Effects of Intravenous Infusions of Feather Keratin: Preliminary Characterization and Evaluation as a Plasma Expander.* (28850)

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Polypeptide solutions of both natural and synthetic origin have been tested as plasma expanders(1,2,3). Although they have been found to be efficacious in treatment of hypovolemic shock in experimental animals, allergic reactions, rapid excretion, and toxic manifestations have discouraged clinical use. It has been suggested that multiple charged amino acid residues are responsible for the toxic effects(2,3).

Feather keratin represents an inexpensive, abundant, and virtually unutilized supply of protein. We have recently evaluated a keratin hydrolysate obtained from chicken feathers for its potential usefulness as a plasma volume expander. Since approximately 50% of the amino acid residues in this hydrolysate carried no charge (pH 3.5), it was hoped that possible toxic effects would be minimized or eliminated. Initial tests gave no evidence of antigenicity and encouraged further evaluation. This report concerns the results of preliminary characterization and toxicity studies.

Materials and methods. Polypeptide solutions of keratin were obtained from the feathers of hybrid chickens by hydrogen peroxide hydrolysis. The crude solutions were then dialyzed against 0.9% saline for 18 hours. Approximately 16% of the biuret-reacting material was insoluble at pH 2.8. This fraction and the acid soluble supernatant were characterized separately. Characterization studies included: Two-dimensional paper electrophoresis (2000 volts, pyridine-acetic acid solution, pH 3.5) and chromatography (n-butal alcohol-acetic acid-water solution); and moving boundary electrophoresis (0.1 ionic strength barbital buffer, pH 8.6).

The hydrolysate was tested for antigenicity in rabbits by the interfacial ring precipitin test and in guinea pigs by passive cutaneous anaphylaxis.

The acid soluble material was used for the initial acute toxicity studies as a 1.4% to 3.6% solution in 0.9% saline. Prior to infusion the solutions were adjusted to pH 6.0 to 7.0 and sterilized by filtration through Seitz pads.

Six mongrel dogs weighing 6.8 to 15.4 kg were fasted 12-18 hours prior to the experiment, but with free acess to water. The animals were anesthetized during infusion with intravenous pentobarbital sodium (30 mg/kg). Ten ml/kg of the polypeptide solution was infused into the femoral vein in 5 or 30 minutes. Temperature, pulse and respiratory rates were recorded, and hematologic and chemical studies carried out before infusion and at 15 minutes, 1, 3, 6, 12 and 24 hours following completion of the infusion.

Venous and arterial pressures, electrocardiographic changes, and respiratory movements were monitored in one animal. Changes in blood volume were evaluated by serial hematocrit, hemoglobin, and serum protein determinations. Methemoglobin and sulfhemoglobin were measured by the method of Evelyn and Malloy(4) and sulfhydryl concentrations by a modification of the nitroprusside reaction(5). Determinations of bleeding time were performed by making a standard incision in the skin of the abdomen. Detection of oxidizing substances was carried out by potassium permanganate titration, ferrocyanide oxidation, and a slight modification of the assay method for catalase(6) which permitted identification of peroxides.

Results. Characterization studies. Two-dimensional paper electrophoresis-chromatographic analyses of the acid-soluble fraction at pH 3.5 demonstrated at least 20 amino acid residues, 4 of which were negatively charged and 5 positively charged. The main portion of the material showed only slight electrophoretic migration with some tailing in the chromatographic run. Areas which

TABLE I. Effects of Hydrolyzed Feather Keratin upon Erythrocytes, Leucocytes, and Platelets *in vitro.*

	Pooled dog blood		Pooled human blood	
	Saline control	Keratin solution	Saline control	Keratin solution
RBC morphology	N*	Sl cr't'n‡	N	N
RBC aggregation	0	0	0	0
Rouleaux	+	+	+++	+++
Corrected ESR (mm/hr)	1.0	.0	4.0	5.0
Hemolysis (mg % Hb)†		+4.0		+5.0
WBC morphology	N	N	N	N
WBC aggregation	0	0	0	0
WBC motility	N	N	N	N
Platelet morphology	N	Altered	N	Altered
Platelet aggregation	0	++	0	++

* Normal.
† Represents difference between control and test solutions.
‡ Slight crenation.

stained acidic probably represented cysteic peptides. Nitro-prusside-cyanide stains for sulfhydryl groups and disulfides were weakly positive while the trephenyl tetrazolium and aniline diphenylamine stains for carbohydrate moieties and reducing substances were negative. The acid-insoluble fraction was mainly electrophoretically inert at pH 3.5, but otherwise the analyses were similar.

On moving boundary electrophoresis about 70% of the material in both fractions moved with a mobility of 6.4×10^{-5} cm^2 sec^{-1} $volt^{-1}$. The remaining 30% of the material remained at the starting boundary. Qualitative tests for sulfates, sulfites and sulphides were negative. The sulfur content of a lyophilized sample was 43 mg/g of biuret-reacting material.

In vitro *studies.* The effects of a 3% keratin solution (pH 7.4) on pooled specimens of normal human and dog oxylated blood were studied using saline controls. One volume of solution was incubated with 5 volumes of blood at 37°C for 15 minutes. The results are shown in Table I. The absence of red cell aggregation and increased erythrocyte sedimentation rates is in contrast to that observed with synthetic polypeptide solution(2,3) and may be attributed to the presence of multiple non-charged amino acid residues. The slight hemolysis observed in the test solutions was not considered significant. Morphologically the platelets showed a marked decrease of filamentous processes as compared to control samples.

Antigenicity studies. Under the above conditions of testing, the hydrolysate preparation gave no evidence of antigenicity in rabbits or guinea pigs. The test animals exhibited no allergic reactions during or following the acute toxicity studies or with rechallenge 2 weeks later (2 dogs).

Toxicity studies. When the dogs were infused with the hydrolysate the vital signs, arterial pressure, and electrocardiogram remained essentially unaltered. The venous pressure was elevated only during infusion and immediately thereafter.

The transient thrombocytopenia and leucopenia which developed in all test animals following infusion (Fig. 1) has also been observed after infusion of synthetic polypeptides(3). The leucopenia was followed by leucocytosis (neutrophilia) which persisted for several days. The hematocrit, hemoglobin and total serum protein concentration were all elevated to the same degree. Infusion did not result in red cell aggregation or increased erythrocyte sedimentation rates. Blood urea nitrogen and blood sugar determinations were unaltered, and there was no prolongation of bleeding time. Blood sulfhydryl concentrations were not significantly increased by infusion (Table II).

Cyanosis accompanied by coughing and vomiting occurred in 2 animals immediately

TABLE II. Effect of Hydrolyzed Feather Keratin Infusions upon Blood Sulfhydryl Levels.

Dog No.	Before infusion	After infusion (min) 0	15	30	60	180
1	1.82	2.64	2.97	2.81	2.97	2.87
2	3.24	—	2.64	3.10	3.30	3.27
3	2.54	3.46	3.17	—	3.30	3.30

Figures expressed as mg-SH as cysteine/100 ml of blood.

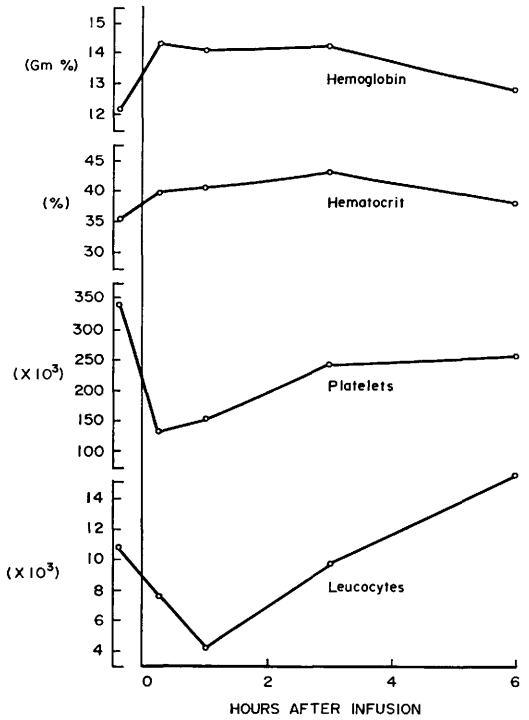

FIG. 1. Hematologic effects following intravenous infusion of hydrolyzed feather keratin in dogs (mean values of 6 exp).

following infusion. Methemoglobin measured in one of the animals was 3.67 g%. There was no sulfhemoglobin. The formation of methemoglobin was confirmed *in vitro* by incubation of acid soluble fractions from various lots of the keratin hydrolysate with fresh dog blood (37°C for 30 minutes, pH 7.0). Heinz body preparations were negative and there was no evidence of hemolysis. An oxidizing agent was not identified and qualitative tests for nitrites were negative. Attempts to produce methemoglobin *in vitro* with varying concentrations of commercially obtained cysteic acid were unsuccessful. Oxidizing substances could not be demonstrated in the acid-insoluble keratin fraction and intravenous infusion of a 3.5% solution in saline, pH 7.0, in one animal did not result in methemoglobinemia. Nevertheless, infusion of this fraction was accompanied by other toxic effects.

Oliguria developed in all animals following infusion as indicated by 6 to 12 hour urinary output studies.

For 2 to 3 days postinfusion the test animals were lethargic and ate poorly, but thereafter appeared clinically well. No deaths occurred during a 4- to 6-week observation period.

Gross and microscopic histopathologic examinations in 2 animals killed 6 hours after infusion were unrevealing except for focal interstitial hemorrhages of the lungs.

Variations in concentration of the hydrolysate infused and rate of infusion did not alter results.

Discussion. The effect of intravenous infusion of hydrolyzed keratin on the formed elements of the blood is a nonspecific reaction pattern ("hemoclastic" reaction) that may be caused by a variety of biological and chemical materials. A similar transient leucopenia and thrombocytopenia has been found during anaphylactic and peptone shock and following intravenous administration of macromolecular substances, particulated matter, heterologous plasma, incompatible blood, and bacterial antigens(7).

The unexplained hemoconcentration observed after infusion indicated loss of intravascular fluid, an effect opposite of that desired of a plasma volume expander. An increase of intravascular osmolality with stimulation of water-conserving mechanisms might account for the postinfusion oliguria seen in these experiments. Indeed, intravenous infusion of saline (30 cc/kg) in one test animal markedly increased urinary output.

The specific mechanism responsible for the methemoglobinemia was not identified.

Summary. A peroxide hydrolysate of feather keratin has received preliminary evaluation as a plasma volume expander. Intravenous infusions in dogs produced acute toxic effects characterized by hemoconcentration, leucopenia, thrombocytopenia, methemoglobinemia, oliguria and lethargy. The results indicate that peroxide hydrolysates obtained from chicken feathers are unsuitable for intravenous use.

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Reaction of *Escherichia coli* Endotoxin with Lithium Aluminum Hydride. (28851)

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Noll and Braude(1) reported that treatment with lithium aluminum hydride ($LiAlH_4$) modified a small portion (about 10%) of Boivin-type endotoxin from *Escherichia coli* to yield a nonpyrogenic and nontoxic but antigenic material (RE fraction). They concluded from infrared absorption data that both the modified portion and the pyrogenic and toxic remainder were free of ester-bound fatty acids. The disappearance of ester absorption bands in the RE fraction was correlated with reduction of pyrogenicity and toxicity. This communication reports the results of studies with the same culture and procedures employed by these workers. We sought to confirm their findings and to obtain additional chemical data on the nature of the changes in bacterial endotoxin produced by treatment with $LiAlH_4$.

Materials and methods. 1) *Culture.* In each experiment, E. coli of serotype 0113, originally isolated from the blood of a patient, was used(1). Organisms from a frozen stock culture (in trypticase-soy broth) were cultivated for 24 hours at 37°C with shaking in synthetic M-9 medium(2) containing 0.5% glucose or without shaking in modified Gladstone medium(3). Three additional transfers were made before the experimental batches were inoculated. These were grown either in 100 ml volumes of M-9 medium in 500 ml Erlenmeyer flasks or in 4000 ml volumes of Gladstone medium in 6000 ml Erlenmeyer flasks. After 18 hours' incubation, the cells grown in M-9 medium were chilled, harvested by centrifugation and washed twice with 0.15 M sodium chloride. The cells harvested after 18 hours from the Gladstone medium were washed with 95% ethanol and then with acetone and were dried in vacuum (1).

2) *Extraction of endotoxin with trichloracetic acid (TCA).* For Experiment I, the wet sediment of saline washed cells grown in M-9 medium was extracted at 0°C for 3 hours with TCA in a final concentration of 0.25 N. The remaining steps were performed at 4-6°C. The extract was clarified by centrifugation and dialyzed. Endotoxin was precipitated by slowly adding ethanol to a final concentration of 68% by volume, and the suspension was allowed to stand overnight. Precipitation was facilitated by adding sodium acetate to a concentration of 0.1 M. The precipitate was collected by centrifugation, dissolved in water, dialyzed, and lyophilized. For Experiment II, dried cells from Gladstone medium were extracted with TCA and refined according to the method used by Noll and Braude(1), except that

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