would dovetail with another potential role for glucose during freezing, that of a fermentable fuel reserve to support energy requirements of the ischaemic state. It is interesting to speculate that multiple actions of glucose (colligative, structural, and metabolic) could make this sugar valuable for artificial cryopreservation of mammalian tissues and organs.

Life in the frozen state

Extracellular freezing imposes an ischaemic and anoxic state on all cells of the body. Circulatory changes during the early stages of freezing may allow remaining oxygen supplies to be used by the most sensitive organs, but when freezing is complete there are no breathing, no heartbeat, and no blood flow.

Energy metabolism in the frozen state is based on fermentation of endogenous fuel reserves in each individual organ. Organs show a decrease in glycogen content with long-term freezing and an accumulation of lactate and/or alanine as end products of anaerobic glycolysis (9). Amino acid fermentation also appears to occur in some organs (notably skeletal muscle; 9). Some small percentage of cryoprotectant glucose may also be fermented, although end-product accumulation is better correlated with glycogen loss (9).

Organ-specific responses to the frozen state are seen. Accumulation of metabolic end products differed in both amount [net lactate plus alanine accumulation was 10-fold higher in heart than in skeletal muscle (Fig. 2)] and pattern [e.g., predominantly lactate in heart, 2.5:1 lactate:alanine in kidney, 1:4.5 lactate:alanine in skeletal muscle (9)]. Differences in total end-product accumulation suggest substantial differences in organ-specific metabolic rates in the frozen state.

Freezing also appears to place varying levels of metabolic stress on individual organs, as judged from the effects of the frozen state on energy status. Skeletal muscle energy reserves (adenylates, creatine phosphate) are minimally affected by freezing, even after 3 days frozen (9). Liver energy status, however, is much more strongly affected by freezing (ATP levels drop by 50% within 18 h), although recovery is complete after several days of thawing (9).

Much remains to be explored to determine the molecular mechanisms involved in freeze tolerance and the specific actions of protectants such as glucose. Freeze-tolerant frogs provide cryobiologists with the first good opportunity to study natural freezing survival on an organ-specific basis. For medical applications these animals provide an excellent model system for the development of organ cryopreservation technology.

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Formate: A Critical Intermediate for Chloride Transport in the Proximal Tubule

Lawrence P. Karniski and Peter S. Aronson

Recent experiments unexpectedly suggest that formate plays a critical role in chloride transport across cell membranes. In particular, active uptake of chloride in the renal proximal tubule cell occurs by chloride-formate exchange. Formate recycles from lumen to cell via nonionic diffusion of uncharged formic acid. In this manner, small amounts of formate can facilitate resorption of large quantities of chloride

The proximal tubule of the mammalian kidney is presented with the enormous task of resorbing approximately 60% of the load of NaCl presented to it by glomerular filtration. In humans this amounts to ~10,000 mmol NaCl (~580 g) resorbed by the proximal tubules each day. New evidence surprisingly indicates that formic acid, a short-chain fatty acid present physiologically in low con-

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centrations, plays a critical role in this process. How does this occur?

Paracellular vs. transcellular Cl^- resorption

The proximal tubule is a "leaky" epithelium. That is, the pathway through the tight junctions between the cells is relatively highly permeable to ions. Thus passive resorption of Cl^- between the cells can occur if there is a favorable electrochemical gradient for Cl^- directed from the lumen across the tubular wall.

In the early proximal tubule the process of Na^+-coupled organic solute resorption (e.g., Na^+ cotransport of glucose, amino acids, etc.) generates an outward flow of positive charge across the epithelium, resulting in a lumen-negative electrical potential difference across the tubular wall. This then serves as a driving force to promote a modest rate of passive Cl^- resorption. However, the Na^+-coupled resorption of organic solutes and HCO_3^- accounts for most of the solute in the nearly isosmotic resorbate in the early proximal tubule. Because of this preferential resorption of HCO_3^-, the luminal concentration of HCO_3^- declines and that of Cl^- rises along the length of the proximal tubule. Hence, in the later proximal tubule there is a lumen-to-capillary concentration gradient of Cl^- that favors its passive resorption. It has been thought that passive Cl^- transport occurring in this manner between the cells accounts for most if not all of the Cl^- resorption in the mammalian proximal tubule (2).

However, several recent studies have suggested that active transport through the cells must also contribute significantly to Cl^- resorption in the proximal tubule (2, 6). For example, substantial transtubular resorption of Cl^- can take place in the absence of a passive driving force. Moreover, even in the presence of an outwardly directed transtubular concentration gradient of Cl^-, inhibitors of cellular metabolism or active Na^+ transport greatly reduce the rate of Cl^- resorption. Finally, intracellular Cl^- activity in the proximal tubule cell is above the level at which Cl^- would be in electrochemical equilibrium across the luminal membrane. Taken together, these findings indicate that substantial active Cl^- resorption through the cells takes place in the proximal tubule and that the step of Cl^- uptake across the luminal membrane of the proximal tubule cell must be an uphill process. But what is the mechanism for this uphill Cl^- transport?

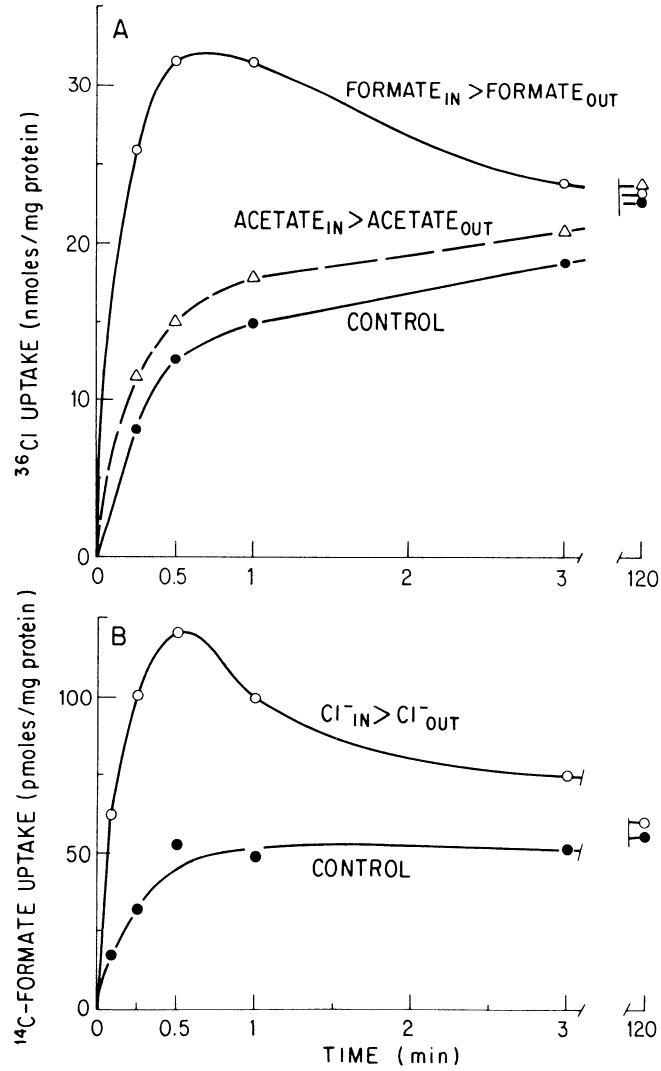

FIGURE 1. Effect of an outward formate gradient on Cl^- uptake (*A*) and an outward Cl^- gradient on formate uptake (*B*) into rabbit renal microvillus membrane vesicles. (Adapted from Karniski and Aronson (4).)

Studies on membrane vesicles implicate Cl^--formate exchange

The mechanisms of Cl^- transport have been studied using luminal (microvillus, brush border) membrane vesicles isolated from the rabbit renal cortex. This technique allows examination of transport events at the luminal membrane independently of cellular metabolism or transport processes at the basolateral cell surface.

A general strategy with this technique is to test whether imposing transmembrane ion gradients can drive the uphill accumulation of a solute of interest. A positive result indicates the presence of a coupled transport process, such as cotransport or exchange, that can mediate secondary active transport of the solute of interest. This strategy failed to detect the presence of Na^+-Cl^- cotransport or Na^+-K^+-$2Cl^-$ cotransport as possible mechanisms of uphill Cl^- transport in rabbit renal microvillus membrane vesicles (8). Although Cl^--OH^- exchange had been described in these membranes (9, 10), we (4, 8) and others (3) were unable to confirm this finding. Thus none of the mechanisms for uphill Cl^- transport previously observed in other epithelia was clearly demonstrable.

However, although we could not demonstrate appreciable Cl^--OH or Cl^--HCO_3^- exchange, we found a high activity of Cl^--Cl^- exchange in

these membrane vesicles (4). Obviously, Cl^--Cl^- exchange is a futile process resulting in no net transport. We reasoned that it would make no sense for such a transport system to exist unless it were capable of mediating net transport. We assumed that the observed Cl^--Cl^- exchange must reflect an anion transporter capable of exchanging some other anion for Cl^-. We therefore screened a large number of anions for their ability to share this anion exchanger.

After an extensive and mostly frustrating search, we obtained the findings shown in Fig. 1*A*. In this experiment, imposing a 10:1 outward formate gradient stimulated the initial rate of Cl^- uptake two- to threefold and induced a transient uphill accumulation of Cl^- above its eventual level of equilibrium uptake, indicating the presence of a Cl^--formate exchange process (4).

An identical outward gradient of acetate stimulated the rate of Cl^- uptake only minimally and did not induce uphill Cl^- accumulation. Similar negative results were obtained when 10:1 outward gradients of other anions such as propionate, butyrate, HCO_3^-, lactate, and sulfate were imposed. Further confirming the presence of Cl^--formate exchange was the finding (Fig. 1*B*) that imposing a 10:1 outward Cl^- gradient induced the transient uphill accumulation of formate (4). Thus these experiments demonstrated the presence of a novel and totally unexpected transport system capable of mediating the specific exchange of Cl^- for formate across the luminal membrane of the proximal tubule cell.

Formate recycling

Resorption of a significant fraction of filtered Cl^- by exchange for formate would result in the net secretion of thousands of millimoles of formate into the urine, unless a mechanism existed for recycling secreted formate back across the luminal membrane into the proximal tubule cell. In fact, such a mechanism is present.

Imposing an inside-alkaline pH gradient caused the accumulation of formate against its concentration gradient in renal microvillus membrane vesicles (4), consistent with the transport of formate via nonionic diffusion of uncharged formic acid. Whereas uptake of formate in exchange for Cl^- was sensitive to inhibition by high concentrations of disulfonic stilbenes, well-known inhibitors of carrier-mediated anion exchange, the uptake of formate driven by an inside-alkaline pH gradient was not inhibitor sensitive, consistent with passive nonionic diffusion of formic acid.

As illustrated by *steps 2* and *3* in Fig. 2, entry of uncharged formic acid into the cell by nonionic diffusion followed by exchange of intracellular formate for luminal Cl^- results in a net uptake of H^+ and Cl^- inward across the luminal membrane. In essence, formate serves as a coupling factor to allow H^+-coupled Cl^- uptake.

In fact, when ^{82}Br was used as a tracer for Cl^-, its uphill accumulation in renal microvillus vesicles was stimulated when an inside-alkaline pH gradient was imposed in the presence of a physiological concentration of formate (i.e., 0.2 mM) but not when the same pH gradient was imposed in the absence of formate (4). Ives and Verkman (3) found that imposing an inward Cl^- gradient caused acidification of the intravesicular space in the presence but not in the absence of formate, confirming the presence of a formate-dependent H^+-coupled Cl^- transport process as predicted by the model in Fig. 2.

To the extent that active H^+ secretion across the luminal membrane occurs by a Na^+-coupled mechanism such as Na^+-H^+ exchange (*step 1* in Fig. 2), as is largely the case in the proximal tubule, the net effect is equivalent to an electroneutral Na^+-coupled Cl^- transport process. This process is driven by the inward Na^+ gradient that is present across the luminal membrane due to the primary active extrusion of Na^+ across the basolateral membrane via the Na^+-K^+-ATPase.

Evidence for Cl^--formate exchange in the intact epithelium

A central issue not addressed by the membrane vesicle studies was whether Cl^--formate exchange plays an important role in the intact proximal tubule in the presence of physiological formate concentrations (0.2–1.2 mM).

To examine this issue, we collaborated with Schild et al. (7) to test the effects of formate on the rate of NaCl transport in the rabbit proximal tubule. Addition of 0.25 or 0.5 mM formate to the bath and lumen reversibly stimulated the rate of fluid absorption by 60% in straight (S_2) proximal tubules perfused in vitro with a low HCO_3^--high Cl^- solution similar to that found in vivo in these segments.

Under the conditions of these experiments, the stimulation of fluid absorption was a measure of a proportional increase in NaCl absorption. This stimulation of NaCl absorption by formate was not associated with any change in the transepithelial electrical potential difference, consistent with the operation of an electroneutral mechanism of NaCl absorption as predicted by the model in Fig. 2.

Similar concentrations of acetate failed to stimulate fluid absorption, consistent with the specificity of anion exchange as determined in the membrane vesicle studies. The formate-stimulated component of NaCl absorption was inhibited by the addition of the disulfonic stilbene DIDS (4,4′-diisothiocyanostilbene-2,2′-disufonic acid) to the lumen and by the addition of ouabain to the peri-

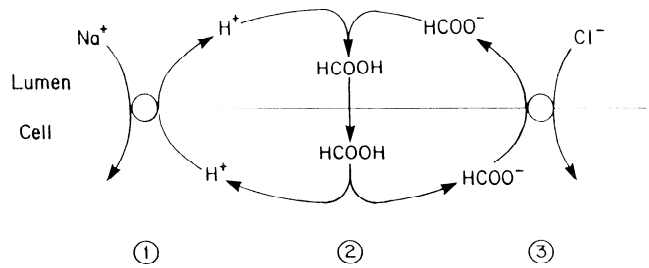

FIGURE 2. Three-step model for Na^+-coupled Cl^- transport involving Cl^--formate exchange and formic acid recycling across luminal membrane of proximal tubule cell. (From Karniski and Aronson (4).)

tubular bath (7). These effects are also predicted by the model in Fig. 2, in which stilbene-sensitive Cl^--formate exchange is indirectly coupled to the luminal membrane Na^+ gradient, which itself arises from ouabain-sensitive Na^+ extrusion by Na^+-K^+-ATPase across the basolateral membrane.

In proximal convoluted (S_1) tubules symmetrically perfused and bathed with a low HCO_3^--high Cl^- solution, formate similarly caused a reversible stimulation of fluid absorption (7). However, when these tubules were perfused and bathed with a high HCO_3^--low Cl^- solution, formate failed to significantly stimulate fluid absorption. The lack of an effect of formate on fluid absorption in this instance correlated with a higher luminal pH along the perfused segment. This finding may indicate that under physiological conditions the pH-dependent recycling of formate from lumen to cell via nonionic diffusion of formic acid is rate limiting for Na^+-coupled Cl^- transport occurring by this mechanism.

In studies of the rat proximal convoluted tubule by microperfusion in vivo, Alpern (1) observed that addition of Cl^- to an initially Cl^--free luminal perfusate caused a much larger acidification of the cell in the presence than in the absence of 1 mM formate. This suggests the presence of a formate-dependent H^+-Cl^- uptake process across the luminal membrane of the rat proximal tubule cell, as predicted by the model of Cl^--formate exchange in parallel with the nonionic diffusion of formic acid shown in Fig. 2. Taken together, the studies on rabbit and rat tubules strongly support the concept that Cl^--formate exchange is a major mechanism for transcellular Cl^- resorption in the intact proximal tubule under physiological conditions.

Origin of formate in the body

Formate is the smallest and probably least studied of the short-chain fatty acids. Found in human plasma at concentrations ranging from 0.2 to 1.2 mM, formate arises from ingestion, intestinal fermentation, and metabolism.

Although certain foods such as coffee contain millimolar concentrations of formate, the major source of intestinal formate is bacterial fermentation of plant cell wall polysaccharides. Human fecal concentrations are 1–2 mM, although the levels are probably much higher in the proximal colon and distal ileum where formate is formed and then transported across the mucosa by unknown mechanisms.

Formate is a product of the metabolism of several compounds. For example, formate arises from the oxidation of methyl groups, particularly from methionine. It is also a product of tryptophan degradation. In addition, formate is the major toxic metabolite in methanol intoxication, with reported plasma levels exceeding 30 mM. Once formed, formate is transferred to tetrahydrofolate by the enzyme formyltetrahydrofolate synthetase, yielding 10-formyl tetrahydrofolate. From here, formate takes part in a number of one-carbon transfer reactions, particularly the formation of purine rings and methionine. Excess formic acid is oxidized to CO_2 via a reaction catalyzed by formyltetrahydrofolate dehydrogenase.

Directions for future studies

What is the physiological advantage of carrying out Na^+-coupled Cl^- resorption in the proximal tubule by a roundabout mechanism involving formate recycling rather than by a more direct mechanism such as Na^+-Cl^- cotransport? Is it possible that the local concentration of formate is regulated as a means of modulating proximal tubule NaCl resorption?

Even in the absence of added formate, a component of active, transcellular Cl^- resorption has been observed in the proximal tubule, as already discussed. Does this reflect the presence of additional pathways for Cl^- transport across the luminal membrane, or is endogenous production of formate by proximal tubule cells sufficient to maintain a modest rate of Cl^--formate exchange even in the absence of added formate?

Answering these questions requires studies of the metabolic production and/or disposal of formate in proximal tubule cells and the concentration of formate in luminal fluid, in peritubular blood, and in proximal tubule cells as a function of maneuvers to alter proximal tubule NaCl resorption.

Is Cl^--formate exchange of physiological importance only in the proximal tubule? In fact, exchange of Cl^- for formate has been demonstrated in microvillus membrane vesicles isolated from the rabbit ileum (5). Current evidence suggests that Na^+-coupled Cl^- absorption in the ileum results from Cl^--HCO_3^- exchange operating in parallel with Na^+-H^+ exchange. But this does not exclude the possibility that Cl^--formate exchange operating in parallel with formic acid diffusion and Na^+-H^+ exchange may significantly contribute to NaCl absorption in this epithelium. Studies of the effect of formate on NaCl absorption in the intact ileum are required to answer this question. The possible presence of Cl^--formate exchange in other epithelia capable of uphill Cl^- transport should also be examined.

Finally, it should be noted that Cl^--formate exchange, diffusion of formic acid, and Na^+-H^+ exchange operating in parallel together represent a process for net solute uptake across the plasma membrane. Na^+-H^+ exchange is a ubiquitous transport process present not only in epithelial cells but also in many types of nonepithelial cells including neurons. If Cl^--formate exchange is present in parallel with Na^+-H^+ exchange in the plasma membrane of nonepithelial cells, such cells might swell in the presence of excess formate. Could this be a mechanism contributing to the cellular toxicity of formate in methanol intoxication?

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Glucose Sensors in Viscera and Control of Blood Glucose Level

Akira Niijima and Noël Mei

In addition to the well-known glucose-responsive neurons in the hypothalamus, recent studies of sensory signals from the visceral area have brought us a new understanding of the mechanisms that control the blood glucose level. The activity of efferent fibres to the pancreas and the liver is precisely modulated, not only by central hypothalamic glucoreceptors but also by peripheral (hepatic and intestinal) glucoreceptors.

Despite the difficulties inherent in the study of the autonomic nervous system (thinness of the relevant fibres, lack of morphological specialization of visceral receptors, depressive effect of anesthesia on functional condition of viscera, and so on), recent investigations have brought to light an amazing variety and complexity of sensory signals from the vegetative area. Available data have modified classical concepts and raised several questions, including that of the normal role of the visceral afferents. In particular, the control of blood glucose level by the intestinal and hepatic receptors has received much attention.

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Richness of sensory innervation of viscera

It was classically thought that sensory fibres are rare in the autonomic nervous system and that visceral nerves are mainly motor nerves. In fact, sensory fibres are found in all the nerves of both the sympathetic and parasympathetic systems. Moreover, they are abundant, often more so than motor fibres, which demonstrates that the visceral nerves mainly subserve sensory activities. This underestimation is easily explained by the fact that the unmyelinated fibres that prevail in visceral nerves cannot be properly investigated with light microscopy. For example, the vagus nerves contain about 80% of such fibres, the majority of which are sensory (1, 7).

Complexity of sensory signals from the visceral area

The use of the single-nerve technique and the microelectrode technique applied to the cranial and spinal ganglia has revealed the complexity of the messages conveyed by the visceral afferents.

In addition to painful signals, the autonomic nervous system carries a large range of physiological information that can be triggered by a variety of interoceptors, including different kinds of mechanoreceptors, thermoreceptors, and chemoreceptors (3, 5, 12).

Although they have been studied only in certain viscera including the digestive system, interoceptors seem to be present in all parts of the vegetative territory. Some are very specific, such as chemoreceptors that are stimulated by one substance or a group of substances, whereas others (multimodal receptors) are nonspecific and respond indiscriminately to chemical, thermal, and mechanical stimulations.

It is now possible to ascribe various physiological roles to visceral afferents. They may be classified into different categories, depending on the type and location of receptors and of central structures. They involve the control of visceral motility, the maintenance of homeostasis, and the regulation of behaviour. Among these mechanisms, the regulation of blood glucose level is a good example of the importance of the physiological role played by the visceral afferents.

Regulation of blood glucose

It is generally recognized that hormones play a major part in the control of the blood glucose level. Insulin is a key substance in controlling entry of glucose into the cells and its utilization. Another effect of insulin is to increase storage of glycogen in the liver and other tissues.

After a meal the increased secretion of insulin results in an acceleration of glucose utilization by body cells, which in turn causes a decrease in the blood glucose level. During starvation there is an increase in the rate of secretion of glucagon, epinephrine, and norepinephrine, which facilitates release of glucose from the liver, thereby contributing to the maintenance of the blood glucose concentration. Therefore, the endocrine pancreas, the adrenal medulla, and the liver play

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