FURTHER OBSERVATIONS ON THE MECHANISM OF RETICULOENDOTHELIAL BLOCKADE*

By DAVID J. DRUTZ,‡ M.D., M. GLENN KOENIG,§ M.D., and DAVID E. ROGERS, M.D.

(From the George Hunter Laboratory, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee 37203)

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The term "reticuloendothelial blockade" (RE blockade) denotes the impaired blood stream clearance which follows intravenous injections of large doses of particulate material. Such blockade has been generally considered to result from physical saturation of RE cells by phagocytosis of the particles injected (1–3). However, a number of studies have indicated that blockade is quite specific and is demonstrable only when the material used to test for the presence of blockade has the same surface characteristics as the blockading agent (4–8).

Two other theories have thus been proposed to account for this specificity: first, that the blockading dose results in the depletion of serum opsonins specific for the surface of the particle under study (5, 9); or second, that there are "particle-specific" macrophage clones in the RE system (6). Experiments reported from this laboratory in 1965 could not confirm either of these hypotheses (7). In these studies RE blockade induced by gelatin-stabilized colloids could not be explained by saturation of RE cells or by the depletion of serum opsonins but was instead dependent on the simple continuing circulation of the gelatin-blockading dose.

Most investigations of RE blockade have employed colloidal materials, such as carbon or gold which are gelatin stabilized (1–3, 5, 7, 9). Because gelatin persists for long periods in the circulation, the present studies were undertaken to characterize blockade in a system free of exogenous stabilizing material. For these purposes a simple, inert, particulate material, chromic phosphate, suspended in 5% dextrose, was employed.

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‡ United States Public Health Service Postdoctoral Fellow, National Institute of Allergy and Infectious Diseases.

§ United States Public Health Service Research Career Development Awardee, National Institute of Allergy and Infectious Diseases.

Materials and Methods

Experimental Animals.—Female Wistar albino rats weighing between 225 and 325 g were used for all experiments.

Particulate Materials.—Both untagged and ^{32}P-labeled chromic phosphate were prepared by the method of Dobson (4). Sodium acetate, chromic nitrate, and disodium phosphate (Fisher Scientific Company, Fair Lawn, N. J.) were appropriately reacted and the resulting purple hexahydrate of chromic phosphate was converted to the dark gray anhydrous and insoluble compound by heating at 650°C for 12–18 hr. Appropriate amounts of ^{32}P-labeled disodium phosphate obtained from Volk Radiochemical Co., Burbank, Calif., were included in the reaction mixture to make the radiolabeled chromic phosphate. Particles of the desired size range were produced by mechanical grinding in a ball mill followed by differential centrifugation. Particle sizes were measured by means of a calibrated eyepiece micrometer mounted on a simple light microscope. Except in specified instances, the particles utilized ranged from 0.8 to 1.0 μ and below. Electron microscopy demonstrated that many particles were smaller than this (see Fig. 1). Particles of this size produced stable suspensions in 5% dextrose in water. The suspensions utilized were sterile and pyrogen-free.

Untagged chromic phosphate (CP) suspended in 5% glucose in water in concentrations of 125–175 mg/ml was used to produce RE blockade. The standard tracer dose of chromic phosphate (^{32}CP) consisted of 0.4–0.7 mg of tracer suspended in 0.1 ml of 5% glucose in water. The specific activity of the tracer at the time of preparation ranged from 30 to 50 μc/mg. In experiments designed to study the clearance characteristics of the blockading dose itself, large doses of chromic phosphate with lesser specific activity of ^{32}P (0.06–0.08 μc/mg) were utilized.

Radioactive colloidal gold (^{198}Au) stabilized with 0.3% salt-free gelatin with a specific activity of 58 μc/ml was obtained from Abbott Laboratories, North Chicago, Ill. The standard tracer dose consisted of 20 μc of ^{198}Au.

Clearance Studies in Living Rats.—Animals were anesthetized by the intraperitoneal administration of 4 mg/100 g body weight of pentobarbital sodium, and a tracheostomy was performed. The left femoral and right jugular veins were cannulated with PE-50-polyethylene catheters and patency maintained with small amounts of heparin. In experiments concerned with the effects of RE blockade, untagged CP was injected via the femoral cannula 60 min prior to the administration of radioactive tracer by the same route. When blockading doses were administered more than 1 hr prior to tracer, the tail vein route was used, and anesthesia and cannula placement withheld until 30 min before tracer injection. 0.2 ml blood samples were withdrawn from the jugular cannula at appropriate intervals following tracer administration and placed in heparinized tubes. 0.1 ml aliquots were transferred to aluminum planchets and ^{32}CP was counted in a gas-flow, thin-window proportional counter. When ^{198}Au was employed, tubes were counted directly in a well-type scintillation counter. Clearance curves were constructed to show the log of the per cent of radioactivity remaining in the blood stream at each sampling period utilizing the 1 min sample as the 100% value.

Clearance Studies in Isolated Perfused Livers.—Both normal and "blockaded" animals were used in isolated perfused liver experiments. The blockaded animals were given an intravenous injection of 1000 mg/kg of untagged CP 1–3 hr before study. Both normal and blockaded animals were then anesthetized with pentobarbital,

the abdomen opened, the liver isolated, and polyethylene inflow and outflow catheters placed in the portal vein and proximal inferior vena cava. The inferior vena cava was clamped just above the renal veins. Krebs-Henseleit buffer solution, pH 7.4, was then used to perfuse the liver employing methods previously described which permitted maintenance of normal portal pressures of 10 cm of water, a temperature of 39°C, and flow rates of approximately 16 ml/min (7). The liver was initially rinsed through with 100–150 ml of Krebs-Henseleit buffer to remove contained blood. 1 ml of the ^{32}CP suspension was then added to 100 ml of the buffer solution and four pairs of simultaneous inflow-outflow samples were drawn as it was perfused. The percentage uptake was calculated from the difference of the averages of these paired inflow–outflow samples as previously described (7). When more than one experiment was performed in a given liver, the liver was perfused with 100 ml of Krebs-Henseleit buffer before the next perfusate was employed.

Opsonization Studies.—(*a*) Homologous serum was obtained by cardiac puncture from ether-anesthetized female Wistar rats. The serum was immediately separated, frozen in dry-ice and ethanol, and stored at −70°C until needed. (*b*) Isologous serum was obtained from femoral cannula bleeding of pentabarbital-anesthetized rats prior to the administration of the untagged CP-blocking dose. Serum was separated, and used within 1 hr.

30 min following administration of the blocking dose, the standard tracer dose of ^{32}CP was placed in 0.33–1.0 ml of either homologous or isologous serum, incubated at 37°C for 30 min on a rotating drum, and employed in the appropriate clearance study.

RESULTS

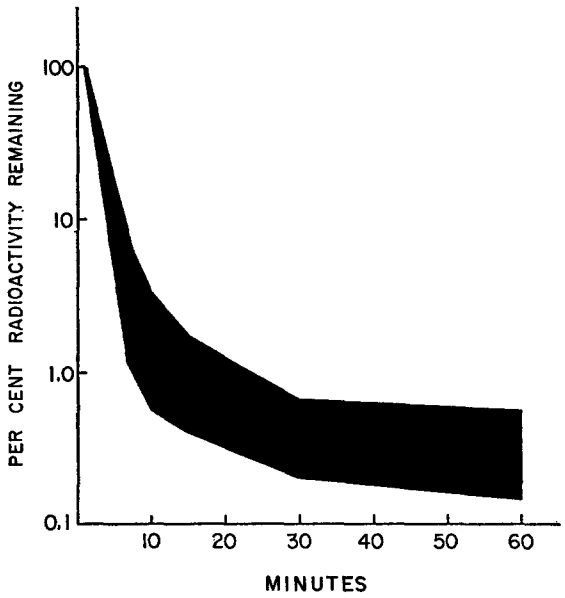
Clearance of the Standard Tracer Dose of Chromic Phosphate in Normal Rats.—Blood stream clearance curves obtained in 10 normal rats given the standard tracer dose of ^{32}P-labeled chromic phosphate (^{32}CP) were uniform and predictable. As noted in Text-fig. 1, clearance appeared composed of at least two exponentials, with an initial rapid phase lasting 10–15 min, followed by abrupt slowing. Less than 1% of the injected CP remained in the circulation at 60 min. Examination of histologic sections showed heavy uptake of chromic phosphate in the liver and spleen; it could not be visualized in other organs.

Chromic Phosphate Blockade.—Injections of 750 or 1000 mg/kg of untagged chromic phosphate produced marked impairment of the ^{32}CP tracer dose clearance administered 1 hr later. These studies are shown in Text-fig. 2. Blockading doses up to, and often including, 500 mg/kg failed to produce significant changes in clearance. To eliminate the possibility of a latent period before blockade was detectable (10, 11), tracer doses were administered from 3 to 24 hr following injection of 100 mg/kg of the untagged material. No evidence of impairment of tracer clearance was obtained in these studies.

The effect of the increasing doses of untagged CP on the clearance half-time ($T\frac{1}{2}$) of ^{32}CP tracer administered 1 hr later are shown in Text-fig. 3. Uniform prolongation of the $T\frac{1}{2}$ was not seen until doses exceeded 500 mg/kg

and then appeared directly related to the blockading dose employed. Blockade persisted in diminishing degree for 48–72 hr.

Specificity of Blockade.—The specificity of CP blockade was studied using radiogold as the test particle. Tracer doses of ^{198}Au administered 1 hr following doses of untagged CP, shown to markedly inhibit tracer ^{32}CP clearance, were cleared normally (Text-fig. 4). It thus appeared that CP blockade had significant specificity and the impairment of clearance of tagged CP could not



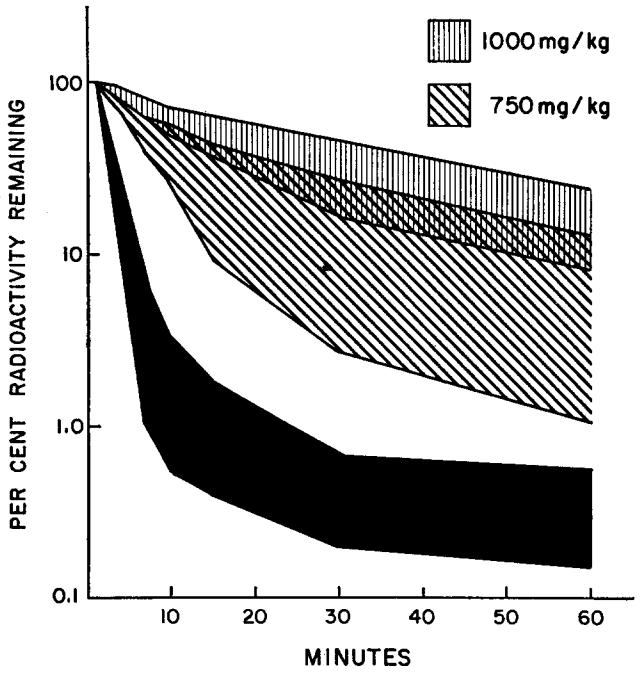
TEXT-FIG. 1. Range of blood stream clearance curves obtained in 10 normal rats given the standard tracer dose of chromic phosphate (^{32}CP).

be explained on the basis of generalized depression of RE phagocytic cell function.

Fate of the Blockading Dose.—Because of the apparent direct relationship between the degree of blockade and the size of the blockading dose administered, the fate of the large doses of CP employed to blockade the RE system was determined. In such studies the clearance of large doses of CP labeled with ^{32}P was examined. As shown in Text-fig. 5, doses below 250 mg/kg cleared at rates closely paralleling those of the standard tracer dose. Above these doses, there was progressive delay of clearance which was proportional to the dose of CP employed. Significant quantities of CP were always in the circulation 1 hr following the injection of doses producing apparent blockade at 1 hr. Microscopic examination of liver sections showed visible CP in phagocytic

cells, free in the vascular system, and lining the endothelium of hepatic sinusoids at the time blockade was demonstrable (see Figs. 2 *a* and 2 *b*).

Studies in the Isolated Perfused Rat Liver.—The above studies suggested that during the first few hours following the injection of the blockading dose, impaired clearance of the tracer ^{32}CP was directly related to the presence of

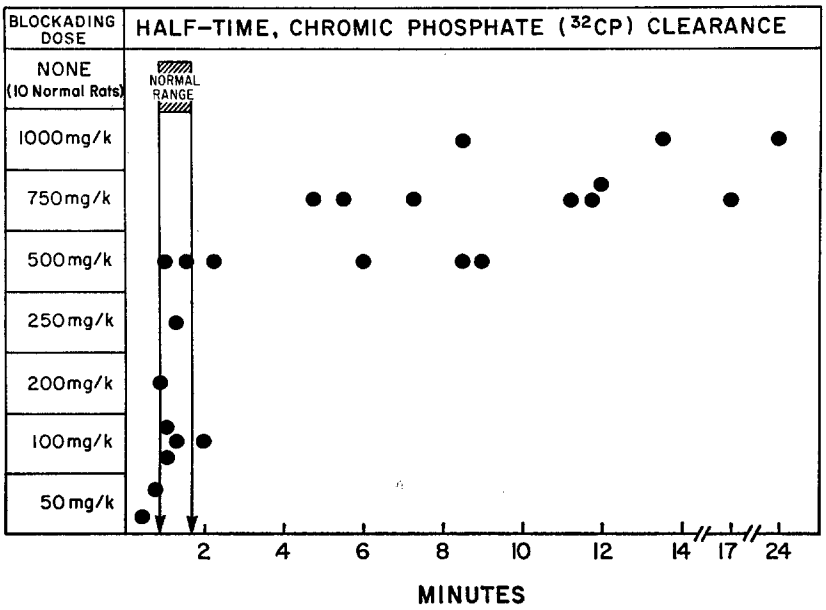


TEXT-FIG. 2. Chromic phosphate blockade. The effect of large doses of untagged chromic phosphate given 1 hr before tracer dose of ^{32}CP. Doses of 750–1000 mg of CP markedly delayed clearance of ^{32}CP.

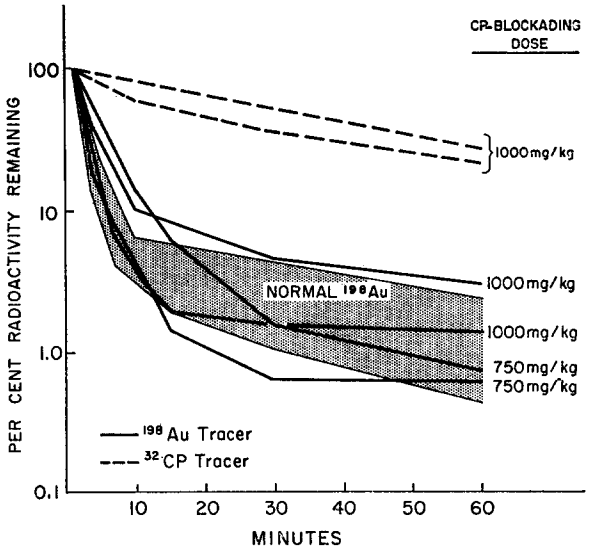
untagged "blocking" CP still in the process of being cleared. To eliminate the possibility that such blockade was due to changes in RE phagocytic cells per se, studies were performed in isolated, perfused livers.

The livers from normal animals were highly efficient in removing tracer from the perfusate and examination of microscopic sections showed both Kupffer's cell incorporation and intravascular trapping of the ^{32}CP (Figs. 3 *a*–3 *c*). Further, as noted in Text-fig. 6, ^{32}CP tracer was removed in an identical fashion and with similar efficiency when perfused through "blockaded" livers.

Effect of Particle Size on Blockade.—The preceding studies suggested that blockade was more closely related to the quantity of blockading material still

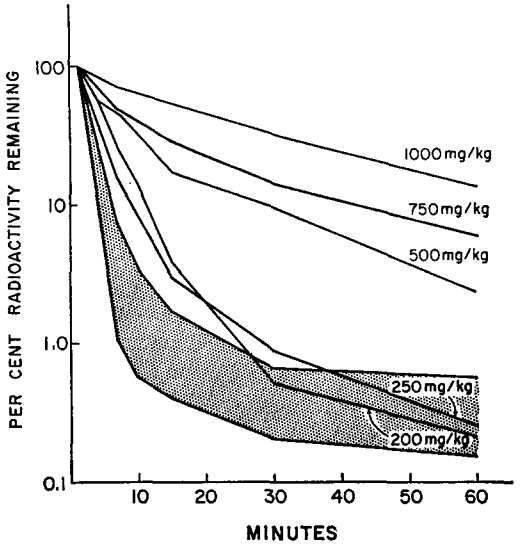


TEXT-FIG. 3. The effect of increasing amounts of untagged chromic phosphate on the clearance half-time ($T\frac{1}{2}$) of ^{32}CP. The tracer dose was administered 1 hr after the blockading dose.

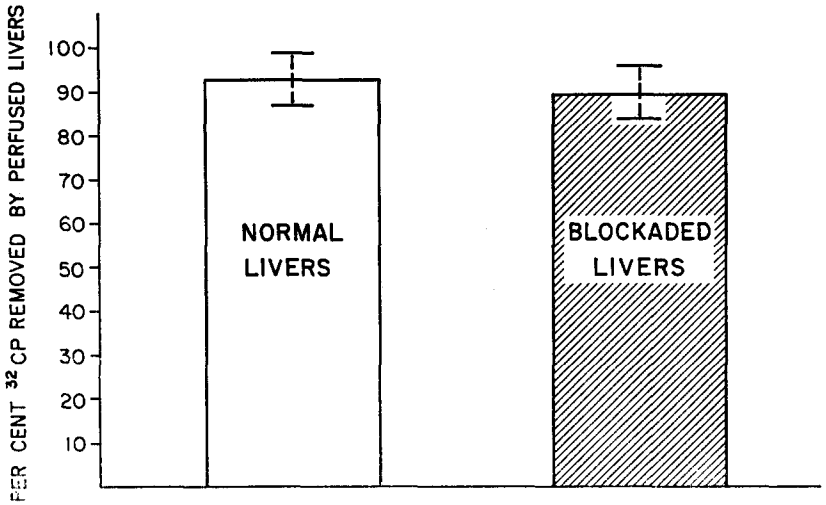


TEXT-FIG. 4. Specificity of blockade. Large doses of untagged chromic phosphate had little effect on clearance of tracer doses of radiogold (^{198}Au). The shaded curve represents ^{198}Au clearances obtained in 10 normal rats. Four rats receiving 750–1000 mg/kg of chromic phosphate had normal radiogold clearances. The two animals which received 1000 mg/kg of chromic phosphate showed markedly impaired ^{32}CP clearance.

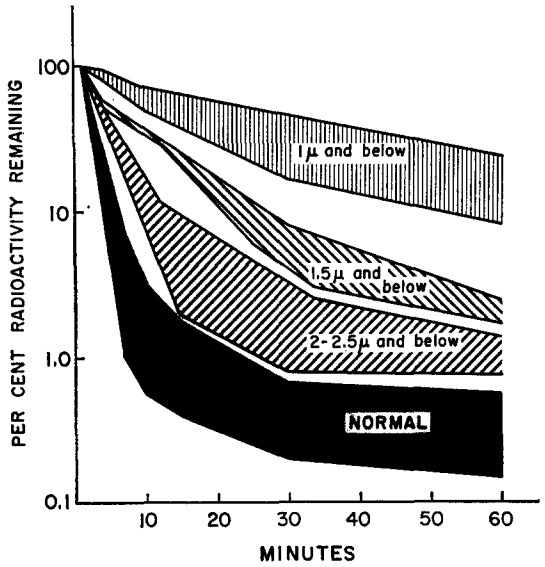
circulating at the time of injection of the tracer than to the amount of untagged CP present in the RE cells. Because other investigators have shown that small particles are cleared from the circulation more slowly than large particles (4, 12–15), it was postulated that small particles might be more effective than



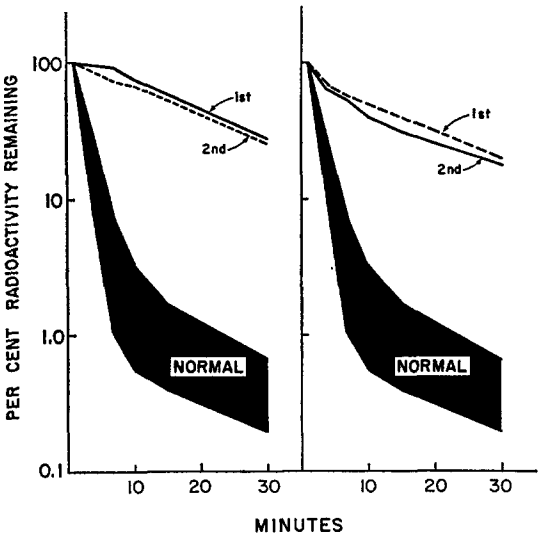
TEXT-FIG. 5. Fate of blockading dose. Significant quantities of the blockading dose were present in the circulation 1 hour after administration of doses of 500 mg/kg or above.



TEXT-FIG. 6. The removal of chromic phosphate by five normal and four blockaded perfused rat livers. The ranges in each group are depicted by the broken vertical lines.



TEXT-FIG. 7. Effect of particle size on blockade. Blockading doses were maintained at 1000 mg/kg, but the particle size was varied. Each curve represents the clearance observed in three or more animals.



TEXT-FIG. 8. Effect of opsonization on blockade. In the experiment on the left, a tracer dose of ^{32}CP was given 1 hour after blockade. This was followed 1 hr later by a second tracer dose of 0.1 ml ^{32}CP, opsonized in 0.9 ml of fresh isologous serum. In the experiment on the right, the sequence of opsonized and nonopsonized tracers was reversed. In neither instance did opsonization alter tracer clearance.

large in producing blockade if persistence of the blockading dose in the circulation was of importance in the process.

As noted in Text-fig. 7, demonstrable blockade was progressively increased as the particle size of the blockading dose was decreased from 2.5 to 1.0 μ. Moreover, it was possible to reduce the dose of CP necessary to produce blockade by progressive reductions in particle size.

Opsonization Studies.—Smaller particles which more easily induced blockade also presented a significant increase in total surface area of CP. It was thus postulated that large numbers of small particles might more readily deplete the blood of opsonins necessary for CP phagocytosis. Therefore, experiments were conducted to test this hypothesis.

No evidence of the importance of serum opsonins in the clearance of CP could be obtained. In four separate experiments performed in four different animals, incubation of the ^{32}CP tracer in fresh isologous serum failed to improve clearance of the tracer in blockaded animals. One such experiment is shown in Text-fig. 8. Similarly, incubation of the ^{32}CP tracer in homologous serum in three separate experiments failed to alter clearance of the tracer in blockaded rats.

DISCUSSION

The demonstration that particulate chromic phosphate suspended in 5% dextrose in water could produce reticuloendothelial blockade indicates that this phenomenon is not dependent on the presence of exogenous stabilizing materials such as gelatin. As in previous studies (4–8), the RE blockade induced demonstrated significant specificity. Thus, blockade could not be explained on the basis of saturation of the entire phagocytic cell population of the RE system. The fact that isolated perfused "blockaded" livers could take up chromic phosphate in a fashion identical to normal livers appeared to eliminate saturation of particle-specific cell clones as a likely explanation for the delays in tracer clearance observed. Blockade could not be explained on the basis of depletion of serum opsonins. Fresh isologous serum utilized to assure the replacement of opsonin identical to that which had presumably been depleted by the blockading dose failed to eliminate impaired clearance of tagged ^{32}CP. Furthermore, the use of nonisologous sera also failed to overcome blockade although it has been suggested that incubation with foreign serum may change the particle surface to one for which opsonins are still present in the blood stream (5). Thus our experience was similar to that of Biozzi and his coworkers, who were unable to demonstrate a role for serum opsonins in RE blockade (3).

For at least the first several hours following its induction, RE blockade with chromic phosphate appeared to relate more to those particles still in circulation *outside* the cells than to those within. This finding is similar to that reported in previous studies utilizing gelatin as the blockading agent (7).

The effect of particle size on blockade was striking. It has been shown that

the efficiency with which particulate material is cleared by the RE system is directly related to the size of the particles injected (4, 12–15). Small particles tend to remain in circulation longer. Thus, the apparent enhancement of blockade resulting from reductions in particle size might relate to the prolonged presence of small particles outside the cells. Perhaps sufficient numbers of particles external to phagocytic cells adversely affect cell membrane recognition phenomena. On the other hand, reductions of particle size are accompanied by increases in particle numbers when dosage is held constant. Thus, it is conceivable that blockade occurs because membrane-binding sites are saturated rather than from physical cellular stuffing when increased numbers of particles are presented to the RE system. The demonstration of normal clearance by blockaded livers in these and previous studies (7) might result from the removal of significant amounts of blockading material from such binding sites by the preliminary buffer perfusion. As a result, binding sites would again become available for subsequent tracer attachment. The presence of specific cellular binding sites for specific particle types might also explain the phenomenon of specificity.

The doses of chromic phosphate required to produce blockade in the present studies were much greater than those utilized by Murray, who demonstrated blockade with 40 mg/kg of untagged and presumably stabilizer-free chromic phosphate (5). Particle size differences may provide the explanation for this disparity. In the present studies blockade was enhanced by reducing particle size and the dose of material required to induce blockade could be decreased with decreasing particle size.

This study has focused on early blockade. Few other investigators have concerned themselves with the fate of the blockading dose itself. The present studies show that early blockade is detectable only while the blockading material is still present in the circulation. Since chromic phosphate remains inert within the RE cell (16) and is not catabolized like albumin or gelatin (6), it is probable that evidence of late "blockade" persisting beyond clearance of the blockading dose has some explanation other than that set forth in these studies.

SUMMARY

The mechanism of the reticuloendothelial "blockade" which followed injection of large quantities of chromic phosphate without exogenous stabilizing material was investigated in Wistar rats.

The RE blockade observed for several hours after induction appeared related to the continuing circulation of the chromic phosphate–blockading dose, and a reduction in the size of the particles used enhanced blockade.

RE blockade appeared to be particle specific and was not related to a generalized depression of RE-phagocytic cell function.

Studies in isolated perfused rat livers appeared to eliminate saturation of particle-specific macrophage clones as a likely explanation of blockade, and blockade could not be explained on the basis of depletion of serum opsonins.

In the system employed, it is postulated that blockade occurs when large numbers of circulating particles saturate specific macrophage cell membrane–binding sites rather than from physical stuffing of RE-phagocytic cells.

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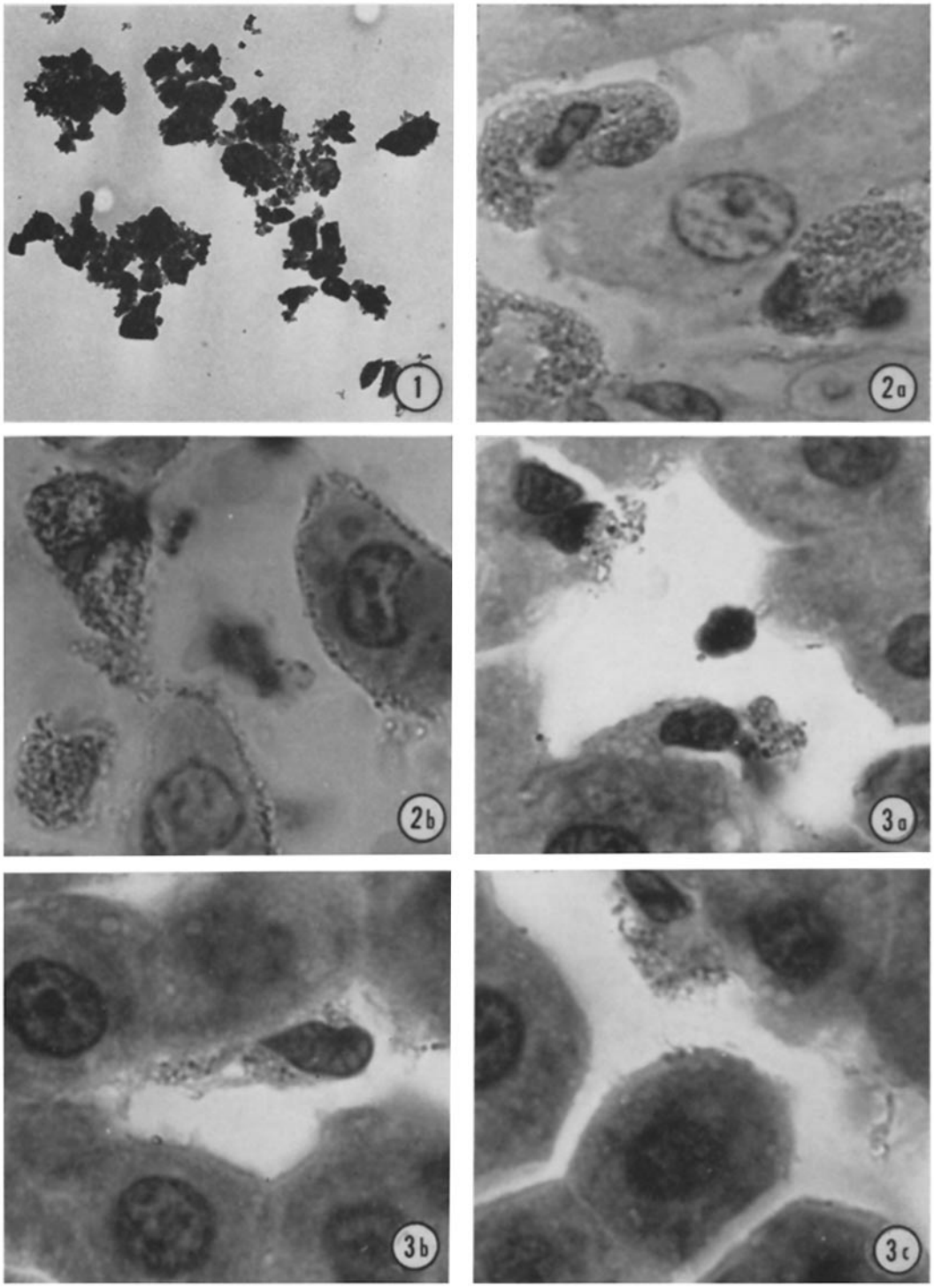
EXPLANATION OF PLATE 85

FIG. 1. Electron photomicrograph of chromic phosphate in distilled water. The heterogeneity of the particle population is evident. The majority of the particles measure approximately 0.9 × 0.9 μ, while the largest are 0.9 × 1.4 μ. Note, however, the presence of many smaller particles. Some clumping occurs in the electron microscopy–fixation process. × 6160.

FIG. 2 *a*. The appearance of the liver 1 hr following the intravenous injection of 1000 mg/kg of untagged chromic phosphate. Note the chromic phosphate particles within Kupffer's cells lining hepatic sinusoids. × 2000.

FIG. 2 *b*. The appearance of the liver 1 hr following the intravenous injection of 1000 mg/kg of untagged chromic phosphate. Note that some chromic phosphate is within Kupffer's cells, some lies free within the lumen of the sinusoids, some adheres to the surface of Kupffer's cells, and some lines the endothelium of the sinusoids. × 2000.

FIG. 3 *a*–3 *c*. The appearance of the isolated perfused liver after perfusion of tracer amounts of radiolabeled chromic phosphate. Note the chromic phosphate within hepatic Kupffer's cells and also adhering to the endothelial lining of hepatic sinusoids. × 2000.



(Drutz et al.: Mechanism of reticuloendothelial blockade)