THE DYNAMICS OF RETICULOENDOTHELIAL BLOCKADE*

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The phagocytic cells of the reticuloendothelial system play a major role in trapping and destroying bacteria which enter the blood stream (1). The importance of this system of fixed phagocytic cells in the control of bacteremic states has led to numerous studies of factors which modify its behavior. During the past 30 years, considerable attention has been directed toward the experimental phenomenon of "reticuloendothelial blockade," or the impaired blood stream clearance which follows large injections of colloidal or particulate substances. Such studies have been of biologic interest because of the possible relationship between reticuloendothelial system malfunction and the increased susceptibility to infection noted in individuals with diseases involving the liver and spleen. The majority of such investigations have concluded that reticuloendothelial system (RES) blockade is the result of saturation of the phagocytic capacity of the system (2–4), or saturation of clones of phagocytic cells which remove the specific particles under study (5). However, recent observations have suggested that RES blockade may be caused by depletion of specific serum opsonins by the preliminary blockading injection (6–8). The current experiments were undertaken to resolve these conflicting views.

The present studies focus on the mechanism of RES blockade. These experiments confirm the specificity of blockade for the particle under study. In the system employed, they demonstrate that blockade is not a result of impaired phagocytic function of specific clones of phagocytic cells or the RES in general. Finally, they suggest that the phenomenon of RES blockade in this experimental model relates in some manner to the persistence of the blockading substance in the circulation rather than depletion of serum opsonins required for RES phagocytosis.

Materials and Methods

Experimental Animals.—White New Zealand male rabbits weighing approximately 3 kg were used for all experiments.

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Particulate Materials.—Radioactive colloidal gold (aurcoloid) with a specific activity of 0.025 mg/mc and stabilized with 0.3 per cent salt-free gelatin was obtained from Abbott Laboratories, Oak Ridge, Tennessee. Non-radioactive colloidal gold containing 2 mg of gold/ml similarly prepared was obtained from the same source.

I^{131}-labeled aggregated human albumin (1 mg/ml) and inactive colloidal human albumin (14.2 mg/ml) was obtained from E. R. Squibb and Sons, New Brunswick, New Jersey.

Thorotrast (colloidal thorium dioxide in aqueous dextrin) was obtained from Testagar Company, Inc., Detroit, Michigan.

Latex particles in the form of Bacto-Latex (0.81 μ), were obtained from Difco Laboratories Inc., Detroit.

Carbon ink, No. C11-1431a was obtained from Günther Wagner, Hanover, Germany. This ink contained approximately 100 mg carbon/ml suspended in a solution of partially hydrolyzed gelatin (fish glue) and phenol. The carbon was prepared as previously described by others (2), and contained approximately 90 mg carbon/ml. This suspension was diluted appropriately to give doses of 32 mg carbon/100 gm of rabbit body weight in volumes of not more than 20 ml. Three different blockading mixtures were prepared: (*a*) carbon diluted in saline, (*b*) carbon to which sufficient 6 per cent gelatin was added to provide a final gelatin dose of 5 mg/kg of rabbit body weight, and (*c*) carbon made up in 6 per cent gelatin to provide a final dose of gelatin and carbon equal to that employed by Biozzi *et al.* (2). This latter preparation provided a final gelatin dose in excess of 200 mg/kg of rabbit body weight.

Gelatin.—The gelatin used in all of the present studies was specially prepared, salt-free, 6 per cent gelatin (No. P-20) from Knox Gelatin Co., Inc., Camden, New Jersey. When this supply of special gelatin was exhausted, attempts were made to use Knox gelatin which contained 0.9 per cent NaCl. It was found impossible to produce blockade in the usual doses with this salt-containing gelatin, presumably due to an alteration in molecular size or configuration. Consequently, all studies reported employed P-20 salt-free gelatin.

Gelatin Assays.—Concentrations of gelatin in serum were measured by differential precipitation of plasma proteins, hydrolysis of the gelatin in the supernate, and assay for 1-hydroxyproline (9). Three ml of 5 per cent trichloracetic acid were added to a 2 ml aliquot of the serum sample to be analyzed. The gelatin containing supernate was removed and evaporated to one-half volume, 2 ml of concentrated hydrochloric acid were added, and the mixture sealed in an acid-washed 5 ml ampule. Ampules were incubated 18 hours in an oven at 100°C. After hydrolysis the contents of the ampule were removed, placed in a beaker in a vacuum desiccator, and allowed to stand until evaporated to dryness. Two ml of water were then added, mixed well, and 1 ml of each sample was assayed for 1-hydroxyproline. The assay was performed by the colorimetric method described by Greenstein and Winitz (10). Gelatin was considered to contain 14 per cent 1-hydroxyproline by weight (11). In all *in vivo* experiments in which gelatin concentrations of serum were measured, suitable *in vitro* standards were analyzed simultaneously. When gelatin in known concentrations was added to serum, recoveries ranged from 95 to 105 per cent.

Clearance Studies in Living Rabbits.—Animals were anesthetized with intravenous pentabarbital sodium and the right jugular vein was cannulated with a PE-160/S12 polyethylene catheter as previously described (12). In experiments concerned with the effects of RES blockade the various blockading agents were administered intravenously through a left marginal ear vein 60 minutes before the tracer dose of radioactive material.

The standard tracer dose of radioactive gold employed contained approximately 170 microcuries of Au^{198}, and gelatin was added to bring the gelatin concentration to the desired amount. Tracer doses of aggregated albumin contained 1 mg of aggregated albumin per kg of rabbit body weight.

In clearance studies, blood samples were removed at appropriate intervals following ad-

ministration of the radioactive tracer dose, placed in heparinized tubes, and the radioactivity in 1 ml blood samples was measured in a well-type scintillation counter. The I^{131}-tagged aggregated albumin was counted after passage through an amberlite IRA-400 anion exchange resin to remove free iodide (13).

Clearance curves were constructed to show the log per cent radioactivity remaining in the blood stream at each sampling period, with the 1 minute samples used as the 100 per cent value. Half-times were calculated from the initial rapid exponential portion of these curves though it was apparent that they represented the sum of at least two exponentials.

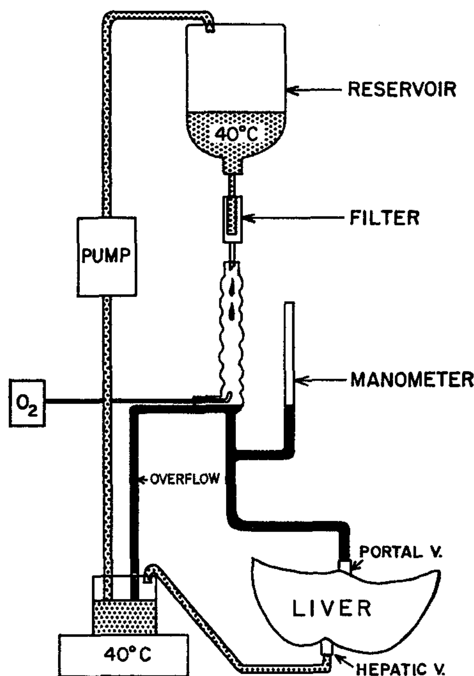

FIG. 1. Liver perfusion apparatus.

Hepatic Catheterization.—A radiopaque catheter was inserted into the right jugular vein and passed into the right or left lobe of the liver by a technique previously described (12). A second catheter placed in the left jugular vein was used to obtain simultaneous blood samples for calculations of splanchnic trapping.

Isolated Liver Perfusion.—Under light pentabarbital anesthesia supplemented with local 0.5 per cent procaine, a long mid-line abdominal incision was made. The common bile duct was isolated and cannulated with a PE-160/S12 polyethylene catheter, allowing bile production to serve as an indicator of liver function throughout the experiment.

The portal vein was then cannulated with a 4 mm diameter polyethylene catheter. Ten ml of 1:1000 heparin in saline was immediately injected into this catheter and slow perfusion of the liver was started. The chest cavity was opened promptly and an "outflow" catheter was tied in the superior vena cava at the level of the diaphragm above the entrance of the hepatic veins. Finally, the inferior vena cava was clamped just above the renal veins to complete the isolation of the liver circulation. The entire procedure required approximately 20 minutes, and

liver perfusion was maintained throughout surgery. In experiments employing a blockaded liver, 150 mg/kg of gelatin was injected intravenously 1 hour prior to surgery. Approximately 1 liter of fluid was used to perfuse each liver to remove residual blood prior to study.

The perfusion apparatus was assembled as shown in Fig. 1. A reservoir allowed fluid to run through a filter leading down a glass condenser in which the perfusate was oxygenated by a continuous flow of oxygen passed into the condenser. Perfusions were carried out at pressures of 12 cm of water, the normal portal pressure of the rabbit, and controlled by the level of the condenser outflow. A Brewer automatic pipetting device served to pump overflow fluid which had not entered the liver back to the reservoir. Rabbit body temperatures of approximately 40°C were maintained in the perfusate by placing the overflow flask in a water bath and by wrapping reservoir and condenser with an electric heating tape. With the exception of the initial experiments, in which the perfusate was continuously recirculated, the perfusate was passed once through the liver, and the liver outflow was collected separately. Overflow flasks were changed as each new perfusate was added to the reservoir.

In the majority of the experiments, Ringer's solution (Cutter Laboratories, Berkeley, California) was used as the perfusion fluid. One hundred mg of heparin and 1 gm of dextrose were added to each liter of solution. The final pH was approximately 6.0 before the addition of serum.

In certain experiments a Krebs-Henseleit buffer solution containing similar amounts of heparin and dextrose was used (14). A pH of approximately 7.4 was maintained by bubbling 5 per cent CO_2 and 95 per cent oxygen through the perfusing fluid throughout such experiments.

Preliminary studies showed that 5 per cent normal serum produced maximum liver uptake in the Ringer's system. In studies to be described, appropriate sera were added to 200 ml volumes of Ringer's or Krebs-Henseleit solutions to give a final concentration of 5 or 10 per cent, and the tracer dose of radioactive colloidal gold was added to each test perfusate. Each tracer dose contained approximately 30 microcuries of Au^{198} and enough added gelatin to provide 5 mg/kg for a standard rabbit weight of 3 kg.

After the initial liver washout with plain perfusion fluid, the reservoir was allowed to drain, a warmed test solution was added to the reservoir, and after 1 minute of passage to allow equilibration, simultaneous samples were drawn from the inflow and outflow catheters at 3 intervals of approximately 1 minute. When different test solutions were used sequentially, approximately 500 ml of plain perfusion fluid were used to wash the system between studies. The order of test perfusates was varied in different experiments. A complete series of studies on a given liver took less than 1 hour. Pressure, temperature, and rates of flow were ascertained at intervals to maintain constant conditions.

The radioactivity of inflow-outflow samples was determined as described, and the per cent removed was calculated by the formula: $\frac{(\text{"inflow" CPM})-(\text{"outflow" CPM})}{(\text{"inflow" CPM})} \times 100$. Because the data obtained from the 1 minute paired samples indicated that complete equilibration had not always occurred in this time period, the results were calculated from the averages of the second and third paired samples.

Serum Absorption Studies.—Attempts to absorb normal rabbit serum with gelatin employed two methods. (*a*) Bacto-latex (0.81 μ) was "coated" by two incubations in 1 per cent gelatin on a rotation drum at 37°C for 1 hour. The latex particles were then washed twice in saline, added to fresh rabbit serum, and allowed to stand at 4°C for 18 hours. The serum was separated from the latex particles by high speed centrifugation (10,000 RPM), and two additional absorptions were carried out by adding fresh "gelatin coated" latex particles followed by rotation at 37°C for 1 hour.

Normal serum absorbed with untreated latex particles in an identical manner was used as

a control. Gelatin assays on the two absorbed sera revealed only trace amounts of hydroxyproline, equivalent to that in normal unabsorbed serum.

(*b*) Rabbit red blood cells were treated with tannic acid as described by Quinn and Lowry (15), and exposed to 0.6 per cent gelatin for 30 minutes at room temperature. The sensitized red cells were washed in saline and added to fresh normal rabbit serum. Normal rabbit sera were absorbed three times with fresh sensitized red cells, once at 4°C for 18 hours and twice at 37°C for 1 hour as with treated latex particles and used as described above.

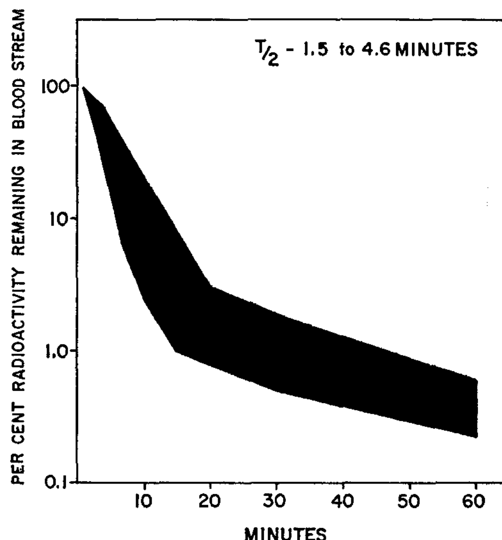

FIG. 2. Range of blood stream clearance curves obtained in ten normal rabbits given radiogold stabilized in 5 mg/kg of gelatin.

RESULTS

Clearance of the Standard Tracer Dose of Gelatin Stabilized Gold in Normal rabbits.—The standard tracer dose of radiogold stabilized in 5 mg of gelatin per kg of rabbit body weight administered to 10 normal rabbits resulted in the range of clearance curves shown in Fig. 2. Half-times ranged from 1.5 to 4.6 minutes, and clearance proceeded rapidly over the first 15 to 20 minutes followed by abrupt slowing. The curves resembled those obtained with bacteria in previous studies (12, 16).

As in studies with bacteria, it could be shown that the splanchnic tissues avidly removed the radiogold during the rapid phase of clearance. As shown in Fig. 3, simultaneous samples obtained from the hepatic and jugular veins showed loss of 50 to 60 per cent of the radioactivity with each passage through the splanchnic tissues during the rapid clearance phase. Hepatic uptake decreased as clearance slowed.

The liver was the principal site of removal of the administered radiogold.

The spleen, because of its small size in the rabbit, contributed little to the total removal. On the basis of liver and splenic weights and counts obtained from ground aliquots of liver and spleen obtained 60 minutes after injection of tracer doses, it was calculated that the liver took up approximately 50 per cent and the spleen 0.1 per cent of the administered radioactivity during this time interval.

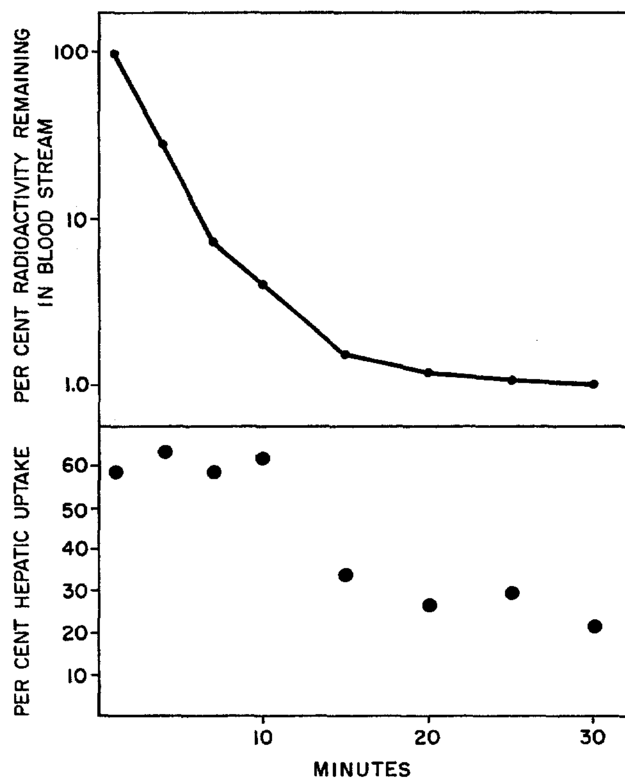

FIG. 3. Blood stream clearance and hepatic uptake of gelatin-stabilized radiogold in the living rabbit.

Effect of Varying Amounts of Gold or Gelatin on the Clearance of Gelatin-Stabilized Gold.—Studies were then conducted to determine the influence of varying doses of radiogold or gelatin on the rate of removal of radiogold from the blood stream. Varying the dose of gold over a thirtyfold range from 0.08 to 2.7 mg/kg by addition of non-radioactive gold colloid while maintaining the dose of stabilizing gelatin at 5 mg/kg of rabbit body weight, produced no significant change in the clearance rates of radioactive gold colloid, as noted in Fig. 4. Accordingly the variation of the specific activity of the gold employed resulting from radioactive decay did not significantly alter clearance rates.

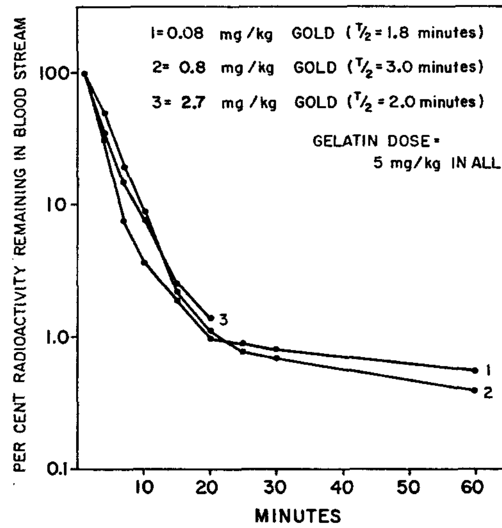

FIG. 4. Effect of increasing amounts of gold on the blood stream clearance of gelatin-stabilized radiogold.

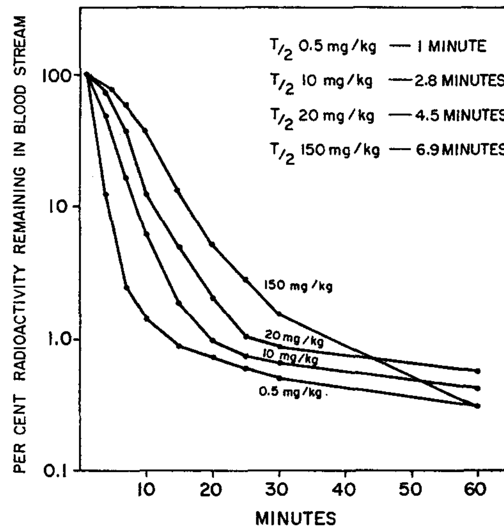

FIG. 5. Effect of increasing amounts of gelatin on the blood stream clearance of gelatin-stabilized radiogold.

In contrast, when a constant quantity of radiogold was stabilized in increasing quantities of gelatin, the clearance of the gold was progressively slowed, as noted in Fig. 5. Under these circumstances clearance half-times were 1 minute when the radiogold was stabilized with 0.5 mg of gelatin per

TABLE I
Gelatin Blockade

Gelatin dose one hour before tracer	T/2 of tracer	Results
mg/kg	*min.*	
None	1.5 to 4.6 (10 normal rabbits) 2.7 ± 0.6*	No blockade
5	1.8	No blockade
20	4.6	No blockade
50	5.8 9.4	Blockade
150	9.0 9.0 9.5 10.5 12.5 14.5	Blockade

* Mean ± 2 standard deviations.

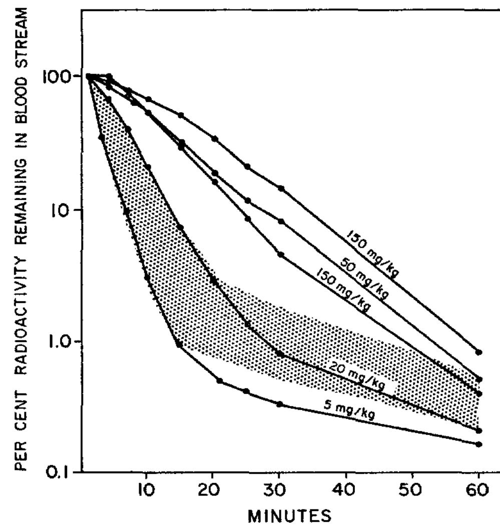

FIG. 6. Gelatin blockade. The effect of various preliminary doses of gelatin on the subsequent blood stream clearance of gelatin-stabilized radiogold.

kg of rabbit body weight, 4.5 minutes in 20 mg of gelatin, and 6.9 minutes when the Au^{198} was stabilized with 150 mg/kg of gelatin.

Gelatin Blockade.—These studies suggested that the amount of stabilizing gelatin employed was the most important determinant of clearance rate. Experiments were then conducted to determine if plain gelatin given 1 hour

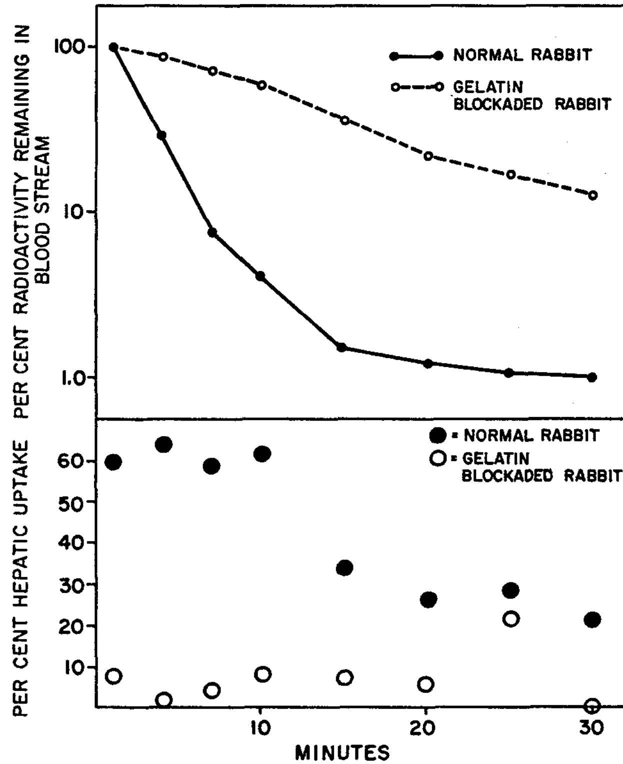

FIG. 7. Effect of gelatin blockade on blood stream clearance and hepatic uptake in the living rabbit.

previously would impair the subsequent clearance of the gelatin-stabilized gold tracer dose. As noted in Table I and Fig. 6, doses of 5 and 20 mg of gelatin/kg of rabbit body weight had little effect on the subsequent removal of the gelatin-gold tracer dose. Injections of 50 or 150 mg per kg of gelatin markedly delayed tracer dose clearance.

Catheters placed so that the simultaneous hepatic inflow and outflow samples could be obtained showed that splanchnic uptake of the tracer particle was markedly decreased in a rabbit given a prior dose of 150 mg of gelatin per

kg. As shown in Fig. 7, splanchnic uptake was markedly reduced when compared with that obtained in a normal rabbit.

Duration of Gelatin Blockade.—Slowing of clearance was maximal shortly after administration of gelatin. Studies performed 1, 6, 12, and 24 hours after

TABLE II

Duration of Gelatin Blockade

Time after 150 mg/kg of gelatin	T/2 of tracer
hrs.	*min.*
0 (10 normal control rabbits no previous injection of gelatin)	1.5 to 4.6
1	14.5
6	6.8
12	5.8
24	3.5
24	2.5

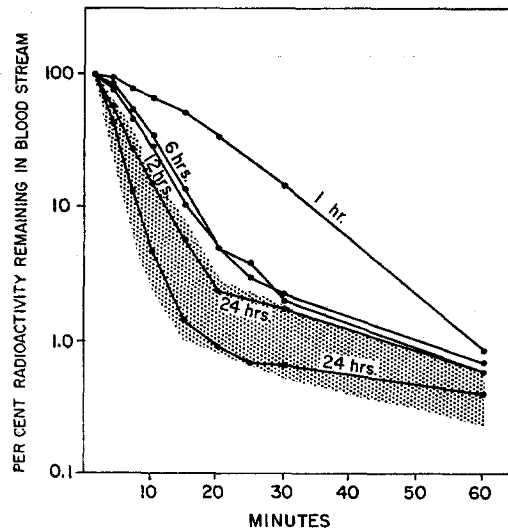

FIG. 8. Duration of gelatin blockade.

injection of 150 mg/kg of gelatin are portrayed in Table II and Fig. 8. As noted, clearance of the tracer dose was delayed maximally at 1 hour, was still apparent at 6 and 12 hours, and had disappeared by 24 hours.

Specificity of Blockade.—In order to determine whether blockade was specific for the particle under study, various blockading agents were injected intravenously and the clearance of the gelatin stabilized tracer was studied 1 hour

later. Substances employed included thorotrast, 2 ml/kg of rabbit body weight; 10^{11} latex particles; carbon in saline, 32 mg/100 gm of rabbit body weight; carbon, 32 mg/100 gm of rabbit body weight stabilized in 5 mg/kg of gelatin; and carbon, 32 mg/100 gm of rabbit body weight stabilized in 2 per cent gelatin as described by Biozzi *et al.* (2).

As noted in Table III and Fig. 9, thorotrast, latex particles, carbon in saline, and carbon stabilized in 5 mg of gelatin/kg of rabbit body weight all failed to alter the subsequent clearance of the gelatin-coated tracer dose. However, if carbon was suspended in large quantities of gelatin, clearance of the tracer dose of gelatin-gold was slowed to the same degree as noted with gelatin alone.

TABLE III

Specificity of Blockade

"Blockading" agent	Tracer	T/2 of tracer	Results
		min.	
None	Gelatin-gold	1.5 to 4.6 (10 normal rabbits)	No blockade
Thorotrast	Gelatin-gold	2.5	No blockade
10^{11} latex particles	Gelatin-gold	1.3	No blockade
Carbon in saline	Gelatin-gold	2.2	No blockade
Carbon in gelatin 5 *mg/kg*	Gelatin-gold	2.2	No blockade
Carbon in gelatin 200 *mg/kg*	Gelatin-gold	30.2	Blockade
Gelatin 150 *mg/kg*	Gelatin-gold	9.0 to 14.5	Blockade

That blockade was not simply a property of gelatin systems could be demonstrated using another particle as the tracer. The clearance of a tracer dose of I^{131}-labeled aggregated albumin (1 mg/kg of rabbit body weight) was studied in a normal rabbit, a rabbit given a blockading dose of 150 mg/kg of plain gelatin, and a rabbit given a blockading dose of non-labeled aggregated albumin (50 mg/kg). As noted in Fig. 10, while aggregated albumin was cleared more slowly than radiogold, a large dose of gelatin 1 hour previously did not alter the clearance of the tracer dose of aggregated albumin. However, a prior injection of a large dose of aggregated albumin greatly reduced the rate of clearance of labeled aggregated albumin.

Studies in the Isolated, Perfused Rabbit Liver.—These observations suggested that blockade was specific and occurred only when the blockading substance and the tracer particle shared similar surface properties. To answer whether such blockade resulted from changes in humoral factors governing RES uptake or changes in phagocytic cell function within the liver, the remaining studies were performed in isolated rabbit liver preparations.

When a normal liver was continuously perfused with Ringer's solution

alone, no uptake of radiogold could be detected. When 5 to 10 per cent normal rabbit serum was added to the perfusate, the tracer dose of gelatin-coated radiogold was rapidly removed from the perfusate, and continuous perfusion resulted in hepatic uptake curves resembling those obtained in living intact rabbits as shown in Fig. 11.

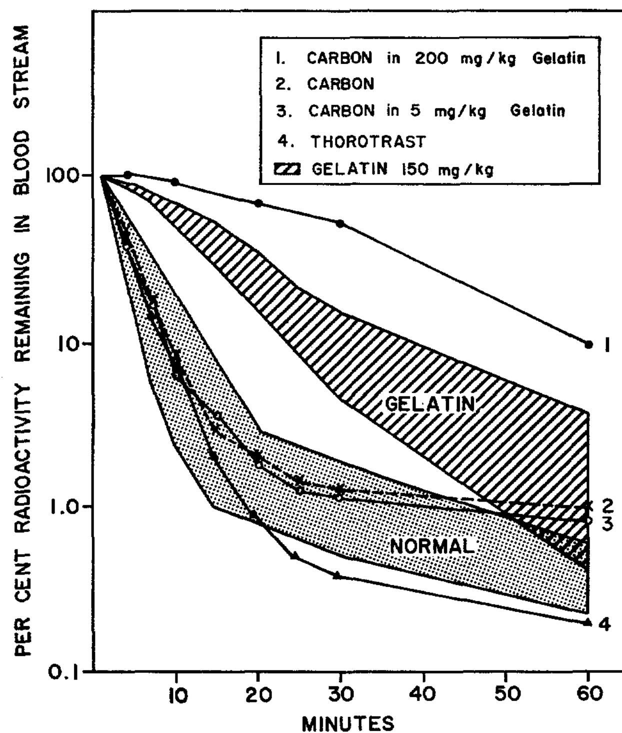

FIG. 9. Specificity of blockade. Only large doses of gelatin or carbon suspended in large doses of gelatin slowed blood stream clearance of gelatin-stabilized radiogold.

In contrast, when the serum employed in the perfusate was obtained from a rabbit given a blockading dose of gelatin 1 hour previously, there was a striking impairment of hepatic uptake of the gelatin-gold tracer dose. These data are shown in Table IV and the left hand part of Fig. 12. As noted here, five normal livers perfused with Ringer's solution containing 5 or 10 per cent normal serum averaged 24.1 per cent removal of the tracer particle at each sampling period. However, when serum from a gelatin blockaded animal was substituted for normal rabbit serum in the perfusate, the same livers showed

definite impairment of radiogold uptake, removing an average of only 8.8 per cent of the tracer particle.

Virtually identical results were obtained when livers from rabbits given blockading doses of gelatin (150 mg/kg) 1 hour previously were employed. As

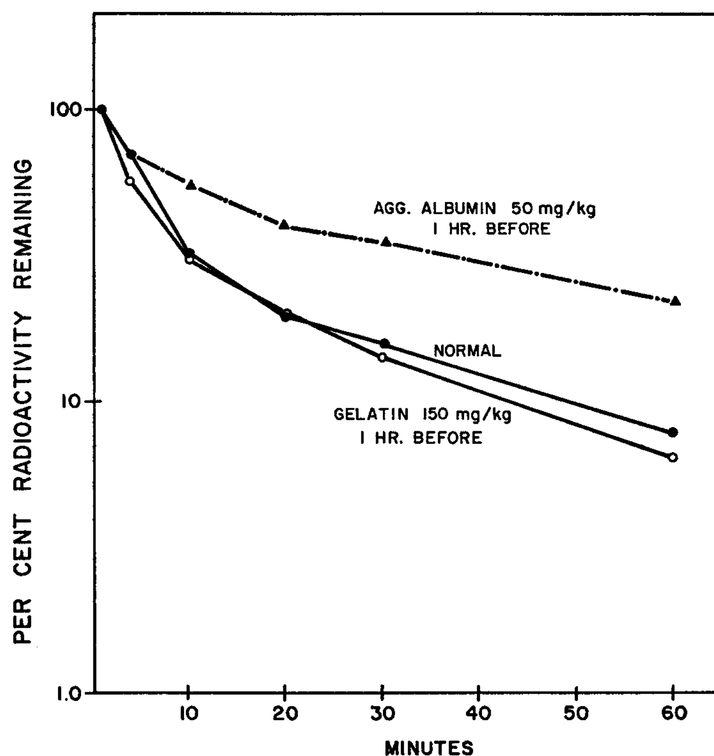

FIG. 10. Specificity of blockade. Blood stream clearance of I^{131}-labeled aggregated albumin in normal, gelatin blockaded, and aggregated albumin blockaded animals.

noted in Table IV and the right-hand portion of Fig. 12, seven blockaded livers removed an average of 30.4 per cent of the radiogold at each sampling period when the perfusate contained normal rabbit serum. Hepatic trapping was suppressed to an average of 10.8 per cent by serum from gelatin blockaded animals. In both normal and blockaded livers, the reduction in hepatic uptake produced by gelatin blockaded serum was statistically significant ($p = < 0.01$, > 0.001), while no significant difference in the performance of blockaded and normal livers was evident. Thus blockade clearly related to the presence or

absence of some factor in the perfusate rather than alteration in the phagocytic function of the reticuloendothelial cells of the liver.

Studies to Define the Humoral Factor Responsible for Blockade.—Because gelatin had once been used as a plasma expander (17–20), it appeared possible that gelatin-stabilized colloids might dissociate in the blood stream allowing

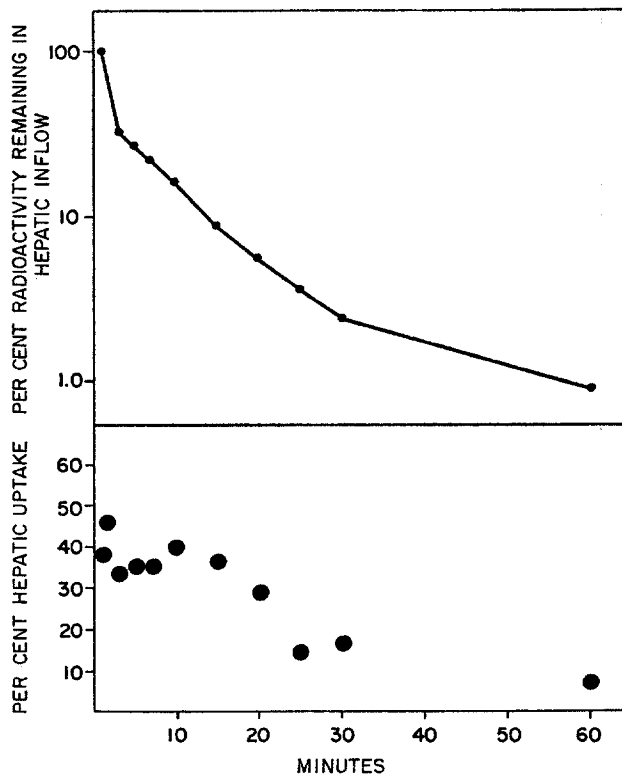

FIG. 11. Removal of gelatin-stabilized radiogold by isolated perfused normal rabbit liver.

gelatin to persist for long periods in the circulation. As noted in the left-hand portion of Fig. 13, when a rabbit was given the usual tracer dose of radiogold stabilized in 5 mg/kg of gelatin, the radiogold (presumably coated with a small quantity of gelatin) was rapidly removed from the blood stream. In contrast, gelatin as separately determined was cleared extremely slowly. This dissociation was even more striking when the same quantity of radiogold was stabilized in 150 mg of gelatin/kg of rabbit body weight. (See the right-hand portion of Fig. 13.) Thus dissociation between the Au^{198} and both large and

small amounts of "stabilizing gelatin" occurred, and circulating gelatin persisted in significant amounts for long periods.

It could further be shown that blockade correlated with the level of circulating gelatin *in vivo*. Rabbits were given a blockading dose of 150 mg/kg of gelatin. At subsequent intervals ranging from 10 minutes to 6 hours the clearance of a standard tracer dose of Au^{198} in gelatin was determined. The results of two such experiments are shown in Table V and Fig. 14. As noted, the

TABLE IV

Uptake of Gelatin-Gold by Normal and Gelatin Blockaded Livers

Livers	Per cent radiogold removed	
	Normal rabbit serum	Blockaded rabbit serum
Normal		
1	37.5	7.5
2	23.2	8.1
3	23.0	13.0
4	19.6	8.9
5	17.5	6.6
Average................	24.16	8.8
Blockaded		
1	53.6	11.7
2	14.3	3.0
3	24.6	11.0
4	35.3	15.0
5	18.4	3.4
6	35.6	21.4
7	31.1	10.1
Average................	30.4	10.8

clearance rates increased as the plasma gelatin concentration diminished. Thus it appeared likely that circulating gelatin was either binding or inactivating a specific opsonin necessary for the RES trapping of gelatin-coated particles, or by its presence *per se* in some way interfered with the clearance of gelatin-stabilized particles. Subsequent studies examined these two possibilities.

Absorption of "Gelatin Opsonins."—Normal rabbit serum was repeatedly absorbed with gelatin-coated latex particles or gelatin-coated tanned red cells to determine whether such absorption would abolish uptake of radiogold by the isolated rabbit liver perfused with Ringer's solution containing 5 per cent of the serum under study. Such sera produced uptake of gelatin-stabilized

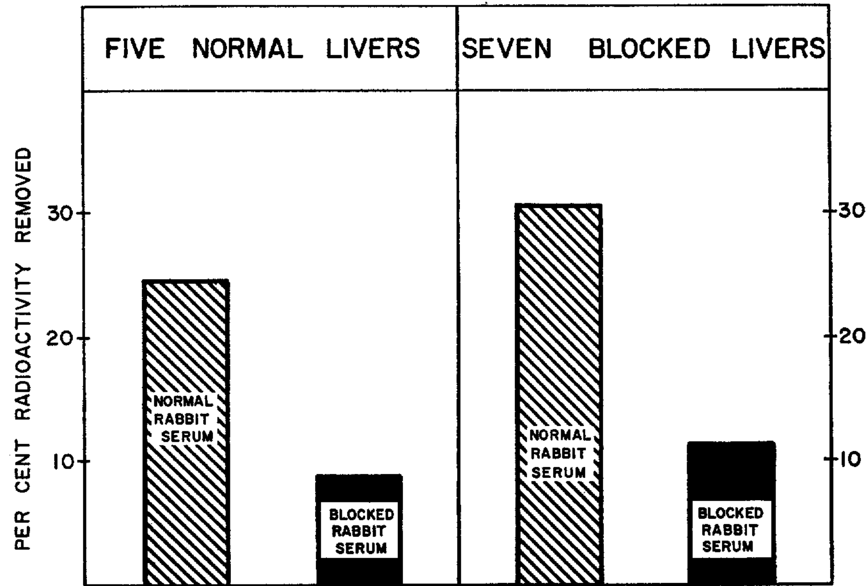

FIG. 12. Removal of gelatin-stabilized radiogold in normal and gelatin blockaded perfused rabbit livers. Hepatic uptake was impaired only when perfusates contained blockaded rabbit serum.

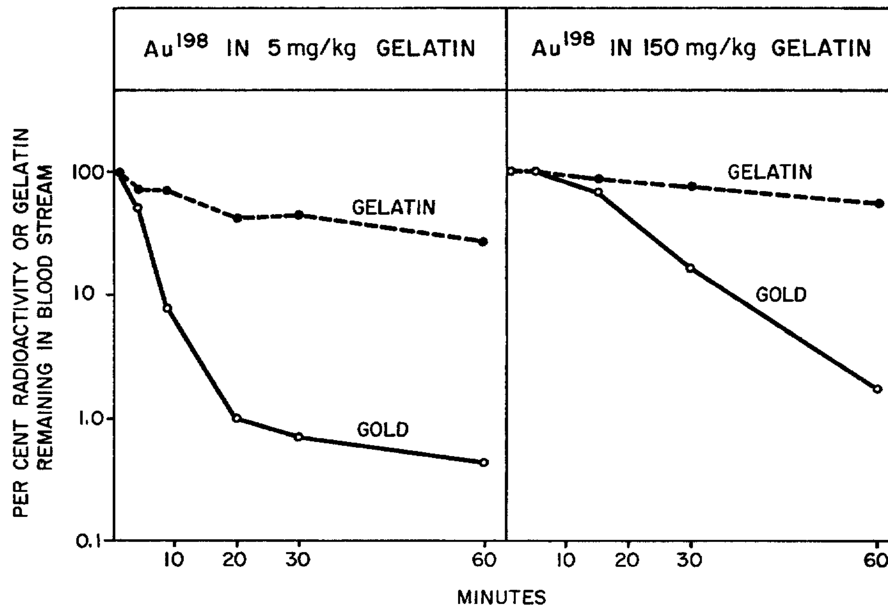

FIG. 13. Dissociation of blood stream clearance of gelatin and radiogold.

TABLE V

Relation of Plasma Gelatin Concentration to Blockade

Rabbit No.	Time after blockading dose of gelatin	Plasma gelatin concentration	T/2 of tracer
		μg/ml	*min.*
1	10 min.	2818	10
	1 hr.	1709	9
	2 hrs.	1632	6.5
	4 hrs.	1062	4.5
	6 hrs.	1170	5.5
2	30 min.	2556	8.8
	1 hr.	2032	9.2
	4 hrs.	1016	4.8
	6 hrs.	723	6.2

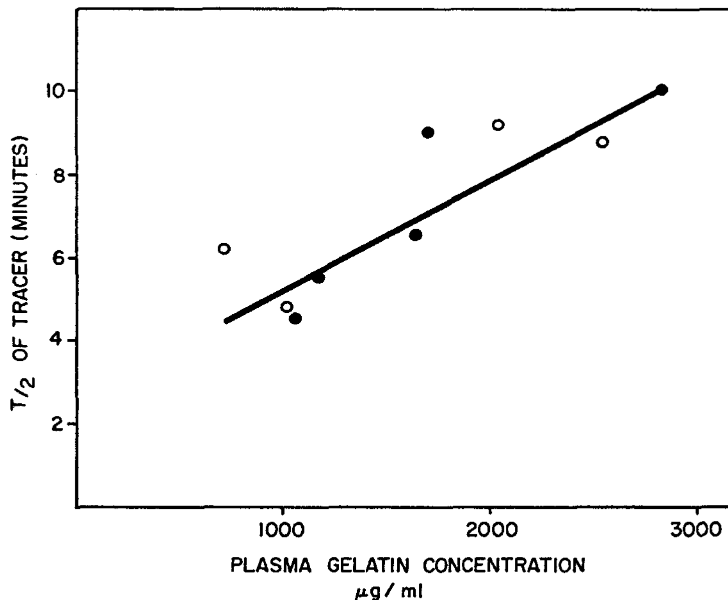

FIG. 14. Effect of plasma gelatin concentration on T/2 of gelatin-stabilized radiogold tracers. Open and closed circles represent results obtained in two separate animals.

radiogold to the same degree obtained with normal rabbit serum. Thus the removal of gelatin opsonins was not demonstrated by these techniques.

Preopsonization of Radiogold in Normal Sera.—It was reasoned that if antibody-radiogold coupling was important for hepatic uptake, preincubation of the tracer dose in normal serum *prior* to the addition of gelatin to the perfusate might abolish the impaired clearance of radiogold. Isolated rabbit livers were thus perfused in sequence with Ringer's solution containing (*a*) the gelatin-

gold tracer dose "preopsonized" by incubation in 20 cc of normal rabbit serum at 37°C for 20 minutes, (*b*) the gelatin-gold tracer similarly preopsonized to which 40,000 μg of gelatin[1] was added immediately before perfusion, and (*c*) 40,000 μg of gelatin[1] preincubated for 20 minutes with 20 cc of normal rabbit serum to which the radiogold tracer dose was added just prior to the perfusion. A final perfusion was performed with Ringer's solution containing the standard gelatin-gold tracer in 20 cc of normal rabbit serum to make certain that the phagocytic function of the liver was maintained throughout the study.

TABLE VI

Uptake of Gelatin-Gold by Isolated Rabbit Liver
(attempts to opsonize)

Perfusate: Ringer's plus:	Hepatic uptake
	per cent
1. Gelatin-gold tracer preopsonized with NRS*	29.0
2. Gelatin-gold tracer preopsonized with NRS* plus Gelatin added just prior to perfusion	10.5
3. NRS* preincubated with gelatin plus Gelatin-gold tracer added just prior to perfusion	5.7
4. Gelatin-gold tracer plus NRS*	34.2

* Normal rabbit serum.

The results of such an experiment are shown in Table VI. As noted, 29.0 and 34.2 per cent of the gelatin-gold tracer dose was removed by the isolated liver when the perfusate contained only normal rabbit serum. However, when gelatin was added to the perfusate, hepatic uptake was profoundly reduced irrespective of whether or not the gelatin-gold tracer was preopsonized with normal serum prior to the addition of gelatin.

Similar attempts were made to supply a missing opsonin by the addition of large amounts of normal rabbit serum to perfusates which contained 5 per cent gelatin blockaded serum or simply gelatin in the amounts found in the serum of blockaded rabbits. In a series of 5 experiments the addition of 50 to

[1] Since perfusates were made up in 200 ml volumes containing 10 per cent serum, it was calculated that this amount of gelatin was equivalent to that contained in 20 cc of blockaded rabbit serum at 1 hour.

85 per cent normal serum to such perfusates did not significantly correct impaired hepatic uptake.

Attempts to preopsonize the gelatin-gold tracer particles with normal serum prior to injection into intact animals receiving a blockading dose of gelatin 1 hour previously similarly met with failure. In this study, 20 cc of blood was obtained from a normal rabbit, the animal was then given a blockading dose of 150 mg/kg of gelatin, and the standard tracer dose of radiogold was administered at 1 hour. Following blood samples at 1, 4, 7, and 10 minutes to ascertain clearance, the second tracer dose, preincubated for 20 minutes at 37°C

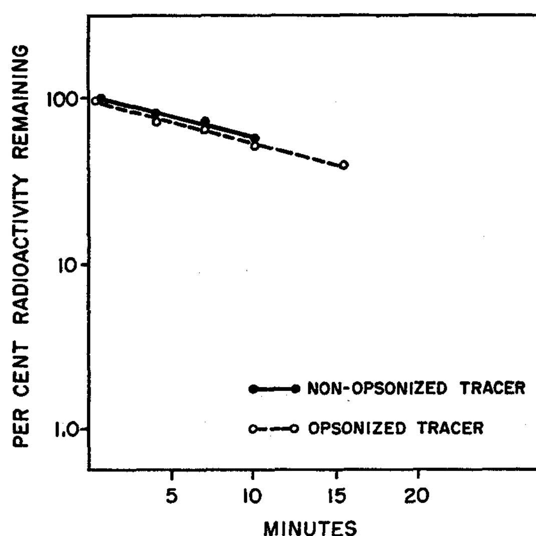

FIG. 15. Failure of opsonization of radiogold tracer to alter blood stream clearance rates in a gelatin-blockaded rabbit.

with 10 cc of the isologous serum obtained prior to blockade, was administered and its clearance determined. As shown in Fig. 15, the opsonized tracer dose was not cleared more rapidly than the non-opsonized particles, suggesting that depletion of serum opsonins played little role in the mechanism of blockade. Because the supply of salt-free gelatin was exhausted, only one such experiment was performed in the intact animal.

Studies of Hepatic Uptake with a Serum-Free Perfusate.—The observations which suggested that excess gelatin *per se* rather than removal of a serum factor interfered with hepatic uptake of gelatin stabilized gold were confirmed by perfusions performed in Krebs-Henseleit buffer. It was observed that this buffered system (pH 7.4) promoted hepatic uptake of the gelatin stabilized gold tracer in the total absence of serum (see Table VII). It therefore appeared

likely that the low pH of the Ringer's solution (6.0) inhibited hepatic removal mechanisms and that the addition of serum to Ringer's solution might enhance hepatic uptake of the tracer particle merely because of its buffering capacity. When isolated livers were perfused with buffered Krebs-Henseleit solution containing radiogold, excellent hepatic uptake was noted. The removal of the gelatin-gold tracer from such perfusates was greatly reduced by the addition of 10 per cent serum from rabbits given blockading doses of gelatin 1 hour before. Uptake of the tracer could be similarly reduced by the simple addition of gelatin to the perfusate in quantities similar to that contained in gelatin-

TABLE VII

Uptake of Gelatin-Gold by Isolated Rabbit Liver

Rabbit No.	Perfusate	Hepatic uptake
		per cent
1	Buffered Krebs-Henseleit	
	plus	
	(*a*) Gelatin-gold tracer	42.1
	(*b*) Gelatin-gold tracer and 10 per cent NRS*	44.8
2	Buffered Krebs-Henseleit	
	plus	
	(*a*) Gelatin-gold tracer	47.7
	(*b*) Gelatin-gold tracer and gelatin	8.0
	(*c*) Gelatin-gold tracer and 10 per cent blockaded rabbit serum	12.1
	(*d*) Gelatin-gold tracer and 10 per cent NRS*	57.2

* Normal rabbit serum.

blockaded serum. The results of such experiments are summarized in Table VII.

Thus in a system in which serum was not required for the removal of gelatin-stabilized gold particles, the simple addition of gelatin greatly reduced hepatic uptake. Identical results were obtained using livers from blockaded animals, indicating that the liver was not contributing serum factors required for opsonization.

DISCUSSION

The present studies viewed in conjunction with the work of Murray (7, 8), Jenkin and Rowley (6), and Wagner and his associates (5) suggest that the whole concept of experimental "reticuloendothelial blockade" as studied in animals and man needs reassessment. The current experiments employed a radiolabeled gold particle stabilized in a gelatin solution. This tracer particle, or carbon particles similarly suspended in gelatin, have been the agents most

commonly used in studies of RES blockade in animals. Because preliminary studies indicated that gelatin alone would produce impaired clearance, a gelatin solution was used as the blockading agent.

Under the conditions examined, these studies indicate that the term "reticuloendothelial blockade" is a misnomer. The impaired clearance which followed large doses of gelatin was dependent on the nature of the perfusate rather than saturation of hepatic cell phagocytic capacity in the classic sense.

In this system it was established that impaired clearance of radiolabeled colloidal gold stabilized in gelatin follows prior injections of gelatin alone. This impaired clearance was due to depressed hepatic uptake of the tracer particle. The duration of blockade was finite and correlated with the amount of gelatin remaining in the circulation. The impairment of clearance which followed gelatin injections was apparently specific for particles with a gelatin surface. Other agents commonly used for blockade, such as carbon in saline, thorotrast, or aggregated albumin produced no impairment in the clearance of gelatin-stabilized radiogold. Conversely, prior injections of gelatin did not alter the clearance of aggregated albumin particles suspended in saline.

Because most phagocytic cells in *in vitro* systems require serum factors for phagocytosis, it was initially our belief that our results might best be explained by binding or depletion by the blockading agent of a serum opsonin required for RES phagocytosis. Subsequent studies did not support this thesis. The evidence obtained suggests instead that "blockade" as observed in the experimental model employed is in some way related to the continuing presence of gelatin in the circulation which in turn affects hepatic phagocytic cell trapping. Repeated absorptions of normal serum with gelatinized latex particles or tanned red cells failed to remove any phagocytosis-promoting factor. Opsonization of the radiogold tracer particle in normal serum did not increase the rate of clearance in a gelatin blockaded animal or reverse the effects of sera containing excess gelatin in isolated perfused livers. Similarly, attempts to supply opsonin by the addition of large amounts of normal serum to blocked serum did not improve hepatic trapping of the tracer particle. Finally, blockade could be reproduced by the addition of serum containing gelatin or simply gelatin alone in the total absence of serum when a Krebs-Henseleit solution which supported tracer particle uptake was employed. In the light of these findings, previous explanations of the phenomenon of impaired clearance produced by prior injections of blockading substances bear review.

It has been recurrently stated that RES blockade follows the injection of a wide variety of substances. Review of these reports indicates that blockade was often inferred from histological examination of tissues, from increased susceptibility to experimental infection, or from suppression of antibody response to a variety of antigenic stimuli. As noted by Jaffe (21), many studies on vital dyes, carbon, and colloidal metals were conflicting. In actuality demonstration

of blockade as defined by impaired blood stream clearance has been rare. However, such blockade of clearance has been observed following injection of carbon (2–4, 8), colloidal iron (22), chromic phosphate (8), gelatin (5, 7, 8), aggregated albumin (5), and thorotrast (23–25). In many of these studies gelatin was utilized as a stabilizing agent.

The careful studies of Biozzi, Benacerraf, and their colleagues (2–4) using carbon particles suspended in gelatin have led to the belief that blockade results from the saturation of the phagocytic cells of the liver, and elimination of blockade has been explained by subsequent RES hypertrophy (4). It should be noted, however, that amounts of gelatin used to suspend carbon particles in their work approximated 200 mg/kg of animal body weight. This amount of gelatin is greatly in excess of that required to produce blockade in the current studies. Thus it appears probable that gelatin rather than the carbon particle was responsible for the results observed, and elimination of blockade was the result of gradual elimination of gelatin. Other evidence also suggests that saturation of RES phagocytic capacity is an unlikely explanation for blockade. Review of past studies would indicate that blockade has rarely been demonstrated to follow injection of simple particulate substances which are rapidly cleared from the circulation. For example, circumstances as taxing as overwhelming bacteremia (12, 26, 27), or the injection of enormous numbers of latex balls (10^{11} particles) as in the present experiments, have not produced detectable blockade of subsequent tracer injections. By the same token, experiments utilizing isolated polymorphonuclear leukocytes suggest that preliminary phagocytosis of particles often *increases* subsequent phagocytic activity or intracellular bacteriocidal digestion (28, 29).

Murray (7, 8), and Jenkin and Rowley (6), have proposed that experimental blockade results from the depletion of serum opsonins bound by the preliminary injection of blockading agent. While this thesis is immunologically attractive, it could not be confirmed in the present experiments. Although it has been established that opsonins play an important role in increasing the speed of RES removal of bacteria (30–33), no evidence could be obtained to show that an opsonin was required for removal of gelatinized radiogold by the rabbit liver.

While our explanations for the results observed differ, our findings in rabbits closely parallel those obtained in dogs and man by Wagner and Iio (5). Their studies show similar specificity and duration of blockade and similar failure of normal serum to opsonize tracer doses of gelatin-stabilized radiogold or aggregated albumin following appropriate blockade. These observations led Wagner and Iio to postulate that different groups of macrophages in the RES had varying avidity for substances of differing surface. The results obtained in the isolated perfused liver in the current experiments render this possibility unlikely. The phagocytic capacity of blocked livers was normal when gelatin was absent from the perfusate, a finding which would not obtain if cells committed to gelatin-coated substances had been saturated by the prior injection.

The ability of the liver to remove gelatinized radiogold in the absence of serum and the specificity of the RES impairment which follows gelatin injections, does, however, direct attention to the reticuloendothelial macrophage *per se*. Phylogenetically it seems reasonable to believe that certain phagocytic cells may possess the ability to "recognize" foreign substances which enter the circulation. A number of previous studies show that the perfused liver can actively remove certain bacteria from perfusates without added serum (34, 35). The phagocytic cells of invertebrates without detectable gamma globulin can ingest infectious agents (36). Antibody forming mechanisms emerge in the primitive vertebrates and may represent an evolutional refinement of host defenses (37). Thus antibody may not be required for primary macrophage recognition and entrapment of certain foreign substances, although this process may be enhanced by serum antibody.

The mechanism by which the continuing presence of gelatin impairs subsequent hepatic uptake of gelatin-coated gold remains uncertain. Changes in circulatory dynamics imposed by the addition of an osmotically active substance appear unlikely. Hematocrits did not change with blockade and the identical results obtained in both normal animals and isolated perfused livers rule against this possibility. Similarly, changes in the circulation of formed elements (platelets, leukocytes) appear eliminated. Injections of gelatin did not produce thrombocytopenia or leukopenia and again identical findings were obtained with cell-free perfusates in the isolated liver. The relationship between serum gelatin concentration and half-times of clearance of gelatin-stabilized radiogold suggests that simple dilution of the tracer particle in a greatly expanded gelatin pool is the most reasonable explanation. If this were the case, the probability of removal of a particular particle in the RES might be decreased. However, the dissociation of clearance of radiogold and gelatin makes it possible that the two substances are handled differently once they enter reticuloendothelial cells.

The use of gelatin as a suspending agent for the particle under study has been common in studies of reticuloendothelial system function and blood stream clearance. The fact that gelatin *per se* rather than the particle in suspension may be the primary determinant of impaired clearance requires that many of these studies be reevaluated. The slow clearance of aggregated albumin and the short duration of aggregated albumin blockade noted in Wagner's studies permits similar interpretation of his results in man (5). Studies in which thorotrast suspended in dextrin has been employed as a blockading agent are difficult to interpret. This agent produces profound thrombocytopenia (which can be duplicated by dextrin alone) and possible direct RE cellular damage. Similar difficulties are encountered in assessing blockading experiments which have employed other agents which damage cells such as saccharated iron (38), lithium carmine (21), and endotoxin (39).

It thus emerges that clear-cut blockade has rarely been demonstrated to fol-

low injections of particulate substances in the absence of a suspending agent or the persistence of the blockading substance in the circulation. The possibility that reticuloendothelial blockade is largely a laboratory artifact induced by the continuing circulation of the blockading agent thus deserves consideration.

SUMMARY

The dynamics of "reticuloendothelial blockade" were studied in living rabbits and isolated, perfused rabbit livers utilizing gelatin as a blockading agent and Au^{198} stabilized in gelatin as a tracer.

Employing the above experimental model, the following observations were made. (*a*) RES blockade was specific and dependent on the surface properties of the particle under study. (*b*) RES blockade was not caused by saturation of hepatic removal mechanisms. (*c*) RES blockade was not caused by depletion of demonstrable serum opsonins. (*d*) RES blockade appeared to correlate with high circulating levels of the blockading agent, *per se*.

Thus, under the conditions employed, the term "reticuloendothelial blockade" was a misnomer. Although specificity of liver macrophage-particle interaction was evident and deserves further study, the data suggest that blockade as usually studied is a laboratory phenomenon induced by the continuing circulation of the blockading agent.

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