a newly developed mammogen extracted from IR (mammogen "C") and lactogenic preparation produced lobule-alveolar development. Extracted mammogen "C" was white, crystalline, apparently salt-free and water soluble. Crude and purified FSH and growth hormone exhibited little or no mammogenic effects when injected with a synergizing dose of estradiol benzoate in ovariectomized mouse.

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Ingestion of Latex Particles by Chicken Fibroblasts in Tissue Culture.* (26788)

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The ingestion of foreign particles by certain cell types both in intact tissue and in tissue culture has been studied extensively and generally referred to as phagocytosis. Cells of the phagocytic type include mammalian granular leucocytes and those of mesoblastic origin commonly called macrophages. These cells not only can ingest bacteria, cell debris and protozoa but also may ingest smaller particles of a colloidal size, this latter phenomenon being called *ultraphagocytosis* or *colloidopexy*. The precise mechanisms of the phagocytic process are not entirely clear and some variations in the manner by which particles are taken in by cells have been described. Thus, in some cases particles appear in cell vacuoles and in others such vacuoles are not seen. Pinocytosis, or "cell drinking," is a second method by which the cell may take in material from its environment. Initially described by Lewis as a mechanism by which fluid enters cells(1), the studies of Mast and Doyle(2), Holter and Marshall(3) and Chapman-Andresen and Holter(4) indicate various materials may enter the cell in this manner. Palade has described a vesicular component of endothelial cells which functions in the pinocytotic process(5) and De Robertis and Bennett(6) have described a similar component for nervous tissue cells. Ferritin molecules are ingested by endothelial cells by the pinocytotic process as reported by Wissig(7), and small (0.014 μ) colloidal gold particles have been reported to be taken in by HeLa cells *in vitro* by Harford *et al.*(8).

No electron microscopic studies have been published of ingestion of foreign inert particles by tissue cells of the fibroblastic type. The present study presents results in which cell uptake of polystyrene latex particles having a diameter of 0.264 μ is demonstrated in tissue cultures of chicken fibroblasts examined by electron microscopy.

Materials and methods. Polystyrene latex suspensions (PSL). Polystyrene latex (PSL) was obtained from Dow Chemical Corp., Midland, Mich., run No. LS-057-A. This lot had a particle size of 0.264 μ and a standard deviation of 0.0060 μ in 577 measurements. Stock suspension of PSL was made in distilled water. PSL content of this suspension determined by direct sedimentation with technics described previously(9,10) was found to be 2.3×10^9 PSL particles/ml. PSL was then diluted further in tissue culture media to obtain the final concentration of inoculum.

Chicken fibroblast cultures. Chicken fibroblast cell suspensions were prepared by trypsinizing tissue of 9-10 day old chicken embryos. These cells were placed in large Rous or T-60 flasks and growth observed

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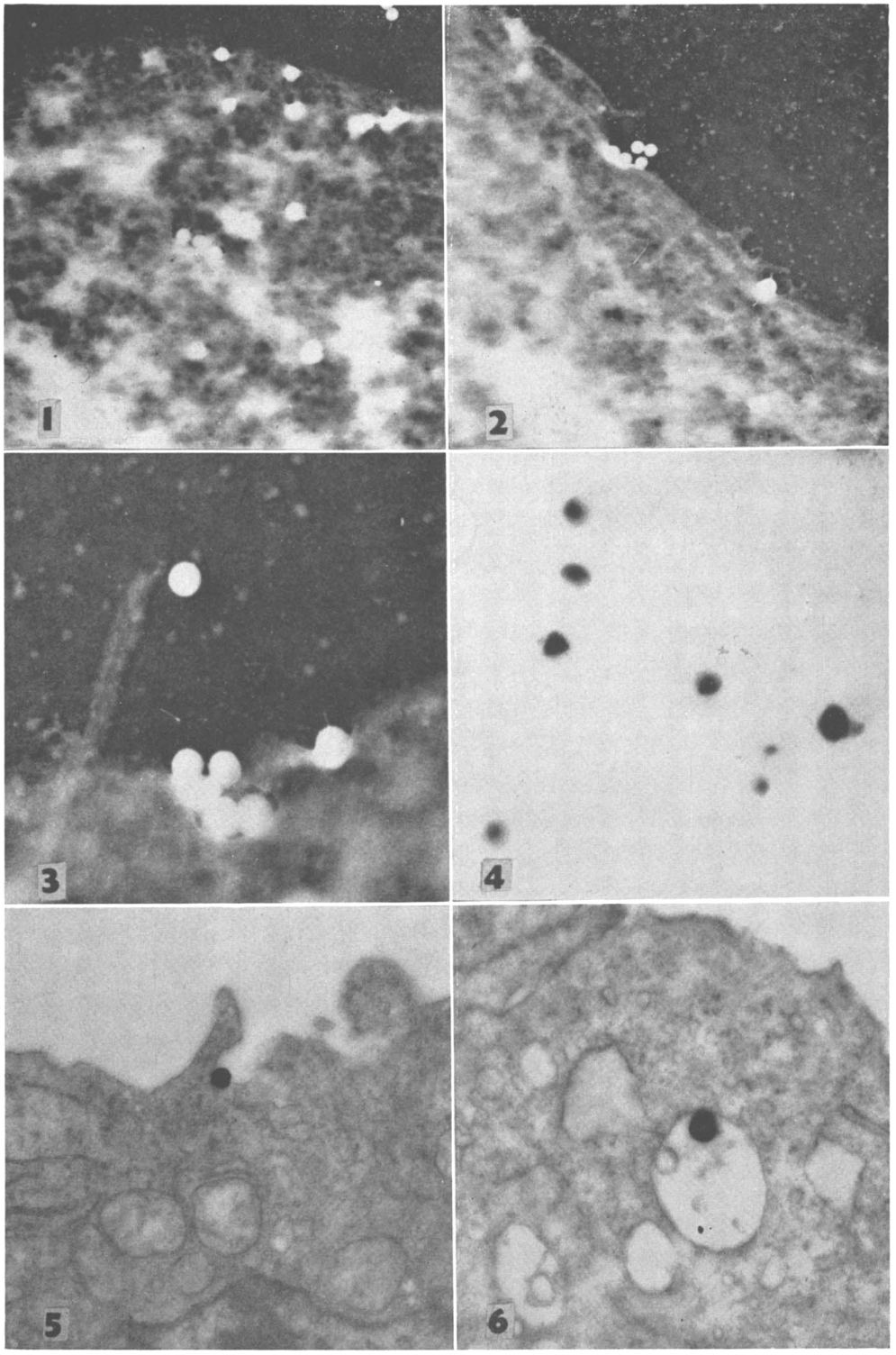

FIG. 1. Portion of chicken fibroblast cell in tissue culture in which polystyrene latex (PSL) particles are seen entering and within the cell. Chromium shadowed. × 7,500.

until monolayers had formed. The cell growth was then trypsinized from the flasks and a suspension diluted to contain 1.5 million cells. This suspension was pipetted into Leighton tubes containing carbon-collodion coated stainless steel grids. PSL was then added in ratios of 50:1 or less latex to cell. Grid mounts were removed, fixed and shadowed as described.

In some instances flask cultures of chicken fibroblasts in a monolayer were inoculated with PSL also in a ratio of 50:1. At various intervals after inoculation of PSL, the cells were trypsinized off the glass, washed 2-3 times and sedimented into a loose pellet. This pellet was then fixed with osmium tetroxide, embedded in araldite and sectioned with a Servall Porter-Blum microtome. Micrographs were taken with an RCA-EML electron microscope.

Results. In the initial experiments, PSL was added to chicken fibroblast cultures and the whole cell mounts removed at the 1 and 30 minute periods showed only a rare particle which appeared to be intracellular. However, cultures removed at the 1 and 2 hour periods or later showed many particles at various stages of entry into the fibroblast as well as large collections of PSL particles in the interior of the cell (Fig. 1, 2, 3). The general condition of cells exposed to polystyrene and observed for periods of 1-4 days showed no changes in their general morphology. Specifically addition of latex to the cultures failed to induce "toxic" changes in the cells.

An additional observation was the finding in whole cell preparations of small spikes of processes on the surface of the PSL particles in the process of engulfment. In some cases where several PSL particles were being engulfed as a group these spikes interconnected these particles. A few particles had very large spikes about 40 mμ in width (Fig. 3); others were considerably smaller. Most PSL balls entering cells showed these processes and none of the extracellular particles or particles not directly or indirectly in contact with the cell showed spikes of any size.

To help resolve the question as to whether the particles observed actually were within the cell, or rather were on top or under it, the following experiments were performed. The cell monolayer was exposed to PSL for 2 hours, trypsinized, washed several times and this cell suspension inoculated into Leighton tubes to allow the cells to reattach to the coated grid surface. These cells were observed until most had put out processes at which time the grid mounts were removed, fixed and shadowed. Photographs of these cells showed a few PSL particles in the processes. Many particles consistent with PSL size and density were seen indistinctly in the interior of many cells. Conversely, no extracellular PSL was observed in any of these preparations indicating that all removable PSL had been washed away leaving only those particles which were inside the cell or very firmly attached to it. In addition, PSL particles sprayed onto fixed cells showed very clear margins and well defined shadows. Thus, the appearance of PSL particles lying on top of cells is quite different from those intracellular particles observed in Fig. 1, 2, & 3.

Various stages of progression of PSL into the cell could be observed. The particles showed no predilection for any particular part of the fibroblast and PSL was observed entering the smooth wall of the cell body as well as the wall of cell processes. Interestingly, out of several hundred cells examined no PSL particles were found entering a microvillus. PSL particles initially appeared to be pressed against the cell wall and in the

FIG. 2. A single PSL particle with a large spike. Also seen is a group of 5 PSL particles in an early stage of ingestion. Chromium shadowed. × 7,500.

FIG. 3. Another group of 5 PSL particles enclosed in a pocket in the wall of a cell. A solitary particle nearby has a very thin spike. Chromium shadowed. × 18,000.

FIG. 4. PSL particles suspended in agar, fixed, embedded in methacrylate and sectioned. There is a varying degree of distortion of PSL particles. × 18,000.

FIG. 5. Early ingestion stage of PSL by chicken fibroblast. Cell culture fixed in buffered osmium and embedded in araldite. × 18,000.

FIG. 6. This micrograph is typical of many PSL particles ingested by chicken fibroblasts in tissue culture. × 18,000.

next stage the cell wall invaginated and the particle was surrounded by the cell wall (Fig. 2 & 3). As the particle was taken deeper into the cytoplasm the wall closed off forming a vacuole. Not all cells appeared to ingest PSL and of those that did some contained far larger numbers than others. In most instances where the PSL particle was definitely within the cell a vacuole could be seen, but in a few instances no vacuole was visible.

PSL in ultrathin sections of chicken fibroblasts. To demonstrate further the uptake of PSL by chicken fibroblasts, cell cultures were exposed to PSL, the cultures then washed, the cells removed by trypsinization, fixed and embedded for thin sectioning. To aid in identification of PSL in sections a heavy suspension of PSL was embedded in 2% agar, fixed and embedded for thin sectioning. Fig. 4 demonstrates the appearance of the PSL in sections. Some distortion of the usual spherical shape occurred and in some cases partial dissolution of PSL was found.

PSL was demonstrated readily in sections of chicken fibroblasts (Fig. 5 & 6) and the particles were usually located in cell vacuoles.

Rate of adsorption of PSL by chicken fibroblasts. In an effort to determine the rate at which PSL was taken up by the fibroblasts the following experiments were done. PSL was added to groups of T-60 tissue culture flasks containing 12 to 30 million cells per flask. Amount of PSL was determined by sedimentation counting(9,10) and concentration per flask adjusted to give a ratio of PSL/cells of 20:1, 5:1 and 3:1. PSL was also inoculated into cell free media for a control. Samples of supernate from each flask were removed at 1 minute, 30 minutes and 2, 4, 7, and 24 hours, pooled and PSL particle counts made on each pool. Each pool represents aliquots from 5 T-60 flasks.

Table I gives results of these experiments. No significant adsorption of PSL could be demonstrated, however, if 15% or less were removed it would not be detectable since this is the standard deviation of this method(9).

TABLE I. Rate of Removal of Polystyrene Latex (PSL) Particles from Tissue Culture Media by Chicken Fibroblasts.

Interval after addition of PSL to culture	PSL content of tissue culture media $\times 10^7$ PSL/cell ratios* 20:1	5:1	3:1
Media control	7.0	7.0	4.0
1 min.	6.5	6.2	3.6
1 hr	7.1	6.5	—
2 "	7.0	6.4	3.3
4 "	6.7	6.3	3.6
7 "	7.2	6.4	—
24 "	6.5	6.0	3.2

* Ratios calculated to result after adding a determined number of PSL particles to flasks of cells. Flask population estimated from cell counts of replicate flasks in same experiment.

The difference in counts between media control and culture samples was not significant. As PSL obviously is taken in by some cells based on visual examination, one can conclude that the overall rate of uptake is relatively low. This could hardly be otherwise since the cells in the monolayer would not be in direct contact with most of the PSL in suspension in the media. Measured uptake of PSL might be demonstrable if the PSL/cell ratio could be reduced to well below 1:1 but this has not been possible to date. The 3:1 ratio was close to the minimum PSL which could be added for accurate sedimentation counting.

Discussion. The present studies clearly demonstrate the capacity of polystyrene latex particles to enter the chicken fibroblast *in vitro* but the mechanisms involved are obscure. Primarily, the question raised is whether the process described represents phagocytosis by a cell generally considered non-phagocytic or pinocytosis in which the PSL particle is taken in during the drinking process. The invagination of the cell wall and vacuole formation surrounding the PSL particles would suggest a pinocytotic process as originally described by Lewis and since investigated by various workers. Palade(5) and Bennett(11) propose a process of "membrane flow and membrane vesiculation" which appears applicable to the present findings. This suggests that particles engaged by the cell wall (by mechanisms unknown)

are trapped in a recess formed as the wall slides around it.

It is of interest that a particle as large as the PSL used is taken into the cell by an apparently pinocytotic process. PSL of 0.264 μ diameter was used because this size is almost identical to that of vaccinia virus (.230 $\times$ .260 μ). In studies of virus particle entry into the cell a model system was sought whereby inert (PSL) and biologically active (vaccinia) material could be compared. It was thought that the inert PSL would remain extracellular but obviously, as the present experiments show, it does not. If the chicken fibroblast can ingest, probably by pinocytosis, PSL particles, it would seem likely that the entry of other particles, including viral, can occur by the same process (*e.g.* pinocytosis). The point of greatest importance, then, would not be whether a particle of 0.264 μ or less can enter a cell but what further activity the particle can stimulate after it becomes intracellular.

The PSL used in the present experiments is larger than most colloidal particles but certainly small enough so that the particles do not settle out over a period of days. PSL added to the media used in these experiments and allowed to stand showed no significant sedimentation after 2 weeks. Moreover, PSL sedimented from media showed no unusual amount of clumping. In the PSL adsorption experiments, therefore, it seems certain that few of the particles settle out during the short period of time involved. Rate of PSL uptake indicates that of the total number inoculated less than 15% are removed from the supernate fluid. Thus, although many PSL particles are seen within some cells this represents only a small portion of the whole number of particles exposed to the culture. Evidence of this type suggests that ingestion of these particles is more passive than active and would support the mechanisms of particle ingestion proposed by Palade and Bennett, and would argue against a phagocytic process.

PSL has many special advantages for studies of particle uptake by cells and has been used by Sbarra and Karnofsky in studies of leucocyte metabolism during phagocytosis(12). The high degree of uniformity of size, shape and density make it easily recognizable in the electron microscope. The PSL used in the present experiments had a variation of only $\pm$ .006 μ in particles whose average diameter was 0.264 μ. Total number of PSL particles can be measured easily by calculation from dry weight of sample, by the spray droplet method of Backus and Williams(13) or by sedimentation counting as was done in the above experiments. Finally, PSL can be obtained in sizes ranging from 80 to 1,000 or more millimicrons per lot. These characteristics make feasible a variety of cell-particle experiments of both a qualitative and quantitative nature relating to the general problem of how extracellular material is ingested by cells.

Summary. Ingestion of polystyrene latex (PSL) particles measuring 0.264 μ in diameter has been demonstrated in chicken fibroblasts *in vitro*. PSL was added to cultures of chicken fibroblasts grown on carbon-collodion coated steel grids and these mounts removed at intervals of ½ to 24 hours. The fixed and metal shadowed whole cell preparations revealed PSL in various stages of entry into these cells. PSL was found in invaginations of the cell wall and in vacuoles in the cytoplasm. Sections of cultured fibroblasts inoculated with PSL revealed PSL particles in cell vacuoles. The rate of particle uptake, however, was too low to be measured by the method used.

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Studies Suggesting the Presence of Intrinsic Factor in Bile.* (26789)

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Evidence has previously been presented suggesting the presence of a "circulating intrinsic factor" involved in selective deposition of B_{12} in the liver(1). The present report exhibits 4 lines of evidence suggesting that intrinsic factor may be present in bile.

Materials and methods. Human bile was collected *via* a mushroom catheter in the gall bladder of a patient with complete occlusion of the common duct by a carcinoma of the head of the pancreas. Rat bile was obtained by means of catheterization,† under temporary anesthesia, of the bile duct of rats thereafter maintained for 6 to 24 hours in a special restraining cage with access to food and water.

Hog intrinsic factor concentrate (HIFC), WES 671 A, and purified HIFC, were obtained from the same source.‡ Their activity in man had been demonstrated by a modified Schilling test(2) showing a standard effect in pernicious anemia with single oral doses of 5 mg and of 0.3 mg, respectively. The Co^{60}-B_{12} used was of specific activity approximately 1 $\mu c/\mu g$.§

The liver homogenate system used has previously been described(3); each of the serial incubations was for ½ hour, in air, at room temperature. Everted sacs of rat small intestine were prepared and incubated during procedures carried out essentially as described by others(4). In all experiments a minimum of 10,000 counts were obtained on each sample. Samples which were less than twice background were counted for 10,000 counts on each of 3 separate occasions to ensure validity.

Antiserum to purified HIFC was prepared by injecting into each footpad of a rabbit 0.5 ml of 0.2 mg/ml purified HIFC weekly for a month, and subsequently harvesting serum at intervals separated by further injections of antigen. Agar double diffusion analysis was performed as described by Ouchterlony (5).

Results. Experiment 1, Table I, indicates that human bile increases the enhancing effect of HIFC on Co^{60}-B_{12} uptake by rat liver homogenate in a sequential incubation system. Exp. 2, shows that human bile by itself enhances Co^{60}-B_{12} uptake by rat liver homogenate. Exp. 3, parts a. and b., suggests that the effect of human bile, like that of HIFC(6), is calcium-dependent; part c. demonstrates that the enhancing effect of human bile following incubation is not due to physical entrapment of Co^{60}-B_{12}-laden bile in the liver homogenate. Thus without incubation liver homogenate showed little more uptake from Co^{60}-B_{12}-laden bile than from NaCl-$CaCl_2$.

Table II indicates that, like HIFC, human

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