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IN VITRO ABSORPTION OF γ-GLOBULIN BY NEONATAL INTESTINAL EPITHELIUM OF THE PIG

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SUMMARY

1. An *in vitro* method, using fluorescent γ-globulin and everted neonatal pig's intestinal slices, for the study of the active transport of large molecules is described.

2. Uptake of γ-globulin occurred within 15 min and required no exogenous substrates.

3. *In vitro* absorption of γ-globulin by intestinal epithelium was limited to the neonatal pig and 5-day-old mouse. No uptake was seen in intestines from a mature mouse, a pig with diarrhoea, a normal pig, a mature rabbit, a guinea-pig, a chick, and a chick embryo. Chick embryo yolk sac readily took up γ-globulin.

4. Rings of everted intestinal epithelium remained active (still absorbed γ-globulin) after incubating for 4–6 hr in balanced salt solution (BSS).

5. Uptake of γ-globulin required oxygen and sodium and was reversibly inhibited by metabolic antagonists such as iodoacetate, arsenate, fluoride, 4,6-dinitro-φ-cresol, phlorrhizin, anaerobiosis and cold. Under the conditions of the test, large colloidal molecules did not inhibit uptake of γ-globulin.

6. Similar results (although not as clear-cut) with metabolic inhibitors were obtained with preparations of chick embryo yolk sacs.

7. Injuring mature pig's intestinal epithelium with surface-active agents did not produce non-specific absorption artifacts that resembled the specific absorption found in immature pig's intestinal epithelium.

INTRODUCTION

It is generally agreed that pinocytosis (cell drinking) is the means whereby large molecules gain entrance to certain cells (Holter, 1959; Wilson, 1962). Even though this process was first described years ago by Lewis (1931), a paucity of information (mostly descriptive) exists concerning this absorption mechanism in mammalian cells (Clark, 1959). Most of the current information comes from extensive investigations using

amoeba (Holter, 1959; Chapman-Andresen, 1964). The reason for this, in part, is the relative ease of working with cell suspension of amoeba versus the difficulty of manipulating mammalian tissues and cells. Also, the problem is magnified in that pinocytosis is an absorption mechanism associated mostly with primitive or immature cells. Thus, it is not easy to demonstrate vigorous pinocytotic activity in mammalian cells except those that are a part of the reticulo-endothelial system. Further complications arise from the difficulty of distinguishing adsorption from absorption and non-specific absorption caused by permeability changes in injured cells (Holtzer & Holtzer, 1960; Ryser, Aub & Caulfield, 1962; Ryser, 1963).

This paper describes the use *in vitro* of a readily available group of mammalian cells that are easily manipulated and demonstrate pinocytosis to a marked degree. Results obtained with this system seem little influenced by the endogenous nutrients of the cells and lend themselves to easy interpretation.

METHODS

Balanced salt solution (*BSS*). Gut was washed and incubated, unless noted otherwise, in a carbohydrate-free BSS (similar to Hanks & Wallace's (1949) tissue culture BSS) of the following m-equiv./l: Na^+, 143; K^+, 5·6; Ca^{2+}, 1; Mg^{2+}, 1·6; Cl^-, 146; PO_4^{3-}, 5·7; SO_4^{2-}, 1·6. The pH was adjusted to 7·4 with a few drops of concentrated NaOH, and 8 m-equiv/l. CO_3^{3-} was added. Na^+-free BSS was made by substituting Tris (trishydroxymethylaminomethane) for the Na^+. Usually all tissues were incubated aerobically at 37° C in a rotating water-bath with a gassing hood. For aerobic conditions, a mixture of 95 % O_2 and 5 % CO_2 was used and for anaerobic conditions 95 % N_2 and 5 % CO_2. A pH of 7·0–7·4 was maintained and monitored by adding 5 mg/l. of phenol red to the BSS.

Preparation of gut. New-born unfed piglets no older than 12 hr and weighing more than 1 kg were used. The neonate was killed by stunning and bleeding to death. The intestines were removed, and the jejunum was everted with a glass rod. The everted jejunum was serially sliced into approximately 5-mm pieces, placed in 500 ml. of BSS and washed twice with BSS (Crane & Mandelstam, 1960; Wilson, 1962).

Testing system. A gut slice, taken at random, was placed in a 30 ml. beaker containing 5 ml. of the appropriate incubation medium. After the prescribed pre-incubation time, the gut ring was tested for its capacity to absorb large molecules by transferring it to another 30 ml. beaker containing an absorption marker of 2 % fluorescent γ-globulin in BSS.

Unless noted to the contrary, the gut was incubated with the absorption marker for 30 min, removed, washed in BSS and fixed overnight in 10 % formalin in phosphate buffer saline, pH 7·0. Having been processed through increasing concentrations of alcohol to xylene, the gut was blocked in paraffin. Sections of 5 μ were cut and picked up on slides. After the paraffin had been removed the sections were mounted in a non-fluorescent mountant (Clearcol, N.W. Clark; Melrose, Massachusetts).

Photomicroscopy. The slides were viewed with a Lietz microscope, using a Lietz high-intensity ultra-violet light source. The light passed through a heat absorbing BG 38 filter, a primary Blau BG 12 filter and a Blau-Abs yellow eyepiece secondary filter. Photographs were taken on Kodak Plus X film exposed for 90 sec through a 54 × oil objective and a 10 × ocular. Standard developing and printing procedures were used.

Preparation of fluorescent γ-globulin (*fluor-γ globulin*). Fifty millilitres of 4 % porcine γ-globulin (Pentex Inc.; Kankakee, Illinois) in 0·5 M carbonate-bicarbonate buffer, pH 9·0,

was stirred overnight at 4° C with 1 g of fluorescein isothiocyanate (10 % on celite) (Nutritional Biochemical Corp.; Cleveland, Ohio). The supernatant from this reaction was dialysed for at least a week against slightly alkaline distilled water at 4° C. The 4 % fluor-γ globulin when diluted with 2 × concentrated BSS gave the concentration that was used (2 %).

Standardization of test

Minimal exogenous substrates. Everted intestinal slices prepared as described above were incubated for 15 and 60 min in 2 % fluor-γ globulin in BSS solution and 2 % fluor-γ globulin in tissue culture medium (TC 1099; Microbiological Associates, Inc.; Bethesda, Maryland).

Age and species specificity. Everted intestinal slices obtained from a 5-day-old mouse, mature mouse, 1-month-old chicken, 18-month-old guinea-pig, 1-year-old rabbit, 12-day-old pig with diarrhoea, 18-day-old normal pig, 14-day-old chick embryo, and 14-day-old chick embryo yolk sac were incubated in fluor-γ globulin-BSS for 30 min. The mice were unfed for 4 hr and the other animals for 24 hr before they were used. The jejunal slices from these different animals were prepared as described for the neonatal pig in the above section,

Preparation of gut.

In vitro *functioning time.* Slices of everted neonatal pig gut were incubated in BSS or tissue culture medium for zero, 0·5, 1, 2, 3, 4, and 6 hr. At each time interval, a gut slice was transferred into another beaker containing the test fluor-γ globulin-BSS solution and incubated for an additional 0·5 hr.

Effect of inhibitors

Large colloidal molecules. Porcine albumin, porcine γ globulin (both from Pentex, Inc.; Kankakee, Illinois), polyvinylpyrrolidone (PVP-K30) (Antara Chemicals; New York, New York) and colloidal carbon (Pelikan Werke; Hanover, Germany) were extensively dialysed before use to remove any small contaminating molecules. In the test, slices of everted neonatal gut were placed in BSS containing either 5 % porcine albumin, 5 % porcine γ-globulin, 5 % PVP, 1 % colloidal carbon or 10^{10} *Escherichia coli*, and incubated for 3 hr, removed and placed into the test fluor-γ globulin-BSS for 30 min.

Metabolic inhibitors. Slices of everted neonatal pig gut were pre-incubated aerobically at 37° C for 20 min in BSS containing 10^{-3}, 10^{-4}, and 10^{-5} M-NaF; 10^{-3}, 10^{-4}, and 10^{-5} M-Na-arsenate; 10^{-3}, 10^{-4}, and 10^{-5} M-Na-iodoacetate; 10^{-3}, 10^{-4}, and 10^{-5} M-4, 6-dinitro-ϕ-cresol (DNC); 10^{-3}, 10^{-4}, and 10^{-5} M phlorrhizin: other slices were pre-incubated aerobically in BSS at room temperature, others under anaerobic conditions in BSS at 37° C and others under aerobic conditions at 37° C in Na^+-free BSS. After the 20 min pre-incubation period, the gut slices were transferred for 30 min to an incubation medium identical to the pre-incubation medium except that it contained in addition the test fluor-γ globulin. To determine whether the effect of inhibitors was reversible, gut slices pre-incubated for 20 min in BSS containing 10^{-4} DNC, in Na^+-free BSS and under anaerobic conditions were transferred to inhibitor-free fluor-γ globulin-BSS and incubated for 30 min.

Pieces of a 12-day-old chick embryo yolk sac (approximately 5 × 5 mm) washed twice with BSS were treated similarly with metabolic inhibitors.

Surface-active agents. Everted gut slices from pig's mature intestinal epithelium (6 weeks old) were incubated for 1 hr in fluor-γ globulin-BSS containing 5 and 20 mM cholate, 5 and 20 mM glycocholate (both from Mann Research Lab.; New York, New York), 5 and 20 mM tween 80 (E.I. DuPont de Nemours; Wilmington, Delaware), 5 and 10 mM lecithin (Nutritional Biochemical Corp., Cleveland, Ohio) 0·5 and 4 mM triton × 100 (Rohm and Hass; Philadelphia, Pennsylvania), Staphylococcus lysate 1:10 and 1:100 (Delmont Laboratory, Inc.; Swarthmore, Pennsylvania), endotoxin 0·1 and 1 mg/ml. (Difco Lab.; Detroit, Michigan) and 2 % corn oil.

RESULTS

Standardization of test

Minimal exogenous substrates. The intestinal epithelium immersed in fluor-γ globulin-BSS (containing no substrates) seemed to absorb the fluorescent γ-globulin as well as the intestinal epithelium immersed in fluor-γ globulin–tissue culture fluid (a complete complex medium). Fluorescent γ-globulin was detected inside intestinal epithelium within 15 min of exposure and the cells were engorged with fluorescing droplets within 1 hr. Thus for the tests, i.e., to determine whether the gut had the capacity to absorb large molecules, it was decided to adopt as a standard the incubation of intestinal slices in 2% fluor-γ globulin in BSS for 30 min.

Species specificity and age of animal. Of the animals tested, only the gut of the 5-day-old mouse absorbed the fluorescent γ-globulin (see fluorescing droplets in 5-day-old mouse epithelium, Fig. 1). It is interesting

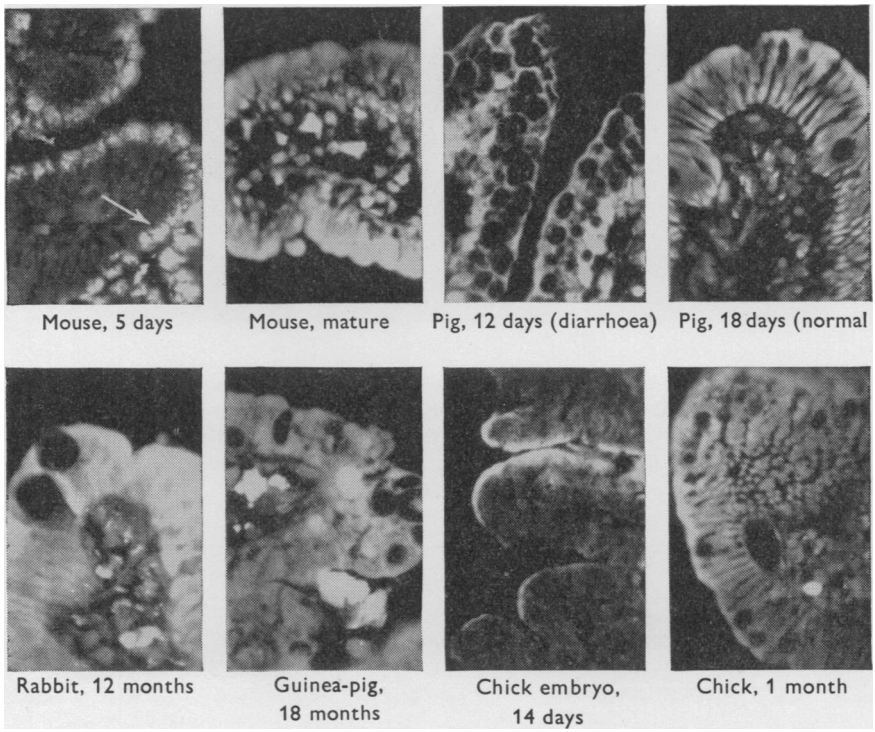

Fig. 1. *In vitro* absorption capacity of intestinal epithelium from various animals and states of maturity. Everted gut rings were incubated aerobically at 37° C for 30 min in 2% fluorescent γ-globulin in BSS. Arrow points to droplets of fluorescent γ-globulin within epithelium.

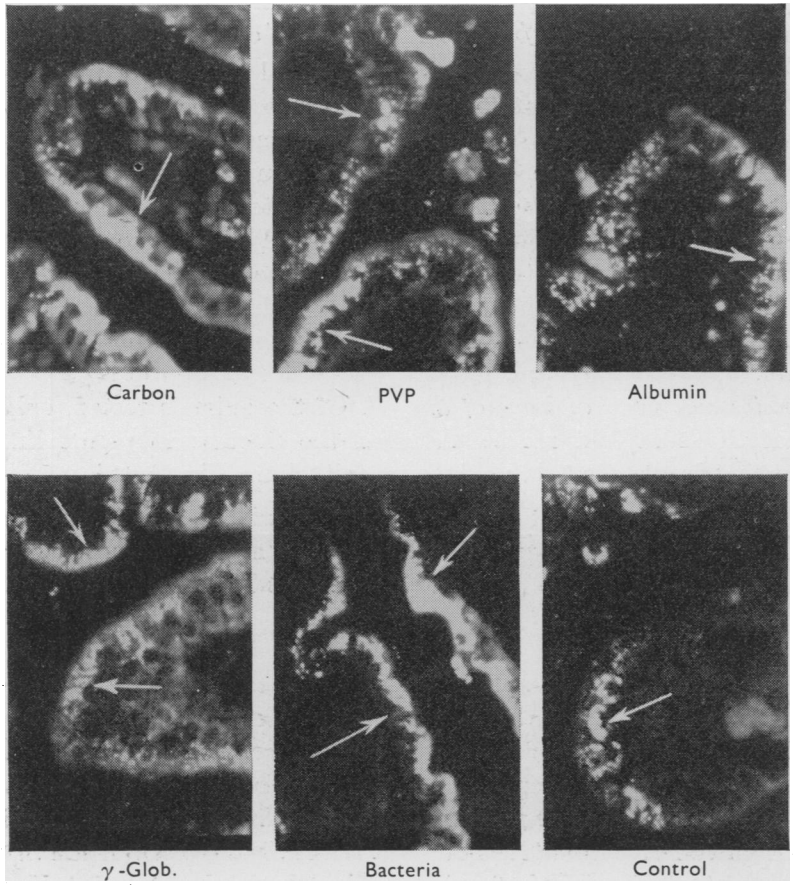

Fig. 2. Effect of large colloidal molecules on the absorption of γ-globulin by neonatal intestinal epithelium of the pig. Everted gut rings were pre-incubated aerobically at 37° C in the colloid-BBS for 3 hr, removed and incubated in 2 % fluorescent γ-globulin in BSS for 30 min. Arrows point to droplets of fluorescent γ globulin within epithelium.

Legend to Fig. 3

Fig. 3. Effect of metabolic inhibitors on the absorption of γ-globulin by neonatal intestinal epithelium of the pig. Everted gut rings were pre-incubated aerobically at 37° C (except where noted) in the inhibitor-BSS for 20 min, removed and incubated in the inhibitor-BSS plus 2 % fluorescent γ-globulin for 30 min. For reversal of inhibition (REV.), the gut rings were pre-incubated for 20 min in the inhibitor-BSS, removed and incubated in inhibitor-free 2 % fluorescent γ-globulin in BSS for 30 min. IAc = iodoacetate, phlor = phloridzin, DNC = 4, 6-dinitro-ϕ-cresol. Arrows point to droplets of fluorescent γ-globulin within epithelium.

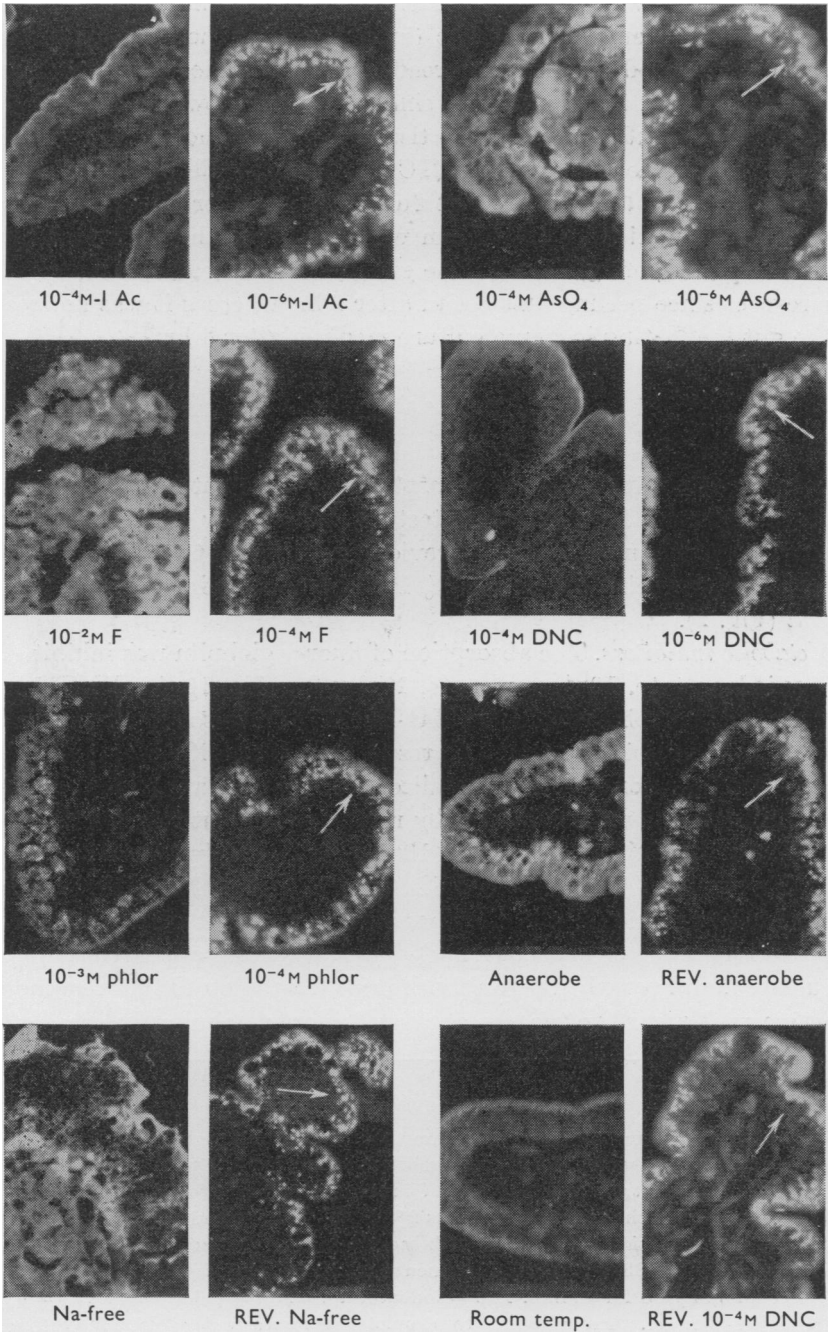

Fig. 3. For legend see opposite page.

that the intestinal epithelium of the piglet with diarrhoea, even though vacuolated, still was without evidence of γ-globulin absorption. The 14-day-old chick embryo yolk sac (in contrast to the 14-day-old chick embryo gut) tested at this time readily took up fluorescent γ-globulin (not shown in Fig. 1 but uptake is similar to that shown in Fig. 4).

In vitro *functioning time.* In gut tissues that had incubated for 6 hr in either BSS or tissue culture media (TC 1099), it was still possible to detect absorption when placed in the test fluor-γ globulin for 30 min. However, even though functioning epithelium was present at this time, there was extensive peeling of epithelium. The gut in BSS seemed more damaged in this respect since peeling first began after 2 hr, whereas it was noticeable in the gut incubating in tissue culture medium after 4 hr.

Effect of inhibitors

Large colloidal molecules. Under the conditions of the test, it was not possible to detect inhibition of absorption after the gut had been exposed to large molecules for 3 hr before testing with fluor-γ globulin. Note the fluorescent γ-globulin in cells previously exposed to 5% porcine γ-globulin, 5% porcine albumin, 5% PVP, 1% colloidal carbon and 10^{10} *E. coli* (Fig. 2).

Metabolic inhibitors. The absorption of fluor-γ globulin was inhibited by 10^{-4} M iodoacetate, 10^{-4} M arsenate, 10^{-2} M fluoride, 10^{-4} M-DNC, 10^{-3} M phlorrhizin, anaerobic conditions, Na^+-free BSS and also room temperature (Fig. 3). The inhibition seemed to result from a specific metabolic block rather than from non-specific generalized damage or death to the cell, since the inhibition was reversible; e.g. by removing the gut from 10^{-4} M-DNC to inhibitor-free BSS (Fig. 3, REV. 10^{-4} M-DNC) and by removing the gut from Na^+-free to Na^+-replete BSS (Fig. 3, REV. Na-free) and from anaerobic to aerobic (Fig. 3, REV. anaerobe). The gut incubated in Na^+-free BSS became oedematous and the epithelium appeared lacy and vacuolated, and yet when placed back into Na^+-replete BSS it regained its function with regard to the absorption of fluor-γ globulin. In some of the

Legend to Fig. 4

Fig. 4. Effect of metabolic inhibitors on the absorption of γ-globulin by 12-day-old chick embryo yolk sac. Pieces of yolk sac (5-mm square) were pre-incubated aerobically (except where noted) at 37° C in the inhibitor-BSS for 20 min, removed and incubated in the inhibitor-BSS plus 2% fluorescent γ-globulin for 30 min. For reversal of inhibition (REV.), the yolk sac pieces were pre-incubated for 20 min in the inhibitor-BSS, removed and incubated in inhibitor-free 2% fluorescent γ-globulin in BSS for 30 min. IAc = iodoacetate, phlor = phloridzin, DNC = 4, 6-dinitro-ϕ-cresol. Arrows point to droplets of fluorescent γ-globulin within epithelium.

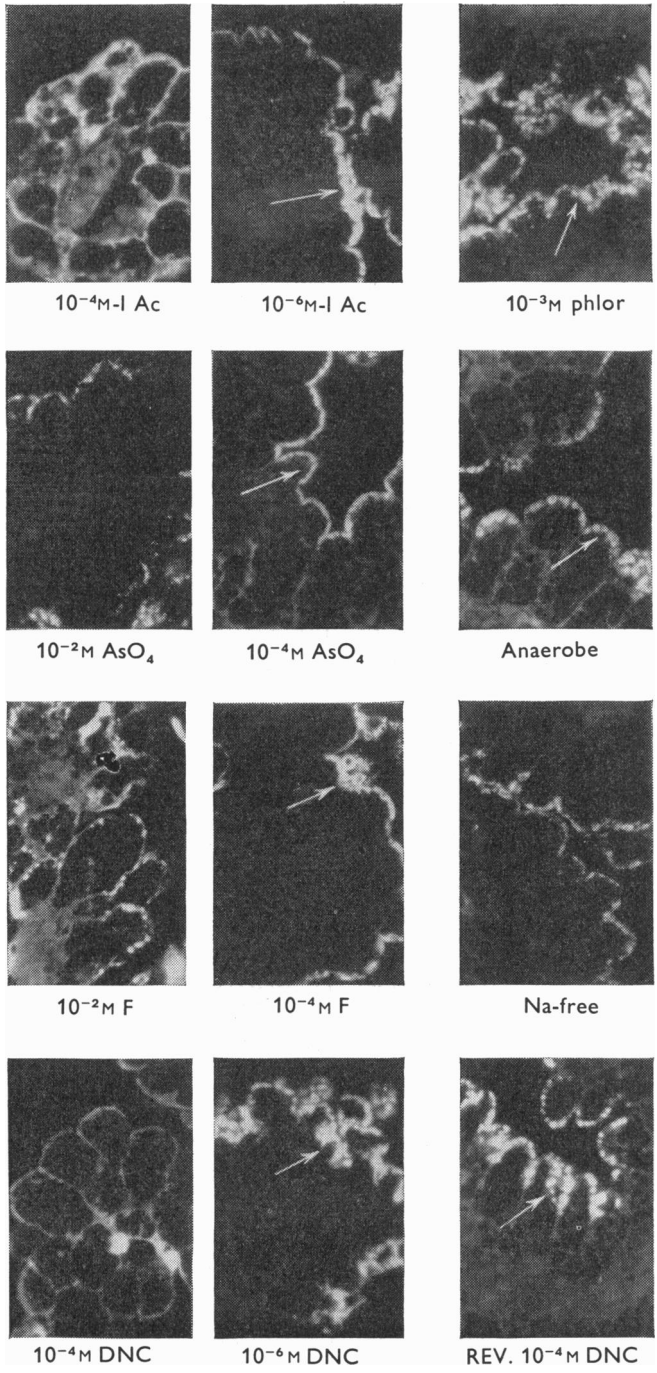

Fig. 4. For legend see opposite page.

preparations, the epithelium appeared to show adsorption of fluor-γ globulin on to the microvilli (see Fig. 3, 10^{-4} M iodoacetate and 10^{-4} M-DNC).

Chick embryo yolk sac treated similarly with metabolic inhibitors reacted like the piglet's gut except that the inhibition was not as obvious nor as complete with the 10^{-2} M arsenate, Na^+-free BSS and anaerobic conditions (Fig. 4). Perhaps this less-than-complete inhibition reflects the presence of much exogenous and endogenous substrate, since the yolk sac cell was not starved and it was not possible to wash away all the yolk material. Note the absorbed fluorescent γ-globulin appeared as a hollow ball, possibly protein adsorbed to a lipid core.

Surface-active agents. There was no evidence that a variety of surface-active agents altered the mature intestinal epithelium so that it could then absorb fluorescent γ-globulin in a manner analogous to the immature neonatal gut. Cholate (20 mM) and triton $\times 100$ (4 mM) were particularly destructive to the villi. Many of the preparations showed adsorption on to the microvilli but not within the cell. In damaged cells, if anything, only a diffuse homogeneous increase in background fluorescence was observed, in no way resembling the discrete fluorescing droplets in the neonatal gut.

DISCUSSION

Results reported here demonstrate that it is possible to obtain actively pinocytosing mammalian cells that are not part of the reticulo-endothelial system. These cells—namely, intestinal epithelium of the neonatal pig gut—are amenable to manipulation *in vitro*, and some of the parameters necessary for such manipulations are delineated.

Results obtained with the use of fluorescent γ-globulin although not quantitative are easy to interpret. Quantitative results obtained with techniques based on the uptake of radioactive, large molecules are not as easily interpreted because of the difficulty of distinguishing the non-specific surface adsorption from the specific absorption into the cell. Also, the 'injured cell reaction' makes quantitative interpretation hazardous since the permeability of an injured cell is altered and manipulated cells are easily injured (Holtzer & Holtzer, 1960; Ryser *et al.* 1962; Ryser, 1963). With the fluorescent γ-globulin technique, any adsorption on to the cell was easily distinguished from absorption within the cell. If there was uptake caused by cell injury (even in the cells treated with strong surfactants), it was reflected by a slight, homogeneous increase in fluorescence—while in marked contrast specific absorption was characterized by intense, green droplets within the cell.

In a previous study dealing with the specificity of absorption of large molecules in the neonatal piglet, such diverse molecules as PVP, various egg white antigens and various bovine colostral antigens were absorbed *in vivo* by the piglet (Lecce, Matrone & Morgan, 1961). Since this absorption was qualitatively non-specific the possibility was considered that simple passive diffusion might account for the transfer of large molecules from the gut to the blood. However, this does not seem to be so. The present results with metabolic inhibitors indicate that this transfer is energy coupled and in that sense truly an active process. Furthermore, it is interesting that the same inhibitors (including the requirement for sodium) that antagonize the transport of a simple small molecule like glucose in intestines of other animals inhibit the transfer of large complex molecules in our system (Crane & Mandelstam, 1960; Czaky & Thale, 1960; Crane, 1962; Bihler & Crane, 1962).

The chick embryo yolk sac is another source of primitive pinocytosing cells; however, it is more difficult to detach these cells from their metabolic and nutritive history, and the results are not so clear-cut. In contrast, gut from the unfed neonatal piglet seems devoid of potentially interfering substrates, although it has sufficient endogenous energy for transport. It is possible that the neonatal piglet derives its energy for active transport from the same source as the unfed hamster; namely, the endogenous energy may come from non-carbohydrate metabolites such as fat or fatty acids (Crane & Mandelstam, 1960).

Although *in vitro* the neonatal pig gut readily takes up large molecules into the intestinal epithelium, we have never seen any evidence for the secretion of this absorbed γ-globulin out of the epithelium into the lamina propria, even in gut incubated for 3 hr in fluorescent γ-globulin in tissue culture media. Perhaps secretion into the lamina propria requires conditions conducive to organ function, while the needs for cellular absorption are not nearly so demanding—requiring mainly the maintenance of cellular integrity.

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