Qualitative and Quantitative Importance of the Constituents Used in Microperfusion Experiments*

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In vivo and in vitro microperfusion techniques are powerful tools by which nephron function can be directly characterized. However, there are several potential sources of error that may be associated with the utilization of these methods. The purpose of the present manuscript is to examine four general areas from which the sources of these errors may arise: 1) adequacy of volume marker; 2) osmotic disequilibrium secondary to evaporation of fluids; 3) identification of normal ultrafiltrate constituents; and 4) preparation of the perfusion solutions.

VOLUME MARKERS

For volume marker to be ideal, we must have complete assurance that 100% of the perfused marker is collected and analyzed without losing the marker either by diffusion through the tubule or by adherence to the glassware used. The volume markers that we have evaluated are: \(^{125}\)I-albumin, \(^{125}\)I-iothalamate, \(^{3}\)H-inulin, and radioactive colloidal gold. Unfortunately, none of these has proved to be ideal. \(^{125}\)I-Albumin sticks to glassware so that the specific activity of the solution coming out of the pipet often is less than what was put into it; \(^{125}\)I-iothalamate is not completely impermeant across the proximal convoluted tubule; \(^{3}\)H-inulin appears to be somewhat unstable and probably liberates small-molecular weight fragments that are permeant across the tubular membrane; and isotopic colloidal gold, in our hands, behaves as if it does not form a homogeneously labeled solution, and in addition, seems to have the undesirable property of adhering to glass. Colloidal gold has been used successfully in some laboratories as a volume

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marker, but in our specific protocol, it has been the least satisfactory of all the volume markers evaluated. We are currently evaluating $^{125}$I-polyvinylpyrrolidone, and our initial impressions are quite favorable. In a small series of studies it neither penetrated the membrane of the proximal convoluted tubule nor the descending limb of Henle, nor did a significant fraction ($<0.5\%$) adhere to the membrane itself.

**EVAPORATION**

Evaporation may cause significant changes in solution osmolalities between the time they were prepared and the actual experimental procedure time. In Fig. 1, the magnitude of the evaporation problem is examined. On the top panel a single 1-mm-long proximal convoluted tubule of rabbit is perfused with isosmolal ultrafiltrate of same serum as the bath. If the perfusion rate is 10 nl/min, then the collection rate is approximately 9 nl/min with a net reab-

![Diagram](image)

**Fig. 1.** Schematics to illustrate effect of osmotic disequilibrium on net transport of fluid out of the proximal convoluted tubule (PCT). A illustrates reabsorptive capacity of the PCT, while B shows the net reabsorption when bath has increased its concentration by 10%. $V_i$ = perfusion rate, $V_{out}$ = collection rate, $C_{active}$ = net reabsorption secondary to active transport, $C_{passive}$ = reabsorption secondary to passive osmotic equilibration, and $C_{total}$ = sum of $C_{active}$ and $C_{passive}$.
sorptive rate of 1 nl/mm/min which, presumably, is secondary to active transport properties of the tubule. Similarly, at greater perfusion rates, for example, 30 nl/min, we found that absolute reabsorption remains unchanged and is independent of perfusion rate at flow rates above 10 nl/min. These results are in agreement with the studies published earlier by Burg and Orloff(1). However, consider the events in the bottom panel in which the concentration of the bath has increased 10% secondary to evaporation for a final osmolarity of 330 mOsm/liter. We have previously determined that the osmotic water permeability of both the proximal convoluted tubule(2) and the descending limb of Henle(3) is such that certainly within 1 mm of tubule length complete osmotic equilibration takes place at all reasonable perfusion rates. Thus, under this circumstance, 10% of the perfused volume will exit due to osmotic equilibration. At perfusion rates of 10 nl/min this amounts to 1 nl/min while at 30 nl/min the magnitude of fluid transported by passive equilibration is 3 nl/min. Under the latter condition, a major fraction of the net fluid is extracted secondary to passive osmotic forces. In the technique where isolated tubules are perfused in vitro, we generally tend to work at flow rates less than 15 ml/min, thus, the effects of hypothesized osmotic disequilibrium are minimized when using isosmolal ultrafiltrate as the perfusion fluid. However, considerable artifactual fluid movements may be introduced both in vitro and in vivo when artificial perfusion solutions are used. The fundamental difficulty arises in the construction of an artificial perfusion solution which has the same chemical concentration of each constituent and the same osmotic pressure as the peritubular fluid.

IDENTIFICATION OF NORMAL ULTRAFILTRATE CONSTITUENTS

A. Osmotically Significant Components

In biological systems there may be great discrepancies between the freezing point osmolality of a solution as compared to the effective osmolality that this solution can generate across a given membrane. Fig. 2 illustrates this principle. In this example the tubule is perfused with pure NaCl isosmolal to the bath as

![Diagram](image_url)

**Fig. 2.** The effective osmotic gradient generated by unequal concentration of fluid constituents.
measured by freezing point depression. Since the osmotic coefficient for NaCl is 0.92, this solution has a NaCl concentration of 163 meq/liter. The effective osmolality here is equal to the reflection coefficient for NaCl, 0.68(2), times the freezing point osmolality and is equal to 204 mOsm/liter.

An examination of the peritubular surface reveals a NaCl concentration of 110 meq/liter with the remainder of the osmolarity composed of NaHCO₃, non-Na salts, and other constituents. If the remainder of the non-sodium salt constituents are assumed to have a reflection coefficient close to 1, then the total effective osmolality is 235.2. The assumption that nonelectrolytes have a reflection coefficient of 1 probably is justified on the basis that the measured reflection coefficient for a small molecular-weight compound, such as urea, is close to 1 or 0.91 ± 0.05(4). Thus, even though the freezing point osmolarities are identical, the effective osmolality of the peritubular side in this example is 15% higher than the lumen, and accordingly, will provide a driving force for net efflux of fluid out of the lumen. The magnitude of this efflux will depend on the perfusion rate.

Table 1 shows that in vivo microperfusion experiments present problems similar to those described for in vitro experiments. This figure shows the composition of the fluid used in the last microperfusion paper noted(5). Two points illustrated by this figure have generally been typical of most other artificial perfusion fluids: (1) All appear to have a lower effective osmolality than the plasma of the hydroscopic rat, and (2) Most of the reported fluids have a much higher chloride concentration than the plasma. The imposed chloride concentration gradient provides yet another passive driving force for net movement of fluid out of the tubule. In this case, chloride would diffuse down its concentration gradient being accompanied by enough water to maintain isosmolar conditions. Thus, it is not surprising that most of the in vivo microperfusion studies are reporting absolute reabsorption rates of the proximal convoluted tubule to be higher than similar measurements made under free flow conditions.

**B. Substituents Necessary for Active Transport**

I would like next to discuss those constituents in artificial solutions that are necessary for complete expression of net active transport properties. Many such constituents might be present in minute concentrations and would therefore be difficult to quantitate. The following studies in which we measured potential differences across the proximal convoluted tubule exemplify this.

| TABLE 1 | **Typical Composition of in Vivo Microperfusion Fluid** |
| --- | --- |
| NaCl | 130 meq/liter | Calculated freezing point osmolality = 288 mOsm/liter |
| NaHCO₃ | 10 meq/liter | Calculated effective osmolality = 191 mOsm/liter |
| NaAC | 10 meq/liter | Na total = 150 meq/liter |
| CaCl₂ | 3 meq/liter | Cl total = 156 meq/liter |
| H₂ inulin trace | | |
When perfusing with isosmolar ultrafiltrate at rates above 10 nl/min, we find that the mean transmembrane potential difference is $-5.8$ mV, with the lumen negative. This potential difference is felt to be generated by some active transport process since it has appropriate temperature responses and reversibly decreases with ouabain(6). In these studies we further noted that the magnitude of the transmembrane potential was dependent upon the perfusion rate down the tubule. At perfusion rates above 10 nl/min, the potential difference was stable and maximum, but at slower flow rates, the potential difference was noted to decrease. The most sensitive range of flow dependence to potential difference was at perfusion rates around 2 nl/min(6).

We have interpreted this to mean that at slow perfusion rates some necessary substituents are utilized in such a way that the maximum potential difference cannot be fully expressed. After prolonged efforts, we have finally made an artificial perfusion solution which nearly approximates the control potential difference when perfusing with ultrafiltrate. Then if we decrease such constituents as would be accomplished by the metabolic processes of the tubule; for example, glucose, amino acids, calcium, bicarbonate, and fatty acids, the potential difference decreases. We currently feel that the potential difference near the glomerulus is negative, perhaps in the 5 to 6-mV range as observed with ultrafiltrate when perfusing at high rates, but further down the length of the tubule preliminary results indicate that the potential difference actually is somewhat positive, in the range of +.5 to +1.0 mV. These results were obtained by removing constituents in a manner that approximated what occurs in the tubule in vivo. The purpose of presenting the potential difference data here, aside from its obvious implications for the electrolyte transport processes, is to point out that it is difficult to decide which constituents to put into the artificial solutions since such unexpected compounds as amino acids and glucose effect the magnitude of the transmembrane potential difference(6). Undoubtedly there are still other unidentified constituents present in normal ultrafiltrate which also have an effect on the potential difference.

**PREPARATION OF PERFUSION SOLUTIONS**

In the preparation and use of perfusion solutions there are three precautionary measures which will minimize the sources of potential error. First, if at all possible, an investigator should use either ultrafiltrate alone or ultrafiltrate which has been modified according to desired protocol as the perfusion fluid. With this technique we can be certain that the majority of the constituents with varying reflection coefficients are in approximately equal concentrations. Second, the studies should be conducted at the slowest possible rates. This has two advantages: (1) The concentration of volume marker is increased in the collected fluid and (2) The contribution due to passive osmotic equilibration is minimized. Thirdly perhaps the most important control that should be followed when effects of two or more artificial solutions are compared, involves dialyzing the desired
fluids against each other prior to their use. To accomplish this, one simply places one solution in a cellophane bag and then places this bag into a flask containing the other solution for 72 hr at 4° while stirring slowly. This procedure will ensure that the osmolality and other small-molecular-weight constituents of the two solutions will be identical. Obviously, this only permits us to study the effect of a compound which does not permeate through the cellophane bag. If we wish to study the effect of some small molecular-weight constituent, then to each of the dialyzed fluids we should add equal volumes and osmolar concentrations of the desired constituents. In one case we would add the control constituent, while in the other case we would add the experimental substituent.

Last year Dr. Imai and I(7) reported that the oncotic pressure of the bath could influence net transport out of the proximal tubule without the necessary juxtaposition of the capillary bed. When bath was made hypooncotic as compared to control, there was a 38% decrease in net reabsorption, and when the protein concentration of the bath was increased to 12.5 g/100 ml, there was an increase of 28% in net transport of fluid. In these experiments ultrafiltrate was used as the hypooncotic bath, while the remaining filtrand with higher protein concentration was used as the hyperoncotic bath. It could be argued that it was something else besides the protein which affected net transport which was filtered out during process of ultrafiltration. The studies in Fig. 3 were designed to examine this question. Initially a large lot of ultrafiltrate was prepared by pressure dialysis using aminco PM-30 membranes(7). To one half of this we added bovine albumin so its final protein concentration was 6.6 g/100 ml, identical to the

![Ultrafiltrate (UF) of Rabbit Serum through Aminco PM-30 membrane](image)

**Fig. 3.** The effect of ambient protein concentration on net reabsorption from proximal convoluted tubule. UF = ultrafiltrate.

| Bath No. 1 | Bath No. 2 | Bath No. 3 |
|------------|------------|------------|
| Control rabbit serum | Reconstituted UF | Dialysed UF |
| Net reabsorption nl/mm min | 0.75 ± 0.07 | 1.02 ± 0.10 | 0.28 ± 0.15 |
| (P < 0.02) | (P < 0.01) | |
control serum. This solution was then placed in a cellophane bag for 72 hr and dialyzed against the other half of ultrafiltrate without protein. Thus, the only possible difference between these two solutions was the protein concentration. The same ultrafiltrate without protein was used as the perfusion solution. You will note that absolute reabsorption using this protein containing reconstituted ultrafiltrate was actually somewhat higher than the control serum. We interpret this increase to the greater number of onctically active particles present in reconstituted ultrafiltrate since all of its protein concentration was made up of albumin and not higher molecular-weight globulins. Net transport again decreased, as in our previous reports, when ultrafiltrate without protein was used as the bath. The thrust of this study was to prove that it was the protein concentration difference per se which effected net transport. We are in a strong position to come to this conclusion since all of the other constituents were identical due to the equilibrium dialysis which was carried out.

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