slice of dry cellulose sponge, and the mixture is kneaded thoroughly into the sponge with a pair of forceps. A section of uncoated cellulose sponge is seen in Fig. 1A. The appearance of a sponge immediately after impregnation with collagen is seen in Fig. 1B. The sponges are placed in petri dishes and dried overnight at 38°C. As the collagen-dispersing medium evaporates, the fibers coalesce, deposit on, and adhere firmly to the cellulose trabeculae as thin sheets of collagen (Fig. 1C). The coated sponges are then washed for 12 to 24 hours in 50 percent methanol containing 0.5 percent ammonium hydroxide, followed by two 1-hour washes in sterile distilled water. The sponges are washed for 1 hour in Hanks's balanced salt solution and then stored in fresh Hanks's solution, *p*H 6.8, at 4°C until ready for use.

The difference in growth of Walker tumor 256 has been studied in three different matrices—cellulose sponge only, collagen sponge (Ethicon) only, and collagen-coated cellulose sponge.

A dense population of the tissue-culture line of Walker tumor 256 (Pgh No. 63–79) growing on the flat surface of a glass culture tube (*5*) was scraped loose in 9 ml of fresh medium (30 percent calf serum and Eagle's minimal essential medium, in Hanks's balanced salt solution). The suspension was divided equally among three large, flat culture tubes, each containing four pieces of one of the types of sponge. Each piece of sponge measured 12 by 6 by 1 mm. The suspension of cells was thoroughly irrigated through each piece of sponge. The recipient tubes were stoppered and incubated at 35°C on a stationary rack for 1 day. Subsequently they were placed on a rocking platform in the incubator and fed appropriate, increasing volumes of nutrient depending on the changes in *p*H produced in the medium by the growing cells.

In the first experiment one sponge in each group was fixed in Bouin's after 2 days, and the remaining three were fixed after 6 days. In the second experiment prepared in the same way, two sponges in each group were fixed after 8 days and two after 13 days.

Both experiments gave similar results. Adhesion of the cells on the bare cellulose trabeculae was meager, whereas collagen-matrix surfaces—either collagen-coated cellulose or collagen sponge alone—were covered with adherent proliferating cells. The difference was striking in less than a week. The results observed in the sponges fixed only 6 days after inoculation are illustrated in Fig. 2, A–C. As growth proceeded in sponges made of collagen alone, the mass of each sponge progressively decreased to less than half of its original size during a 2-week period of cultivation. Histopathologic examination of such older cultures revealed the surface of the sponge, the part in immediate contact with the medium to be covered with viable cells, but the remainder of the matrix consisted of frayed, collapsed trabeculae of collagen and of necrotic cells. In contrast, during the same period the collagen-coated cellulose sponge retained its shape, including the pattern of its open alveolae. Medium permeated the sponge through these connected spaces permitting vigorous growth throughout the matrix.

Collagen-coated cellulose sponge as a matrix for supporting three-dimensional growth in vitro eliminated the major problems of the combined plasma clot and sponge and of collagen sponge alone. It retains the best features of each. The cellulose matrix allows the interstices in the sponge to remain open. The lining of the trabeculae by collagen provides a surface on which a variety of cells adhere. In the interstices of the sponge, lacunae of varying sizes provide foci where groups of cells may more readily retain in their microenvironment the products of their metabolism.

Using several kinds of inocula we have recently observed that collagen-coated cellulose sponge provides an excellent matrix for three-dimensional growth. These include suspensions of HeLa cells and of rodent ascites hepatomas, as well as explants of chick embryonic heart and liver.

This method facilitates the study of organized growth of cell populations under conditions that permit the correlation of observations in vitro with histopathologic examination of material in vivo.

JOSEPH LEIGHTON
GERALD JUSTH
MARTHA ESPER
Department of Pathology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania
RICHARD L. KRONENTHAL
Research Division, Ethicon, Inc., Somerville, New Jersey

References and Notes

1. R. L. Ehrmann and G. O. Gey, *J. Nat. Cancer Inst.* **16**, 1375 (1956).
2. J. Paul, *Cell and Tissue Culture* (Williams and Wilkins, Baltimore, 1965).
3. J. Leighton, *J. Nat. Cancer Inst.* **15**, 275 (1954).
4. ———, *ibid.* **12**, 545 (1951).
5. ——— and M. Esper, *Public Health Rep.* **79**, 642 (1964).
6. Supported by NIH grant CA-02800.

6 December 1966

D-Glucose: Preferential Binding to Brush Borders Disrupted with Tris(hydroxymethyl)aminomethane

Abstract. *The actively transported sugar* D-*glucose binds to brush borders disrupted with tris(hydroxymethyl)aminomethane in preference to* D-*mannose and* L-*glucose, which are not actively transported. This preferential binding of* D-*glucose is not dependent on either added* Na^+ *or adenosine triphosphatase activity stimulated by* Na^+ *with* K^+ *and* Mg^{2+}, *but it is temperature-dependent and is completely inhibited by 0.1 millimolar phlorizin and 1 millimolar mercuric chloride.*

It is currently thought that the mechanism of active transport of sugar by the small intestine comprises two steps: (i) an energy-independent, Na^+-dependent binding of the sugar to a "carrier" and (ii) an energy- and Na^+-dependent process which results in the dissociation of the "sugar-Na^+-carrier" complex, causing the sugar to be accumulated against its own concentration gradient (*1*). Furthermore, it has been proposed that a polyvalent or polyfunctional "carrier" with multiple binding sites may represent an integral portion of the membranes of the microvilli or brush borders of the mucosal cell (*2*) which is responsible for active sugar transport (*3*), rather than a specialized molecule shuttling back and forth within the membrane barrier. Phlorizin can competitively inhibit sugar transport, perhaps by virtue of its greater affinity for a site on the "carrier" within the brush-border membrane (*4*). In addition, data of autoradiographic studies suggest that phlorizin is bound to the mucosal microvilli and does not penetrate the mucosal cell (*5*).

We determined if the actively trans-

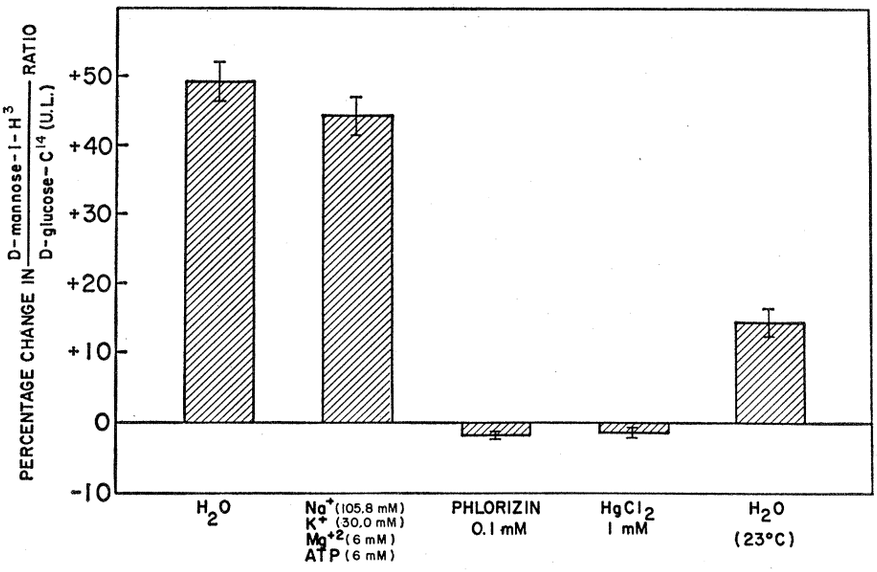

Fig. 1. Percentage change in the DPM ratio of D-mannose-1-H^3 to uniformly labeled D-glucose-C^{14} in the supernatant from tris-disrupted brush borders under various conditions at 37°C, unless noted otherwise. In experiments involving preferential binding of D-glucose to tris-disrupted brush borders in the presence of H_2O, a final Na^+ concentration of 0.27 mmole/liter was measured with a Beckman DU flame photometer from nitric acid preparations. The Na^+ concentration present under these conditions can be attributed to bound Na^+ within the brush border membrane and to a small amount of contamination from the washing solution, disodium EDTA. However, the low Na^+ concentration in these experiments is still much greater than the concentration of either D-mannose-1-H^3 (10^{-5} m*M*) or D-glucose-C^{14} (10^{-3} m*M*). Each bar point represents the mean of at least four experiments. The vertical lines represent one standard error of mean above and below the bar points.

ported sugar D-glucose is bound to isolated brush-border membranes in preference to D-mannose or L-glucose, which are not actively transported but which are similar to D-glucose with respect to molecular configuration. Since preferential binding of D-glucose was established, we studied the nature of this binding in relation to factors relevant to the first step in the active transport of this sugar by the small intestine.

The following procedures were performed in the cold (2°C) unless noted otherwise. The mucosal epithelium was scraped, with a glass microscope slide, from jejunums of young hamsters (70 to 80 g). We used a modification of the methods of Miller and Crane (*6*) and Harrison and Webster (*7*) to isolate the epithelial brush-border membranes or microvilli. These methods yielded a substantially purified preparation of brush borders. We placed the chilled mucosa from each animal in 45 ml of cold 5 m*M* disodium ethylenediaminetetraacetate (EDTA) at *p*H 7.0. A total of 8 to 12 hamsters were used each day. After the mucosa from every two animals were collected, the suspensions were combined and homogenized in a Sorvall Omni-Mixer for 30 seconds at a low speed (control position four). The homogenate was then passed through No. 9 bolting cloth (Dufour-Anchor Brand, 97 meshes per linear inch) and next through No. 25 bolting cloth (200 meshes per linear inch). Finally, the filtered homogenate was passed through glass wool to remove any nuclei that might be present (*7*). The final filtrate was centrifuged at 600*g* for 10 minutes in 100-ml plastic centrifuge tubes. The supernatant was removed, and the precipitated brush borders were washed three times with 5 m*M* EDTA. After the last wash, in which the brush borders were transferred to glass, conical, 15-ml graduated centrifuge tubes, the supernatant was carefully removed with a dropper pipette, and a volume of cold 1*M* tris(hydroxymethyl)aminomethane (tris) (*p*H 8.2 at 2°C), exactly four times the volume of the brush borders, was added. This treatment with tris causes dissociation of the brush borders isolated from the epithelial cells of hamster jejunum (*8*). In a few cases, we substituted cold distilled water for tris to maintain intact brush borders. The microvilli suspended in either tris or distilled water were stirred for 3 minutes at a moderate speed with a Vortex stirrer and then placed on ice for 45 minutes. Exactly 1 ml of the suspension of intact or disrupted brush borders was used for each experiment, which consisted of the following procedure. To a glass test tube was added 1.0 ml of the suspension of intact or disrupted brush borders, 2.2 ml of either plain distilled water or distilled water in which was dissolved various substances, and 0.1 ml of a radioactive sugar mixture containing D-mannose labeled with tritium and an enantiomorph of glucose labeled with C^{14}. The concentration of D-mannose-1-H^3 in the total of 3.3 ml was 10^{-5} mmole/liter and for either uniformly labeled D-glucose-C^{14} or for L-glucose-1-C^{14}, 10^{-3} mmole/liter. The specific activities of these purified labeled hexoses were 700, 3.47, and 4.33 mc/mmole, respectively. The test tubes containing a final volume of 3.3 ml were then incubated for 1 hour at either 37°C or, in a few cases, at 23°C (room temperature). After incubation, they were centrifuged at top speed in an International clinical centrifuge for 5 minutes at room temperature.

Precisely 0.1 ml of the supernatant was added to 15 ml of liquid scintillation fluid—5 g of PPO, 2,5-diphenyloxazole, 0.2 g of POPOP, 1,4-bis-[2-(5-phenyloxazolyl]-benzene, and 100 g of naphthalene dissolved in a liter of spectroquality *p*-dioxane—in vials. The tritium-labeled D-mannose and the C^{14}-labeled D-glucose or L-glucose were counted with a Nuclear-Chicago liquid scintillation system, Series 720, and the disintegrations per minute (DPM) for each hexose was calculated. The ratios of the DPM for H^3 to that for C^{14} were obtained for the controls without the brush borders and for experimentals under various conditions with the membranes. An increase in this ratio indicates a preferential binding of the C^{14}-labeled sugars.

Experiments on intact brush borders suspended in distilled water, or in Na^+ (105.8 m*M*) and K^+ (30.0 m*M*), or Na^+ (105.8 m*M*) with K^+ (30.0 m*M*), Mg^{2+} (6.0 m*M*), adenosine triphosphate (ATP) (6 m*M*) and either D-mannose-1-H^3 and uniformly labeled D-glucose-C^{14} or D-mannose-1-H^3 and L-glucose-1-C^{14} at 37°C demonstrated that no change in the DPM ratios had occurred. Consequently, no preferential binding of any of these sugars was evident under these conditions.

However, with tris-disrupted brush borders incubated at 37°C in the presence of these labeled sugars and under these same conditions, there was almost a 50 percent increase in the DPM ratio of D-mannose-1-H^3 to D-glucose-C^{14} and no change in the DPM ratio of D-mannose-1-H^3 to L-glucose-1-C^{14}. This, of course, indicated a stereospecificity for this binding, since the actively transported sugar, D-glucose, was being preferentially bound to the preparation of tris-disrupted brush border membranes. Incidentally, when the tris–brush border suspension was kept on ice for a period longer or shorter than 45 minutes, less binding of D-glucose occurred.

The percentage change in the DPM ratio of D-mannose-1-H^3 to D-glucose-C^{14} in the presence of tris-disrupted brush borders under various experimental conditions is shown in Fig. 1. It can be seen that binding of D-glucose in plain distilled water is similar to that in Na^+ (105.8 m*M*) with K^+ (30.0 m*M*), Mg^{2+} (6 m*M*) and ATP (6 m*M*), which Faust and Wu (*9*) have shown to stimulate mucosal adenosine triphosphatase activity. Additional experiments also have indicated that the preferential binding of D-glucose under these conditions is unaffected by 0.1 m*M* ouabain, an inhibitor of adenosine triphosphatase activity stimulated by Na^+ and K^+ in hamster mucosal homogenates (*9*). These data suggest that neither added Na^+ ions nor adenosine triphosphatase activity stimulated by Na^+ with K^+ and Mg^{2+} are required for this binding. However, the concentration of Na^+ in the experiments in which Na^+ is not added is still greater than the concentration of uniformly labeled D-glucose-C^{14} (Fig. 1). Some of these Na^+ ions may be closely associated with glucose binding sites. Therefore, it is impossible to test the Na^+-dependency hypothesis for D-glucose binding in these experiments, although it is obvious that an increase in the Na^+ concentration does not produce an increase in D-glucose binding. However 0.1 m*M* phlorizin completely inhibits the binding of D-glucose, presumably by competing with D-glucose for binding sites within the tris-disrupted membranes (Fig. 1). The complete inhibition of D-glucose binding in the presence of 1 m*M* $HgCl_2$ suggests that perhaps protein and sulfhydryl groups (*10*) within the disrupted microvilli preparation are involved in this process. Binding of D-glucose at 23°C instead of 37°C in plain distilled water is reduced by approximately 70 percent, indicating a temperature dependency (temperature coefficient, Q_{10}, of 2.4) for this phenomenon (Fig. 1).

We find that D-glucose is preferentially bound to isolated and disrupted brush borders of epithelial cells from hamster jejunum. The properties of this binding are compatible with the initial step in the proposed mechanism of active sugar transport by the small intestine.

ROBERT G. FAUST
SHIH-MIN L. WU
MARY L. FAGGARD
Department of Physiology,
University of North Carolina
School of Medicine, Chapel Hill

References and Notes

1. R. K. Crane, *Fed. Proc.* **21**, 891 (1962); *Biochem. Biophys. Res. Commun.* **17**, 481 (1964).
2. J. T. Wong, *Biochim. Biophys. Acta* **94**, 102 (1965); F. Alvarado, *Science* **151**, 1010 (1966).
3. D. B. McDougal, Jr., K. D. Little, R. K. Crane, *Biochim. Biophys. Acta* **45**, 483 (1960).
4. B. J. Parsons, D. H. Smyth, C. B. Taylor, *J. Physiol.* **144**, 387 (1958); F. Alvarado and R. K. Crane, *Biochim. Biophys. Acta* **56**, 170 (1962).
5. C. E. Sterling and W. B. Kinter, *Fed. Proc.* **25**, 640 (1966).
6. D. Miller and R. K. Crane, *Anal. Biochem.* **2**, 284 (1961).
7. D. D. Harrison and H. L. Webster, *Biochim. Biophys. Acta* **93**, 662 (1964).
8. A. Eichholz and R. K. Crane, *J. Cell. Biol.* **26**, 687 (1965).
9. R. G. Faust and S-m. L. Wu, *J. Cell. Physiol.* **67**, 149 (1966).
10. A. White, P. Handler, E. L. Smith, *Principles of Biochemistry* (McGraw-Hill, New York, ed. 3, 1964), p. 237.
11. Supported by PHS grant AM07998 and by a grant from the School of Medicine, University of North Carolina.

29 November 1966

Crayfish Muscle: Permeability to Sodium Induced by Calcium Depletion

Abstract. *Membrane of crayfish muscle fibers becomes selectively permeable to sodium when the calcium concentration of the bathing medium is reduced. Removal of calcium or its reduction below 1 or 2 millimole per liter causes large transient depolarizations up to 70 millivolts in amplitude. They resemble prolonged action potentials and occur only in the presence of sodium. The responses are abolished when tris(hydroxymethyl)aminomethane or lithium is substituted for sodium, and are blocked by tetrodotoxin even in the presence of sodium.*

In general the gradedly responsive arthropod muscle fibers derive their depolarizing electrogenesis from the inward electrochemical gradient for Ca (*1*) or Mg (*2*, *3*). The fibers can develop spikes in Na-free media with an increase in the concentration of alkaline earth cations or by treatment with procaine or tetraethylammonium ion (TEA). Tetrodotoxin (TTX) does not affect these spikes (*3*, *4*), whereas it abolishes spikes that require the presence of Na (*5*, *6*). We have observed a depolarizing response of crayfish muscle fibers that is induced by removal of Ca, is dependent on the presence of Na, and is blocked by TTX.

Single fibers were isolated (*7*) from the flexor in the meropodite of the walking legs of crayfish of the genus *Orconectes* sp. They were equilibrated in various media, the standard saline being that of Van Harreveld (*8*). The basic experimental variable was a change in the concentration of Ca; in the experiments illustrated, the muscles were challenged with a Ca-free medium. In some (Fig. 1), all the Na was substituted stoichiometrically with Li or tris(hydroxymethyl)aminomethane (tris HCl). In others, the control medium was free of K (Fig. 2). In still another series the medium for equilibration was free of Cl, the latter being substituted stoichiometrically by propionate, to which the muscle fibers are effectively impermeable (*9*); in these experiments (Fig. 3) the medium was also free of calcium, and ethylenediaminetetraacetate (EDTA) was added in amounts between 1.0 and 0.01 mmole per liter. In certain experiments of each series, TTX (5×10^{-7} g/ml) was added. The muscle fiber was held in a lucite chamber which had a volume of 1.5 ml. The solution was changed at a rate of 3 to 4 ml/sec, and the flushing was continued for at least 2 seconds.

The changes in membrane potential when the bathing medium was changed were recorded with an intracellular microelectrode. In some experiments a second microelectrode was also introduced into the fiber to apply polarizing currents for resistance measurements (Fig. 1C). The electrophysiological equipment was standard for the