

METABOLIC AND MORPHOLOGICAL OBSERVATIONS ON THE EFFECT OF SURFACE-ACTIVE AGENTS ON LEUKOCYTES

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ABSTRACT

Morphological and metabolic observations have been made on the effects of endotoxin, deoxycholate, and digitonin (at less than 50 $\mu\text{g}/\text{ml}$) on polymorphonuclear leukocytes and mononuclear cells. The agents stimulate the respiration and glucose oxidation of these cells in a manner similar to that seen during phagocytosis. Electron microscopy revealed no morphological changes with the first two agents, but dramatic membrane changes were seen in the case of digitonin. Here tubular projections of characteristic size and shape formed on and split off the membrane. All the agents stimulated uptake of inulin, but efforts to demonstrate increased pinocytosis by electron microscopy have not so far succeeded, probably due to limitations in present experimental techniques.

Studies of the biochemical changes that accompany phagocytosis have shown that the ingestion of solid particles by polymorphonuclear leukocytes (PMN) and mononuclear cells (MC) is accompanied by a stimulation of metabolism (1-4). Through the use of conventional metabolic inhibitors, it has been possible to establish which increases in metabolism are essential to the phagocytic process, and which appear to be only concomitants (1, 3, 4). In the case of PMN and MC, it has been shown that, although respiration may more than double during phagocytosis, this is merely incidental to the process of ingestion, and not essential. The approximately sevenfold increase in the oxidation of glucose-carbon-1 to CO_2 is also an accompaniment to, rather than an essential part of, the process in PMN (1). Glycolysis is actually the energy-providing pathway for phagocytosis by both PMN and MC (1, 4). In these two types of cell, another important stimulation of

metabolism has been noted during uptake of particles, i.e., increased incorporation of $^{32}\text{P}_i$ into complex phosphatides (4-6).

It might be assumed that these changes in metabolism are associated with the involvement of the cellular membrane in phagocytosis, but it is clearly rather difficult to obtain rigorous evidence concerning the interrelationship between activities of the membrane and metabolic perturbations. The possibility of stimulating phagocytic cells without using solid phagocytizable objects has been considered as a potential aid to a better definition of the situation.

On another level, there is a question as to whether the process of pinocytosis requires the expenditure of metabolic energy by the cell in the same way that phagocytosis does (7). This phenomenon has been thought of by some as not being different, in principle, from phagocytosis, but data have not been available (7). Recently,

Chapman-Andresen has shown that interference with aerobic respiration, the presence of metabolic inhibitors, or a decrease in temperature, depresses pinocytosis in the amoeba (8). Further comparison of pinocytosis and phagocytosis in various cells at the biochemical level is clearly desirable. Concurrent metabolic and morphological observations during stimulation of cellular uptake, in vesicles, of external fluid might indeed provide a means for establishing some of the similarities of, or differences between, pinocytosis and phagocytosis.

In line with the two points made above, we have been interested in the reports of Cohn and Morse concerning the activity of endotoxin in stimulating glycolysis (but not respiration) of rabbit PMN (9), and of Strauss and Stetson concerning the action of endotoxin or antigen-antibody complexes in stimulating the oxygen uptake of samples of whole blood (presumably by leukocytes) (10). We have determined that the effects of endotoxin at the concentrations used may not necessarily be specific to these substances, but could be the result of their surface-active properties. A number of surface-active agents, and in particular digitonin and

deoxycholate, exhibit the same properties as endotoxin with respect to the metabolism of leukocytes. In this study, we have attempted to determine whether the metabolic changes that are induced by the substances mentioned above, and which are qualitatively very similar to those observed during phagocytosis, are accompanied by characteristic changes in the cell membrane.

EXPERIMENTAL

Cells

PMN and MC were obtained from the peritoneal cavities of guinea pigs, as has been described previously (4). In most experiments, the eliciting agent for PMN was polyvinylpyrrolidone (7.5% (w/v) in 0.9% saline). Although the yield was less with this agent than with neutral caseinate, the cells were more suitable to our purposes in that they appeared from the electron microscopy to be in a better state of preservation. In several experiments, mouse peritoneal macrophages were also studied. These were obtained without use of an eliciting agent, by washing the peritoneal cavities of mice of the Harvard strain with physiological saline. Cells were centrifuged in

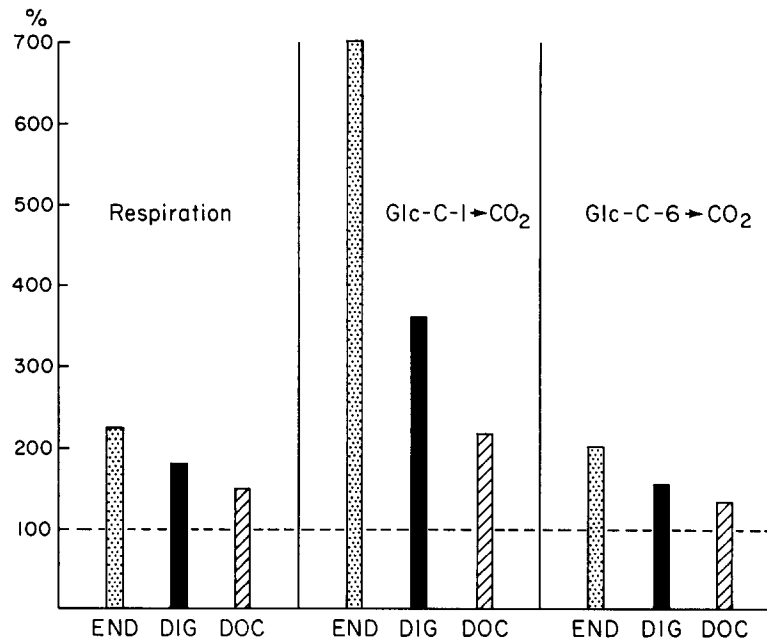


FIGURE 1 Effects of endotoxin (*END*), digitonin (*DIG*), and deoxycholate (*DOC*) on respiration and oxidation of glucose in PMN. The values for control cells are set at 100. The concentration of endotoxin and deoxycholate was 50 $\mu\text{g}/\text{ml}$; that of digitonin, 25 $\mu\text{g}/\text{ml}$. Conditions were as in the Experimental Section. The experiments are representative, except that in this experiment the effects with endotoxin are more marked than usual.

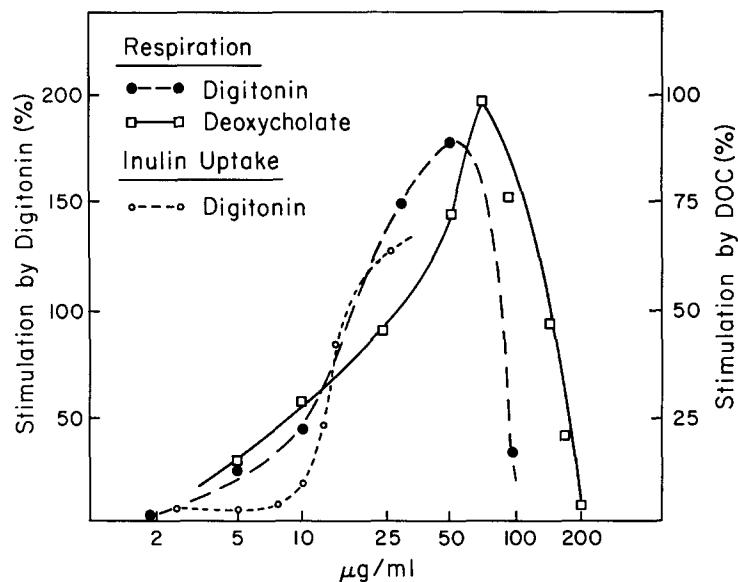


FIGURE 2 Dose-response curve for digitonin and deoxycholate with respect to respiration of PMN. (The curve for the effect of digitonin on the uptake of inulin is also given). The respiratory data were obtained with the Warburg respirometer under conditions given in the Experimental Section. The cell monolayer technique was used in the experiments with inulin- ^{14}C , and this was present at 0.3 mg/ml. It should be noted that the molar amounts of digitonin (mol. wt. 1229) were about three times less than those of deoxycholate (mol. wt. 392). The approximate molarities for 50% stimulation of respiration are 1×10^{-5} M for digitonin and 1×10^{-4} M for deoxycholate.

the cold at low speeds (ca. 100 g) and washed twice with Krebs-Ringer phosphate medium, pH 7.4, before use. They were finally suspended in that medium at a concentration of 10–15%.

Chemicals and Biochemical Agents

Polyvinylpyrrolidone (mol. wt. 360,000) was obtained from Sigma Chemical Co., St. Louis. Endotoxin of *E. coli* was obtained through the generosity of Dr. O. Westphal.¹ Digitonin was purchased from Merck and Co., Inc., Rahway, N.J.; deoxycholic acid was obtained from Fisher Scientific Co., N.Y., and also from Dr. H. Danielsson, who most generously supplied a selection of bile-acids. Allodeoxycholic acid was the kind gift of Dr. A. F. Hofmann.

Glucose-1- ^{14}C , glucose-6- ^{14}C , and inulin- ^{14}C (carboxyl-labeled) were obtained from New England Nuclear Corp., Boston. Phosphate- ^{32}P (as inorganic orthophosphate in 0.1 N HCl) was purchased from IsoServe, Inc., Cambridge, Mass. All other chemicals

¹ In a series of experiments that was carried out in a different context, it was noted that endotoxin prepared from *S. enteritidis* manifested properties similar to those reported here for the endotoxin of *E. coli*.

used were analytical grade, or the best grade procurable.

Chemical and Radioactive Determinations

Glucose was determined by the glucose oxidase method (11) and phosphorus (12) or protein (13) by the methods of Lowry and his collaborators, on a suitable scale. Radioactivity was measured either on planchets, using a Nuclear Chicago Counter Model No. 186A, or by liquid scintillation techniques, using a Packard Tri-Carb Liquid Scintillation Spectrometer. The scintillation fluid was that described by Buhler (14).

Metabolic Measurements

Measurements of respiration were performed as has been described (4). In brief, 1 ml of a 10–15% suspension of the cells in question was added to a Warburg flask together with appropriate labeled substrates, i.e., glucose-1- ^{14}C or -6- ^{14}C . The center well contained 0.2 ml of 20% NaOH. The final volume in each flask was a total of 3.2 ml. The stimulatory agent (or, for controls, an equal volume of Krebs-Ringer phosphate medium) was usually placed in the side arm and tipped after equilibration of the flasks (10 min). Where desired, chemical measurements were made at the termination of the

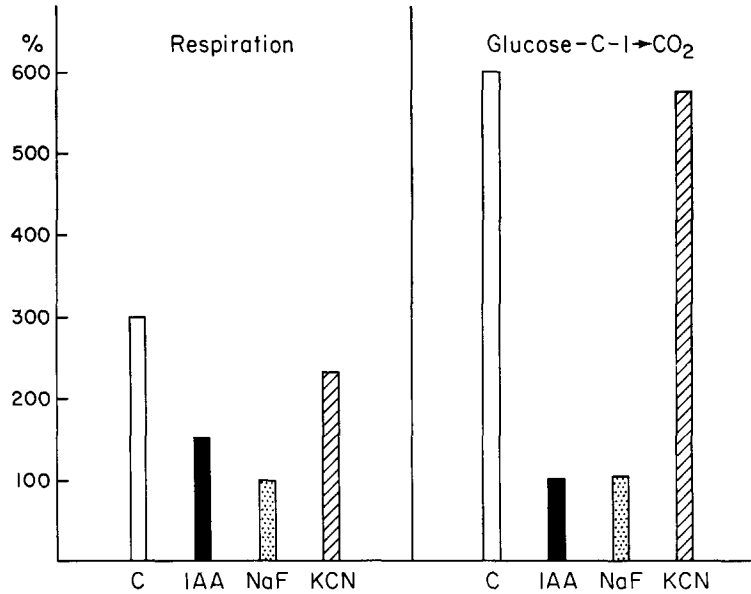


FIGURE 3 Effect of metabolic inhibitors on respiration and glucose oxidation by PMN stimulated with endotoxin ($50 \mu\text{g}/\text{ml}$). Values for cells in the absence of endotoxin are set at 100. *C* represents the control, i.e., cells stimulated with endotoxin, but in the absence of inhibitors. *IAA* represents iodoacetate 2×10^{-5} M; *NaF* is sodium fluoride 2×10^{-2} M; *KCN* is potassium cyanide 1×10^{-3} M.

incubation. When the incorporation of inorganic ^{32}P into phosphatides was examined, cells were incubated in Erlenmeyer flasks on a Dubnoff constant temperature bath at 37° . The proportions of cells and substrates, etc., were as in the respiratory experiments; the radioactive phosphate was added just prior to the aliquot of stimulating agent (or for controls, an equal volume of medium). Approximately $500 \mu\text{c } ^{32}\text{P}_i$ was suitable for 10 ml of final medium. At the end of the incubation, the cells were recovered by transferring the flask contents to a 50-ml centrifuge tube and diluting with a threefold volume of cold nonradioactive Krebs-Ringer phosphate medium. The cells were centrifuged at less than 100 *g*. They were washed twice with volumes of Krebs-Ringer phosphate medium, each equal to the original volume of the incubation medium, and the lipids were then extracted and purified according to the methods of Folch et al. (15). Phosphorus determinations were made on the lipid (12), and aliquots were plated and counted. Individual phosphatides were separated by thin-layer chromatography according to the "basic plate" procedure of Skipski, Peterson, and Barclay (16). Thin-layer plates were stained by exposure to iodine vapor, and the appropriate yellow zones separated and analyzed for phosphorus by a combination of the methods of Lowry et al. (12) and of Berenblum and Chain (17). The absorbancy of the final isobutanol solution of

the phosphomolybdous acid was determined, and aliquots were plated on planchets and counted. Details of these determinations will be given elsewhere².

Entry of Inulin into Cells

The effect of various agents on the uptake of extracellular fluid into cells was determined by the use of radioactive inulin- ^{14}C . Two techniques were used. In the first, cells were incubated in suspension, and after the incubation with the radioactive inulin (ca. 0.3 mg/ml) were harvested by centrifugation and washed three times with a cold solution of non-radioactive inulin (1.0 mg/ml) in physiological saline. The cells were finally suspended in distilled water and thoroughly dispersed. Aliquots were plated, counted, and analyzed for protein. This procedure has been described previously (18).

In the second technique, monolayers of cells were used. These monolayers were made in plastic Petri dishes for tissue culture (Falcon Plastics, Los Angeles, Cal.) in a fashion similar to that previously used for alveolar macrophages (4). The washed PMN or MC obtained in the usual way from the guinea pigs were suspended in Krebs-Ringer phosphate medium with

² A. W. Shafer, E. A. Glass, and M. L. Karnovsky. Effects of various agents on phosphatide metabolism in leukocytes. To be submitted.

10% serum at a concentration of 1×10^7 cells per ml. 1.5 ml of the suspension was then placed in each Petri dish (area 8 cm²) and the dishes were covered. They were incubated for 60 min at 37°C and very gently rocked every 15 min. The liquid was then gently poured off, and the whole dish carefully immersed, successively, in three beakers each containing about 50 ml of Krebs-Ringer phosphate medium. The dishes, held in forceps, were gently agitated in each washing medium. After the third wash, the dishes were inverted and drained on paper towels for 30 sec. The components of the final medium (total volume 1.5 ml), i.e., solutions containing glucose, inulin, and where appropriate the agent under study, were carefully added from pipettes. The final concentrations of all components in the medium were identical to those used in the studies with suspended cells. The experiment was then performed at 37°C, with gentle agitation from time

using a total volume of 9 ml for the system, the cell suspension was diluted to 30 ml with ice-cold Krebs-Ringer phosphate medium, mixed, and centrifuged gently (80 g) for 3 min. The supernatant fluid was then decanted, and 3 ml of cold 1.3% OsO₄ in *s*-collidine buffer (19) pH 7.2, containing 150 mg of sucrose and 5 mg of CaCl₂ per 10 ml, was added to the pellet. The cells were resuspended in the fixative by gentle agitation, and fixed in the cold for 90 min. They were then washed in distilled water, dehydrated in ethanol, and embedded in Epon 812 or Araldite (20). In some experiments, the cell suspension was gently centrifuged after each step in the dehydration and embedding processes. In other experiments, clumps of cells which formed during fixation were dehydrated and embedded without further centrifugation. The use of clumped cells provided a less satisfactory sample, but the cells generally showed less evidence of mechanical damage than did those

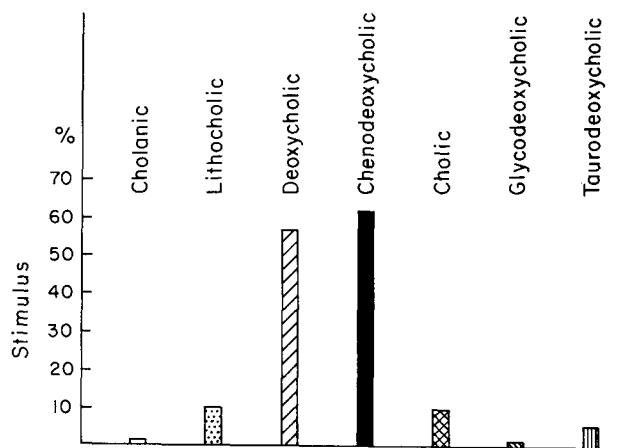


FIGURE 4 Specificity in the bile acid series for respiratory stimulation in PMN. The test substances were studied at 30 $\mu\text{g/ml}$ and at 50 $\mu\text{g/ml}$, with similar results. The incubation period was 1 hr.

to time. To terminate the incubation, the dishes were again drained, and passed through five beakers of a washing medium consisting of physiological saline containing nonradioactive inulin (0.5 mg/ml), to remove extracellular labeled inulin. Each beaker contained about 50 ml of washing fluid, and the dishes were gently moved about in each for about 15 sec. After washing, the dishes were allowed to air dry, and an aliquot (2 ml) of 0.5 N NaOH was added. After standing overnight, an aliquot (0.2 ml) from each dish was analyzed for cellular protein, and another aliquot (0.2 ml) was counted in 10 ml of Buhler's fluid on a scintillation counter. The data from both methods were calculated as cpm per mg cellular protein, and were corrected for zero-time values.

Preparations for Electron Microscopy

After incubation for up to 15 min in a fashion similar to that of the metabolic experiments, but

subjected to repeated centrifugation. Thin sections were cut on a Porter-Blum MT-1 ultramicrotome or an LKB Ultratome. They were then stained with alkaline lead (21) or with saturated aqueous uranyl acetate followed by alkaline lead, and examined in an RCA EMU 3D, EMU 3F, or an AEI EM6B electron microscope.

RESULTS

Fig. 1 illustrates the effects of endotoxin, digitonin, and deoxycholate on some metabolic functions of polymorphonuclear leukocytes. It is clear that all three agents, like phagocytosis, cause an increased respiration and a large increase in the oxidation of carbon-1 of glucose to CO₂. It should be noted that the ratio of glucose carbon-1 to that of carbon-6 in the CO₂ from cells at rest was about 10 and rose to values of about 40 during stimulation. In the case of mononuclear cells (MC), the agents

mentioned cause very similar effects, except that the increase is not so marked with respect to carbon-1 compared to carbon-6 of glucose (cf. phagocytosis) (4). In Fig. 2 is shown a dose response

curve of PMN with respect to respiration, when digitonin or deoxycholate was present. These curves are examples taken from several trials, and it appears that the cells are more responsive to

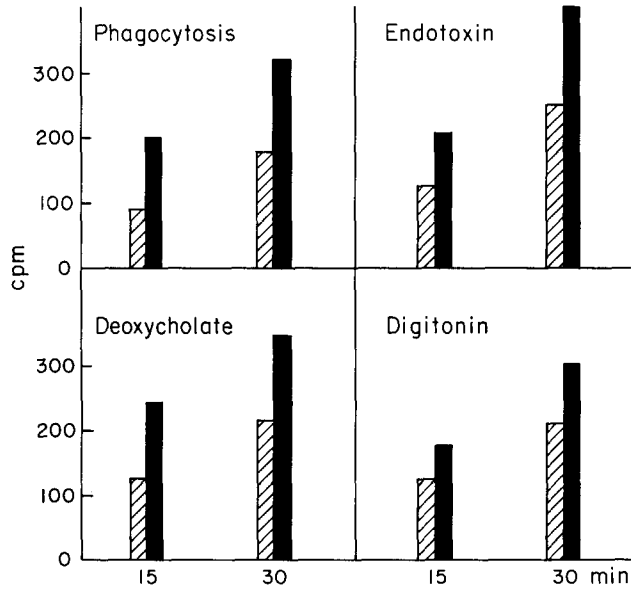


FIGURE 5 Influence of phagocytosis, endotoxin, deoxycholate, and digitonin on the uptake of inulin-¹⁴C. Conditions as in Fig. 1 and Experimental Section. Inulin-¹⁴C was present at a concentration of 0.3 mg/ml. The data are expressed per mg of cell protein. The hatched bars are controls; the solid bars represent cells stimulated in various ways.

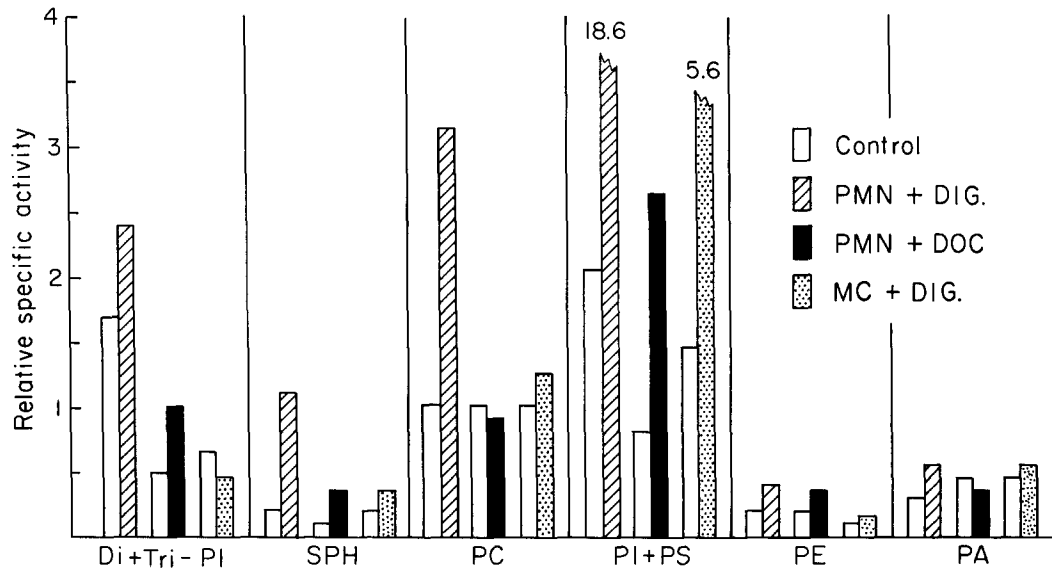


FIGURE 6 The effect of digitonin or deoxycholate on the relative specific activity of various phosphatide classes. The values for the specific activity of the lecithin of control cells are set at 1.00 in all cases. (The following abbreviations are used: *Di-* and *Tri-PI*, di- and triphosphoinositides; *SPH*, sphingomyelin; *PC*, phosphorylcholine [lecithin]; *PI* and *PS*, inositol phosphatide and serine phosphatide; *PE*, phosphatidylethanolamine; *PA*, phosphatidic acid.) The open bars are controls; hatched bars represent PMN stimulated with digitonin (25 μ g/ml). Solid bars are PMN stimulated with DOC (50 μ g/ml), and dotted bars are MC stimulated with digitonin (25 μ g/ml). Incubations were for 1 hr.

digitonin than to deoxycholate. If the quantities of stimulating agent exceed a given amount, there is a dramatic inhibition of oxygen uptake, presumably due to disruption of the cells. The respiratory activity for control cells and cells stimulated with deoxycholate, digitonin, and endotoxin has been found to be linear for at least 30 min, after which the rates of respiration or digitonin-stimulated cells decreased slightly. Fig. 3 demonstrates that the pattern obtained with conventional inhibitors of metabolism with respect to the stimulation of metabolic activity induced by endotoxin is the same as that previously noted in the case of phagocytosis, i.e., iodoacetate and fluoride eliminate the effects, which are insensitive to cyanide or, in fact, to any interference with the cytochrome system or oxidative phosphorylation, e.g. by dinitrophenol or Antimycin A (1). The effects

with digitonin and deoxycholate were identical to those with endotoxin. Furthermore, the effects of inhibitors on MC in the presence of the stimulating agents were also analogous to those noted during phagocytosis (4).

Specificity in the Bile Acid Series

In order to determine whether there was any specificity to such effects as those just mentioned, the bile acid series was chosen. Respiratory activity of polymorphonuclear leukocytes in the presence of these substances is shown in Fig. 4. It is interesting to note that the stimulatory activity correlates with the number of hydroxyl groups present on the steroid nucleus, but not with their position (cf. deoxy- and chenodeoxycholic acids). A few studies with allodeoxycholic acid indicated that this substance had somewhat less activity than

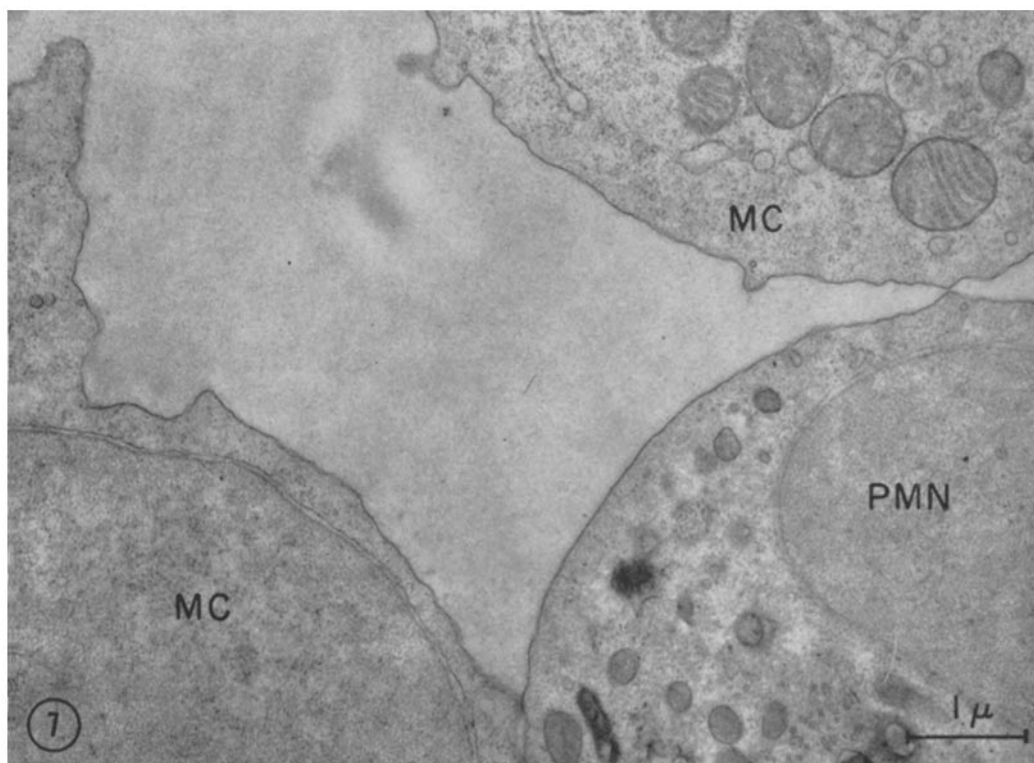


FIGURE 7 Portions of polymorphonuclear leukocyte (PMN) and two mononuclear cells (MC) from a control preparation incubated without digitonin, as described in the Experimental Section. The cells appear intact and no cylindrical surface membrane projections are present. These cells are representative of those seen in control preparations, except that the number of vesicles and vacuoles is somewhat less than is usually seen. $\times 16,000$; marker = 1μ .

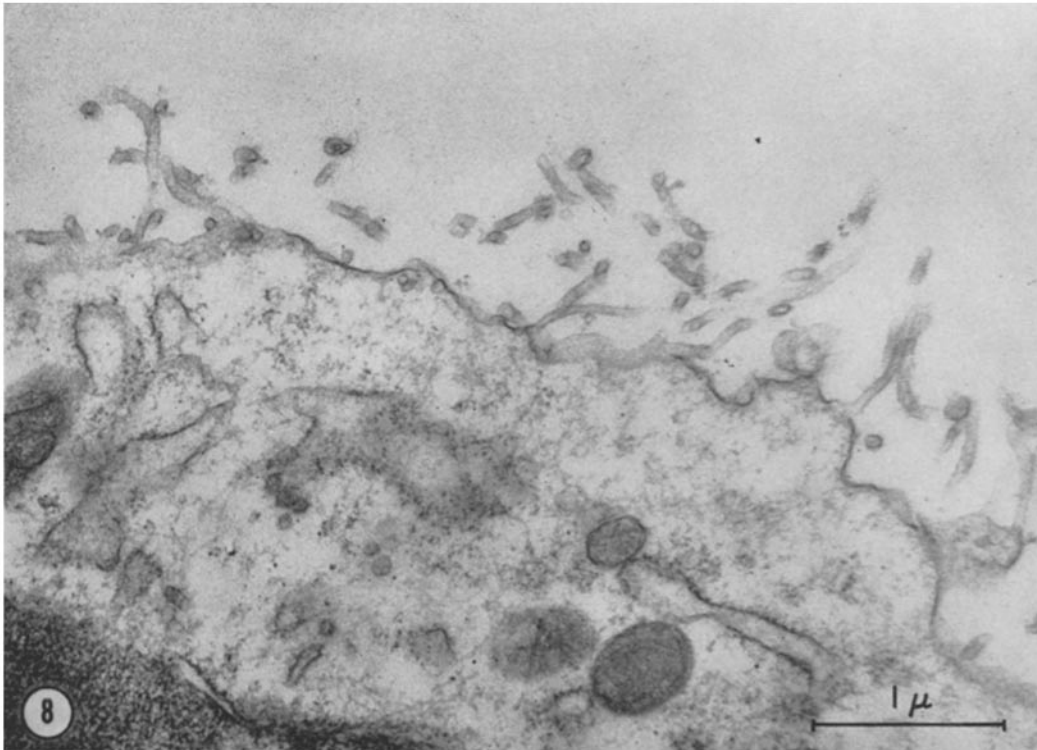


FIGURE 8 A portion of a mononuclear cell incubated with digitonin, showing numerous typical cylindrical projections arising from the cell membrane. The cell shows no other obvious alterations. $\times 25,000$; marker = 1μ .

deoxycholic acid, suggesting the possibility of some stereochemical specificity. Conjugation of the carboxyl group of deoxycholic acid with glycine or taurine virtually eliminated activity at the concentrations studied here.

Uptake of Inulin in Cells Treated in Various Ways

Fig. 5 illustrates the effects of endotoxin, digitonin, and deoxycholate on the uptake of inulin- ^{14}C by polymorphonuclear leukocytes. The situation that pertains during phagocytosis has been discussed elsewhere (18), and is included in the figure for comparative purposes. It is clear that, in all cases, the passage of extracellular fluid (indicated by transfer of inulin) into the stimulated cells was increased. Attempts to determine whether the increased uptake of inulin caused by endotoxin, deoxycholate, and digitonin was sensitive to inhibitors of glycolysis were not conclusive because the inhibitors themselves (iodoacetate, NaF)

caused changes in the entry of extracellular fluid (inulin) into the cells. In the case of phagocytosis, of course, the uptake of solid particles is very susceptible to the presence of inhibitors of glycolysis (1).

The results obtained are compatible with, and even suggestive of, the possibility that increased vesiculation is occurring as a result of the stimulation, and that inulin is entering the cells as a consequence. It will be noted that "resting" cells show a considerable baseline level of uptake of inulin, and this will be discussed later.

Labeling of Phosphatides

Phagocytosis is accompanied by increased incorporation of $^{32}\text{P}_i$ into the cellular phosphatides. In view of the fact that most of the lipid of PMN is bound to protein, the probability has been discussed that this effect on complex phosphatides may represent alterations in the cellular membrane (6, 7). All three of the agents studied here

cause effects on the PMN similar to those that accompany phagocytosis. The situation is exemplified in Fig. 6. It should be noted that the chromatographic method used here (thin-layer chromatography) to separate the phosphatides was different from that which was used when the effect of phagocytosis was originally examined (column chromatography). The only important difference observed in the behavior of the phosphatide fractions pertains to phosphatidic acid which appears to be little affected in the present experiments—in contrast to those previously reported for phagocytosis (6). We have repeated experiments on the effect of phagocytosis on these cells, using thin-layer chromatography to separate the lipids. In these experiments, the patterns obtained are entirely similar to those of Fig. 6. It has recently been shown that, in column chromatography on silicic acid, inositol phosphatides may contaminate the phosphatidic acid fraction, and this may explain the discrepancy we have observed (22).

The experiment shown in Fig. 6 for PMN with digitonin is an extreme case; all fractions showed increased specific activities. However, even here the stimulation was most notable, as in all cases, i.e. stimulation with phagocytosis, deoxycholate, or endotoxin, with respect to the phosphatidyl-inositol and -serine fraction. It should be emphasized that we have not detected changes in the composition of the lipids isolated from stimulated cells, compared with normal cells, but differences in labeling of phosphatide entities from $^{31}\text{P}_i$.

Effect of Cholesterol on the Respiratory Stimulations Due to Digitonin or Deoxycholate

When cholesterol was dispersed by ultrasonic means or in solutions of Tween-80 (50 mg %) and added to PMN, it was found that, at final concentrations of cholesterol from 25 to 150 $\mu\text{g}/\text{ml}$ incubation medium, the respiratory stimulation due to digitonin was depressed. For example, when the

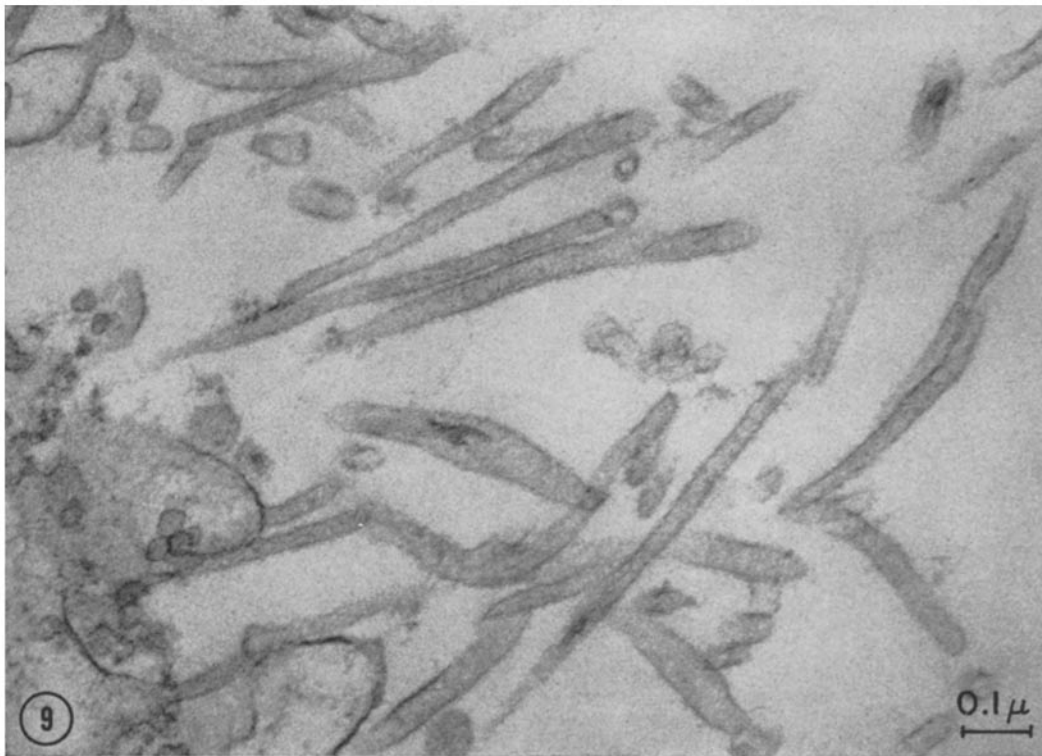


FIGURE 9 Cylindrical projections from the membrane of a mononuclear cell incubated with digitonin, shown at a higher magnification. The surface membranes limiting the projections are somewhat less electron opaque than is the cell membrane proper. $\times 79,000$; marker = 0.1μ .

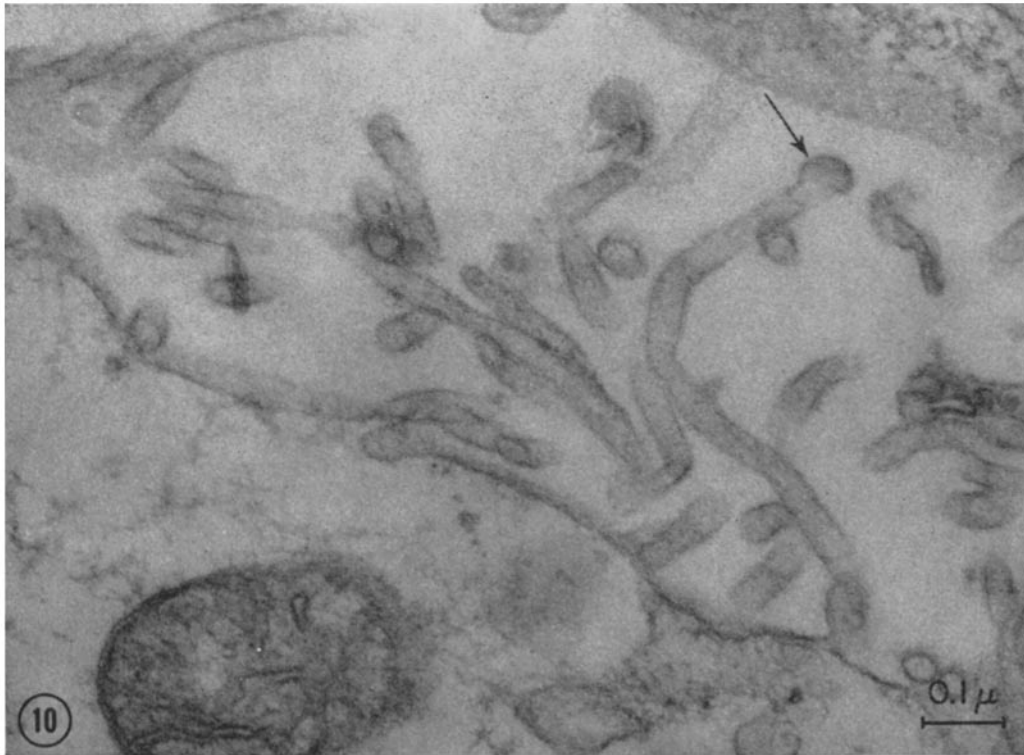


FIGURE 10 Cylindrical projections from a mononuclear cell incubated with digitonin. One projection appears to have a bulbous end (arrow). $\times 87,000$; marker = 0.1μ .

usual conditions pertained in the respiratory experiments, and $25 \mu\text{g/ml}$ digitonin was added, the rate of respiration increased threefold. Cholesterol, if present at $30 \mu\text{g/ml}$, depressed this increment by 50%; at $90 \mu\text{g/ml}$ cholesterol, the depression was 75%. These effects were not noted with regard to respiratory stimulations that accompanied the presence of deoxycholate, nor during phagocytosis.

Effects of Other Surface-Active Agents and Detergents

In view of the striking effects of digitonin and deoxycholate, a range of substances was examined in the region of final concentration around 10–50 $\mu\text{g/ml}$. Respiration was followed as the most convenient variable. The following summarizes the findings.

Neutral substances such as Triton X-100, (Rohm and Haas Inc.) Tween-80, (Nutritional Biochemical Corp.) or Cutscum (Fisher Scientific Co.) (commonly used as dispersing agents for

lipid substances in enzymatic reactions) and 1-octanol were essentially without effect. Positively charged agents such as cetyltrimethylammonium bromide, octadecylamine, or cetylpyridinium chloride were without effect, or depressed respiration of cells. Negatively charged substances, e.g. palmitate, lauryl sulfate, tocopheryl phosphate, and dicetylhydrogen phosphate exhibited very slight stimulatory effects on respiration, whereas octadecylphosphates (mono- and di- were more effective. Among steroid- and steroid-containing substances, the bile acids have been discussed in some detail; saponin (which, as will be noted below, has been extensively studied in model systems) was moderately active, and digitonin represented an extreme of activity.

ELECTRON MICROSCOPIC RESULTS

Controls

Most of the cells observed in these preparations were polymorphonuclear leukocytes (PMN) or

mononuclear cells (MC). There also were occasional erythrocytes and rare eosinophiles. The MC were of several types, the most common of which was a large cell with many clear vacuoles and numerous pseudopodia. Less frequently, cells resembling the blood monocyte were observed.

Control cells, as well as cells incubated in the experimental media, often contained large numbers of vesicles and vacuoles. Since these also were observed in cells fixed without incubation, it seems clear that most of them formed before the start of experimental manipulations. Unfortunately, these "background" vesicles and vacuoles were numerous enough to make it impossible to detect small changes in the extent of pinocytosis which might have been induced by the agents studied in the experimental preparations.

Cylindrical projections such as those seen after incubation with digitonin (to be described below) were not observed in control cells (Fig. 7). Control macrophages sometimes exhibited multiple small pseudopodia. However, these pseudopodia differed

from the cylindrical projections associated with digitonin in that the former (*a*) had a greater and more variable diameter, (*b*) were often more irregular in shape, and (*c*) were bounded by a membrane of the same electron opacity as the plasma membrane of the cell proper.

Effects of Digitonin

PMN and MC incubated in media containing digitonin displayed distinctive morphological changes. The changes in cells of both types were similar, but the MC appeared much less liable to disruption. The difference is of questionable significance, since in control preparations the mononuclear cells seemed more resistant to damage during the preparatory procedures. The most characteristic feature was the presence of cylindrical projections on the outer membranes of the cells (Figs. 8-15). These projections, which were quite uniform in size and appearance, were 450-700 Å in diameter and 3-4 μ or more in length. The projections were enclosed by a 3-layered membrane

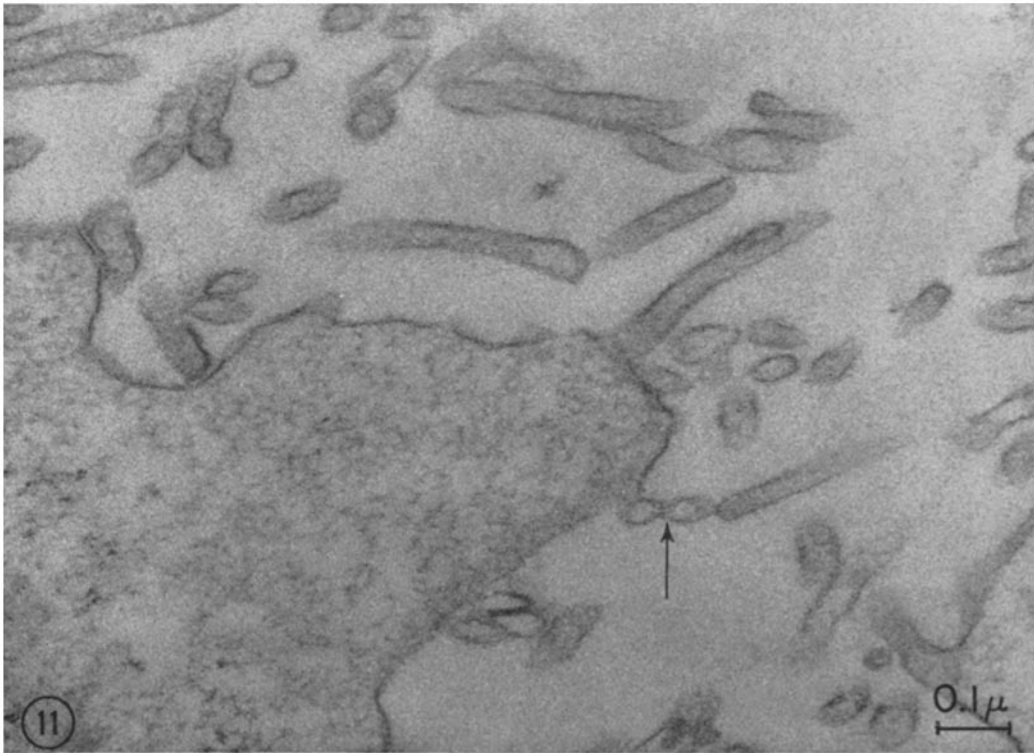


FIGURE 11 Cylindrical projections from a mononuclear cell incubated with digitonin. The base of one projection has a beaded appearance (arrow). $\times 87,000$; marker = 0.1μ .

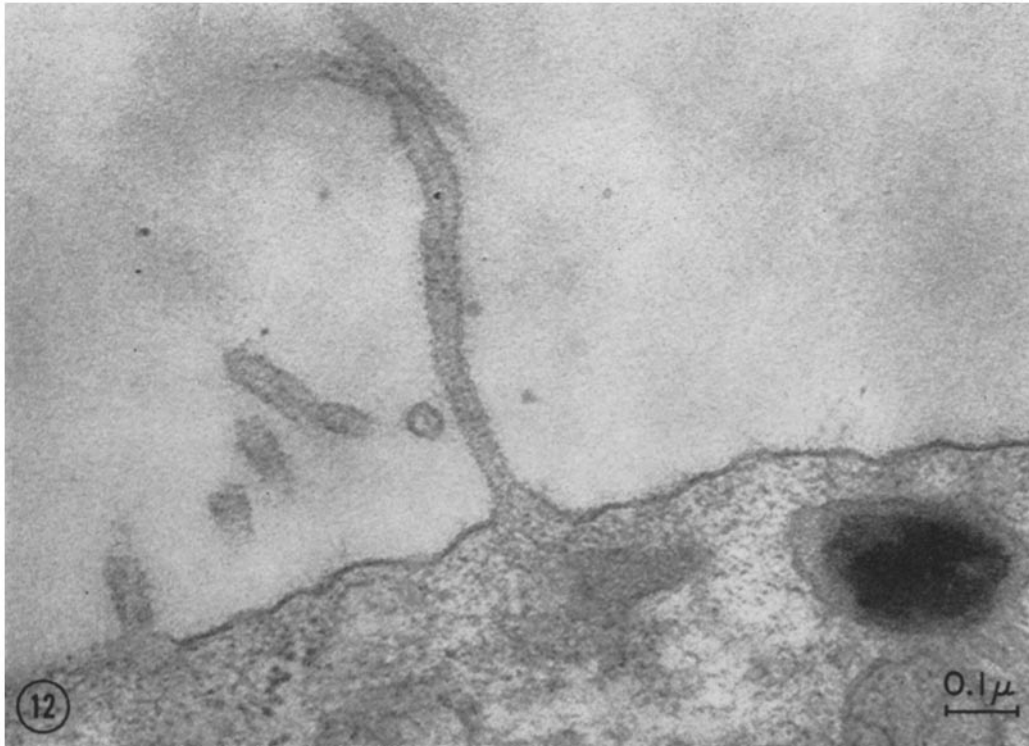


FIGURE 12 Cylindrical projections from a polymorphonuclear leukocyte incubated with digitonin. The surface membrane surrounding the projections appears less electron opaque than the cell membrane proper. A central linear structure is apparent within the projections, but resolution is inadequate to determine whether it is tubular or filamentous in nature. The cell appears otherwise intact. $\times 93,000$; marker = 0.1μ .

which resembled the plasma membrane of the cell proper, except that the membrane surrounding the projections was less electron opaque (Figs. 9–13) (as mentioned above, the membranes surrounding the usual pseudopodia in controls have an electron opacity similar to that of the plasma membrane). An opaque structure was sometimes apparent within the projections, oriented parallel to their long axes (Fig. 12). The degree of resolution obtainable with our material was insufficient to clarify whether this structure was filamentous or tubular in nature. The magnitude of the morphological changes varied greatly from cell to cell. Some cells were indistinguishable from cells in control preparations. Some had a few scattered cylindrical projections, but appeared otherwise intact. Other cells were markedly affected, with many projections lining the outer membrane and presenting a fringelike appearance. Various intermediate stages were observed. Cells with only a few projections often appeared otherwise intact

(Figs. 8, 10–12), but when large numbers of projections were present, the cells generally showed obvious signs of disruption (Figs. 14, 15). Some appeared swollen; in others, the plasma membrane was fragmented and in the cell interior only a conglomerate of nucleus, granules, and mitochondria remained. Granules which appeared morphologically intact remained in most of the disrupted polymorphonuclear leukocytes (Fig. 14). It is uncertain, of course, whether the digitonin effect actually caused disruption of the cells during incubation, or whether it merely rendered them more susceptible to disruption during the subsequent preparatory procedures.

Effect of Metabolic Inhibitors on Morphological Changes

The presence of iodoacetate, fluoride, or deoxyglucose, all of which inhibit glycolysis in leukocytes, had no detectable effect on the formation of

cylindrical projections in cells incubated with digitonin. In Fig. 15, a cell incubated with digitonin in the presence of iodacetate shows an advanced stage of the digitonin effect. The presence of cyanide also had no detectable effect on the morphological changes evoked by digitonin.

Incubation with Deoxycholate and Endotoxin

No definite morphological changes were observed after incubation of cells with deoxycholate (Fig. 16) or endotoxin for periods up to 15 min. As mentioned previously, the present system proved unsuitable for the accurate morphological quantification of pinocytosis because of the large amount of pinocytic activity in control preparations. Cylindrical projections, such as those seen after incubation with digitonin, were, as mentioned above, never observed after incubation with deoxycholate or endotoxin.

Addition of Ferritin to Incubating Medium

Cells were incubated in media containing 0.5% ferritin in the hope that the presence or absence of

ferritin in vesicles and vacuoles would permit the differentiation between those forming during incubation and those which had formed previously. In both control and experimental cells, a minority of vesicles and vacuoles were labeled, confirming the impression that the majority of these structures formed before incubation and that pinocytosis was occurring during incubation of control cells. Quantitative differences in ferritin uptake between control cells and cells incubated in media containing digitonin, deoxycholate, endotoxin, or starch particles could not be detected. It is interesting that much greater amounts of ferritin adsorbed to the digitonin-induced projections than to the cell membrane proper.

Effect of Omission of Ca^{++} and Mg^{++}

The omission of Ca^{++} and Mg^{++} from all solutions used in washing, incubating, and fixing the cells did not prevent the formation of characteristic projections upon incubation with digitonin. Since omission of these ions resulted in inferior structural preservation, particularly with respect to the cell

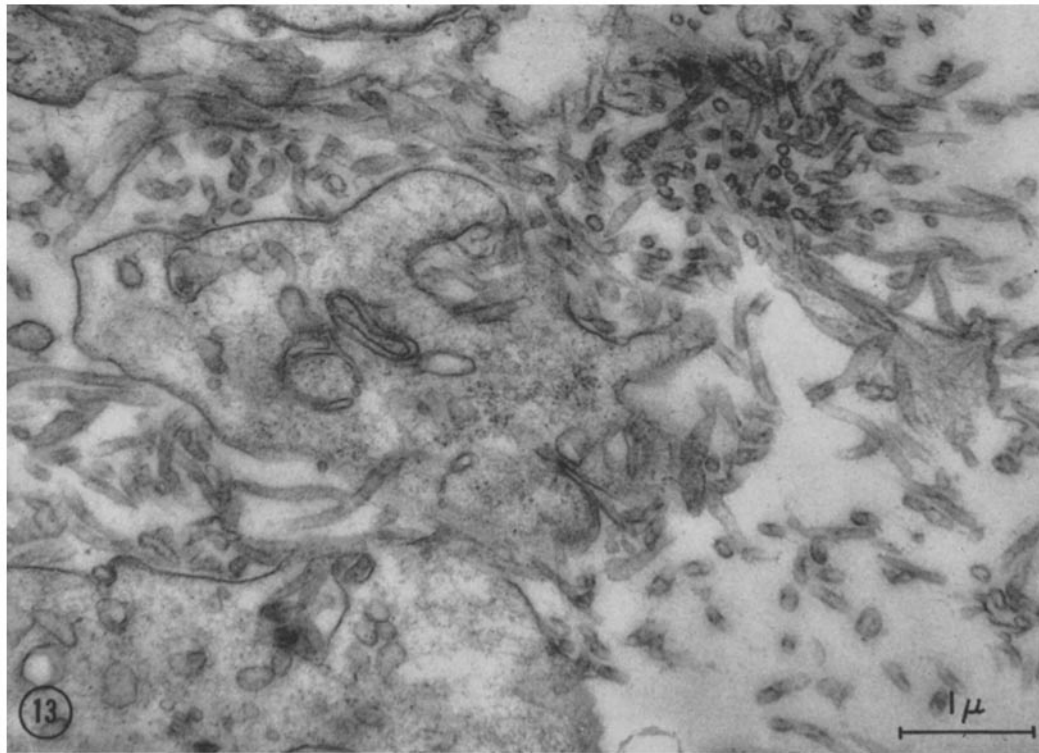


FIGURE 13 A large clump of tubular projections from a preparation incubated with digitonin. Their uniformity in size and shape is apparent. $\times 18,000$; marker = 1μ .

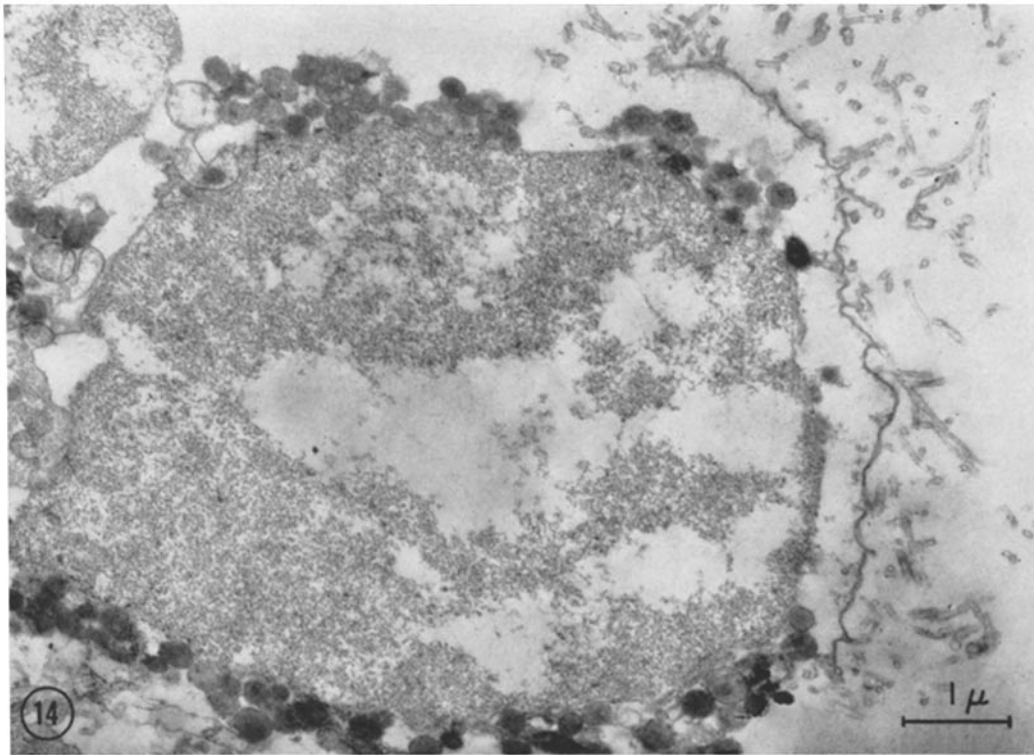


FIGURE 14 A polymorphonuclear leukocyte at an advanced stage of the digitonin effect. The cell membrane, showing numerous cylindrical projections, appears to have ruptured. Numerous granules, most of which appear morphologically intact, are clustered about the nucleus. $\times 14,000$; marker = 1μ .

membranes, quantification of the digitonin effect in these cells was not possible.

In experiments in which metabolic effects were determined, we observed that omission of Ca^{++} and Mg^{++} from the medium, and addition of just enough ethylenediamine tetra-acetic acid to balance intracellular divalent cations, eliminated the metabolic stimulations due to digitonin and deoxycholate.

DISCUSSION

To facilitate discussion of various points, the major observations made in these experiments may be summarized as follows:

1. Endotoxin, digitonin, and deoxycholate cause metabolic perturbations in leukocytes that resemble those noted during phagocytosis.
2. Under the influence of the agents mentioned, there is a marked increase in the uptake of extracellular fluid by the cells—as indicated by entry of inulin- ^{14}C .

3. It has not been possible to reveal definitively by electron microscopy whether endotoxin or deoxycholate causes increased pinocytosis or accumulation of extracellular fluid by vesiculation. Morphological changes were, as has been mentioned above, not detectable in the presence of these two substances because of the large “background” activity.

4. Digitonin causes dramatic changes in the cell membrane. These are not detectably inhibited by the presence of inhibitors of glycolysis, which are known to eliminate phagocytic activity, i.e. the engulfment of solid objects (and presumably the changes of the cell membrane characteristic thereof).

The cylindrical projections observed on peritoneal exudate cells after incubation with digitonin have apparently not been described previously. However, several similar phenomena have been observed. When erythrocytes are subjected to osmotic hemolysis, many of them give rise to so-

called "stromatolytic forms" (23). These are long, cylindrical tubes 200–1000 Å in diameter and up to several microns in length. No internal structure is apparent in these tubes, which are thought to contain hemoglobin. Some of the stromatolytic forms are long and straight, whereas others are bulbous, segmented, or branched. Although rare digitonin-induced projections appear bulbous (Fig. 10) or segmented (Fig. 11), branched forms have not been observed here.

The filamentous forms of influenza and Newcastle disease viruses also bear a certain resemblance to the digitonin-induced projections (24–26). The membranes surrounding these viruses are derived from the outer membrane of the infected host cell. It is interesting that, in the presence of vitamin A alcohol or certain surface-active agents, filamentous forms are produced with strains of virus which ordinarily are released in globular form (24, 26). Moreover, in the presence of vitamin A alcohol, uninfected chick chorioallantoic

membranes release particles with long villous protrusions (24). On the other hand, ultrastructural studies of vitamin A alcohol-induced hemolysis of erythrocytes showed striking surface indentation and vacuole formation, but no evidence of projections from the cell membrane (27). In the present studies, we noted that vitamin A alcohol had no effect on the metabolism of PMN under conditions similar to those used by Glauert et al. with erythrocytes (27).

The mechanism by which digitonin induces the formation of cylindrical projections is unknown. Willmer (28) has suggested that some of the properties and functions of the cell surface (e.g., the type of pseudopodia formed) may be partly dependent on the ratio of cholesterol to phospholipid, and on the way these molecules "pack" in the cell membrane. It is possible that digitonin, with its strong affinity for cholesterol, interferes with the relationship of free cholesterol to phospholipid in such a way as to predispose to the forma-

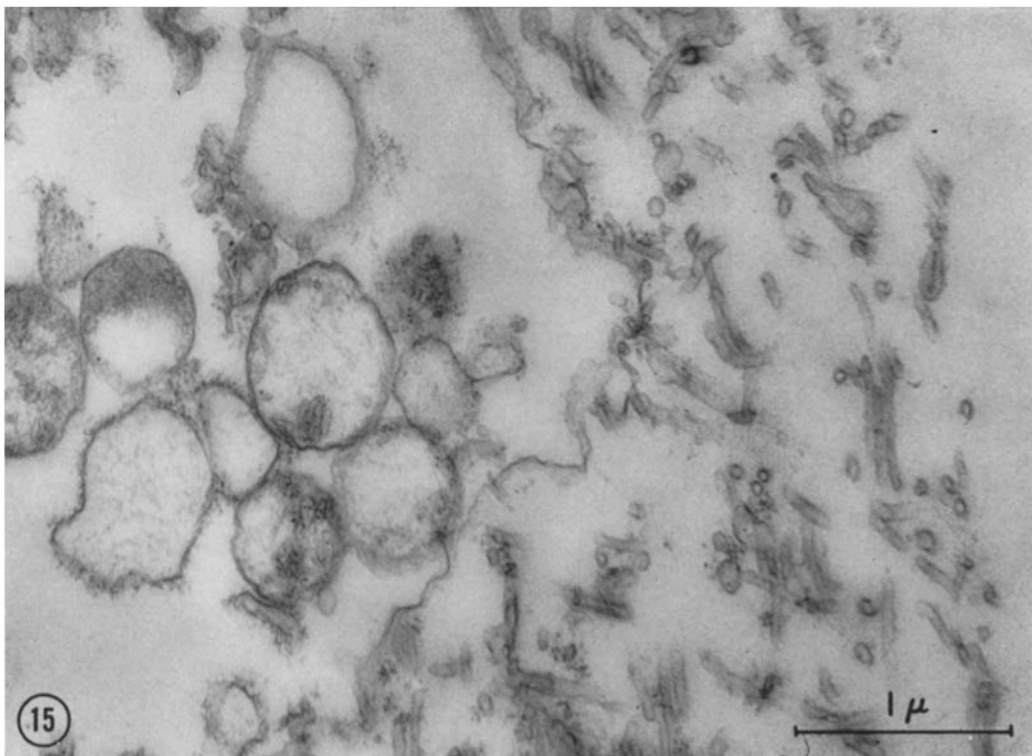


FIGURE 15 A mononuclear cell incubated with digitonin in the presence of iodoacetate, which shows an advanced stage of the digitonin effect. The cell membrane forms numerous cylindrical projections, and the interior of the cell appears swollen and disrupted. $\times 28,000$; marker = 1μ .

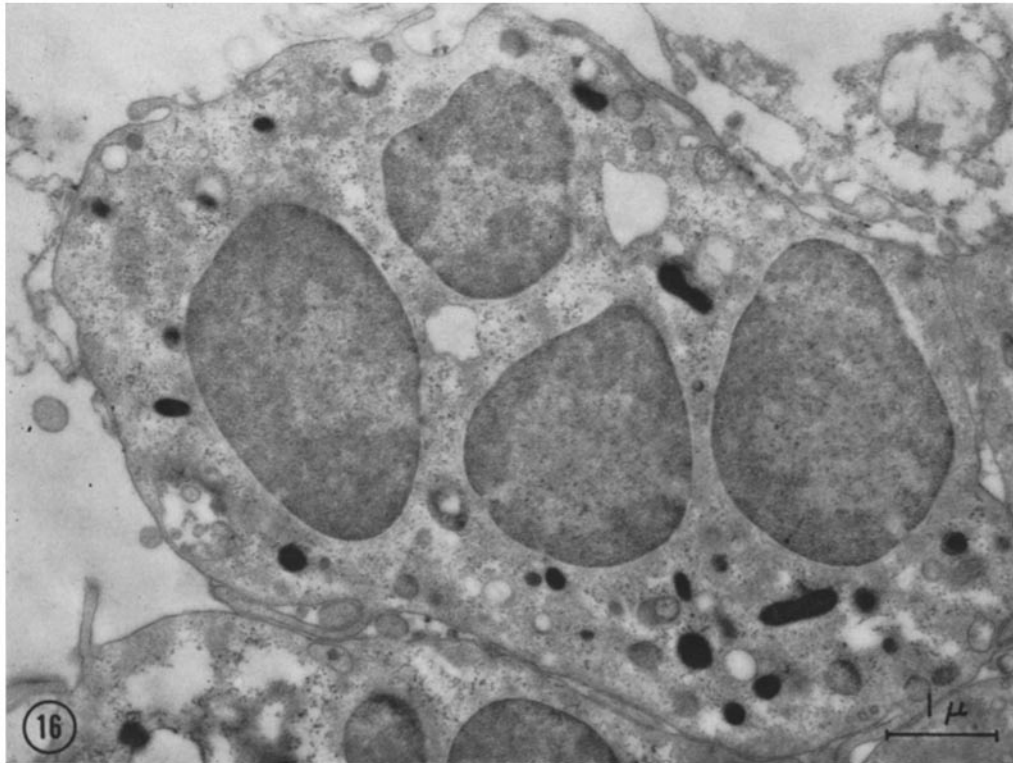


FIGURE 16 A polymorphonuclear leukocyte incubated with deoxycholate, showing no apparent difference from cells in control preparations. A similar number of vesicles and vacuoles often was seen in controls $\times 16,000$; marker = 1μ .

tion of membrane projections of the type observed. We have observed that suspensions of cholesterol in the medium block the respiratory stimulation due to digitonin. One might question this inhibition of the digitonin effect as perhaps being due to formation of digitonin-cholesterol complexes in the medium—thus diminishing the effective concentration of digitonin—but this does not diminish the probability that the digitonin causes its effects by interaction with the cholesterol of the cell membrane. It should be noted that cholesterol has long been known to protect erythrocytes against lytic agents (29).

A good deal of interest has been recently directed to the effects of saponin (a mixture of steroid and triterpene glycosides) on cell membranes. For example, the reaction of saponin with biological membranes has been examined at the electron microscopic level (30). Bangham and his collaborators (31, 32) and Lucy and coworkers (33, 34) have extended concepts of the nature of the phe-

nomena that occur to a more physicochemical realm and have provided models for understanding the manner in which saponin could interact with the ordered components of cell membranes to yield micellar structures that might explain some natural phenomena. Digitonin, a substance similar in type to saponin, with a strong affinity for cholesterol, would be expected to have powerful activity in binding to cellular membranes or altering the physicochemical characteristics of monolayers containing cholesterol. It represents an extreme case in our studies, in that cells were finally disrupted under its influence. Although we were not successful in identifying the morphological effects of deoxycholate, it might be expected to have effects on membrane structures, perhaps also by interference at the level of cholesterol, although cholesterol added to the medium did not inhibit its action on leukocytes. A detailed study was made nearly 30 years ago by Berliner and Schoenheimer (35) on the structural relationships

between bile acids that cause hemolysis and sterols that protect red cells against these agents. The activity of endotoxin at the cellular level could, by analogy, be considered generally in the same light as that of deoxycholate, although, to our knowledge, physical studies of these substances using model lipid monolayers and surface-pressure methods have not been done. There remains, thus, an open question as to whether both deoxycholate and endotoxin exert their action by mechanisms identical to or different from that of digitonin at the molecular level. In this connection, it might be germane to mention the action of polyene antibiotics (such as Nystatin, Filipin, and Amphotericin B) on cellular membranes. These substances are known to alter the cellular membrane so that it loses some of its important biological properties of selectivity. The mode of action has been traced to interaction with the cholesterol of membranes (36), and physicochemical models have demonstrated this in a rather striking fashion (37, 38). In the present work, we tested Amphotericin B and Nystatin, and found that, in the range of concentrations used in these experiments, there was no change in respiratory activity of the leukocytes. Kinsky (36) observed that there is competition between polyene antibiotics and digitonin for ergosterol in the membranes of fungi.

Uptake of Inulin by Stimulated Cells

The entry of inulin into polymorphonuclear leukocytes during phagocytosis is readily understandable (18). Previous data have indicated that about 2.5% of the volume of intracellular fluid is brought in from outside in 30 min (18). Phagocytosis increases this value by about 50%. Since inulin was the marker for extracellular fluid, the mode of entry into the cells was considered to be vesicular.

When cells are stimulated with endotoxin or deoxycholate, the same phenomenon was noted. However, the baseline levels of vesiculation and inulin uptake in unstimulated cells were very high, as has been previously indicated, and pinocytosis in "resting" leukocytes has been demonstrated (39). We can thus report only a tentative impression at the morphological level that vesiculation was increased by these agents.

It is perhaps surprising that increased inulin uptake was noted also in digitonin-stimulated cells, when one considers the morphological patterns. However, an explanation is available. We have noted that in the inulin uptake experiments (done

with monolayers as described above) the dishes treated with digitonin lost considerable cellular protein—which might be expected from the cytological observation. Presumably, those cells that remained (ca. 75%) were stimulated submaximally, took up additional extracellular fluid, and remained attached to the dish. This is consistent with the electron microscopy which gave evidence of unevenness of morphological change in a given cell population. Disrupted cells and those heavily stimulated may have been washed off the dishes, accounting for the loss of protein observed.

In the case of phagocytosis by PMN and MC, inhibition of the glycolytic pathway blocked the actual ingestion of particles, i.e., the results were consistent with the concept that phagocytosis and the associated membrane movements are dependent on cellular metabolic energy. In the case of stimulation of these cells with digitonin, inhibition of glycolysis eliminated the metabolic perturbations (i.e. respiratory stimulation, increased oxidation of glucose) but did not discernibly block the morphologically detectable effects. It must be stressed again that quantitative electron microscopic evaluation of any but very considerable morphological changes would not be possible, as has been stated for the case of possible increases in vesiculation due to deoxycholate and endotoxin. On the other hand, it is entirely possible that changes in morphology of the cell membrane due to the action of digitonin on the cholesterol component of the membrane might not require cellular metabolic energy. The elimination of the respiratory stimulation and that of glucose oxidation when glycolysis was blocked would follow from the biochemical mechanisms believed to underlie those phenomena (40).

The question of whether perturbations in the cellular membrane lead to metabolic perturbations, or whether at least some of the gross membrane changes are dependent upon metabolic perturbations, must await further work that allows more precise evaluation of the situation at the morphological level.

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