THE AFFINITY OF THE RETICULO-ENDOTHELIAL SYSTEM FOR VARIOUS MODIFIED SERUM PROTEINS

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THE reticulo-endothelial (RE) cells of the liver and spleen phagocytose heat denatured proteins (Benacerraf, Biozzi, Halpern and Stiffel, 1956) or heavily iodinated proteins (Biozzi, Benacerraf, Stiffel, Halpern and Mouton, 1957) with great avidity. This phenomenon can be demonstrated by following the fate of ^{131}I labelled proteins after intravenous injection or, indirectly, by measuring the interference which these modified proteins exert on the rate of clearance of a standard dose of carbon particles from the blood by the RE system.

In the present study these observations are extended to the behaviour upon injection of other modified protein preparations derived from bovine serum albumin (BSA) in order to investigate the effect of physico-chemical changes and chemical modifications on the uptake of these proteins by the RE system.

Since considerable information is already available from the studies of Maurer, Sri Ram and Ehrenpreis (1957), Maurer and Sri Ram (1958) and Maurer (1959) on the correlation between antigenicity of BSA and modification of this protein by various chemical and physical means, these products have been used in these experiments.

MATERIALS AND METHODS

Animals.—The experiments were performed on male mice, weighing 25–35 g. All injections were given intravenously.

Protein preparations.—The chemically modified preparations (deaminated, acetylated, guanidinated, esterified) were prepared from Armour's crystalline BSA according to the previously published procedures (Maurer *et al.*, 1957 ; Sri Ram and Maurer, 1958 and 1959). The preparations modified by physicochemical treatments (including urea and ultraviolet (U.V.) treated) were prepared as outlined by Maurer (1959).

Sedimentation constants.—The S_{20w} of 1 per cent protein solutions in pH 7·4 phosphate buffer, $\mu = 0{\cdot}1$, were determined in a Spinco Model E ultracentrifuge run at a speed of 56,100 r.p.m.

Viscosity measurements.—Viscosity measurements were carried out at $25 \pm 0{\cdot}01°$ in Ostwald-Fenske viscometers as described by Ehrenpreis, Maurer and Sri Ram (1957).

Electrophoretic mobilities.—The mobilities were determined by paper electrophoresis in a veronal buffer of pH 8·6, $\mu = 0{\cdot}05$ as described by Ehrenpreis *et al.* (1957). Mobilities are reported relative to the value obtained for BSA.

Interference with phagocytosis of carbon particles.—Carbon preparation C11–1431a (manufactured by Gunther Wagner, Hanover) was used in these experiments. It was prepared as described by Biozzi, Benacerraf and Halpern (1953) and injected into mice in a dose of 16 mg./100 g. The carbon clearances were determined, according to the methods described, by drawing at regular time intervals 0·025 ml. blood samples from the retroorbital venous plexus with a calibrated glass pipette. previously rinsed with heparin. The blood samples were lysed in 2 ml. of 0·1 per cent sodium carbonate and the carbon concentration measured

spectrophotometrically at 675 mμ. The rate of clearance of carbon particles follows an exponential function of the time according to the equation $\frac{\log C_2 - \log C_1}{T_1 - T_2} = K$ in which C_1 represents the concentration of carbon in the blood at time, T_1, C_2 the concentration at time T_2. The rate constant K, called the phagocytic index, varies inversely with the injected dose of carbon in the dose range where the experiments were performed, illustrating the saturating effect of phagocytosed particles on the RE system. If, during the clearance of carbon particles, another colloidal suspension such as denatured protein, which is phagocytosed by the RE system with comparable avidity, is injected, a competitive effect on the clearance of carbon results and the rate of carbon clearance (K_1) is modified (K_2). The ratio of K_1 to K_2 measures the competitive effect on the carbon clearance and indicates the relative avidity with which various substrates are phagocytosed by the RE system as compared with carbon. After a sufficient number of blood samples had been obtained to measure the clearance rate K_1, the substance to be investigated was injected in the dose of 2·5 or 12·5 mg. per 100 g. body weight, and several more blood samples were withdrawn to allow measurement of a change in the clearance rate of carbon (K_2). Each protein preparation was investigated in 4 mice and mean values for K_1/K_2 are recorded in the Table.

Labelling of protein preparations with ^{131}I.—To 10 mg. of each protein preparation while kept at 0–5°, 0·15 ml. of a carbonate buffer (pH 10) and 0·28 ml. of a KI_3 solution containing 0·5 mc. of ^{131}I were added dropwise while shaking. The KI_3 solution contained 123 mg I_2 and 157 mg KI per 100 ml. distilled water. After an hour, the solutions were dialyzed against 0·85 per cent NaCl for 48 hr. The amount of iodine bound to protein was found to vary with the different preparations from 0·09 mg. to 0·41 mg. per 100 mg. of protein. The preparations which had been denatured by heat treatment seemed to bind less iodine (around 0·1 per cent) than the other preparations (around 0·3 per cent).

Clearance of ^{131}I *labelled proteins.*—Mice were injected i.v. with a standard dose of 5 mg. per 100 g. body weight of each ^{131}I labelled protein preparation. Samples of 0·05 ml. blood were drawn from the retro-orbital venous plexus at regular time intervals for a period of 30–90 min. after the injection, depending on the rate at which the protein disappeared from the circulation. The samples were placed on filter paper and mounted on glass slides. The radioactivity was measured with a Geiger-Müller counter and the results plotted as a percentage of radioactivity present immediately after the injection, against time. In order to study the fate of these modified proteins in the body, mice were injected i.v. with a standard dose of 1 mg. per 100 g. body weight of each labelled protein preparation and sacrificed 10 min. later. This short time interval was chosen to avoid metabolism of the phagocytosed protein and release of the label from the cells (Benacerraf, Halpern, Biozzi, Stiffel and Mouton, 1957 ; Biozzi, Halpern, Stiffel and Mouton, 1958). The γ-radioactivity of the spleen, liver, lung and kidneys was measured with a scintillation counter and expressed as a percentage of the injected radioactivity.

RESULTS

The fate of the various modified BSA preparations, injected intravenously, was determined by a study of the comparative speed of elimination from the blood, and of the distribution in various organs. The degree to which these preparations were able to interfere, by competition, with the phagocytosis of carbon particles by the RE system was studied and correlated with the extent to which they were removed by the liver.

These findings were further correlated with various physical constants, such as sedimentation constants, relative viscosity and electrophoretic mobility (Table I, Fig. 1 and 2).

Substances, which are phagocytosed with great avidity by the RE system, are characterized by : a relatively fast disappearance rate from the blood, a high degree of liver uptake and a marked competitive effect on phagocytosis of carbon particles *in vivo*. Among the derivatives of BSA showing this behaviour we can list the following : U.V. denatured, deaminated-P, N-acetyl BSA, urea treated

TABLE

Protein prep. of BSA	K_1/K_2*	Organ distribution† Liver	Spleen	Kidneys	Lung	Electroph. mobility	Relative viscosity 1 per cent pH 7·5	Sedim. constants S_{-0} w
Guanidinated (1)	1·4	19	0	6·5	5	1·05	1·051	92 per cent : 4·1 8 per cent : 6·3
Deam. SIII (2)	1·3	10·5	0	9·5	2	—	1·041	100 per cent : 3·3
Deam. P (3)	2·6	76	4	17	0	80 per cent : 1·34	1·100	65 per cent : 3·8 35 per cent : 5·3
n-acetylated (4)	5·0	52	1·9	6·9	2·8	75 per cent : 1·71	1·081	85 per cent : 3·1 15 per cent : 2·1
Heat denatured guanidinated (5)	5·0	76	4	17	0	70 per cent : 1·04	—	70 per cent : 18·8 25 per cent : 4·3 5 per cent : 9·0
Irreversibly urea denatured (6)	1·6	29	2	28	0	—	1·065	90 per cent : 2·0 10 per cent : 8·4
Urea denatured heat denatured (7)	2·7	67	2	13	0	—	1·182	37 per cent : 3·4 34 per cent : 1·4 29 per cent : 9·3
Ultraviolet denatured (8)	2·8	74	0	6	3	—	1·025	83 per cent : 5·9 10 per cent : 2·8
Alkali denatured (9)	1·9	29	2	40	0	—	1·054	90 per cent : 2·0 10 per cent : 10·0
Acid denatured (10)	1·2	10	2	45	2	—	1·035	100 per cent : 1·5
Methylated‡ (11)	—	74	2·4	2	2·7	—	1·35	77 per cent : 3·6 23 per cent : 9·6
Normal (12)	1	—	—	—	—	1·00	1·040	92 per cent : 4·0 8 per cent : 5·8
Normal‡ (13)	—	—	—	—	—	—	1·29	85 per cent : 3·7 7·5 per cent : 5·4 7·5 per cent : 2·4

Competition with carbon clearance (K_1/K_2) and percentage uptake by the liver as correlated with physicochemical properties of various modified BSA preparations.

* Dose of carbon for clearance : 16 mg./100 g. ; dose of protein 1–5 : 12·5 mg./100 g. ; 6, 7, 9, 10 : 10 mg./100 g. 8 : 6 mg./100 g.

† Determined at 10 min. after i.v. injection of 1 mg./100 g.

‡ Physicochemical data obtained in 0·02 N HCl as solvent.

heat denatured, and heat denatured guanidinated. All these preparations show about 70 per cent of liver clearance, with a great degree of efficiency, and considerable interference with the phagocytosis of carbon : $K_1/K_2 > 2·5$ in all cases. These preparations, however, are not homogeneous, since the rates of clearance from the blood do not follow an exponential function of time as they would with substances which are homogeneously phagocytosed by the RE system. This inhomogeneity is also apparent from the data on sedimentation constants.

With the majority of these substances there is a correlation between the degree of aggregation and the rate at which they are removed by the RE system. Among these, heat denatured guanidinated BSA has a $S_{20} = 18·8$ for 70 per cent of the material, urea denatured heat denatured a $S_{20} = 9·3$ for 29 per cent, and U.V. denatured a $S_{20} = 5·9$ for 83 per cent. Other preparations such as deaminated-P preparation which has a $S_{20} = 3·8$ for 65 per cent and N-acetylated BSA which has a S_{20} of 3·1 for 85 per cent and of 2·1 for 15 per cent, are also phagocytosed by the Kupffer cells of the liver without evidence of considerable aggregation accompanying the denaturation of these products. The extent of aggregation and/or denaturation is also indicated by changes in relative viscosity and may account for the large competitive effect on carbon clearance of heat denatured

guanidinated and acetylated BSA (both $K_1/K_2 = 5$). The former product shows considerable aggregation, while the latter shows a marked increase in negative net charge.

Other BSA derivatives show a rapid clearance from the blood, and yet, small or negligible interference with phagocytosis of carbon and uptake by the liver.

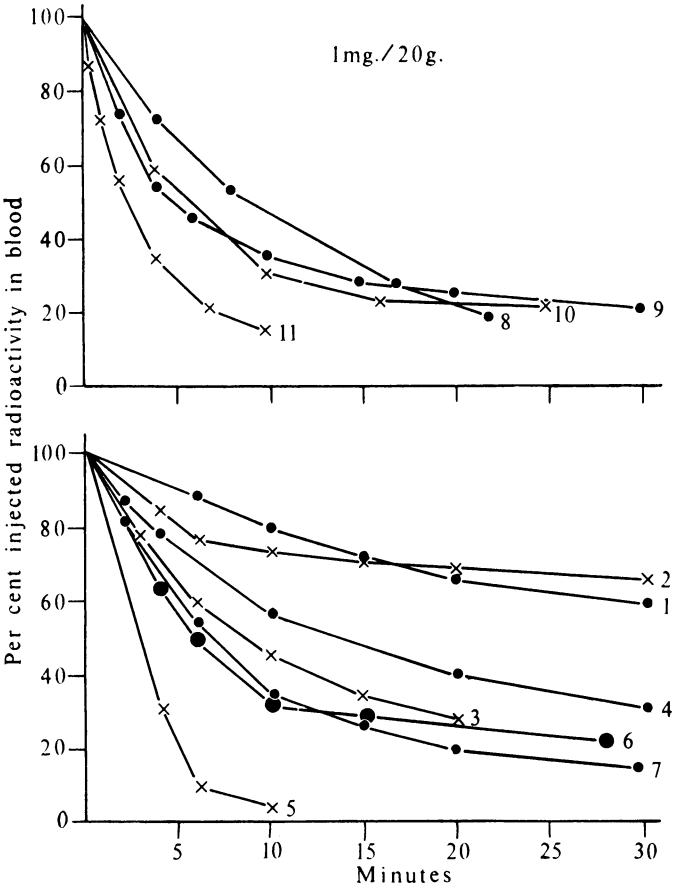


FIG. 1.—Rate of disappearance of intravenously injected ^{131}I labelled modified BSA preparations (5 mg./100 g.) from the blood of mice. The numbers refer each to a different preparation: (1) guanidinated, (2) deaminated SIII, (3) deaminated P, (4) *n*-acetylated, (5) heat denatured guanidinated, (6) irreversibly urea denatured, (7) urea denatured heat denatured, (8) ultraviolet denatured, (9) alkali denatured, (10) acid denatured, (11) methylated.

Among these, we find acid denatured, alkali denatured and urea denatured BSA. Alkali denatured and urea denatured BSA, which both have 10 per cent of an aggregated component, show K_1/K_2 ratios of 1·0 and 1·6 respectively, and 29 per cent clearance by the liver only. However, it is clear from the analysis of the organs, that the kidneys are largely responsible for the rapid clearance of these three products which have been hydrolyzed during the preparation, as evidenced by sedimentation constants considerably smaller than the ones for normal BSA

(acid denatured 100 per cent: 1·5; alkali denatured 90 per cent: 2·0; urea denatured 90 per cent: 2·0).

Guanidinated BSA and the SIII fraction of deaminated BSA which have been changed very little by chemical treatment are not eliminated very rapidly from

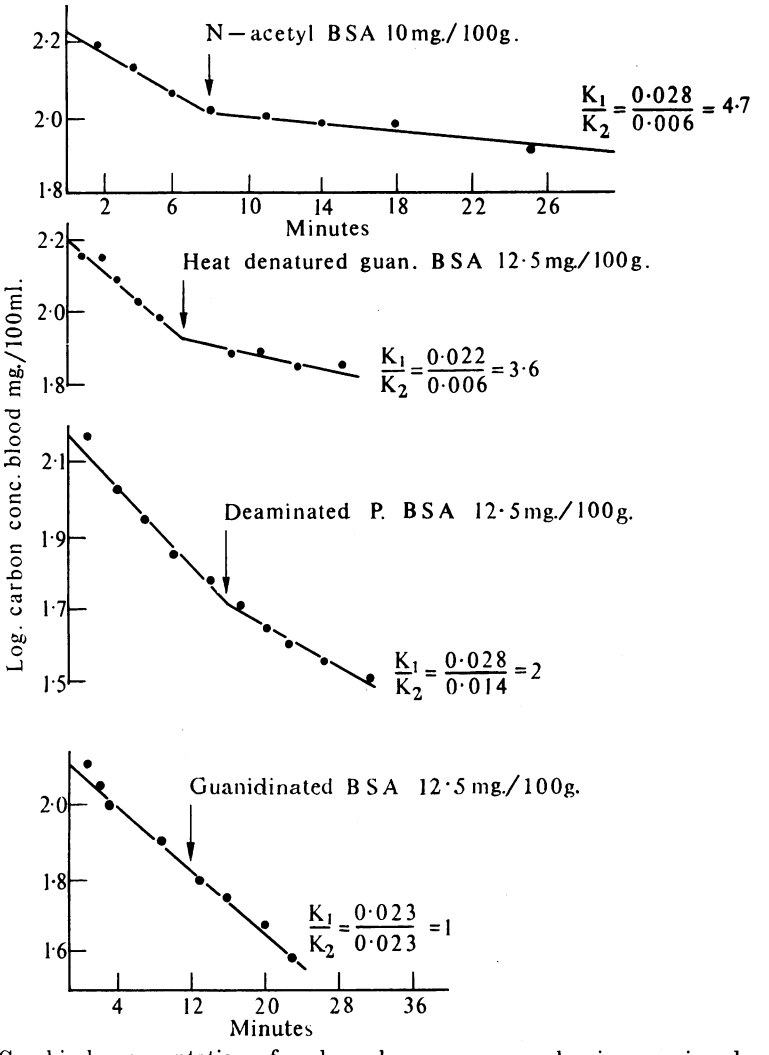
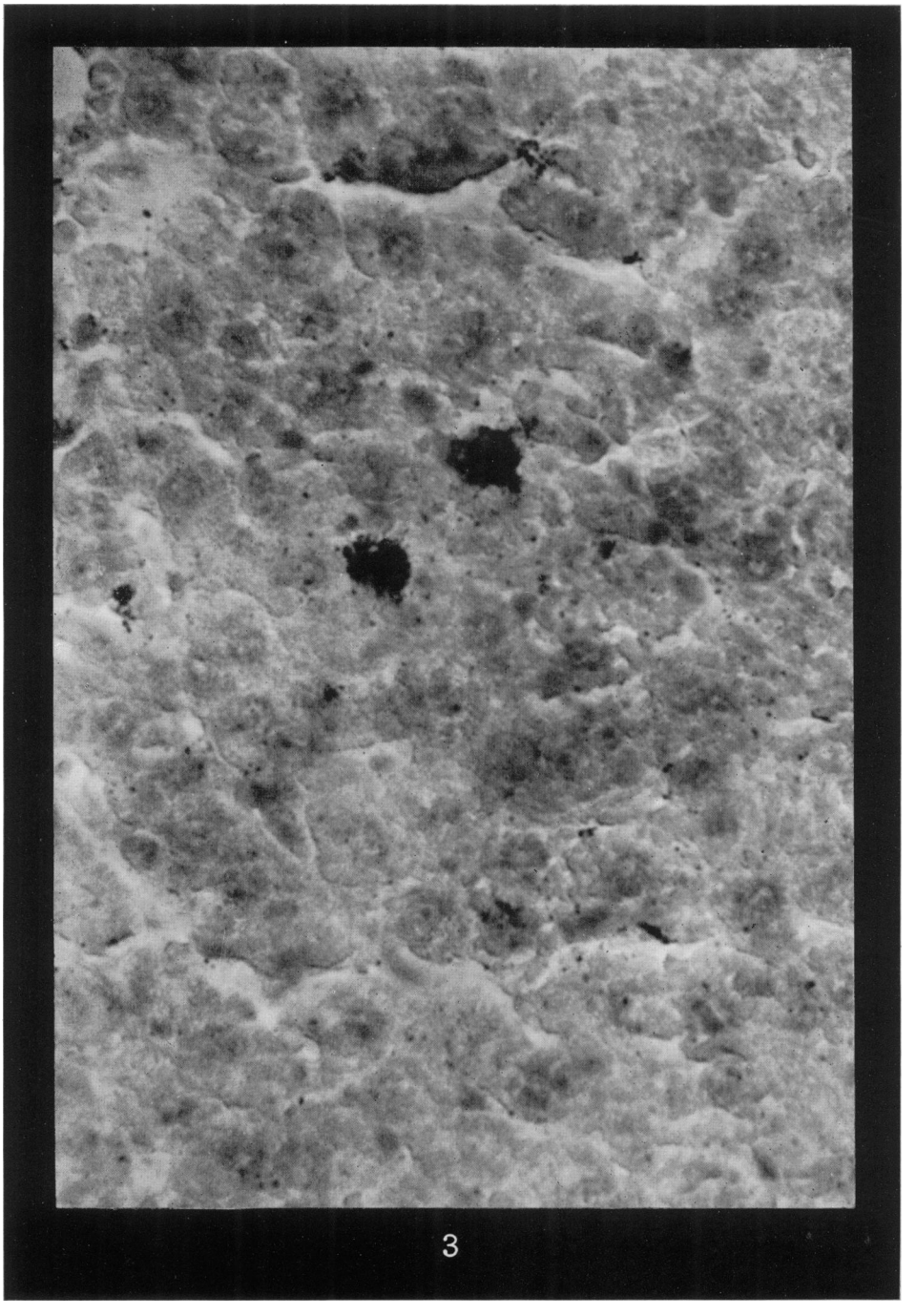


FIG. 2.—Graphical representation of carbon clearance curves showing varying degrees of interference by the injection of modified BSA preparations. Arrows indicate time of protein injections.

EXPLANATION OF PLATE.

FIG. 3.—Autoradiography of liver section of a mouse injected with ^{131}I labelled *n*-acetylated BSA, showing localization of radioactivity in Kupffer cells. Autoradiograph and photograph were prepared by Dr. R. T. McCluskey, Department of Pathology, New York University College of Medicine.



Thorbecke, Maurer and Benacerraf.

the circulation and, as expected, are not recovered, in any significant amount, in the organs when the animals are sacrificed 10 min. after the injection. They also do not show any appreciable effect on the rate of carbon clearance since any ratio $K_1/K_2 < 1{\cdot}5$ must be considered to be of questionable significance and within the limits of error of this technique.

All the modified proteins studied heretofore are negatively charged at the pH of blood. When a positively charged derivative of BSA is injected, such as esterified (methylated) BSA, the preparation is cleared rapidly from the blood and 74 per cent recovered in the liver after 10 min. If methylated BSA is injected in the course of clearance of carbon from the blood, a sharp decrease in carbon concentration is observed. The behaviour of this preparation *in vivo* can be explained by the reaction which it shows with serum *in vitro*. This positively charged colloid precipitates rapidly with the negatively charged serum proteins (Sri Ram and Maurer, 1959). It reacts similarly with negatively charged carbon particles. Because of this, positively charged soluble proteins are precipitated in the blood. It is impossible, therefore, to investigate by our techniques whether such proteins can be phagocytosed by the RE system from a colloidal solution.

All modified BSA preparations were less antigenic than native BSA (Maurer *et al.*, 1957 ; Sri Ram and Maurer, 1957 and 1958 ; Maurer and Sri Ram, 1958). Most comparable to native BSA, in this respect as well, were guanidinated BSA and the SIII fraction of deaminated BSA. A correlation between the tendency of these substances to localize in the RE system of the liver and their degree of antigenicity was not found. The only preparation which was not antigenic in the rabbit when injected as an alum precipitate was acid denatured BSA.

DISCUSSION

The term denaturation refers to several physicochemical changes which can occur during modification of a native protein (Putnam, 1953). Upon " denaturation " of a protein various masked polar groups, such as —SH, tyrosyl and amino groups may become exposed because of " unfolding " and/or expansion of the molecules. Aggregation as a result of reassociation of exposed groups is a secondary phenomenon dependent on the electrokinetic potential of the denatured protein particles. The solubility of denatured proteins is always less than that of normal globular proteins, particularly at the isoelectric point.

Among the several changes that various treatments induce in normal BSA, and that modify the fate of these products upon intravenous injection, a change of molecular size seems to be of major importance. The proteins that have been subjected to partial hydrolysis, such as acid, alkali or urea treated BSA showed a rapid clearance by the kidney, while the ones that were aggregated were phagocytosed by the liver with great avidity. However, in addition to aggregation of the molecules a change in the negative surface charge of the molecules enhanced the affinity of these substances for the RE system as well (deaminated-P fraction, N-acetylated BSA).

What information may one derive from these studies about the factors that are responsible for the greater affinity of some of these products for the RE system? Substances to be phagocytosed have to be adsorbed to the RE cell membranes for a sufficient length of time to initiate the process which transports them inside the cell. The criteria for specific adsorption onto the cell surface

may thus be of major importance in determining the degree of phagocytosis. It seems that the large molecular size of the denatured proteins, irrespective of the method of denaturation, greatly enhances their affinity for the RE cells. On the other hand, such a large molecular size is not the only property which renders proteins capable of being phagocytosed, as some preparations which were not aggregated were avidly phagocytosed. Other factors, such as surface charge and diminished solubility at the pH of blood plasma, may therefore also be of great importance in determining adsorption of these products onto phagocytic cells.

SUMMARY

The fate of modified BSA preparations was studied in mice after intravenous injection. The rate of disappearance from the blood of lightly ^{131}I labelled protein preparations and their distribution in various organs (liver, spleen, kidneys, lung) at 10 min. after an intravenous injection were studied. Further information about the affinity of these substances for the RE system was obtained by studying the interference exerted by the modified proteins on the clearance of a standard dose of carbon particles.

An attempt was made to correlate these results with the physicochemical properties of the preparations, such as relative viscosity, electrophoretic mobility and sedimentation constants.

It was found that partially hydrolyzed protein preparations like acid denatured, alkali denatured and urea denatured BSA, were rapidly cleared from the blood by the kidney, while substances which had not been changed appreciably physicochemically, such as guanidinated and lightly deaminated BSA behaved similarly to normal BSA.

The RE system was shown to remove selectively highly aggregated protein preparations such as heat denatured guanidinated and urea denatured heat denatured BSA, and also proteins which were not aggregated much upon physicochemical treatment, but which had significantly changed negative surface charge, like strongly deaminated and *n*-acetylated BSA.

No correlation was found between uptake by the RE system of the liver and the degree of antigenicity of the protein preparations.

The factors influencing the affinity of modified proteins for the RE system are discussed.

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