Acta vet. scand. 1967, *8*, 76—82.

From McGill Unit of Cell Metabolism, McGill University, Montreal, Canada.

A METHOD FOR THE PREPARATION OF SUSPENSIONS OF INTESTINAL MUCOSAL CELLS BY MEANS OF CALCIUM CHELATORS

(A preliminary report)

By

*Erling Søgnen**)

A logical extension of the in vitro methodology is the isolation of single epithelial cells (*Wilson* 1962). The first report on a successful solution of this problem was given by *Harrer et al.* (1964) who used a method of trypsinisation as described by Moscona. This preparation has recently been reported to have maintained considerable metabolic activity (*Stern & Reilly* 1965) and transports glucose actively (*Stern & Jensen* 1966). Cell "ghosts" which did not respire or consume glucose have recently been prepared by *Clark & Porteous* (1965).

The present paper describes a method for the preparation of a suspension of single cells of the intestinal mucosa by means of EDTA and the more specific calcium chelator EGTA (Ethylene glucol-di-(aminoethyl)-tetra acetic acid). The presence of EDTA causes fragmentation of the intestinal mucosal epithelium in vitro (*Hansen* 1959, *Erichsen & Søgnen* 1964).

The fact that glucose utilization in everted sacs of small intestine was only slightly decreased when disintegration of the epithelium takes place in presence of EDTA, led to the suggestion that this compound might be used for the purpose of isolating viable intestinal mucosal cells (*Søgnen* 1965). EDTA, a non-fat-

*) Present address: Department of Pharmacology and Toxicology, Veterinary College of Norway, Oslo.

soluble substance with a molecular weight of 372, is unlikely to penetrate cell membranes. This is confirmed by the fact that EDTA is very poorly absorbed from the intestinal tract and is found to be confined to the extracellular fluid in volume distribution experiments (*Foreman & Trujillo* 1954). Furthermore, it is likely to be an advantage that Ca^{++} and Mg^{++} removed by EDTA during the disintegration process can be replaced before the cells are used for experimental purposes.

METHOD

Unfasted, male hooded rats of the local strain weighing 200—300 g were killed by a blow in the neck. The abdomen was opened, a cannula was introduced at flexura duodeno-jejunalis, and the small intestine was rinsed out with 50 ml ice cold oxygenated saline, removed, everted as described by *Wilson & Wiseman* (1954), and filled with 20 ml Ca^{++}- Mg^{++}-free Krebs Ringer phosphate solution, containing 2 mM EDTA.

The filled loop was incubated in 50 ml of the same solution in a 250 ml Erlenmeyer flask, which was packed in ice in a polythene beaker, oxygenated for 4 min., sealed and shaken gently (40 oscillations/min.) for 1 hr. The flask was finally shaken by hand for 30 sec. at a rate sufficient to detach the epithelium from the loop. The loop was removed and discarded. The outer fluid containing the suspension of mucosal tissue was taken up twice in a 50 ml pipette with a narrow opening to disintegrate the epithelium. The suspension was centrifuged for 1 min., and the supernatant with the overlaying layer of fat and mucus was discarded. The mucosal tissue was washed twice with 50 ml ice cold saline containing twice the normal concentration of Ca^{++} and Mg^{++}. Finally the cells were suspended in 10 ml Krebs Ringer phosphate solution, transferred to a 12 ml graduated centrifuge tube by means of a Pasteur pipette, through which they were passed repeatedly to increase the disintegration. The cells were packed by centrifugation for 2 min. at $500 \times g$, the volume was noted, the supernatant discarded, and the cells were resuspended in Krebs Ringer phosphate solution usually in dilutions 1:10 or 1:20. All solutions used during the procedure were kept packed in ice.

Immediately after the preparation of the cell suspension aliquots were transferred to Warburg flasks, or 25 ml Erlenmeyer flasks, with or without center well, depending on the type of

experiment. The transference of cell suspension, and addition of substrate, were carried out while the flasks were kept on ice under continual oxygenation, through polythene tubes, which were kept above the surface of the incubation fluid. When the water bath shaker was used, the flasks were sealed with rubber stoppers (Fisher Catalogue 14-126) through which TCA could be injected to stop reactions.

Preliminary examinations of the mucosal tissue, before the final disintegration, revealed nicely detached, more or less disintegrated, epithelium, but very few single cells. After the disintegration the cells were totally dispersed and could be counted in a hemocytometer when stained with methylene blue or nigrosin.

RESULTS AND DISCUSSION

The cell yields varied from 1—2.5 ml packed cells containing from 160×10^6 to 220×10^6 cells per ml. The cells had an average dry weight of 20 % and a protein weight of 7 %. Oxygen uptake and glucose utilization were recorded at 37, 32 and 27°C. At 27°C there was a tendency to an increase of the period in which the respiration curve was linear and glucose utilization and respiration was reduced with about 10 %. The initial steps of the procedure ought to be carried out very rapidly, but a lag time up to 90 min. between the preparation and the final incubation of the cells did not affect their respiration and glucose utilization. Transport experiments were carried out as described by *Tenenhouse & Quastel* (1960) for Ehrlich ascites cells. In several experiments a swelling of the cells took place during the incubation.

Table 1. Tissue medium distribution of glycine-C^{14} in a suspension of intestinal mucosal cells after 15 min. incubation under various conditions.
Initial concentration of glycine in the medium: 1 μmol/ml.

		Glycine conc. in the cell water / Glycine conc. in the medium
Control		1.91
DNP	0.1 mM	1.92
Control		1.63
Anaerobic		1.48
Control		1.48
Choline		1.34

The calculations were, therefore, based on the volume of cell water at the end of the experiment.

Experiments show that the ratio of glycine concentration between the tissue and the medium (T/M) varies from unity to about 2. Anoxia, dinitrophenol, and replacement of sodium with choline do not abolish the cumulation of glycine in the cells (Table 1). When, however, sodium is replaced entirely with potassium, glycine transport is markedly affected (Table 2). The same effect has previously been observed on glucose transport (*Riklis & Quastel* 1958), and on amino acid transport in everted rings of small intestine (*Bronk & Parsons* 1965). T/M values for methionine are similar to those obtained with glycine. In repeated experiments with different concentrations of methyl glucose no T/M value higher than 1 has been observed.

Table 2. Effects of replacement of Na^+ by K^+ on glycine-C^{14} uptake in mucosal cells in Krebs Ringer medium buffered with 10 mM Tris-HCl pH 7.4.
Initial concentration of glycine in the medium: 1 μmol/ml.
Duration of incubation 30 min.

Exp. no.	Predominant cation	T/M	Final conc. μmol/ml		Per cent decrease of conc. in cell water
			Medium	Cell water	
1	Na	1.78	0.94	1.67	33
	K	1.18	0.96	1.12	
2	Na	2.73	0.82	2.23	50
	K	1.17	0.95	1.11	
3	Na	2.53	0.81	2.13	36
	K	1.46	0.94	1.37	
4	Na	1.3	0.90	1.18	16
	K	1.04	0.96	0.99	

The fact that there is a difference in glycine uptake in the cells in presence and absence of sodium indicates that the cells have maintained their ability to transport actively.

A further indication for the existence of normal membrane function in the cells is the effect of phlorizin on the glucose metabolism (Table 3).

It has been shown that phlorizin accumulates in the brush-border of the cells in mucosal preparations, but does not penetrate appreciably beyond this region (*Stirling & Vinter* 1966). It is generally agreed that phlorizin inhibits the entry of glucose into

Table 3. Effect of phlorizin on rate of oxygen uptake and glucose utilization by intestinal mucosal cells.
Concentration of glucose in the medium 5 μmol: 60 μg atoms C/ml.

Conc. of phlorizin M/l	Q_{O_2}	μg atoms C^{14} from glucose-U-C^{14} per 100 mg wet weight	Per cent inhibition
Nil	12.5	7.95	
5×10^{-6}	12.0	6.90	15
5×10^{-5}	11	5.10	36
5×10^{-4}	6	1.62	80

columnar cells by altering the brushborder membrane permeability (*Newey et al.* 1959). It is therefore likely that the inhibition of glucose metabolism in presence of phlorizin is a consequence of reduced uptake into the cells.

From this result it is apparent that mucosal cells obtained from the rat small intestine by the procedure described possess a metabolic activity which compares favourably with all intestinal preparations previously in use.

One of the important features of this method is that it opens up the possibility of making similar preparations from other larger animal species. This should permit more accurate comparison of intestinal biochemical processes in different species than has been possible hitherto.

ACKNOWLEDGMENT

I wish to thank Professor *J. H. Quastel* for the opportunity to work in his unit and for valuable advice from him and his coworkers. Thanks are also due to Professor *C. P. Leblond* and Mr. *G. Altmann* for their preliminary histological examination of the cells, and to Mr. *Joav Prives* for his interest in the biochemical aspects of the preparation. My stay in Canada has partly been financed by a grant from the Norwegian Research Council for Sciences and Humanities.

REFERENCES

Bronk, J. R. & D. S. Parsons: Accumulation and incorporation of amino acid in rat intestine in vitro. Nature (Lond.) 1965, *208*, 786—787.

Clark, B. & J. W. Porteous: The isolation and properties of epithelial cell "ghosts" from rat small intestine. Biochem. J. 1965, *96*, 539—551.

Erichsen, S. & E. Søgnen: Unpublished data 1964.

Foreman, H. & T. T. Trujillo: The metabolism of C^{14}-labeled ethylenediaminetetraacetic acid in human beings. J. Lab. clin. Med. 1954, *43*, 566—571.

Hansen, I. A.: Effects of Ca^{++} and Mg^{++} on cell adhesion. J. Sci. indust. Res. 1959, *180*, 84—85.

Harrer, D. S., B. K. Stern & R. W. Reilly: Removal and dissociation of epithelial cells from the rodent gastrointestinal tract. Nature (Lond.) 1964, *203*, 319—320.

Newey, H., B. J. Parsons & D. H. Smyth: The site of action of phlorrhizin in inhibiting intestinal absorption of glucose. J. Physiol. (Lond.) 1959, *148*, 83—92.

Riklis, E. & J. H. Quastel: Effects of cations on sugar absorption by isolated surviving guinea pig intestine. Canad. J. Biochem. 1958, *36*, 347—362.

Stern, B. K. & R. W. Reilly: Some characteristics of the respiratory metabolism of suspensions of rat intestinal cells. Nature (Lond.) 1965, *205*, 563—565.

Stern, B. K. & W. E. Jensen: Active transport of glucose by suspensions of isolated rat intestinal cells. Nature (Lond.) 1966, *209*, 789—790.

Stirling, C. E. & W. B. Vinter: Phlorizin-H^3 distribution in mucosa from hamster intestine during inhibition of sugar transport. Fed. Proc. 50th Ann. Meet. 1966, *25*, Part 1, 640.

Søgnen, E.: Intestinal transport of glucose and sulphanilamide in vitro at low calcium concentrations and in the presence of calcium-binding substances. Acta pharmacol. (Kbh.) 1965, *22*, 61—68.

Tenenhouse, A. & J. H. Quastel: Amino acid accumulation in Ehrlich ascites carcinoma cells. Canad. J. Biochem. 1960, *38*, 1311—1326.

Wilson, T. H. & G. Wiseman: The use of sacs of everted small intestine for the study of the transference of substances from the mucosal to the serosal surface. J. Physiol. (Lond.) 1954, *123*, 116—125.

Wilson, T. H.: Intestinal Absorption. W. B. Saunders, London 1962, p. 34.

SUMMARY

A method is described for the preparation of a suspension of single cells of the intestinal mucosa by the calcium chelators EDTA and EGTA.

Preliminary experiments with oxygen consumption and glucose-utilization indicate a considerable metabolic activity of the cell suspension.

The difference in glycine uptake in presence and absence of sodium and the effect of phlorizin on the glucose metabolism of the cells indicates the existence of a qualitatively normal membrane function.

The application of a similar method for the purpose of obtaining viable in vitro preparations from intestinal tissue of larger animals is suggested.

ZUSAMMENFASSUNG

Eine Methode zur Herstellung von Suspensionen von intestinalen Mucosazellen mit Hilfe von Kalziumchelators.

Eine Methode zur Herstellung von Suspensionen von intestinalen Mucosazellen mit Hilfe von kalziumbindenden Substanzen EDTA und EGTA wird beschrieben.

Ergebnisse der preliminären Respiration- und Glucoseausnutzungsversuchen ergeben, dass diese Zellen eine bedeutende metabolische Aktivität zeigen.

Der Unterschied, was die Glycinaufnahme der Zellen anbetrifft bei Anwesenheit oder Abwesenheit von Natrium, und die Einwirkung von Phlorizin auf den Glucosemetabolismus lässt auf eine annähernde intakte Membranfunktion schliessen.

Wenn diese Methode zur Isolierung von Mucosazellen grosser Tierarten angepasst werden kann, wird vermutet, dass sie sich, bei komparativen Studien von intestinalem Transport und Metabolismus nützlich zeigen kann.

SAMMENDRAG

En metode til fremstilling av suspensjoner av intestinale mucosaceller ved hjelp av kalciumchelatorer.

En metode til fremstilling av suspensjoner av intestinale mucosaceller ved hjelp av de kalciumbindende substanser EDTA og EGTA beskrives.

Resultater av preliminære respirasjons og glukoseutnyttelsesforsök viser at cellene har betydelig metabolisk aktivitet.

Forskjellen i cellenes glycinakkumulering i nærvær og fravær av natrium og effekten av phlorizin på cellenes glukosemetabolisme peker i retning av en tilnærmet intakt membranfunksjon.

Hvis metoden kan tilpasses for isolering av mucosaceller fra store dyrearter, antas den å kunne bli nyttig bl. a. ved komparative studier av intestinal transport og metabolisme.

(Received December 5, 1966).