cholesterol levels than those grown on normal sera. 3. In the group of normal human sera there was significant correlation between (a) age of the donor and cholesterol content of cells, (b) serum cholesterol level and cholesterol content of cells and (c) age of the donor and serum cholesterol level. 4. In the atherosclerotic human sera there was significant correlation between serum and cell cholesterol but none with age of the donor. 5. It appears from these results that in an older group of human atherosclerotics at least, there is no evidence that the higher cholesterol content of cells grown on the serum is due to factors other than the increase in serum cholesterol which occurs with aging.

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Increase of Plasma Lysozyme Activity Following Injections of Typhoid Vaccine.* (26699)

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Kerby has shown that white cells incubated *in vitro* with endotoxin from Gram-negative bacteria leak lysozyme into the fluid in which they are suspended(1). The studies reported here were undertaken to see whether injections of endotoxin into rabbits resulting in leukopenia were followed by an increase in plasma lysozyme activity.

Materials and methods. The lysozyme activity of rabbits' plasma was measured after intravenous injection of endotoxin or Newcastle disease virus (NDV). Blood was obtained from unanesthetized rabbits by repeated cardiac puncture using a syringe containing 0.3 ml of 5% sodium ethylene diamine tetraacetic acid/10 ml of blood.† Plasma was obtained free of cells by 2 centrifugations at 1000 rpm for 20 minutes at 4°C, and was stored at 4° until the assay of lysozyme activity was performed. Most of the assays were done within 8 hours after collection of blood, and none was delayed more than 24 hours. Pyrogen free glassware and syringes were used in preparation of plasma.

Lysozyme activity was measured by the rate at which plasma samples cleared turbid suspensions of *Micrococcus lysodeikticus*. Irradiated cell walls of *M. lysodeikticus* ("lysozyme substrate"—Difco Corp., Detroit, Mich.) were diluted in phosphate buffer pH 6.2 ("lysozyme buffer"—Difco) to a concentration of 0.5 mg/ml. Plasma was diluted in buffer 1/20, 1/40, or 1/80. Standard solutions containing 0.8 and 1.0 μg/ml of crystalline egg white lysozyme (Difco) were prepared in buffer.

Four ml of substrate and 4 ml of diluted plasma or standard solution were mixed in a cuvette and optical density was determined at 5 minute intervals in a Bausch and Lomb Spectronic 20 at 540 mμ wavelength. All tests were caried out at 32°C and the reactants were allowed to reach this temperature before mixing. The lysozyme concentration of plasma was determined by comparing the

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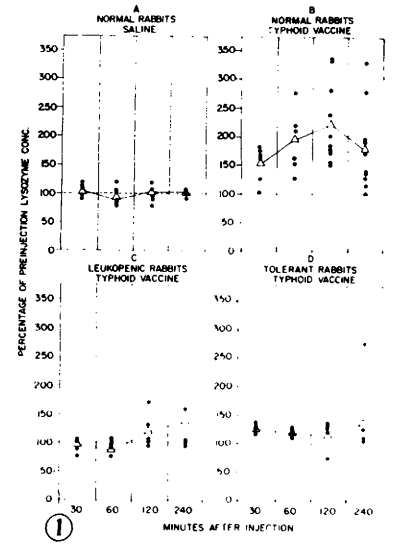
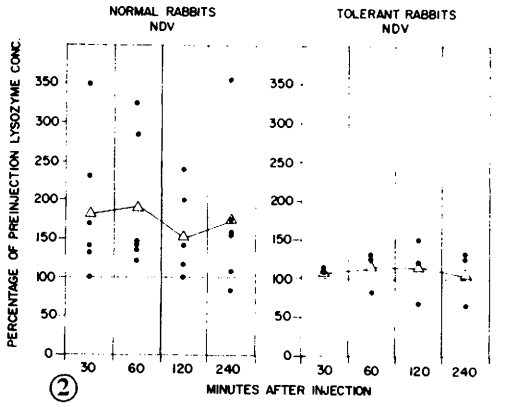
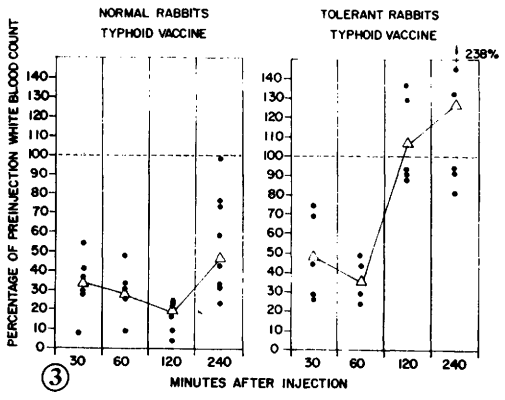
† Plasma of citrated blood had almost no detectible lysozyme activity.







● = individual observations
△ = avg of observations

FIG. 1. Plasma lysozyme concentrations at intervals after intrav. inj. of saline or typhoid vaccine into rabbits.

FIG. 2. Lysozyme concentrations after intrav. inj. of Newcastle disease virus into rabbits.

rate of lysis observed with known amounts of purified egg white lysozyme.

White blood cells were counted in the blood removed from the heart for lysozyme assay.

Results. Injection of endotoxin into normal rabbits. The resting lysozyme concentration in 27 rabbits varied from 4 to 36 μg/ml with an average of 11.1 μg/ml $\pm$ 8.0 (S.D.). Because of the variation the data are recorded as percentage of pre-injection lysozyme concentrations.

Intravenous injection of 4×10^8 heat killed *Salmonella typhosa* in 1.0 ml of saline into 13 rabbits was followed without exception by leukopenia (Fig. 3) and by an increase in plasma concentration of lysozyme (Fig. 1B). The maximum increase was noted 2 hours after injection, and by 4 hours the concentration of lysozyme had declined slightly. The increases in lysozyme concentration in animals injected with typhoid vaccine roughly paralleled the degree of leukopenia observed. Three rabbits injected with 1.0 μg of a purified endotoxin from *Salmonella abortus equi* (supplied by Dr. Otto Westphal) showed similar increases in lysozyme concentration suggesting that endotoxin was the agent in the typhoid vaccine responsible for the rise in concentration. The rabbits injected with pyrogen free saline had little change in lysozyme concentration (Fig. 1A) and no change in white blood cell count.

Leukopenic rabbits. Because of the studies of Kerby(1) and the correlation of the increased lysozyme concentrations with development of leukopenia it seemed likely that the white blood cells were a major source of the lysozyme released into the plasma. Five rabbits were made leukopenic by a single intravenous injection of 6.0 mg of nitrogen mustard ("Mustargen"—Merck, Sharp & Dohme). When the concentration of circulating granulocytes in the blood was less than 1000/mm^3 (usually about 48 hours after injection of nitrogen mustard) the animals were injected intravenously with typhoid vaccine in the same dose used for

FIG. 3. White blood counts in blood of normal and tolerant rabbits following inj. of typhoid vaccine.

normal rabbits, and lysozyme concentrations were measured. The pre-injection lysozyme concentration in the plasma of these rabbits was in the normal range. At 30 and 60 minutes after injection of typhoid vaccine there was *no increase* in lysozyme concentration. At 120 and 240 minutes there was only slight increase to an average of 120% of the preinjection value (Fig. 1C). These results suggest that the lysozyme released into the plasma comes from white cells, damaged in some way by typhoid vaccine.

Tolerant rabbits. Rabbits repeatedly injected with endotoxin gradually become refractory to many of the actions of endotoxin, and it seemed possible that lysozyme release might also be decreased in tolerant rabbits. Five rabbits made tolerant by daily intravenous injections of 4×10^8 heat-killed *S. typhosa* for 2 weeks were studied for the effect of a single injection of typhoid vaccine on lysozyme concentration in the plasma. Three days elapsed between the last injection of the 2-week course and the study of changes in lysozyme concentration brought about by another intravenous injection. There were small increases in lysozyme concentration in the plasma of tolerant rabbits but much less than in non-tolerant rabbits (Fig. 1D). An incidental finding was the short duration of leukopenia in tolerant as compared to non-tolerant rabbits (Fig. 3).

Injection of Newcastle disease virus. It seemed of interest to see whether lysozyme concentration changed in rabbits following injection of other agents. Preliminary studies on rabbits injected intravenously with 512 hemagglutinating units of NDV showed an increase in plasma lysozyme activity as great, in some rabbits, as the increase in rabbits injected with endotoxin (Fig. 2). The peak of the rise was 60 minutes after injection and was not accompanied by a marked leukopenia. Animals made tolerant to the pyrogenic action of NDV by an injection of 512 hemagglutinating units 24 hours before challenge responded with a smaller increase in lysozyme concentration (Fig. 2).

Discussion. These data indicate that plasma lysozyme concentration can be increased by injections of endotoxin from Gram-negative bacteria. It seems likely that the source of the enzyme is damaged granulocytes in view of the finding by Kerby(1) of a release of lysozyme from granulocytes incubated *in vitro* with endotoxin. The lack of increase in lysozyme concentration in granulocytopenic rabbits after endotoxin injection adds further support to this view.

Atkins and Wood(2) have shown that after injection of endotoxin into rabbits an "endogenous pyrogen" appears in the serum that can cause fever when injected into other rabbits. This substance was not found when endotoxin was injected into granulocytopenic rabbits and they hypothesized that white cells were the source of "endogenous pyrogen." The experiments reported here give further indication that white cells in animals injected with endotoxin are damaged and leak some of their contents.

Tolerant rabbits do not show an increase in lysozyme activity after injection of endotoxin. This may result merely from the rapid removal of endotoxin from the blood of tolerant rabbits making it inaccessible to white blood cells. Another possibility put forward by Cluff(3) is that white cells from tolerant animals are relatively resistant to damage by endotoxin. It is interesting that the endotoxin-induced leukopenia in tolerant rabbits is of shorter duration than that in non-tolerant rabbits even though the immediate leukopenia is just as marked. If white cells of tolerant rabbits are not damaged by endotoxin they may be only sequestered outside the circulating blood (accounting for the immediate leukopenia) but not destroyed. The undamaged leukocytes then may return to the blood stream. On the other hand cells from non-tolerant rabbits may be sequestered and destroyed resulting in a prolonged leukopenia.

Summary. 1. Intravenous injection of typhoid vaccine, purified endotoxin and Newcastle disease virus into rabbits results in elevation of plasma lysozyme concentrations. 2. This elevation of lysozyme activity after injection of typhoid vaccine is not seen in leukopenic rabbits or in rabbits made tolerant to endotoxin. 3. Leukopenia in non-

tolerant animals injected with endotoxin is prompt and is still present at 4 hours after the injection. In tolerant rabbits the leukopenia is apparent immediately, but by 2 hours after injection the white cell count has returned to normal levels.

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Various Types of Calcinosis Induced by Egg Albumen or Yolk Following Sensitization with Dihydrotachysterol (DHT).* (26700)

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In rats pretreated with dihydrotachysterol (DHT), it is possible to induce intense local calcinosis anywhere on the skin surface by topical application of mechanical trauma(1) or subcutaneous injection of $FeCl^3$(2). The lesions so induced are somewhat reminiscent of the calcifying form of scleroderma (the "sclérodermie calcaire" of French dermatologists). Recently we found that subcutaneous injections of either egg white or egg yolk are likewise highly effective in eliciting cutaneous calcinosis, following DHT sensitization, although neither of these substances produces any significant local damage (*e.g.*, inflammation, hemorrhage or necrosis). It appears that the active principles of the egg —like $FeCl_3$—act somewhat like mordants, in that they prepare tissues for subsequent uptake of calcium salts. Even when administered intraperitoneally or intravenously, both egg white and egg yolk (unlike $FeCl_3$) are well tolerated by rats; hence, they enabled us to study the influence of systemically applied vital mordants for calcium upon DHT-sensitized animals.

Material and methods. Fifty female Holtzman rats, with a mean initial body weight of 97 g (range: 92-105 g), were subdivided into 5 equal groups. All animals were given 1 mg of DHT ("Calcamin," Wander), in 0.5 ml of corn oil, by stomach tube, once on the first day. The rats of *Group I* acted as controls and received no other treatment, while those of the remaining groups received solutions of either egg white or egg yolk, 24 hours after the DHT. The solutions were prepared by mixing the albumen or yolk of the eggs of domestic fowl with an equal amount of distilled water. The resulting thick fluids tend to form stringy precipitates upon standing and do not pass readily through ordinary filter paper; hence, they were filtered first through cotton and then through tissue paper ("Kleenex"®). The individual dose was invariably 5 ml, egg white being given intraperitoneally to *Group II* and intravenously to *Group III*, while egg yolk was administered intraperitoneally to *Group IV* and intravenously to *Group V*. The animals were kept on "Purina Fox Chow" and tap water throughout the experiments and all survivors were killed with chloroform after 4 days. At autopsy their organs were inspected with a stereoscopic loupe, representative specimens of various tissues being fixed in alcohol-formol for the subsequent histochemical demonstration of calcium with von Kóssa's silver nitrate technic.

Results. Five animals of the group receiving egg yolk intravenously died during the period of observation, but all the other rats supported these injections well, except that egg white produced the usual anaphylactoid reaction during the first few hours after its administration. Even this response was mild, since it is well known that large amounts of

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