tion of migration of fibroblasts with 10, 25, 50, and 100 μg/ml quantities of cortisone (free alcohol) and hydrocortisone and concluded that amount of inhibition was directly related to concentration of the steroids.

The formation of cytoplasmic vacuoles in fibroblasts grown together with cortisone or hydrocortisone has been observed by other workers. Paff and Stewart(7), who precipitated crystals of cortisone (free alcohol) by evaporation on coverslips before addition of nutrients and chick embryonic dermal fragments, reported that the first apparent reaction of the fibroblast to the hormone was formation of cytoplasmic vacuoles. Steen(1), using hanging drop cultures containing cortisone acetate, also observed cytoplasmic vacuoles in fibroblastic outgrowth from chick embryo heart. The presence of large fat vacuoles in an unusually early stage of growth was noted by Grossfeld and Ragan(3) in chick embryo cultures submitted to the action of soluble hydrocortisone.

The failure of cortisone or hydrocortisone to influence the mitotic index of rat fibroblasts or alter distribution of the 4 phases of mitosis accords with the work of Grossfeld and Ragan(3), who found no arrest of mitosis in any particular phase.

Summary. 1) The effect of cortisone acetate and hydrocortisone (free alcohol) on growth of L strain mouse fibroblasts was studied. 2) Addition of 10 μg/ml of either steroid to the culture fluid, after 11 days of incubation, resulted in a decrease in total cell count when compared with counts in control cultures. Hydrocortisone was the more inhibitory agent. 3) The effect of 3 concentrations of hydrocortisone (10, 20, and 50 μg/ml) on growth of fibroblasts over a 14-day period was established. The most marked inhibitory effect was produced during the first 7 days of culture. Fifty micrograms per ml were approximately twice as inhibitory as the lesser amounts of hydrocortisone. 4) Deviations from normal morphology of fibroblasts were seen in the steroid-treated cells. The cell processes were shortened, cells were swollen and flattened and tended to cohere in sheets. A great number of giant cells, often multinuclear or with nuclei irregularly shaped, were present. The cytoplasm contained many vacuoles.

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Phagocytosis of Periodate-Treated and Virus-Coated Red Blood Cells By White Blood Cells *in vitro*. (23224)

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Hemolytic anemia has been reported in virus diseases, such as infectious mononucleosis, virus pneumonia, Coxsackie A infection, influenza A infection and measles (reviewed by Dacie(1)). Several viruses are known to be adsorbed to red blood cells, and cause irre-

versible changes in their membrane(2). It has been shown that treatment of red cells with virus may render them antigenic and agglutinable by normal and immune serum(3, 4). Recently it was reported by Stewart, Petenyi and Rose(4) that canine erythrocytes treated with influenza virus had a shortened lifespan when infused into normal dogs. In some viral diseases, infectious mononucleosis, virus pneumonia and encephalitis due to poliomyelitis virus(5,6) leucocytes containing red cells have been found in the peripheral blood. Mabry and co-workers(7,8) demonstrated that treatment of human and rabbit erythrocytes with influenza or Newcastle disease virus increased their susceptibility to phagocytosis by rabbit spleen macrophages in tissue culture in the presence of serum opsonic factors.

The present study reports effect of influenza-virus treatment of human red cells on their subsequent susceptibility to phagocytosis by human white blood cells *in vitro*.

Methods. Leucocyte suspension. 5 ml samples of heparinized venous human blood were incubated at 37°C for 45 minutes in test tubes (100 × 13 mm) slanted at 45°, allowing the red cells to sediment. The supernatants were pooled and transferred into a siliconized (Dow Corning D.C. 200) test tube and centrifuged (1500 rpm for 30 seconds). The leucocyte sediment was washed twice with a 10-fold volume of phosphate-buffered saline (pH 7.2) and resuspended in the buffered saline to a concentration of about 1.5×10^7 leucocytes per ml. The leucocyte suspensions were kept at room temperature and used within 3 hours of drawing of the blood. *Red cell suspension.* The sedimented red cells were washed twice with buffered saline and a 2% suspension prepared. *Treatment of red cells with periodate.* To 1 ml of a 2% red cell suspension was added 1 ml of a 0.01 M sodium periodate solution in buffered saline. The mixture was kept at 4°C for 20 minutes and then 0.05 ml of 10% glucose was added to arrest the action of periodate. After 3 minutes at room temperature the cell suspension was centrifuged, the sediment washed with 2 ml buffered saline and resuspended in 1 ml of this medium. *Serum deficient in periodate-agglutinin.* Periodate-treated human red cells are agglutinated by compatible human serum(9,10). The periodate agglutinin responsible for this phenomenon was removed from the serum by repeated adsorption at 4° C for 15 minutes with periodate-treated red cells at a final concentration of 2% (generally 8 to 9 adsorptions were required). *Destruction of virus receptors on red cells by receptor destroying enzyme (R.D.E.).* Red cells were treated with R.D.E. according to Burnet and Stone(11). *Virus.* FMl strain of influenza virus in form of infected allantoic fluid (50th-80th chicken embryo passage) or red cell eluate in buffered saline was used. The virus was concentrated and purified according to the method of Francis and Salk (12). The hemagglutinin titers of the suspensions were determined in serial dilution of 1.5-fold steps with a 1% human O-red cell suspension. The virus suspensions were stored at 4°C as long as the titer did not change (not more than 5 days). *Adsorption, elution and inactivation of the virus.* For adsorption of the virus on the red cells, to be used in the phagocytosis experiments, a 2% suspension of red cells was incubated in the virus suspension for 20 minutes at 4°C. When desired, the virus suspension was eluted from the red cells as follows: the cells with the adsorbed virus were centrifuged in the cold, the supernatant was replaced with fresh phosphate buffered saline and the suspension incubated for one hour at 37°C. Subsequently the cells were thrown down, the supernatant was discarded and the cells resuspended for phagocytosis. The supernatants were titrated for presence of hemagglutinins, to insure that adsorption and elution were successful. Inactivation of the virus by heat was done by incubating the virus suspension for 30 minutes at 56°C. *Erythrophagocytosis experiment.* One drop of the red cell suspension was mixed with one drop of the leucocyte suspension and one drop of serum on a watch glass. The mixture was incubated in a moist chamber at 37°C. Smears were prepared from the sedimented cells and stained with Giemsa. The results were expressed as erythrophagocytic index, *i.e.* the percentage of granulocytes plus monocytes with at least one

red cell engulfed. A total of 500 granulocytes plus monocytes was examined in fields where clumping of white cells and of red cells was minimal.

Results. Erythrophagocytosis of virus-treated red cells. Normal red cells or red cells onto which virus had been adsorbed, were not phagocytized by leucocytes in the presence of compatible (in regard to A, B agglutinin) serum, when incubated at 37°C for periods up to one hour. Similarly red cells from which the virus had been eluted or red cells treated with receptor destroying enzyme, were not phagocytized. In order to prevent elution of the virus from the red cells, during the phagocytosis experiment, three different methods were used. a) *Decreased temperature.* No phagocytosis of virus-coated red cells occurred on incubation of the erythrophagocytic system at 21°C for 20 minutes; at this temperature and time the elution of virus was negligible. At 21°C leucocytes phagocytized red cells in the presence of incompatible serum (erythrophagocytic index 12%). b) *Heat-inactivation of virus.* Red cells coated with heat-inactivated virus were not phagocytized. c) *Pretreatment of the red cells with periodate* (Table I). Red cells treated with periodate were readily ingested in the presence of compatible whole serum; in some experiments the erythrophagocytic index reached 40%. It was found that for the phagocytosis of periodate-treated red cells, the presence of serum was essential. The periodate-treated red cells in the erythrophagocytic system showed marked agglutination. Further it was found that serum made deficient in periodate agglutinin had lost its opsonic property for periodate-treated red cells. When serial dilutions of whole serum were made with either saline or serum deficient in periodate agglutinin, the agglutinating and opsonic activities disappeared at about the same dilution.

Adsorption of virus on periodate-treated red cells did not increase the erythrophagocytic index in the presence of whole serum, over that of red cells treated with periodate only; actually a decrease of the erythrophagocytic index was observed, probably due to massive clumping of the periodate-treated, virus-coated red cells, occurring in the presence of periodate agglutinin. However, in the absence of periodate agglutinin, coating with virus of the periodate-treated red cells induced marked erythrophagocytosis, while without virus periodate-treated red cells were ingested to a small extent only or not at all.

Disappearance of the virus in the erythrophagocytic system. When red cells (*not* treated with periodate) onto which virus was adsorbed, were incubated with leucocytes and serum for periods up to one hour at 37°C, no virus could be detected in the supernatant of such a mixture, although during this period elution of the virus from red cells proceeded. The disappearance of the eluted virus could be due to its adsorption on the leucocytes or to phagocytosis. The experiments of Boand, Kempf and Hanson(13) point to the second possibility.

Discussion. The observations show that neither the presence of influenza virus nor destruction of viral receptors on the red cell was sufficient to induce erythrophagocytosis by white blood cells *in vitro*. However when adsorbed onto periodate-treated red cells, the virus had a marked erythrophagocytosis promoting effect, manifest in the absence of periodate agglutinin. In view of the experiments of Nelson(14) on phagocytosis of bacteria adsorbed onto red cells, it seems quite feasible that in the erythrophagocytic system, containing reversibly adsorbed or heat-inactivated virus, the virus was phagocytized off the red cells. Nelson(15) had indeed suggested that virus adsorbed on red cells would be more readily phagocytized than virus free in suspension. It is possible that in the case of periodate-treated, virus-coated red cells, to which the virus is more firmly attached, the leucocytes try to engulf the virus, but cannot detach it from the red cells and ingest them both together. Final proof for such a mechanism may be possibly furnished by electron microscopy. Another explanation may be that the periodate-treatment and the adsorption of virus damage the red cells to an extent that the phagocytes ingest them as foreign bodies.

The role of specific antibodies in the phagocytosis of virus-coated red cells has not been satisfactorily elucidated. In all sera tested

TABLE I. Phagocytosis of Periodate-Treated, Virus-Coated Red Cells by White Blood Cells *In Vitro.*

Supplemental treatment of periodated red cells	Compatible serum	Erythrophagocytic index, % Exp. 1	Exp. 2	Exp. 3
None	Whole	26	39	38
Virus adsorbed at 4°C	"	19	30	20
None	Periodate-agglutinin deficient	0	3	4
Virus adsorbed at 4°C	*Idem*	11	20	21

by us a certain amount of influenza antibodies (hemagglutination-inhibition) was present. When such antibodies were removed by adsorption with virus-coated red cells the phagocytosis decreased. It was however subsequently found that the antibody adsorption resulted in removal of complement, too. The relative role of specific antibodies and of complement in promoting erythrophagocytosis is still under investigation.

The results obtained here with white blood cells differ from those obtained by Mabry and co-workers(7,8) with rabbit tissue macrophages in tissue culture, as macrophages readily ingested red cells to which influenza virus was reversibly attached. Whether this difference is due to the type of phagocyte or to the medium is not known. Nor is it known whether in the human body irreversible adsorption of virus to the red cell is required for the erythrophagocytosis observed in the peripheral blood in various diseases.

Summary. The presence of influenza virus or destruction of viral receptors on human red blood cells did not induce erythrophagocytosis by human white blood cells in presence of compatible serum *in vitro.* Influenza virus adsorbed onto periodate-treated red cells induced marked erythrophagocytosis in the presence of periodate-agglutinin deficient serum, while periodate-treated red cells without adsorbed virus were not phagocytized.

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