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Cell Volume Regulation and Ischemic Tissue Damage

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Over years of friendly meetings with Professor Aharon Katzir-Katchalsky, many topics of mutual interest were discussed. He was the ideal person to come to with a problem. After being subjected to his critical, analytic mind, most research problems seemed simple, more clearly defined and understandable. His broad biologic and scientific background grew from an apparently insatiable interest in all natural phenomena. He generously shared his knowledge and imparted his wisdom with a share of his own infectious excitement. He was quick to sense the significance of understanding of biological processes to their practical application. For this reason it seems appropriate to relate the progress made in the understanding of cell volume regulation, which had been discussed on several occasions with him, to its possible significance as a factor in disease processes.

Studies with isotopes have revealed that cell membranes are permeable to water and to all the small solutes of the extracellular fluids bathing the cells. All cells contain proteins and other macromolecules which must exert an osmotic pressure tending to draw fluid into the cells. Why cells do not swell and burst, thus becomes a valid question. The answer lies in the distribution of small ions between the cells and extracellular fluid.

It has been known for a long time that the intracellular fluids have a high content of potassium but little sodium or chloride whereas the extracellular fluid has a high concentration of sodium and chloride but a low concentration of potassium. Fig. 1 schematically represents the means by which this characteristic ionic distribution affects the cell volume. The intracellular macromolecules are indicated by A^{n-} as most are anionic at the pH of intracellular fluids. Sodium ions are continuously diffusing "down hill" into the cells but are maintained at a low concentration within the cell by an active outward extrusion accomplished by the cell membrane. Since

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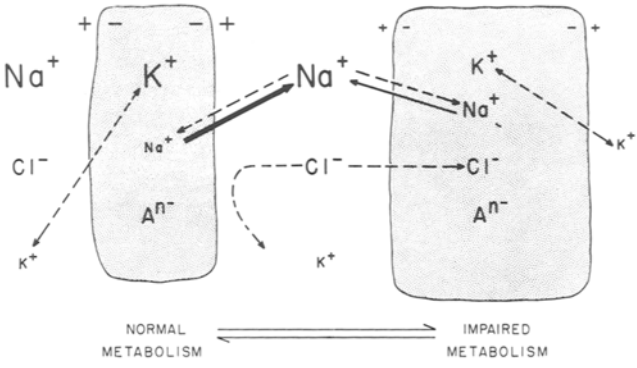

Fig. 1. A schematic representation of the factors involved in the regulation of cell volume as described in the text

sodium ions are positive, their removal from the cell leaves the cell interior electrically negative to the extracellular fluids. This electrical gradient results in an accumulation of potassium ions within the cells so that potassium is close to electrochemical equilibrium on the two sides of the cell membrane. Actually there is some active uptake of potassium by cells so the membrane potential may also be regarded as a diffusion potential resulting from the outward leakage of potassium from the cells. In either case, the membrane potential is so oriented as to exclude chloride ions from the negative cell interior.

With the nondiffusible macromolecules restricted to the cell interior this would leave an imbalance of forces, as mentioned, drawing water into the cell. However, the high extracellular sodium concentration just balances the intracellular osmotic pressure and thus the cell volume is stabilized. But the cell membranes are not impermeable to sodium ions but rather sodium which is continuously moving down its electrochemical gradient into cells is as continuously being pumped out of the cell by active membrane processes. The outward transport of sodium from cells is an energy-requiring process and the energy for this process arises from the metabolism of the cells. When insufficient metabolic energy is available to remove sodium from cells at its rate of entry then a characteristic sequence of events occur which leads to swelling of cells [8, 9]: sodium entering the cell accumulates causing depolarization of the cell membrane potential and this causes loss of potassium from the cell and entry of negative chloride ions into the cell. The gain of sodium and chloride will exceed the loss of potassium and the net gain of intracellular solutes will draw water into the cell causing it to swell. The process is reversible so long as the supply of energy for sodium

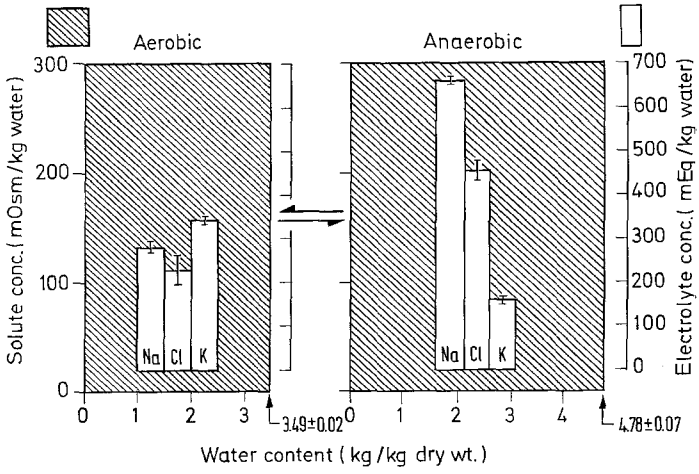

Fig. 2. Water and electrolyte composition of kidney cortex slices under aerobic and anaerobic conditions (30 min anaerobic). The hatched area represents the noninulin space water content of kidney cortex slices. The columns are the content of sodium, chloride and potassium of the same slices. All values are related to dry weight of tissue

transport is re-established before cell death occurs. Fig. 2 shows the actual changes in water, sodium, chloride and potassium content of renal cortex slices which occur when the energy supply is interrupted by 30 min of anoxia [8].

Thus, cell volume is stabilized by the activity of transport mechanisms for sodium present in the plasma membranes of all animal cells. This means of preserving cell volume and an optimal ratio of surface-to-volume for cells with high rates of metabolism allows very low cell membrane tensions to exist. This in turn is compatible with deformity of cell membranes and motility of cells. The plant kingdom which chose the alternative method of preventing cell swelling, namely to encase each cell in a tough cellulose compartment sufficiently rigid to withstand the large osmotic swelling pressures involved, is forever committed to a sessile existence as a result of this "choice". Furthermore, the energy available in the ionic gradients which we think originally were developed to maintain cell volume have been exploited by the specialized cell membranes of the nervous system to conduct nervous impulses.

During life, a variety of factors may affect the ability of cells to regulate their volume. One such common clinical phenomenon is transient tissue ischemia which may result from the blockage of a blood vessel, severe vasoconstriction, or a period of hypotension caused by blood loss, cardiac failure or other major insult to the circulation [10].

It has been known for many years that following such a period of transient ischemia to the kidney, blood flow does not return when the cause of the ischemia is removed. This phenomenon of persistent reduced blood flow to the kidneys has been investigated by many workers and has been termed "no-reflow" by Sheehan and Davis [11]. One factor playing a role in this no-reflow is cell swelling occurring during the initial ischemic period which obstructs small vessels within the kidney thus perpetuating the ischemia beyond the initial period [5]. Final death of cells may result from the vicious cycle of ischemia → cell swelling → further obstruction of small vessels → more ischemia → more cell swelling, etc., resulting finally in death of the kidneys. Such a sequence of events is similar to that postulated by Ames and associates [1, 3, 4, 7] to account for their experimental studies on transient cerebral ischemia.

The initial studies utilized the rat and dealt with the no-reflow phenomenon following a period of total obstruction of the renal artery. Fig. 3 illustrates the no-reflow phenomenon. On the left is seen a normal kidney with its vasculature injected intra-arterially with silicone rubber to yield a cast of its blood vessels. The middle and right kidney were injected in a similar manner but after 10 min, and 30 min of reflow of blood, respectively, following the 60 min of obstruction of the renal artery. The failure of blood flow to all vessels of the kidney after release of the renal artery is evident and the residual ischemia is patchy.

If the hypothesis is correct, that cell swelling, which occurred during the hour of obstruction of the renal artery was now responsible for the patchy intrarenal ischemia, then shrinking the swollen cells by any means should relieve the patchy ischemia. Fig. 4 shows the effect on the vascular pattern of an intravenous injection of hypertonic mannitol during the final few minutes of the one hour during which the renal artery was clamped. Sufficient mannitol was infused to increase the osmolality of the rat's plasma from 304 ± 4 to 360 ± 10 mOsm/kg of water 5 min following the injection. It is evident that the hypertonic mannitol relieved the patchy ischemia. In another group of animals, isotonic saline was injected in an amount calculated to expand the extracellular fluid volume to an extent equal to or greater than that resulting from the hypertonic mannitol. Volume expansion alone produced no improvement in the renal vascular pattern during no-reflow. Other poorly penetrating solutes, however, when added in concentrated solution to the extracellular fluids produced the same improvement observed with mannitol.

A more direct test of the hypothesis that cell swelling caused patchy renal ischemia was afforded by electron-microscopy. Fig. 5 contrasts the

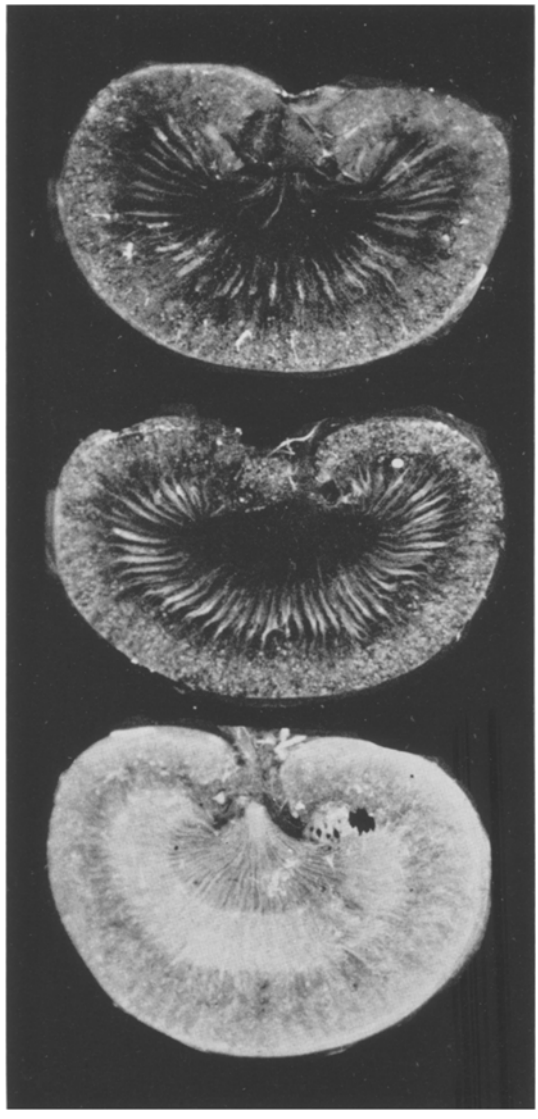

Fig. 3. Vascular pattern of hemisections of kidneys visualized by silicone rubber injected into renal artery. At the left is shown the vascular pattern of a normal kidney, while middle and right kidneys show the vascular pattern after 60 min of obstruction of the renal artery followed by 10 to 30 min of reflow of blood to the kidneys, respectively, after release of the arterial obstruction. The blood vessels are filled with white silicone rubber and the dark areas represent portions of the kidney which were ischemic

appearance of subcortical tissue from a normal kidney, an ischemic kidney 15 min after release of the renal artery and a similarly prepared ischemic kidney treated by an intravenous infusion of hypertonic mannitol, as described. Ischemia alone has caused readily visible swelling of all the cellular elements as compared with the control kidney. Surprisingly, even the endothelial cells seem swollen and the erythrocytes appear wedged into the narrowed vascular channels. Following the administration of hypertonic mannitol the appearance of the electron-micrograph has reverted to that of the normal tissue.

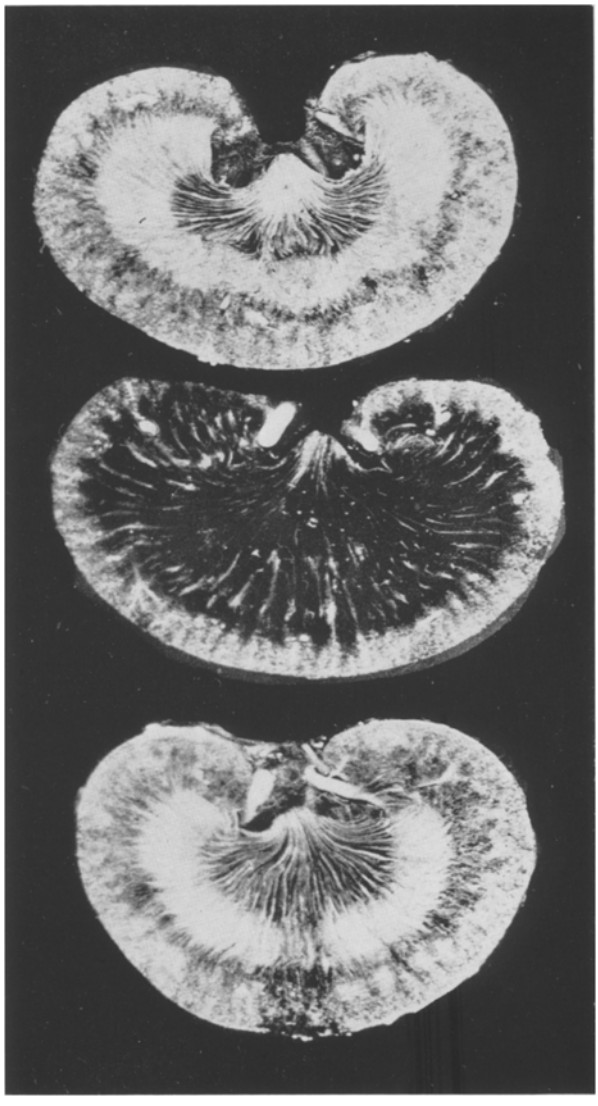

Fig. 4. Vascular pattern of hemisection of kidneys after 120 min of obstruction of the renal arteries with and without infusion of hypertonic mannitol. On the left is a normal kidney. The middle and right kidneys were injected with silicone rubber after 120 min of ischemia but hypertonic mannitol was administered in the case of the kidney on the right

To provide a quantitative assessment of the changes in cell volume produced by ischemia and ischemia plus hypertonic mannitol, two different measurements were made. The outer and inner area of all vessels of 10 μ radius or less were measured and from these two measurements a mean endothelial thickness was calculated. Fig. 6 shows the results of such measurements and indicates the definite increase in endothelial thickness in the post ischemic period. Hypertonic mannitol effectively reduced the endothelial swelling. Next, the circumference and area of all interstitial cells in the sections were measured. From these two values the "per cent circularity"

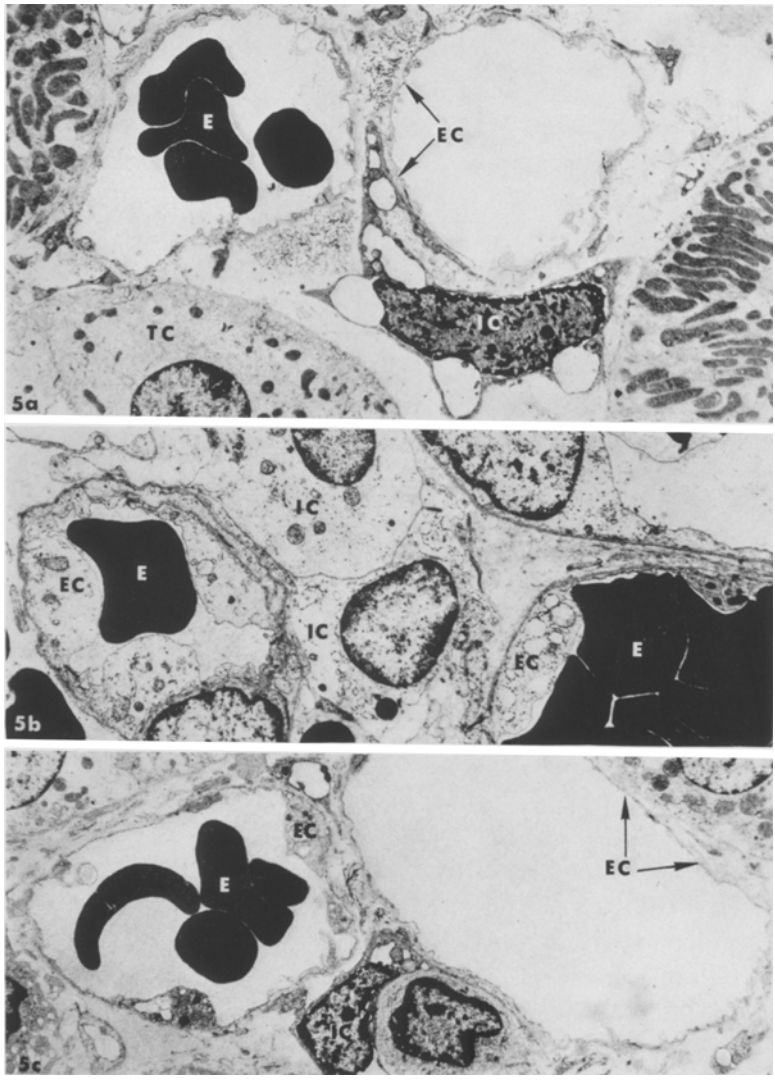

Fig. 5. Comparative electron-micrographs from the sub-cortical zone of normal (*a*) and ischemic kidneys treated with isotonic saline (*b*) and hypertonic mannitol (*c*). (*a*) Normal kidney showing patent vessels, flat endothelial cells *EC* and an interstitial cell *IC* with a very irregular profile, erythrocyte *E*, tubular cell *TC*. (*b*) Ischemic kidney treated with isotonic saline showing swollen endothelial cells *EC*, and interstitial cells *IC*; the smaller vessel to the left suggests strongly the role of endothelial swelling in restricting blood flow, while larger vessels (to the right) are filled with sludged blood. (*c*) Ischemic kidney treated with hypertonic mannitol. It is evident here that endothelial cells *EC* have regained the more normal thickness. The interstitial cells appear considerably less swollen. 5250 ×

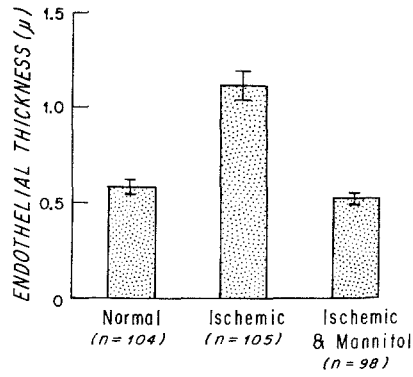

Fig. 6. Mean endothelial thickness of renal capillaries (radii <10 μ)

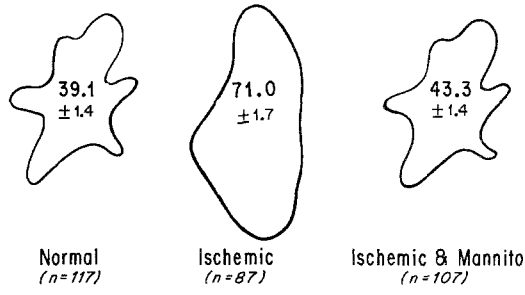

Fig. 7. Mean per cent circularity of renal interstitial cells

could be calculated, that is the per cent of the area of a circle having as its circumference the perimeter of the interstitial cell that the actual area of the interstitial cell comprised. Fig. 7 shows that the crenated normal interstitital cells did, in fact, round out during ischemia and this swelling was reversed by the hypertonic mannitol. Thus, direct morphologic evidence is provided that cell swelling does occur following transient renal ischemia and that hypertonic mannitol which improves the vascular pattern of the post ischemic kidney also shrinks the swollen renal cells.

That the treatment with hypertonic mannitol also improved renal function is shown in Fig. 8. The serum creatinine was measured 24 hr after the period of renal ischemia and this measure of renal function indicates that in all instances ischemia impaired renal function but less so in the animals treated with mannitol. Hypertonic mannitol has been used in treatment of acute renal failure for the past decade; however, attention has been focused on its ability to sustain urine flow whereas its major beneficial effect may be to correct the patchy ischemia caused by cell swelling.

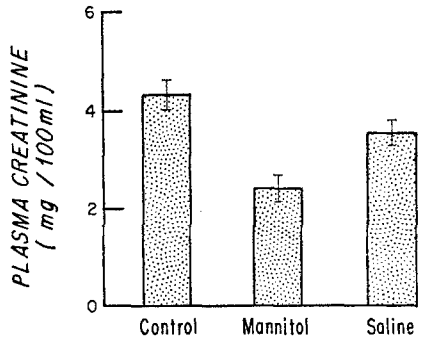

Fig. 8. Plasma creatinine levels 24 hr after clamping both renal arteries for 60 min

From the appearance of erythrocytes in the small vessels of the ischemic kidney one has the distinct impression that they are actually occluding the narrowed arteriolar lumen. The experiments described were all performed with the animals fully heparinized so that intravascular thromboses would be excluded. However, the conditions which prevail within an ischemic organ are just those which will exhaust the supply of metabolic substrates to the red blood cells as well as to the tissue. This will result in a fall in red cell ATP levels and with this a loss in the flexibility of the erythrocytes [15]. In fact, Summers and Jamison [12] found that if the red cells were washed out of the kidney before the ischemic period, the no-reflow was prevented as indicated by the distribution of carbon particles to all of the vasculature of the post ischemic kidney. If blood was first reinjected followed by the carbon black then the latter again revealed the patchy ischemia. The silicone rubber injection also filled the complete vascular bed of the kidney after an hour of ischemia provided the blood had first been washed out of the kidney with a physiologic saline solution. Although the lumen of the small blood vessels underwent the same narrowing during ischemia, the small particulate silicone rubber still entered all the vessels whereas the larger erythrocytes having lost their normal maleability can no longer pass through the narrowed vessels.

Recently, we have examined the effects of transient renal ischemia produced by hemorrhagic hypotension to determine if cell swelling also played a role in this situation. This condition is more closely related to the usual clinical setting in which acute renal failure develops than is clamping of the renal artery. Furthermore, humoral, as well as neurogenic factors, may modify the findings with the preparation. It has previously been demonstrated that hemorrhagic hypotension causes an increase in the total water

content of the kidney with an increase in its content of sodium and chloride and a decreased content of potassium [14].

Sufficient blood was removed from a carotid artery of an anesthetized rat to lower its arterial pressure from approximately 100 to 40 mm of mercury. This requires removal of about 40% of the animal's estimated blood volume. The rat was then kept in this hypotensive state for a period usually of 2 hr at the end of which 70% of the blood was reinfused slowly over a period of 12 min together with either saline or a variety of other test solutions. This reinfusion raised the arterial blood pressure back to its normal level. Morphologic studies revealed that ischemia caused by hemorrhagic hypotensions produced cell swelling similar to that which resulted from clamping the renal artery.

Measurements of renal blood flow with Xenon-133 were performed in the post ischemic period [6] according to a modification of the method of Thorburn *et al.* [13]. Whereas the normal renal blood flow in the rat by this method was 384 ± 20 ml per 100 grams of tissue per minute, 30 min following the 2 hr of hemorrhagic hypertension the mean renal blood flow was only 145 ± 10 ml per 100 g of tissue per minute in the control animals reinfused with saline. When 25% mannitol replaced saline in the reinfusion, the mean flow increased to 182 ± 8 ml per 100 g of tissue per minute. Other solutes were tested to determine whether a larger molecule might be more effective in correcting the renal blood flow. Twenty per cent dextran (mol wt 40,000) reinfused instead of the saline did increase the mean blood flow to 230 ± 10 ml per 100 g of kidney tissue per minute.

Using the intraarterial injection of silicone rubber to allow visualization of the renal vasculature, it was apparent that hemorrhagic hypotension, like clamping the renal artery, resulted in patchy ischemia in the post hypotensive period. Injections of 25% mannitol and of 20% dextran in place of the saline, following reinfusion of 70% of the blood removed from the animal, improved the renal vascular pattern.

Morphologic studies of the kidney following hemorrhagic hypotension revealed generalized cell swelling in the saline-infused kidneys. Thus, hypertonic mannitol shrank the swollen cells but the effect was incomplete while dextran produced a greater and more generalized shrinkage of cells.

It is not entirely clear at this time why the large molecular dextran appears to be more effective than the smaller molecule of mannitol in correcting the patchy ischemia of the no-reflow state. At least two possibilities suggest themselves. First, cell membranes will become increasingly permeable to small solutes as cells swell. Mannitol, which normally penetrates cell membranes slowly, may have such a decrease in reflection coefficient

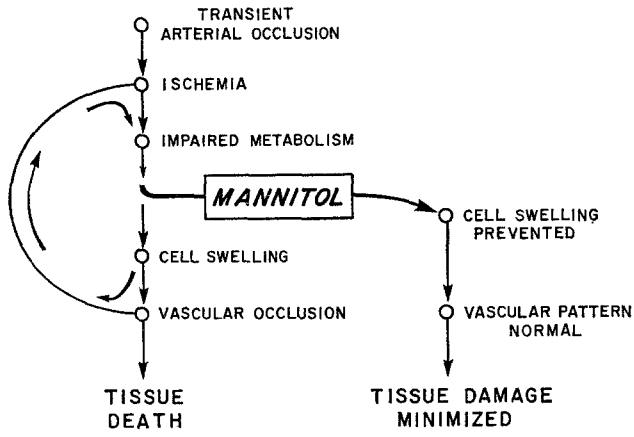

Fig. 9. Schematic representation of the effect of transient arterial occlusion on tissue ischemia and the corrective effect of hypertonic solute (mannitol)

as to be ineffective in withdrawing water from cells. Dextran, being a larger molecule with a high reflection coefficient, however, might still be effective. Second, the beneficial effects of dextran may depend on some rheological property or its ability to reverse the inflexibility of erythrocytes and thus reestablish the circulation through the narrowed vessels.

It must be apparent that the factors involved in the no-reflow phenomenon should not be restricted only to the kidney. The same sequence of events which are postulated here and summarized in Fig. 9 must occur in other tissues as well as in the kidney. In fact, Ames and associates [1, 3, 4, 7] before us had demonstrated a similar phenomenon in the brain. Ames had found that the isolated rabbit retina would still show evidence of function after a period of total anoxia for 2 hr. This seemed paradoxical in the light of common knowledge that obstruction of blood flow to the brain for even a few minutes will result in brain death. Ames and associates infused carbon black into the carotid artery following a 5-min period of arterial occlusion. The carbon particles failed to reach all portions of the vasculature of the brain but instead showed a patchy distribution with many small areas remaining unperfused. The cause of this patchy ischemia was the apparent swelling of perivascular astrocytes which narrowed the small blood vessels. Some beneficial effects on this patchy ischemia were obtained with injections of hypertonic mannitol. Thus, cell swelling during transient ischemia of brain may cause or contribute to the subsequent no-reflow ischemia with ultimate death of brain cells.

A similar phenomenon seems to affect the heart also in response to transient ischemia. Thus, Willerson *et al.* [16] found that mannitol protected

the myocardium from the usual consequences of clamping the left anterior descending coronary artery. In the presence of hypertonic mannitol, the S – T segments determined with a probing electrode placed directly over the muscle supplied by the left anterior descending artery failed to show the usual early elevation indicative of myocardial damage, the contractility of this muscle was preserved and measurements of regional blood flow into the ischemic myocardium via collateral vessels was greater in the presence of the mannitol. Studies are currently in progress to determine whether these beneficial effects of hypertonic mannitol are, in fact, based on the ability of the mannitol to shrink swollen cells in the ischemic myocardium.

In conclusion, it now seems that the energy-requiring transport processes present in plasma membranes of all animal cells play an important role in the regulation of cell volume. When the supply of metabolites or of oxygen to tissues is interrupted, as may occur during periods of transient tissue ischemia, the volume regulation of cells fails, sodium, chloride and water enter the cells causing cell swelling. This in turn may cause obstruction to blood flow in small blood vessels sustaining the tissue ischemia after the initial transient ischemic insult has passed. This secondary ischemia may be the ultimate cause of cell or organ death. Methods to reverse the cell swelling are being sought with the hope that their clinical application may disrupt the vicious cycle and prevent the ultimate death of cells.

Permission from the *Journal of Clinical Investigation* to reproduce Figs. 1, 3, 4, 5, 6, 7, 8 and 9 is gratefully acknowledged.

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