Active Sugar Accumulation by Isolated Intestinal Epithelial Cells. A New Model for Sodium-Dependent Metabolite Transport*

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ABSTRACT: The properties of active sugar transport by a preparation of isolated intestinal epithelial cells have been examined with special emphasis on evaluating the sodium gradient transport hypothesis. In common with other systems, the isolated cells accumulate sugars by a sodium-dependent process which is inhibited severely by ouabain or oligomycin at concentrations known to inhibit active sodium transport. The onset of inhibition by these agents is somewhat more rapid than might be expected for dissipation of the cellular Na gradient. Moreover, cells loaded to about 50 mM intracellular Na^+ are able to actively accumulate sugar within a 1–2-min interval when placed in a medium containing 20 mM Na^+. It can be shown that intracellular sodium is greater than 20 mM during the entire 2-min interval. Furthermore, the increment in sugar uptake is as great during the first 2 min as at any subsequent 2-min interval. Finally, cells loaded with 50 mM Na^+ and 2.5 mM 3-*O*-methylglucose begin to accumulate additional sugar immediately when placed in media with 20 mM Na^+ and 1.25 mM 3-*O*-methylglucose. No extrusion of sugar from the cell is observed in this case, even during the initial 15-sec interval. These observations are totally inconsistent with the sodium gradient hypothesis. A model has been devised which is more consistent with these data as well as other properties of sodium-dependent nonelectrolyte transport. It is based on the premise that energy for a number of energy-dependent processes may be derived from a common intermediate which is generated by the [$Na^+ + K^+$]-activated ATPase. A close analogy is drawn between this concept and one suggested to describe energy coupling in isolated mitochondria.

A vital role for sodium ion in the active transport of certain nonelectrolytes such as sugars and amino acids has been demonstrated by a wide variety of experimental techniques (Csaky, 1961, 1963; Bihler and Crane, 1962; Crane *et al.*, 1965; Schultz *et al.*, 1967). Although much of this effort has been directed at mammalian renal (Kleinzeller and Kotyk, 1961; Fox *et al.*, 1964; Hauser, 1969) and intestinal tissue (Bihler and Crane, 1962; Csaky, 1963; Crane *et al.*, 1965; Schultz *et al.*, 1967), it is apparent that a large number of other tissues possess similar sodium-dependent systems particularly for amino acids (Holdsworth and Wilson, 1967; Inui and Christensen, 1966; Wheeler and Christensen, 1967; Goodman, 1966; Riggs *et al.*, 1968). At present it is recognized that a number of features are characteristic for the process regardless of the tissue or origin. These include: (1) a high degree of dependence on sodium ion for accumulation of metabolite against a concentration gradient (Csaky, 1961, 1963; Bihler and Crane, 1962; Crane *et al.*, 1965; Schultz *et al.*, 1967; Kleinzeller and Kotyk, 1961; Fox *et al.*, 1964; Hauser, 1969; Holdsworth and Wilson, 1967; Inui and Christensen, 1966; Wheeler and Christensen, 1967; Goodman, 1966; Riggs *et al.*, 1958); (2) dependence (perhaps indirect) on a functioning energy conservation system (Darlington and Quastel, 1953; Crane and Mandelstam, 1960); (3) inhibition by ouabain and other cardiac glycosides (Csaky *et al.*, 1961; Csaky and Hara, 1965; Schultz *et al.*, 1966); (4) influx of sodium ion which is to some degree proportional in rate and extent to the transport of the nonelectrolyte (Schultz *et al.*, 1967; Schultz and Zalusky, 1964; Goldner *et al.*, 1969); (5) a high degree of correlation between the activity of the monovalent ion transport system and the transport of sugars or amino acids (Csaky *et al.*, 1961; Csaky and Hara, 1965; Schultz *et al.*, 1966; Schultz and Zalusky, 1964; Goldner *et al.*, 1969; Curran, 1965); and (6) inhibition of maximal metabolite transport by increased K^+ concentrations (Csaky, 1963; Bosackova and Crane, 1965; Riggs *et al.*, 1958). All of these observations have led to the general concept that the cellular sodium–potassium and sugar–amino acid transport systems are in some manner obligatorily linked. One of the most generally accepted hypotheses in this regard is the so-called sodium gradient hypothesis of Crane (for reviews, see Crane, 1965, 1968). According to that model, the energy necessary for active sugar accumulation is dependent upon and a consequence of an inwardly directed sodium gradient. The model envisions a mobile membrane carrier which has binding sites for both sodium ion and a sugar molecule. Moreover, when the sodium site is filled the carrier is thought to have a higher affinity for sugar (Crane *et al.*, 1965; Crane, 1968). Therefore in the high sodium environment at the outer surface of the cell a sodium–sugar–carrier ternary complex is readily formed, while at the relatively sodium poor environment of the inner membrane surface the complex readily releases its load of sodium and sugar. The sodium ion brought in by the sugar carrier is then actively extruded by the so-called sodium pump and this keeps the system poised for further sugar entry.

Two fundamental concepts are implicit in the above model. First, any asymmetry in sugar distribution able to be

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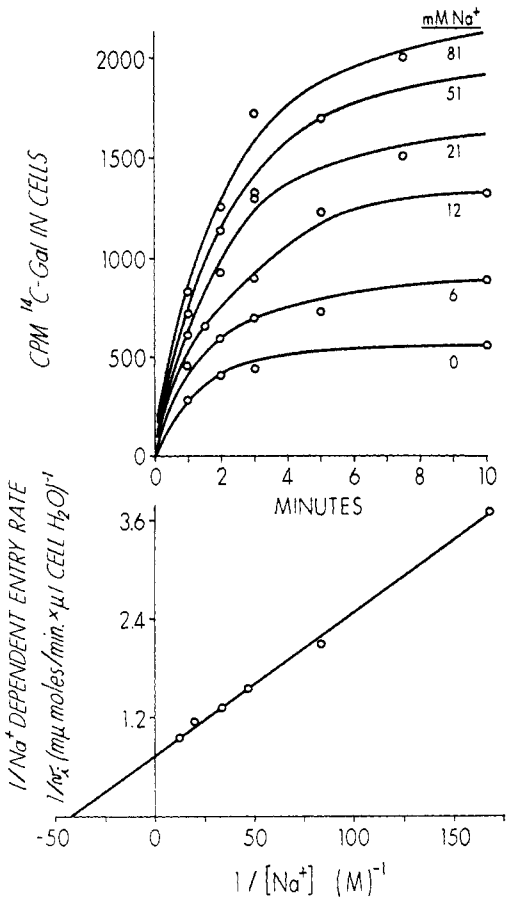


FIGURE 1: Upper: accumulation of [^{14}C]galactose by isolated intestinal cells at various concentrations of Na^+. Varying amounts of mannitol were used to replace NaCl in the standard medium to maintain uniform osmolarity. Cells (8.7 mg of protein) were incubated in 4.0-ml total volume at 37°. Sampling technique is described in the text. Lower: reciprocal plot of initial galactose transport velocity *vs.* sodium concentration. Only entry which was dependent on Na^+ was considered in constructing the plot. Accumulation during the first 1-min interval was taken as initial velocity.

generated across the cell membrane is a result of an opposite asymmetry in sodium distribution. Energy for the generation of the sugar gradient is supplied from energy inherent in the established sodium gradient. Second, the metabolic energy input required by the system occurs only at the site of sodium extrusion, there being no direct energy input at the sugar carrier itself. As a corollary to these two aspects the model further predicts that active sugar uptake should be possible in the absence of a metabolic energy input if the normal sodium gradient is maintained. In fact, reversal of the normal sodium gradient should cause an active extrusion of sugar from the cell if the Crane model is functionally correct.

In this regard, the data to be presented in this paper are of particular importance. Using isolated epithelial cells from the chicken small intestine we have shown that even when the normal sodium gradient has been reversed, sugar transport into the cell continues at the same rate and to the same extent. A new model has been devised which accommodates this fact as well as other literature data regarding the coupled nature of sodium and nonelectrolyte transport. This model is developed and described in some detail in the text.

Methods

Cells were prepared from small intestine of 1–6-week-old chickens by the method cited in the previous paper (Kimmich, 1970). All experiments were performed in media containing 20 mM Tris-Cl (pH 7.4), 3 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml of BSA,[1] and 0–80 mM NaCl. Sufficient mannitol was added to make the total osmolarity 300 mosmolar in each case. For convenience media are designated simply by their NaCl content. Thus 80 mM Na medium has 80 mM NaCl and 80 mM mannitol, while sodium-free medium has only 240 mM mannitol in addition to the other components. Unless otherwise stated sugars were used at a concentration of 1.25 mM, with 0.25 μCi/ml of the appropriate [^{14}C]sugar. All experiments were performed at 37°, and space measurements were performed by the methods cited earlier (Kimmich, 1970).

Uptake of sugar was monitored as described previously by the Millipore filtration technique (Kimmich, 1970). In some experiments cells preloaded with ^{22}Na were employed. In these instances, the cells were incubated for at least 15 min at 0° with radioactive sodium at the appropriate specific activity. These preloaded cells could then be added to sodium-free media in order to generate a situation in which the normal cellular sodium gradient is reversed (*i.e.*, high intracellular–low extracellular sodium). Most experiments of this nature were performed by adding 1.0 ml of cells "loaded" at 80 mM NaCl to 3.0 ml of mannitol medium so that extracellular Na^+ became 20 mM. Extrusion of cellular sodium could then be monitored by the same procedure used for sugar accumulation. Cells could be preloaded with [^{14}C]sugar by a similar procedure in which case they were added to medium containing the same concentration of sugar at identical specific activity. This technique allowed us to monitor sugar flux in cells initially at equilibrium with the suspending medium with regard to sugar distribution. It is important to remember that in these cases flux of [^{14}C]sugar represents net movement of sugar since loading and incubation were performed with sugar solutions of identical specific activity.

All salts, inhibitors, and reagents were obtained from commercial suppliers and of reagent grade quality. ^{22}Na and [^{14}C]sugars were purchased from Amersham-Searle and New England Nuclear Corp., respectively.

Results

The fact that ouabain severely inhibits sugar and amino acid transport by isolated intestinal cells (Kimmich, 1970) suggests that sodium ion is important to their sugar transport capability, as expected. This idea was demonstrated more directly by monitoring sugar accumulation at several levels of medium sodium concentration (Figure 1). Accumulation of galactose in the absence of sodium represents only equilibration of cell water with the medium as can be determined

[1] Abbreviations used are: BSA, bovine serum albumin; DNP, dinitrophenol; Gal, galactose; 3-OMG, 3-*O*-methylglucose.

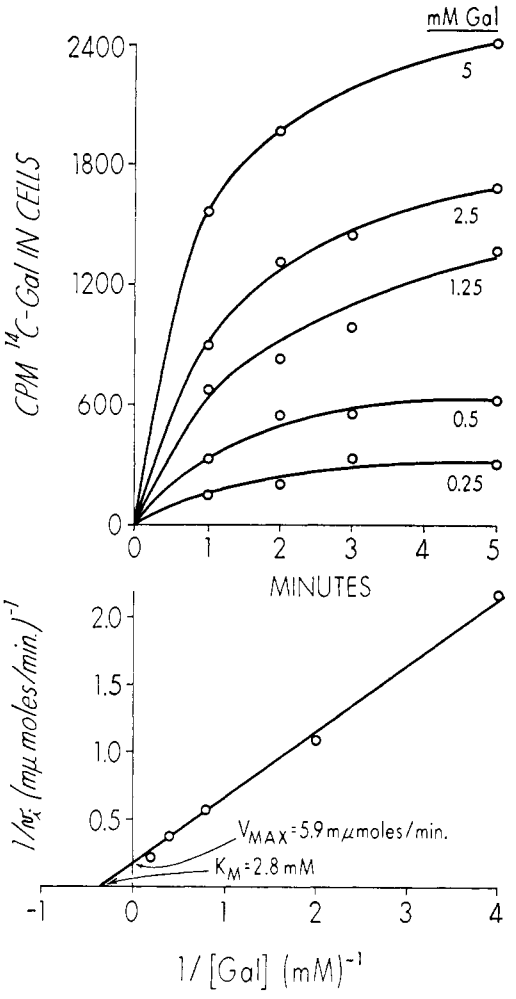


FIGURE 2: Upper: accumulation of [^{14}C]galactose by intestinal cells isolated in Na^+-free medium, and incubated in 80 mM Na^+ medium with varying concentrations of galactose. Mannitol was used to maintain osmolarity at 300 mosmolar in each medium; 7.3 mg of cell protein was used in 4.0-ml incubation volume; temperature, 37°. Lower: reciprocal plot of initial galactose transport velocity *vs.* galactose concentration. The indicated V_{max} refers to maximal rate for only those cells in a 200-μl sample. V_{max} = 300 mmoles/l. of cell H_2O × hr. Initial velocity was taken as entry during the first minute of the experiment.

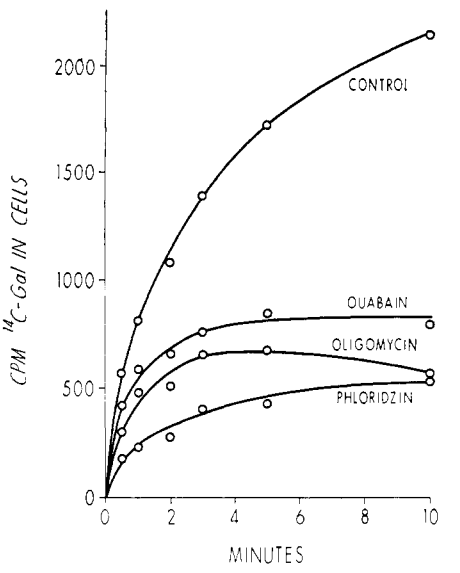


FIGURE 3: Effect of ouabain (0.5 mM), oligomycin (10 μg/ml), and phloridzin (0.2 mM) on accumulation of [^{14}C]galactose by isolated intestinal cells. Cells (12.8 mg of protein) were incubated in 4.0-ml volume at 37°. Galactose concentration = 1.25 mM.

from the values obtained for intracellular space. However, as sodium concentrations were increased there was a parallel increase in galactose transport rate which was half-maximal at 24 mM Na^+ as determined from the reciprocal plot shown in the lower half of the figure. V_{max} for 1.25 mM galactose occurs at infinite sodium concentration and was calculated to be 84 mmoles/l. of cell H_2O per hr. As expected this value is less than V_{max} calculated at optimal sodium and infinite galactose concentrations (250 mmoles/l. of cell H_2O per hr) (Kimmich, 1970). Only sodium-dependent entry rates were used in determining the indicated V_{max} since sodium-independent entry probably represents diffusional uptake or entry unrelated to the active transport process.

If the isolated cells are to be of value for evaluating the role of sodium in active sugar transport it would be helpful if the effects due to changes in medium sodium concentration are fully reversible. Complete reversibility would allow one to predict behavior of the sugar transport process at any given sodium concentration regardless of the sodium content of a previous environment. Accordingly, cells were prepared by the normal procedure except mannitol completely replaced sodium chloride in the isolation medium. After washing with the sodium-free medium several times, the sugar transport capability of the cells was measured at 80 mM Na^+ and several different galactose concentrations. The data are illustrated in Figure 2 along with graphical determinations of the kinetic parameters for galactose accumulation. The K_M of 2.8 mM and V_{max} of 300 mmoles of galactose/l. of cell H_2O per hr are in excellent agreement with those values obtained with cells prepared in the standard 80 mM NaCl medium (V_{max} = 250 mmoles/l. of cell H_2O per hr and K_M = 2.5 mM). This fact indicates that although sugar transport is regulated by the concentration of sodium in the suspending medium, the effects of this ion do not represent nonspecific, irreversible changes in cellular capability. The same conclusion has been reached by other investigators using various preparations of more intact intestinal tissue (Bihler and Crane, 1962; Schultz *et al.*, 1967; Goldner *et al.*, 1969; Csaky and Zollicoffer, 1960).

One observation which seemed inconsistent with the sodium gradient hypothesis was the rapid onset of ouabain inhibition of cellular sugar and amino acid accumulation (Kimmich, 1970). According to the hypothesis, nonelectrolyte transport should be prevented only when the sodium gradient is completely dissipated. Therefore, ouabain ought to allow an initial period of active sugar accumulation which then disappears as sodium equilibrates across the cell membrane. Instead, as shown in Figure 3, even at intervals as short as 30 sec, ouabain is severely inhibitory to galactose uptake, and the small gradient of sugar which is produced does not dissipate but is maintained for the entire duration of the experiment. Phloridzin allows only diffusional entry of sugar, and uptake in the presence of that inhibitor provides

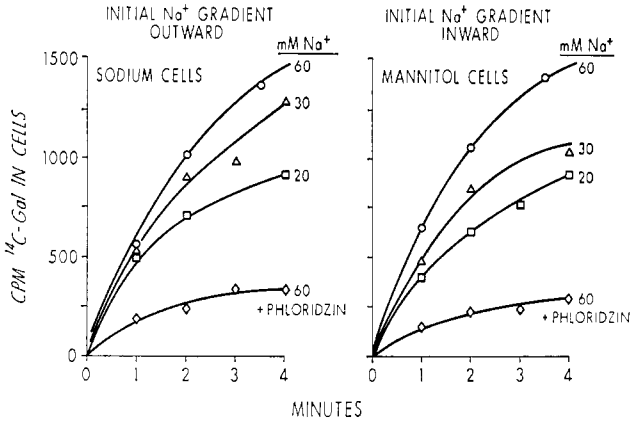


FIGURE 4: Accumulation of [^{14}C]galactose by intestinal cells preincubated (0°) in 80 mM Na^+ medium (left) or Na^+-free medium (right) and incubated (37°) in 20, 30, or 60 mM Na^+ medium. The initial Na^+ gradient across the cell membrane is opposite in the two cases as described in the text. Cells equal to 7 mg of protein were used in each case.

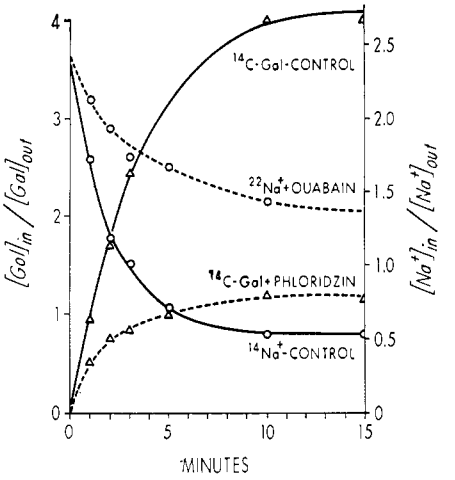


FIGURE 5: Accumulation of [^{14}C]galactose by cells preloaded (0°) with nonradioactive Na^+ in 80 mM Na^+ medium and extrusion of $^{22}Na^+$ by cells preloaded under the same conditions with radioactive Na^+. Galactose concentration was 1.25 mM and [Na^+] was 20 mM in each case during the incubation phase, and incubation temperature was 37°. Cell protein = 12 mg in a 4.0-ml incubation volume.

a reference value for nonactive entry (Kimmich, 1970). Figure 3 also shows that oligomycin markedly inhibits galactose accumulation by the cells. In fact, significant concentration gradients of galactose are never developed in the presence of that inhibitor. To our knowledge these data represent the first published report of inhibition of Na^+-dependent sugar transport by oligomycin, although the inhibitory effects of that agent on monovalent ion transport are well known (Whittam *et al.*, 1964; Blake *et al.*, 1967). Unless the cellular sodium gradient is dissipated extremely rapidly it is difficult to explain the short-term (30–60 sec) inhibitory effects of oligomycin in terms of the Crane hypothesis. A normal sodium gradient should be maintained for at least a short interval unless the cell membranes are exceptionally leaky to monovalent ions. If the latter possibility is allowed, one must envision the unlikely situation of a cell rapidly dissipating energy by endeavoring to maintain a sodium gradient across a membrane which is permeable to that ion.

Another observation inconsistent with the Crane hypothesis was established while studying the sugar transport ability of cells preloaded with sodium as compared to nonloaded cells. A preparation of cells isolated in sodium-free medium was divided in two portions. One portion was resuspended in 80 mM Na medium and the other kept in mannitol medium. Both portions were placed in ice for 15 min. This procedure allows at least partial equilibration of sodium between medium and cell water as will be shown subsequently. The experiment was initiated by adding an aliquot of sodium loaded cells to sodium-free medium, and an aliquot of mannitol cells was placed in medium containing sodium. Extracellular sodium was equal in the two cases after adding the cells, but in one case the sodium gradient was inward (mannitol cells), and in the other it was outward (sodium cells). Experiments were run with each group of cells at three different Na^+ concentrations. Each diluting medium contained [^{14}C]-galactose so that samples could be taken for comparing relative sugar transport ability of the two cell populations. Surprisingly, the rate and extent of galactose transport were identical in the two populations as shown in Figure 4. According to the Crane model one would have expected a greatly reduced rate of galactose uptake in the sodium-loaded cells (particularly at the lower extracellular Na^+ concentrations), with no active uptake occurring until an inward sodium gradient had been reestablished. Phloridzin was employed in this experiment to identify the point at which sugar equilibrates between medium and cell water. The data show active uptake of galactose has occurred in each situation as early as one minute after the start of the experiment.

Active accumulation of sugar while the sodium gradient is reversed was demonstrated more directly by using cells preloaded with radioactive sodium at an external Na^+ concentration of 80 mM. At $T = 0$ the cells were diluted in mannitol medium so that external [Na^+] fell to 20 mM. Figure 5 shows that under these conditions a very rapid extrusion of sodium from the cell can be demonstrated. Extrapolation of the earliest samples to $T = 0$ indicates the cells had loaded to an intracellular [Na^+] of 50 mM. Within 2–3 min, intracellular sodium has fallen to the same concentration as in the medium, and after that time the uninhibited cells actively extrude sodium. Ouabain-treated cells, on the other hand, lose Na^+ only by diffusion and are not capable of establishing a normal Na^+ gradient. Of greatest importance, cells preloaded with cold sodium under the same conditions are accumulating [^{14}C]galactose against a concentration gradient even before the 2–3-min interval required for dissipating the reversed sodium gradient. Again phloridzin was used to determine the extent of sugar uptake in the absence of active transport capability. Space determinations were made as described earlier for calculating concentration gradients generated by the cells. At $T = 2$ min the sugar gradient (in/out) is already equal to 1.7, but intracellular Na^+ (25 mM) is still greater than extracellular (20 mM). Furthermore, the increment of sugar entry over the first 2-min interval is greater than at any succeeding

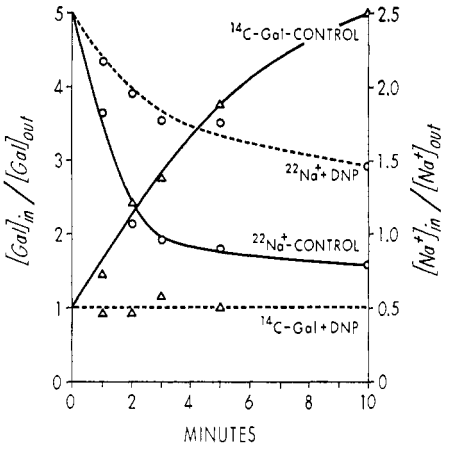


FIGURE 6: Extrusion of $^{22}Na^+$ and accumulation of [^{14}C]galactose by intestinal cells preloaded at 0° with 1.25 mM [^{14}C]galactose in 80 mM Na^+ medium, or with $^{22}Na^+$ (total [Na^+] = 80 mM) and 1.25 mM unlabeled galactose. Incubation was carried out at 37° at 20 mM Na^+ and 1.25 mM galactose. Cell protein = 13 mg in 4.0-ml volume.

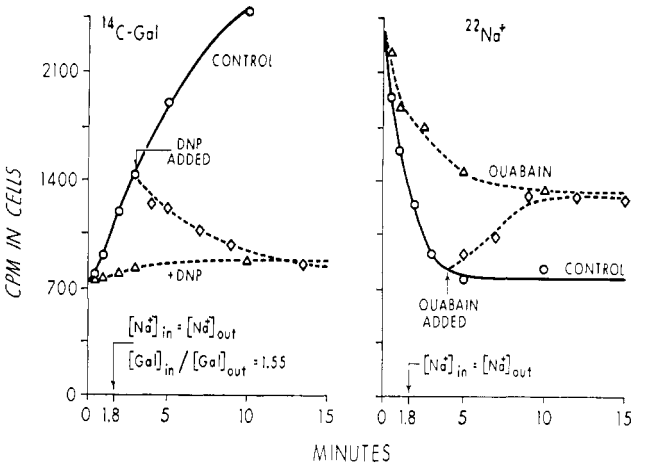


FIGURE 7: Extrusion of $^{22}Na^+$ and accumulation of [^{14}C]galactose by cells preloaded with $^{22}Na^+$ or [^{14}C]galactose. Effect of ouabain and dinitrophenol before and after active flux has been allowed. Cells were "loaded" in medium containing 1.25 mM galactose and 80 mM Na^+, and incubated at 37° in media containing 1.25 mM galactose and 20 mM Na^+. Cell protein = 19.2 mg.

2-min interval, yet the Na^+ gradient is reversed throughout the initial 2 min.

A better way to demonstrate the same process is to start with cells preloaded to equilibrium with labeled galactose as well as with sodium. In this situation if the sodium gradient hypothesis is correct one would expect a net loss of sugar from the cells during the early part of the experiment while intracellular sodium is higher than that in the medium. When the outward sodium gradient is dissipated, internal and external galactose should be equal in concentration and only when an inward gradient of sodium is re-forming should active galactose uptake be possible. Instead, Figure 6 shows the accumulation of galactose against a concentration gradient is detectable at the earliest time of sampling (1 min) even though active extrusion of sodium against a concentration gradient does not begin for more than 2 min. Furthermore the increment in galactose accumulation is as great during the first 2 min as at any subsequent 2-min interval. In this experiment DNP was used in place of ouabain and phloridzin to block active transport of sodium and galactose, respectively.

Of course, all of the above conclusions depend on an accurate determination of intracellular space on which to base the calculation of intracellular concentrations. In each of the experiments described thus far space was determined by two independent methods as described earlier, and the agreement was within 10%. It should be pointed out, however, that the same conclusion can be drawn from a space independent measurement. The data are illustrated in Figure 7. In this experiment cells preloaded with sodium and galactose were again employed. After a 3-min interval of sodium and galactose flux had been allowed, dinitrophenol was added to prevent further active transport and allow only diffusional flux. After that time, galactose moved out of the cells indicating a concentration gradient of sugar had already been generated. Moreover, this outward flux continued until extracellular galactose was at the same level as when dinitrophenol was present from the beginning of the experiment (*i.e.*, equilibrium between cell water and medium). In the control situation even after 1 min intracellular galactose was higher than this level; yet the cellular sodium concentration was significantly higher than extracellular for 1.8 min. At 1.8-min intracellular galactose is 50% higher than extracellular.

The data in Figure 8 illustrate a still more compelling demonstration that active sugar accumulation is possible while the normal sodium gradient is reversed. In this experiment the cells were preloaded with 2.5 mM [^{14}C]-3-*O*-methylglucose and 80 mM NaCl, and then introduced to sodium-free medium containing 0.8 mM [^{14}C]-3-OMG at the same specific activity. Extracellular sodium and 3-OMG fell

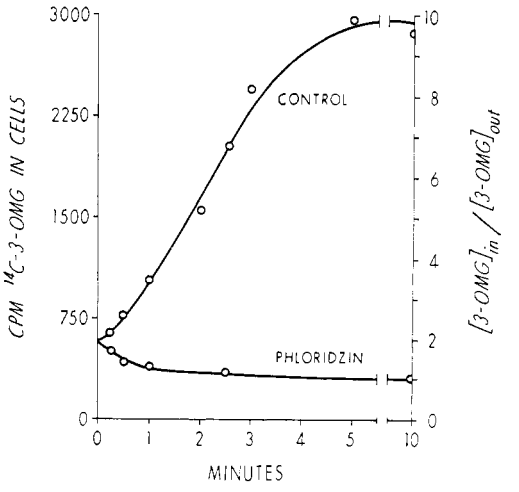


FIGURE 8: Accumulation of [^{14}C]-3-OMG by intestinal cells preloaded (0°) with Na^+ and [^{14}C]-3-OMG. Cells were loaded in media containing 2.5 mM [^{14}C]-3-OMG and 80 mM Na^+, and incubated in media with 1.25 mM [^{14}C]-3-OMG and 20 mM Na^+. Initially the intracellular concentration of both Na^+ and [^{14}C]-3-OMG was higher than the concentration in the medium. The earliest samples were taken 15 sec after adding cells to the medium. Cell protein = 15.8 mg in 4.0-ml volume; temperature, 37°.

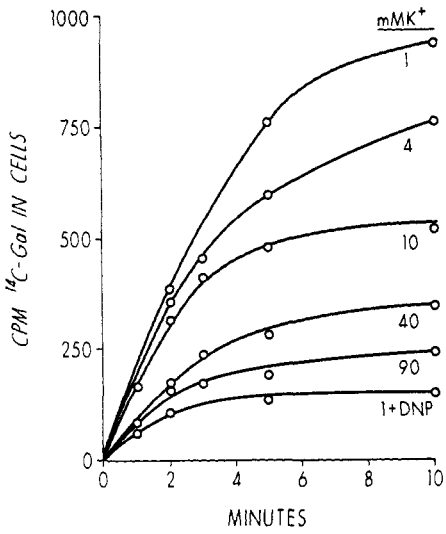


FIGURE 9: Effect of K^+ on accumulation of [^{14}C]galactose by isolated intestinal cells. The incubation medium contained 20 mM Na^+. Cell protein = 12.2 mg in 4.0 ml at 37°. Galactose concentration = 1.25 mM.

to 20 and 1.25 mM, respectively. In this situation, the sodium gradient hypothesis would predict an initial loss of cellular sugar both by diffusion and by extrusion due to the reversed sodium gradient. On the other hand, if energy input for sugar accumulation is direct and independent of the sodium gradient, one might expect immediate further accumulation of 3-OMG against a concentration gradient. The data show that the cells do indeed accumulate sugar even at the earliest times of sampling. In this experiment samples were taken as early as 15 sec, and 3-OMG was used to ensure that metabolism of the sugar substrate is fully prevented. Furthermore, those cells introduced to medium with phloridzin lost exactly half of their initial sugar content by diffusion as expected. Parallel experiments with ^{22}Na-loaded cells indicated again that about 2 min was required for intracellular Na^+ to fall to external levels. By this time, a 4-fold gradient of 3-OMG has been established.

The data in Figure 9 show another aspect of the characteristics of active sugar transport by intestinal cells. Potassium ion at concentrations as low as 4 mM significantly inhibits accumulation of [^{14}C]galactose when the sodium concentration is 20 mM. As little as 10 mM potassium inhibits the rate and extent of uptake by 58%. Inhibition by potassium ion is characteristic of metabolite transport in more intact intestinal preparations (Csaky, 1963; Bosackova and Crane, 1965). These data will be discussed in more detail in the next section with regard to a model for active sugar transport.

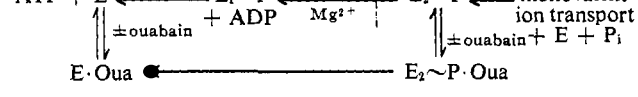
Discussion

The fact that active galactose accumulation can be demonstrated in isolated intestinal cells while the usual sodium gradient is reversed in direction in itself renders the sodium gradient hypothesis untenable. Also, since the rate and extent of metabolite transport seems identical regardless of the direction in which the sodium gradient is imposed, it seems doubtful that even a part of the driving force for transport is normally derived from the ion gradient. A reasonable alternative to the Crane hypothesis was therefore sought.

One possibility which must be considered is that a direct energy input for sugar transport exists *via* an ATP-energized carrier. Such a mechanism is consistent with a wide variety of other energy-dependent biochemical processes which derive their energy from ATP expenditure. It is not a satisfying alternative, however, for it fails to directly explain any of the characteristics of transport mentioned earlier, *e.g.*, sodium dependence, ouabain and oligomycin sensitivity, and a general correlation between the transport of sugars and monovalent ions. In order to adequately explain these and other aspects one must ascribe an entire set of properties to the imagined carrier which in number alone render the premise unlikely.

A more convincing mechanism was derived from a consideration of the properties a workable model must satisfy. As already mentioned, there is a great deal of evidence which suggests a close parallel in properties and function of the transport system for sodium–potassium and that for sugars. These observations led to a consideration of the nature of the mechanism thus far elucidated for cellular monovalent ion transport.

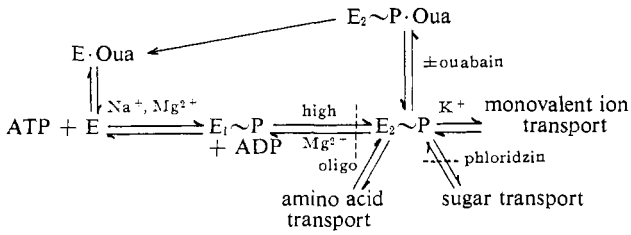
The elegant work of Skou (1965), Post *et al.* (1969), Sen *et al.* (1969), and Fahn *et al.* (1966) has been most important in this regard. Membrane bound [$Na^+ + K^+$]-activated ATPase is thought to form an integral element of the ion-transport process (Skou, 1965; Glynn, 1968). This enzyme has been shown to generate at least two intermediates during catalysis of ATP hydrolysis which are thought to be important in energy transfer for ion translocation (Sen *et al.*, 1969; Fahn *et al.*, 1966). Both intermediates are phosphorylated and likely represent a high-energy complex in different conformational states (Sen *et al.*, 1969; Fahn *et al.*, 1966). The first is generated from ATP and "free" enzyme (E) in a Na^+- and Mg^{2+}-dependent reaction, while higher levels of magnesium lead to a transition from $E_1\sim P$ to $E_2\sim P$. Oligomycin inhibits [$Na^+ + K^+$] ATP-ase activity, but enhances Na^+-dependent ATP–ADP exchange (Fahn *et al.*, 1966), and thus its action might be to prevent conversion between the two phosphorylated intermediates. Ouabain seems to have multiple points of interaction (Post *et al.*, 1969; Sen *et al.*, 1969; Fahn *et al.*, 1966; Glynn, 1968), combining with both free enzyme or the $E_2\sim P$ complex. Potassium ion leads to hydrolysis of the second intermediate and in some undefined way the energy of hydrolysis can be harnessed to drive monovalent ion transport (Sen *et al.*, 1969; Fahn *et al.*, 1966). The entire sequence of events can be represented schematically as



Each of the reactions has been indicated as freely reversible in accord with the work of Garrahan and Glynn (1967b) who demonstrated an increased incorporation of [^{32}P]P_i into ATP in glycolytically inhibited red cell ghosts during collapse of an imposed ion gradient. They suggest the inter-

mediates may be generated either from ATP or by reversal of the ion pump at the expense of a previously generated ion gradient.

With the above sequence of events in mind an interesting model for sodium-dependent sugar transport can be constructed which encompasses the set of characteristics necessary for a workable alternative to the sodium gradient hypothesis. The major premise of the model rests on the assumption that the energy inherent in the $E_2 \sim P$ complex can be utilized either for monovalent ion transport or for active sugar accumulation as shown in



The scheme also indicates the $E_2 \sim P$ intermediate may furnish the energy for active accumulation of amino acids in keeping with the remarkable similarity of properties in the two transport systems (Schultz *et al.*, 1967; Fox *et al.*, 1964; Inui and Christensen, 1966; Wheeler and Christensen, 1967; Csaky *et al.*, 1961; Curran, 1965; Schultz and Curran, 1969).

An attractive feature of such a coupling concept is that it explains the high degree of correlation which has been observed between sodium and nonelectrolyte transport. Because each process is dependent on the formation of a common intermediate one would expect each to be sensitive to those factors which alter the rate of generation of that intermediate. Hence, sugar transport is sodium and magnesium dependent and inhibited by ouabain or oligomycin just as is monovalent ion transport. Furthermore, both the sugar and ion transport systems should show a similar sensitivity toward the inhibitors and an identical K_M for sodium ion. In this regard, it is noteworthy that 24 mM extracellular sodium ion allowed half-maximal rates of active sugar accumulation by the isolated cells (Figure 1). When extracellular Na^+ concentration is 24 mM the intracellular concentration is approximately 12 mM (Figure 5), in good agreement with a reported K_M of 10 mM for sodium in the $[Na^+ + K^+]$-activated ATPase obtained from nerve tissue (Skou, 1957). Likewise, 10 μg/ml of oligomycin was completely effective in preventing active sugar transport (Figure 3) in accord with its reported effects on Na^+ transport. Since either ouabain or oligomycin acts directly on the energy input for sugar accumulation one expects and observes immediate inhibitory effects of these two agents (Figure 3). In contrast the sodium gradient hypothesis would predict a delay in onset of significant inhibitory effects due to the time required to partially dissipate the normal sodium gradient.

The sodium gradient model would also predict a rather tight stoichiometry between *active* sugar entry and coupled sodium entry. Function of the sugar carrier in active accumulation rests on the premise that the ion site must be filled with sodium. A sodium-free carrier is thought to allow only facilitated diffusion. Hence, that portion of sugar entry which is active should be coupled to an equivalent entry of sodium ion. While this has been demonstrated in one case (Goldner *et al.*, 1969), other data indicate a rather variable stoichiometry might be involved (Crane *et al.*, 1965). Entry ratios for Na^+ and amino acids have been examined more closely, and found to be variable and a function of the external sodium concentration (Schultz *et al.*, 1967; Schultz and Curran, 1969). The present model predicts a variable stoichiometry since energy diverted from $E_2 \sim P$ for sugar entry is no longer available for ion transport, and this leads to a partial collapse of the sodium gradient. The extent of collapse is of course dependent on the magnitude of the gradient (*i.e.*, external sodium concentration), and sugar-dependent sodium entry therefore varies. Still, a proportionality exists between sodium and sugar or amino acid entry because the extent of metabolite accumulation determines the degree of energy diversion from the ion-transport mechanism and this in turn determines the extent of collapse of the sodium gradient. Note that from this viewpoint it is not even necessary that the sugar carrier have a binding site for sodium, although it of course does not rule out that possibility.

As a corollary to the model described above one would also expect a partial collapse of the potassium gradient during sugar transport. As a consequence potassium should leak from the cell under these conditions. In fact, potassium efflux induced by sugar or amino acid accumulation has been observed in a number of different systems (Riggs *et al.*, 1958; Brown and Parsons, 1962; Koopman and Schultz, 1969). If the sodium gradient model were correct, however, one might expect a sugar-induced potassium influx because the sodium pump should be turning more rapidly to extrude sodium brought in with the sugar carrier.

It should be pointed out that the sodium binding site of Na^+–K^+-activated ATPase has been shown to be at the inner surface of the cell plasma membrane (Glynn, 1962; Whittam, 1962; Garrahan and Glynn, 1967a). Therefore, if the view presented above is correct it is intracellular Na^+ which is important for sugar and amino acid transport rather than extracellular. However Schultz *et al.* (1967) has demonstrated that extracellular sodium is most likely required for alanine transport, and others tend to favor this viewpoint (Crane *et al.*, 1965; Crane, 1968). Furthermore, cationic amino acids seem to have a less strict dependence on sodium ion for active transport (Munck and Schultz, 1969). This may indicate a cation binding site on the amino acid carrier has been partially satisfied by the ionized side chain (Munck and Schultz, 1969). Both observations may indicate a direct interaction of the nonelectrolyte carrier with Na^+. On the other hand, it is of course impossible to alter extracellular sodium concentration without concomitantly changing cellular sodium to some degree (Schultz *et al.*, 1967). Dependence on exogenously added Na^+ for active sugar or amino acid transport thus does not specify the locus of Na^+ interaction. It is noteworthy that Schultz *et al.* (1967) have demonstrated that incubation of intestinal sheets in choline medium following incubation at high sodium concentration leads to only partial inhibition of alanine transport as compared to sheets incubated in sodium medium continuously. Perhaps the cells retain sufficient intracellular sodium to satisfy the requirements for active amino acid transport. In addition, Csaky has shown that intestinal sheets preincubated in

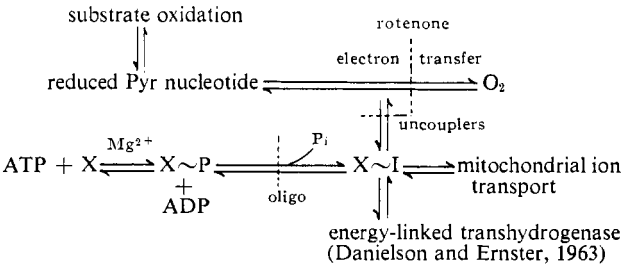
mannitol regain their sugar transport capability only slowly when placed in a sodium medium (Csaky, 1963). Again this suggests the site of sodium interaction may not be extracellular. Clearly more information is needed to determine the exact locus of the site for sodium interaction with the sugar-transport assembly. It is possible there are sites which require Na^+ in addition to that which we postulate as necessary for energy transfer *via* Na^+–K^+ ATPase activity.

The present model also accommodates a number of other reported observations concerning the nature of Na^+-dependent metabolite transport. For instance, considering the postulated energy-flow diagram one would predict potassium ion to be inhibitory to active sugar transport, again by causing a diversion of available energy. Figure 7 shows that at relatively low Na^+ concentration as little as 10 mM potassium inhibits galactose transport by 50%. Conversely, a transportable sugar or amino acid inhibits active potassium influx (or stimulates efflux) as already mentioned. Phloridzin, which can block energy flow for Na^+-dependent sugar transport (Parsons *et al.*, 1958), prevents sugar-induced changes in transmural ion flux (Schultz and Zalusky, 1964) as might be predicted from a consideration of the energy-flow diagram presented earlier. Phloridzin also slightly enhances amino acid accumulation (Kimmich, 1970), as expected if a common intermediate is tapped for transport of both sugars and amino acids. Mutual inhibition between sugars and amino acids thus represents competition for an energized intermediate rather than competition for a multifunctional carrier as suggested by Alvarado (1966). This competition is not in the same sense as the Smyth proposal, which suggests limitation of cellular energy reserves in the form of ATP as a basis for competitive inhibition between two transportable species (Hardcastle *et al.*, 1968).

It is well established that an increase in transmural potential difference occurs when an actively accumulated sugar is introduced at the mucosal surface of intestinal tissue (Schultz and Zalusky, 1964; Lyon and Crane, 1966; Quay and Armstrong, 1969). This change in potential is thought to be due to an increased asymmetry in sodium distribution across the epithelial cell layer. In order to account for it in terms of the new model it is only necessary to envision epithelial cells with the complete energy-coupling system described earlier at the mucosal surface, but only those components for monovalent ion transport at the serosal surface. Sugar transport would then lead to a differential effect on sodium extrusion at the two cell surfaces. The mucosal sodium efflux would be partially inhibited with the serosal extrusion rate remaining unchanged. This would result in a net flux of sodium from mucosal to serosal surface and an increased transmural potential difference. Phloridzin would of course prevent the sugar-induced change in potential as has been observed (Schultz and Zalusky, 1964).

Finally, Eddy (1968) has recently shown that in ATP-depleted ascites cells, amino acid accumulation can be demonstrated at the expense of a sodium gradient. This has been cited as compelling evidence in favor of the sodium gradient hypothesis (Schultz and Curran, 1969). It is important to remember, however, that the [Na^+ + K^+]-activated ATPase activity is apparently reversible. Garrahan and Glynn (1967) have demonstrated sodium gradient dependent labeling of ATP with [^{32}P]P_i in red cell ghosts. This suggests that the high-energy intermediates important to ATPase activity are able to be generated either from ATP or at the expense of energy derived from an established ion gradient. If energy derived from one of these intermediates is tapped for sugar transport, one might expect that under the proper conditions a sugar or amino acid gradient might be generated from a preexisting ion gradient. This in no way implies, however, that the normal sequence of events is initial generation of a sodium gradient which then allows formation of the sugar gradient.

A striking analogy may be derived between the model described above and a model proposed for energy coupling in isolated mitochondria. In these organelles, high-energy intermediates have been proposed which allow coupling between substrate oxidation and the mechanism for oxidative phosphorylation (Chance *et al.*, 1955; Chance and Williams, 1956; Ernster *et al.*, 1967). It has also been suggested that energy from one intermediate may be tapped to provide the driving force for mitochondrial ion transport according to (Rasmussen *et al.*, 1965; Rasmussen, 1966)



Note that each reaction is indicated as freely reversible in accord with data showing that mitochondrial electron transfer (Chance, 1961b) and ion transport (Bielawski and Lehninger, 1966) can be driven by ATP, as well as by substrate oxidation. Oligomycin blocks either process when ATP supplies the energy (Bielawski and Lehninger, 1966; Chance, 1961b), but inhibits only phosphorylation of ADP when the energy is derived from substrate oxidation (Rossi and Lehninger, 1964). Recently Cockrell *et al.* (1967) and Cockrell (1969) have shown that a net synthesis of ATP can be demonstrated in rotenone-blocked mitochondria at the expense of a transmembrane potassium gradient. Rotenone prevents substrate oxidation from supplying the necessary energy (Chance and Hollunger, 1963). Thus, one can only conclude that in this case also the ion gradient can supply the energy necessary to generate energy-rich membrane intermediates which are then used for energy-dependent processes. Again this fact in no way implies oxidative phosphorylation is normally driven by a potassium gradient, but only that when proper conditions are imposed energy from this source can be used *in lieu* of energy derived from substrate oxidation.

In summary, a model for sodium-dependent metabolite transport which envisions a direct energy input seems more consistent with available data considered *in toto* than does an indirect energy input *via* the sodium gradient. Furthermore, a direct energy input model *via* [Na^+ + K^+]-activated ATPase shows striking similarities to membrane energy-coupling processes which have been postulated to occur in isolated mitochondria. It is tempting to speculate that energy for a variety of membrane transport systems is derived from a group of membrane-bound energy-rich intermediates which

are produced by a set of identical or closely analogous chemical reactions.

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