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Reactions Between Human Serum Albumin and Several Hemoglobins.* (30355)

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Components of human serum form both soluble and insoluble complexes with hemoglobin. Soluble complex formation has been the most studied, and has been demonstrated between hemoglobin and the haptoglobins (1), hemophylline(2), and between the heme group of hemoglobin and albumin(3).

Recently, the ability of human serum to form insoluble complexes with hemoglobin in agar gels has been described(4-6). It has been suggested that the serum participant in this reaction is albumin(4,6) or an α_2 globulin(5). This report will present a technique for quantification of this precipitin reaction, and will offer evidence that albumin is the serum reactant. Reactions involving several different natural hemoglobins as well as several chemically altered hemoglobins will be described.

Materials and methods. Fresh hemolysates (7) as well as 2× crystallized hemoglobins (Pentex, Inc., Kankakee, Ill.), and human albumin prepared by ethanol fractionation (E. R. Squibb & Sons, New York) or by starch block electrophoresis of fresh human serum(8) were used as reagents. Fresh human serum, and lyophilized serum (Pentex, Inc., Kankakee, Ill.) were also used. "Buffered-Agar" consisted of 0.7% Agar-Noble (Difco Lab., Detroit, Mich.) in glycine (25 g/l), sodium barbital (8.25 g/l) buffer at pH 7.7. Sodium chloride (8.5 g/l) and merthiolate (1:10,000 parts by weight) were added. For study of conditions of precipitation, the pH and salt concentration of the agar were varied, and sodium barbital and glycine were omitted.

Diffusion ring test was performed in Petri dishes filled with agar containing varied concentrations of hemoglobin. The agar medium was first melted, then cooled to 50°C; hemoglobin was then added, the medium mixed, and the plate immediately poured and cooled. Wells 0.6 cm in diameter were then cut out of the gel, and serum or albumin samples (0.15 ml) were pipetted in. After 16 to 20 hours at room temperature, the outer diameters of the resulting rings of precipitation which formed around the wells were measured with calipers from their images pro-

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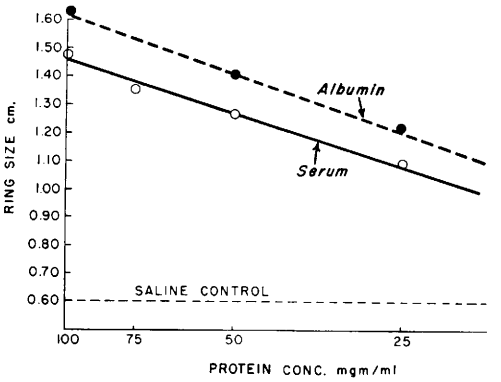

FIG. 1. Diffusion ring test. Relation between ring diameter and protein concentration. Medium: "Buffered-agar" containing 0.5 mg/ml human hemoglobin. Incubated 18 hr at 24°C. Commercial, lyophilized human serum and albumin were used.

jected by a photo-enlarger—together with that of a semitransparent metric scale. The average of 2 perpendicular diameters was recorded for each precipitin ring.

Immunoelectrophoresis(9) was carried out in 1% agar in veronal buffer (pH 8.6, μ 0.075). Protein concentration was determined by the modified Folin-Ciocalteau method(10). Hemoglobin concentration was determined in this manner as well as by measurement of the optical density of solutions at 540 mμ, or by paper electrophoresis (pH 8.6, μ 0.075) in a Durrum type cell (Spinco Div., Beckman Inst., Inc., Palo Alto, Calif.) with densitometric analysis of bromphenol blue stained paper strips.

Results. I. *Precipitin reactions and diffusion ring test.* All normal human sera tested formed precipitin bands in agar gels when diffused against human hemoglobin. Reactions occurred in this manner between serum and hemoglobin derived from the same individual. With the use of the diffusion ring test, these precipitin reactions were quantified. When the agar contained a small concentration of hemoglobin (0.5 mg/ml), and human serum (0.15 ml) was placed into wells cut into this medium, precipitation occurred in a ring around the well. The precipitin ring diameter was proportional to the logarithm of the serum protein concentration (Fig. 1). Maximal precipitation occurred between pH 6 and 8. There was marked inhibition of precipitation at pH values below 4 and above 9.5. Precipitation was maximal when the agar contained 0.01 to 0.10 M NaCl. Above this salt concentration, precipitation was inhibited, with 50% inhibition occurring at approximately 2.2 M NaCl. The reaction was abolished at 5 M NaCl.

II. *Evidence that albumin is the serum reactant.* Fig. 2 shows the results of a modified immunoelectrophoresis of normal human serum. The trough to the left contained a rabbit antiserum to whole human serum. Diffusion and immune precipitation from this trough have outlined the albumin, and globulin regions of the human serum, which had previously undergone electrophoresis from the center well. A solution of human hemoglobin (10 mg/ml) was placed in the trough on the right. A precipitin arc is seen to have formed between the hemoglobin and that portion of serum with the electrophoretic mobility of albumin. The potency of all sera tested in the diffusion ring test was directly related to their albumin content, and serum albumin had a higher specific activity (120-170%) than whole serum (compare Fig. 1). Further, no other fractions of serum, derived by preparative starch block electrophoresis, had the property of precipitating hemoglobin in agar gels.

III. *Reactions with several natural hemoglobins.* Diffusion ring tests, with varied concentrations of a standard human serum, were made in agar gels containing several human and animal hemoglobins. Ratios of the resulting precipitin ring sizes to those formed with adult human hemoglobin are shown in Table I. It can be seen that the best reactions were obtained between human serum and the human hemoglobins. Rabbit, dog, and horse hemoglobins were the most reactive hemoglobins of non-human origin, while pork, guinea pig and chicken hemoglobin had lesser reactivity.

IV. *Reactions with chemically altered hemoglobin.* Diffusion ring tests with varied concentrations of a standard human serum, were made in agar gels containing samples of human hemoglobin which had been treated with several chemical agents. Ratios of these resulting precipitin ring sizes to those formed with the control hemoglobin sample are shown

TABLE I. Reactivity of Several Natural Hemoglobins with Human Serum.

Type	Ratio of reactivity* (%)	S.D.
Fetal-human[a]	121	± 11.6
Adult-human[b]	100	
C-human[c]	92.5	± 10.9
Sickle-human[d]	88.6	± 9.2
Rabbit	73.1	± 2.1
Dog	68.1	± 6.5
Horse	64.1	± 1.0
Pork	35.3	± 4.4
Guinea pig	20.6	± 5.2
Chicken	15.7	± 8.1

Tests were made in "buffered-agar" containing 0.5 mg/ml hemoglobin incubated 20 hr, 24°C.

* Ratio, in %, of precipitate ring sizes formed by several concentrations of a human serum in agar containing each hemoglobin type relative to ring sizes formed by the same concentrations of this serum with agar containing adult human hemoglobin.

[a] 84% Hgb F (cord blood). [b] 95% Hgb A. [c] 96% Hgb C (homozygous pt.). [d] 93% Hgb S (homozygous pt.).

in Table II. Conversion of hemoglobin to cyanmethemoglobin by treatment with ferricyanide plus cyanide abolished reactivity, whereas treatment with ferricyanide or cyanide alone had little effect. Similarly, treatment of hemoglobin with carbon monoxide, azide, or β-mercaptoethanol had no detectable effect. After treatment, all hemoglobin samples (except those treated with carbon monoxide) were dialyzed overnight against 3 changes of 0.15 M NaCl before testing.

Discussion. The specificity of the reaction between human serum albumin and hemoglobin in agar gels is determined, in part, by the globin portion of the hemoglobin molecule. This is inferred from the fact that the several animal hemoglobins differ from each other by the globin part of their molecules, and have differing reactivities. Hemoglobins of human origin are most reactive with human serum. The heme grouping probably also plays a role, however, since conversion to the cyanmet derivative abolishes reactivity of hemoglobin. Also heme, in other circumstances, has been shown to bind albumin(3). Precipitate formation is optimal near neutral pH, and is inhibited by high salt concentration. This agrees with the findings of Metzgar and Grace(6), and with data of Tomasi(11) who demonstrated a reaction in agar between human albumin and a soluble tissue component.

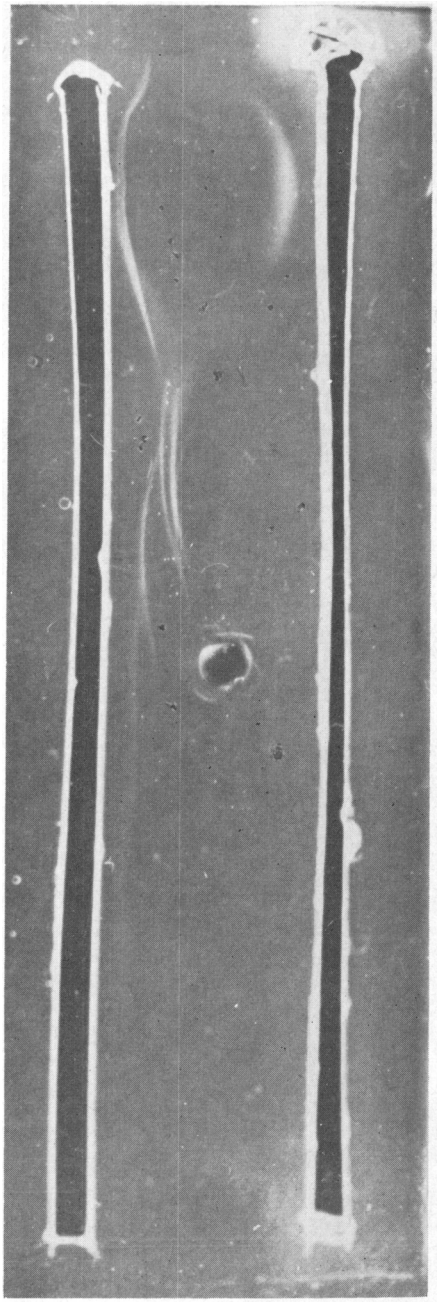

FIG. 2. Modified immunoelectrophoresis of normal human serum. Medium: 1% agar, veronal buffer (pH 8.6, μ 0.075), current 9 m.a., 2 hr. Center well: normal human serum. Trough on left: rabbit antiserum to whole human serum. Trough on right: human hemoglobin 10 mg/ml. Anode: above.

TABLE II. Reactivity of Human Hemoglobin After Chemical Treatment.

Treatment	Ratio of reactivity with human serum* (%)
Hemoglobin + .02 M NaCN + .2 M potassium ferricyanide	0
" + .5 M potassium ferricyanide	77.5
" + .5 M NaCN	100
" + .5 M sodium azide	100
" + .1 M β-mercaptoethanol	100
" (2.0 cc) + carbon monoxide (500 cc, research grade, room temp & pressure) †	100
" + .15 M NaCl (control)	100

Adult hemoglobin, 25 mg/ml, (95% Hgb A) was used. Samples were reacted at room temperature for one hour, then dialyzed against 3 changes of 0.15 M NaCl, 18 hr at 4°C before testing. Tests were made in "buffered agar" containing 0.5 mg/ml of the treated hemoglobin, incubated 20 hr, 23-24°C.

* Ratio, in %, of precipitate ring sizes formed by several concentrations of human serum in agar containing each hemoglobin sample relative to ring sizes formed by the same concentrations of this serum with agar containing the control hemoglobin.

† This sample was not dialyzed before testing.

Thus far the precipitate reaction between albumin and hemoglobin has been noted only in agar medium, and several attempts to demonstrate it in liquid medium, or in gelatin gels have failed(6,12).

Summary. Human serum albumin precipitates in agar gels with hemoglobin. This reaction can be quantified by means of a diffusion ring test, described, and is maximal between pH 6 and 8 when the medium contains 0.01 to 0.1 M NaCl. Several human and animal hemoglobins display varying reactivities. Conversion of hemoglobin to cyanmethemoglobin abolishes reactivity. It is suggested that the specificity of the reaction depends on both the globin and heme moieties of hemoglobin.

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Release of Permeability Factors from the Blood Platelet.* (30356)

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Increased vascular permeability is a characteristic of intravascular immune reactions in which there are platelet and leukocyte aggregates in the lumen(1). Polymorphonuclear

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