonstrates a very great affinity of the phosphatide and protein fractions of the lipoprotein for protamine but does not enable us to specify which of these moieties might be the binding site in the whole cell. Such affinity would be followed by electrostatic alteration and possibly aggregation of the key structural elements, fibrils and lipid plaques, which would distort their regular arrangement and their normal permeability (15). Previous workers have demonstrated the affinity of lipoproteins for basic proteins and for other charged molecules and the inhibitory effects thus produced (16, 17).

It appears that protamine produces hemolysis by an interaction with the anionic components of the cell surface which may reside in the lipoprotein component. Hemoglobin release follows distortion of the normal electrostatic structure and possibly structural alteration. Protamine, therefore, appears to fulfill the requirements for a surface-active agent.

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