The Accumulation of D-Galactose against a Concentration Gradient by Slices of Rabbit Kidney Cortex*

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The excretion of a glucose-free urine is believed to be the result of the presence, in the epithelial cells of the proximal convoluted tubules of the vertebrate kidney, of a process for the reabsorption of glucose from the glomerular filtrate against the concentration gradient (1). This belief is substantiated by analyses of the glucose concentration in samples of tubular fluid obtained by micropuncture at various points along the length of the tubules (2, 3). It is also substantiated by studies of the influence of the concentration of glucose in the blood on the rate of glucose reabsorption (4). The data from these studies in vivo are consistent with the idea that sugar reabsorption is mediated by an energy-dependent process which has a restricted specificity and which is susceptible to inhibition by the glycoside, phlorizin.

In these respects, the phenomenon of kidney reabsorption of sugars is similar to intestinal absorption which has been successfully demonstrated in vivo and which is susceptible to inhibition by the glycoside, phlorizin. The influence of the concentration of glucose in the blood on the rate of glucose reabsorption is consistent with the idea that sugar reabsorption is mediated by an energy-dependent process which has a restricted specificity and which is susceptible to inhibition by the glycoside, phlorizin. The data from these studies in vivo are consistent with the idea that sugar reabsorption is mediated by an energy-dependent process which has a restricted specificity and which is susceptible to inhibition by the glycoside, phlorizin.

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The substance of the present report is the demonstration that p-galactose, a sugar which has been reported to be reabsorbed by the kidneys of several species (8), is accumulated against an apparent concentration gradient in slices of rabbit kidney cortex incubated under appropriate conditions.

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**Experimental**

**Tissue**—The kidneys were removed from rabbits weighing 2 to 4 kg., and were placed in Krebs-Henseleit phosphate buffer (9) equilibrated with 100 per cent oxygen. Thin slices of cortex were cut freehand and placed in a dish of the same buffer, kept at room temperature to avoid the swelling which kidney cortex slices undergo when chilled (10).

**Compounds**—p-Galactose-1-C14 with a specific activity of 2 μc. per mg. was obtained from the National Bureau of Standards. Nonradioactive p-galactose was obtained from Pfanziel Laboratories, Inc., and was recrystallized from 80 per cent ethanol. Phlorizin was obtained from Dr. T. P. Nash, Jr., and was recrystallized from water. 4,6-Dinitro-o-cresol was obtained as the sodium salt from Distillation Products Industries.

**Methods**—Incubations were made in Warburg vessels with two side arms. The main chamber contained 1.83 ml. of a buffer solution (11) consisting of 1.14 ml. of 0.3 M sodium chloride, 0.09 ml. of 0.3 M potassium chloride, 0.3 ml. of 0.1 M sodium acetate, 0.1 ml. of 0.02 M calcium chloride and 0.2 ml. of 0.1 M potassium phosphate, pH 7.3. One side arm contained 0.1 or 0.2 ml. of a solution of galactose-1-C14 (5 μe. per ml.) plus the amount of nonradioactive galactose required to make the desired final concentration, and 0.2 ml. of 0.2 M raffinose. Inhibitors were added to the main chamber or, when their addition during incubation was desired, to the opposite side arm. Water was added to the main chamber to give a final total volume of 2.7 ml.

Approximately 100 mg. wet weight of freshly cut slices were placed in the main chamber of the vessels. The vessels were gassed with 100 per cent oxygen at room temperature, sealed, and placed in a water bath where they were shaken at a rate of 100 to 110 oscillations per minute for 10 minutes. The galactose solution was tipped into the side arm and incubation was continued for the desired length of time. The temperature of the bath was 25°C unless otherwise stated.

When incubation was completed, the vessels were removed from the bath. Then, as rapidly as possible, the slices were lifted out, blotted on filter paper, weighed on a torsion balance, and placed in a water bath where they were shaken at a rate of 100 to 110 oscillations per minute for 10 minutes. The galactose solution was tipped in from the side arm and incubation was continued for the desired length of time. The temperature of the bath was 25°C unless otherwise stated.

When incubation was completed, the vessels were removed from the bath. Then, as rapidly as possible, the slices were lifted out, blotted on filter paper, weighed on a torsion balance, and homogenized together with 3.0 ml. of 0.19% zinc sulfate in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. The suspension was again homogenized after the addition of 3.0 ml. of 0.3 M barium hydroxide. An aliquot, 1.0 ml. of the medium from each vessel was taken and mixed with 2.0 ml. of zinc sulfate and 2.0 ml. of barium hydroxide. Clear solutions were obtained by centrifugation.

The galactose in the deproteinized solutions was converted to mucic acid in the following way. 2.0-ml aliquots of the solutions obtained from the slices or 1.0 ml. of the solutions from the media plus 1.0 ml. of water were placed in 13 × 100-mm test tubes. 0.4 ml. of 0.5 M galactose and 1.2 ml. of concentrated nitric acid were added and mixed in by stirring. The tubes were then heated on a boiling water bath for two hours. The tubes were removed from the bath and their contents were reduced to a volume of 0.2 to 0.3 ml. by means of a jet of dry air. The tubes were set aside at 4°C for 12 hours to permit complete crystallization of the mucic acid. The crystalline pre-
The concentration of galactose in the experiments below is expressed as net counts per minute (net c.p.m.) or as moles per ml. of intracellular water, calculated in the following way:

\[
\text{net c.p.m. per ml. intracellular water} = \frac{\text{c.p.m./slices} - (\text{c.p.m./ml. medium})(\text{ml. raffinose volume})}{\text{ml. total water} - \text{ml. raffinose volume}}
\]

The values expressed as net counts per minute were converted to moles of galactose by multiplying by the reciprocal of the specific activity of the galactose-1-C\textsuperscript{14} in the medium.

**Validity of Method**—Owing to the utilization by the kidney

<table>
<thead>
<tr>
<th>Incubation temperature</th>
<th>Initial galactose concentration</th>
<th>Medium radioactivity</th>
<th>Tissue radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Wet weight</td>
</tr>
<tr>
<td>(25^\circ) C (10^{-4}) M</td>
<td>2.7</td>
<td>60,000</td>
<td>38,500</td>
</tr>
<tr>
<td>25</td>
<td>2.7</td>
<td>60,000</td>
<td>35,500</td>
</tr>
<tr>
<td>25</td>
<td>1.3</td>
<td>30,000</td>
<td>16,300</td>
</tr>
<tr>
<td>25</td>
<td>0.7</td>
<td>15,000</td>
<td>8,400</td>
</tr>
<tr>
<td>25</td>
<td>0.7</td>
<td>15,000</td>
<td>5,000</td>
</tr>
<tr>
<td>4</td>
<td>1.3</td>
<td>30,000</td>
<td>28,600</td>
</tr>
</tbody>
</table>

**RESULTS**

**Accumulation of Galactose**—When slices of rabbit kidney cortex were incubated as described above with galactose-1-C\textsuperscript{14} for a period of time, they were found to contain more radioactivity than would be expected solely from the free diffusion of galactose from the medium into the slice. This fact is illustrated by the data in Table I. At the end of 80 minutes of incubation, the ratio of the intracellular concentration of galactose to the final concentration in the medium varied with the concentration used from a low of about 4.2 to a high of about 7.1. When the slices were incubated at 4\(^\circ\), galactose was not accumulated and, in fact, the intracellular concentration failed to equal the medium concentration at the end of 80 minutes. This failure, striking when compared to the high concentrations attained at 25\(^\circ\), suggests that there is at low temperatures a marked reduction in the rate of all quantitatively important modes of entrance of galactose into kidney tissue cells similar to that observed previously with ascites tumor cells (16).

The utilization of galactose at low external concentrations, as shown by the difference between the initial and the final values for the media, represents an appreciable fraction of the total galactose in the vessels. Utilization was routinely observed in these experiments and rough estimates of its magnitude could be made from the difference between the initial and final concentrations of galactose-1-C\textsuperscript{14} in the media. Use of these
values was made in experiments where vessels were incubated for different periods of time. All the data of such an experiment were multiplied by the factor, initial concentration/final concentration, in order to normalize the data to the same external concentration.

In Fig. 1 the amount of intracellular galactose found after various periods of incubation in the presence and in the absence of 4,6-dinitro-o-cresol is plotted. The horizontal broken line represents the value expected from diffusion equilibrium, if all of the slice water is available for diffusion, and it may be noted that the amount of intracellular galactose found after 45 minutes in the presence of dinitroresol is close to this value. If it is correct to assume that this value represents diffusion equilibrium, the amount found in excess of it represents the extent to which the tissue has accumulated galactose; if accumulation arises from active transport, the magnitude of this excess is related to the rate of active transport.

**Inhibition by 4,6-Dinitro-o-cresol**—If we may assume that the accumulation of galactose arises from transport against a concentration gradient; energy is required. Inhibition by 4,6-dinitro-o-cresol would then suggest that this hypothetical energy is derived from aerobic metabolism since this compound is one of a series of substituted phenols known to inhibit, at low concentrations, the process of aerobic phosphorylation (17-19). However, other sites of substituted phenol inhibition are known (20-22) and it is not possible in these experiments to localize their influence. Dinitroresol was used at concentrations between 1.8 and 7.3 x 10^{-5} M, a range in which nonspecific effects in other tissues are minimal (18, 23). In its presence, galactose was found to be distributed, at equilibrium, in an average of 89 per cent of the intracellular water volume in 12 experiments. On the assumption that the kinetics of galactose entrance into the kidney cells in the presence of dinitroresol are the same as the kinetics of the entrance of sugars into ascites tumor cells (16), a rate constant of entrance has been calculated by the same method. In four individual experiments not shown here, k, min.^{-1}, had a value of 0.10 ± 0.02, at an incubation temperature of 25°C.

In addition to its effect in preventing accumulation, dinitroresol added after accumulation had occurred caused the release of galactose to the medium. In the experiment shown in Fig. 2, accumulation was allowed to proceed. At intervals, dinitroresol was added to paired flasks and incubation was continued. Analyses were then made at intervals after the addition of dinitroresol. These showed considerable variation and the curves drawn through the points in Fig. 2 are necessarily arbitrary. Nonetheless, it is evident that the intracellular concentration fell, in the flasks to which inhibitor was added, at a rapid rate and approached, though it did not attain within the time period chosen, the level expected from diffusion equilibrium. The results suggest that a given gradient is maintained only when the process resulting in accumulation proceeds as rapidly as an opposing leakage of galactose from the tissue.

**Inhibition in Absence of Oxygen**—When slices were incubated in an atmosphere of nitrogen (Linde, HP) instead of oxygen, intracellular accumulation of galactose did not occur (Fig. 3). Under nitrogen, galactose entered the tissue at about the same rate as in the presence of dinitroresol but it appeared to come to equilibrium with a slightly greater volume; a volume of approximately 100 per cent of the slice water.

**Inhibition by Phlorizin**—When the glycoside, phlorizin, was added at concentrations which have been reported to produce glycosuria in vivo, i.e. at concentrations from 10^{-3} M to 10^{-4} M, a significant reduction was observed in the amount of galactose accumulated within a given time period. A representative experiment is shown in Fig. 4. In this experiment, phlorizin
was added at two concentrations. With the lower concentration, galactose accumulated but to a lesser extent than in the control. With the higher concentration, galactose did not accumulate, nor, indeed, did the amount to be expected from diffusion equilibrium enter the cells. This result suggested the possibility that phlorizin could inhibit the entrance of galactose into the tissue cells under conditions where no accumulation occurred, as has been found in the ascites tumor cell (16) and the erythrocyte (25, 26) which do not accumulate sugars against a concentration gradient. In order to test this possibility, an experiment was carried out in which dinitrocresol was added to all flasks, thus preventing accumulation. Under these conditions, phlorizin inhibited the rate at which galactose entered the tissue, as shown in Fig. 5. Also, the degree of inhibition was dependent upon the phlorizin concentration. If it may be assumed that inhibition is noncompetitive with respect to sugar, as it seems to be with ascites tumor cells (16), a $K_i$ value for phlorizin inhibition of $1.7 \pm 0.3 \times 10^{-4} \text{M}$ may be calculated using the 15-minute values for percentage of distribution from Fig. 5.

**Influence of External Galactose Concentration**—When kidney slices were incubated in graded concentrations of galactose for 60 minutes in the presence and absence of dinitrocresol, an influence of the external galactose concentration on accumulation was found (Fig. 6). In the presence of dinitrocresol, accumulation did not occur and the amount of galactose in the tissue was a linear function of the external galactose concentration. In the absence of dinitrocresol, accumulation occurred and the amount of accumulated galactose approached a maximum with increasing concentrations of external galactose. It was then assumed that galactose accumulation resulted from the operation of a catalytic process and an apparent Michaelis constant for this process was calculated from the data in Fig. 6, upon the further assumption that the rate-limiting step in the process producing accumulation was entirely absent when dinitrocresol was added. The difference between the two curves was taken to equal the rate of the process. Owing to the marked deviation of the points at the highest concentration of galactose from the pattern of the rest of the data, the values for the 5 lower concentrations only were used. These difference values were then plotted against the concentrations of galactose, both in the reciprocal form (27), to give Fig. 7. From the extrapolated intercept with the abscissa (28) a value for $K_m$ of $1.2 \times 10^{-3} \text{M}$ was obtained. In two other experiments values for $K_m$ of 2 and of $4 \times 10^{-3} \text{M}$ were obtained. The degree of confidence which one may have in a $K_m$ value obtained in a complex system such as this one is low. There is no practical way, for example, of

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** The effect of phlorizin on the accumulation of galactose-C$^{14}$. 

![Fig. 5](http://www.jbc.org/)

**Fig. 5.** The effect of phlorizin on the entrance of galactose-C$^{3}$ in the presence of 4,6-dinitro-o-cresol.

![Fig. 6](http://www.jbc.org/)

**Fig. 6.** The effect of the concentration of galactose on the accumulation of galactose-C$^{14}$.

![Fig. 7](http://www.jbc.org/)

**Fig. 7.** Lineweaver-Burk plot of the data of Fig. 6, obtained as explained in the text. $1/V$ is given in arbitrary units; $1/S \times 10^3$ is given in liters per mole.
knowing how much influence the rate of outward diffusion of accumulated galactose, the rate of its utilization, and the degree to which the process resulting in accumulation may have established an equilibrium, will have on the calculated value for $K_a$. Its use is justified solely by the fact that it is the only means available at present for comparing, in a quantitative sense, the effect of several substrates on a catalytic process.

**Inhibition by Glucose**—When glucose was added, a significant reduction in galactose accumulation was observed and the extent of the reduction depended in an approximate way upon the relative concentrations of the two sugars, as shown by the data in Table II. Again, if it is assumed that a catalytic process is involved in galactose accumulation, $K_i$ values for glucose (given in Table II), can be calculated. These were found to be highly variable; they ranged from a low of $3 \times 10^{-3}$ M to a high of $2.5 \times 10^{-2}$ M. In the presence of dinitrocresol, the rate at which galactose entered the slice was also inhibited by comparable concentrations of glucose, but to a lesser degree. No inhibition was observed with $2.2 \times 10^{-4}$ M galactose and $7.4 \times 10^{-4}$ M glucose and about 30 per cent inhibition was observed with $1 \times 10^{-3}$ M galactose and $7.4 \times 10^{-2}$ M glucose. Some of the variability of glucose inhibition in both types of experiment probably results from variations between experiments in the extent to which the sugars were utilized during the experimental period. The concentrations given in Table II are initial concentrations.

**DISCUSSION**

The phenomenon of the accumulation of a substance if it occurs against a concentration gradient is, by definition (29), active transport, inasmuch as some force other than that of diffusion is required to maintain an asymmetric system. The unequivocal demonstration of active transport, however, requires proof that the intracellularly accumulated substance is in the same molecular form as the extracellular source. In the present studies, we have been able to demonstrate that the substance accumulated is isolable under mild conditions as $\alpha$-galactose and that it probably has not been metabolically altered. However, when the active cells must be modified or destroyed in order to recover the accumulated substance, it is never possible rigorously to exclude as a major factor in the phenomenon the formation of dissociable bonds between the accumulated substance and an intracellular component. Evidence against this possibility, of whatever kind and however strong, must always be inferential. In this instance, dissociable bonds, if they exist, must have all the characteristics of the system, namely, sensitivity in their formation or disruption to dinitrocresol, to oxygen deprivation, to the presence of glucose, and to the external galactose concentration. For this reason, their information as a part of a specific mechanism seems unlikely. In addition, such bonds are not a factor in the analogous demonstration in this laboratory that segments of hamster intestine accumulate high relative concentrations (20 to 1) of free 6-deoxy-$\alpha$-glucose when incubated with this sugar in the medium. Contrary to the situation with kidney slices, intestine can be divided surgically into an active portion (the mucosa) and an inactive portion (the muscularis), and this division can be made before or after incubation. When it is made before, only the mucosa contains a high relative concentration of sugar; when it is made after, both contain high concentrations.

### Table II

**Inhibition of accumulation of galactose by simultaneous presence of glucose**

<table>
<thead>
<tr>
<th>Initial concentration</th>
<th>Galactose $K_i$</th>
<th>Glucose $K_i$</th>
<th>Inhibition</th>
<th>$K_i$ for glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-3}$ M</td>
<td>0.96</td>
<td>11.0</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>1.7</td>
<td>11.0</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>0.22</td>
<td>7.4</td>
<td>58</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>5.6</td>
<td>62</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.20</td>
<td>5.6</td>
<td>28</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>5.6</td>
<td>61</td>
<td>3</td>
</tr>
</tbody>
</table>

As for the values which have been obtained in the present studies for the 3 parameters of galactose accumulation, namely, Michaelis constant, rate, and intracellular concentration, possibly none of them represents a true value. The probable errors in the $K_i$ values have been discussed above. The values for the rate of galactose accumulation are undoubtedly low, owing to utilization and to diffusion from the tissue to the medium. The concentrations of intracellular galactose have been calculated on the basis of total slice water. It is not known whether galactose is localized in its concentration, in which case the values are low, or whether it is more or less uniformly distributed throughout the tissue. The latter seems more likely by analogy with the hamster intestine experiments mentioned above. Moreover, studies of renal transport of glucose in dogs (30) have been interpreted to mean that the site of the transport mechanism is the cell membrane bordering the lumen. Sugar transported at this site could diffuse out of the opposite border of the cell into the surrounding tissue.

The action of phlorizin on the renal transport of glucose has been considered to be highly specific. Recent experiments, however, have uncovered actions of phlorizin which are not clearly related to one another and which indicate that the action of phlorizin on different systems may not always be the same one. Phlorizin inhibits the entrance of sugars into cells which do not possess a mechanism for the accumulation of sugars against the concentration gradient (16, 25, 26). Phlorizin inhibits, in an apparently competitive way, the nucleotide-requiring enzymes, phosphorlyase $\alpha$ (31), Ehrlich ascites tumor hexokinase, and rat kidney hexokinase (32), as well as aerobic phosphorylation in a homogenate of guinea pig kidney cortex (33). Phlorizin has also been reported to inhibit the enzyme, mutarotase (34). That action of phlorizin which has been observed in the present study appears to be the one observed with erythrocytes and ascites tumor cells, which we have interpreted previously as an action upon free diffusion. A restraint of free diffusion alone, by phlorizin, if it prevented access of sugar to the transport mechanism, could explain the production of renal glycosuria in intact animals and the inhibition of accumulation in the present experiments. It is possible, however, that the membrane sites through which sugar enters a cell are the same whether its transport is passive or active, coupling with an energy source being implied for the latter. The site of phlorizin

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2. R. K. Crane, unpublished observations.
inhibition could then be the same in both instances. The following observations show how great is the need for more precise information on the site and mechanism of renal tubular transport of sugar in order to clarify the role of phlorizin. The ascites tumor cell is impermeable to phlorizin at concentrations which inhibit sugar entrance (16) and inhibition of sugar entrance presumably occurs at the membrane. In the kidney, on the other hand, phlorizin has been reported (33) to be secreted into the intracellular site.

That have been reported for the reabsorption of sugar in vivo galactose by rabbit kidney cortex slices are remarkably like those not to expect an accumulation of galactose in the experiments in vivo. There seems to be no clear way, at present, to reconcile our data with Keston's hypothesis.

**SUMMARY**

Experiments are reported which indicate that slices of rabbit kidney cortex incubated in *vivo* may serve as a test system for the renal reabsorption of sugars in *vivo*. d-Galactose-1-C\textsuperscript{14} added to the medium is accumulated by the slices against an apparent concentration gradient. The following properties of this system have been observed: (a) In the absence of oxygen or in the presence of 4,6-dinitro-o-cresol the amount of galactose entering the cell is limited to that expected from diffusion equilibrium. (b) The amount of galactose accumulated in a given time period is a function of the external galactose concentration and it is reduced by the simultaneous presence of glucose. The degree of reduction depends upon the relative concentrations of the two sugars. (c) Phlorizin inhibits the entrance of galactose into the tissue cells not only under conditions in which accumulation can occur, but also when accumulation has been inhibited by dinitro-o-cresol.

**REFERENCES**


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