Use of Sodium Ferrocyanide as Glomerular Indicator
To Study the Functional Heterogeneity of Nephrons

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Mammals show a large anatomical heterogeneity in their nephron population. In most species, the glomeruli of the outer cortex are smaller and give rise to shorter proximal tubules than those of the inner cortex. Associated with this anatomical heterogeneity is a functional heterogeneity illustrated in Fig. 1. This figure presents data from one I'sammomys undergoing a salt diuresis and demonstrates that the single nephron glomerular filtration rate (SNGFR) is correlated either to the length of the proximal tubule or to the glomerular volume. In the rat(1–8) and the dog(9) also exists a well-known graded distribution of SNGFR within the cortex. Since this distribution is capable of changing in the rat, with regard to the physiological state of the animal(10,11), it appears that extrapolation to the whole kidney of findings obtained on the superficial nephron population alone, as is the custom in most micropuncture studies, could lead to an erroneous conclusion about the overall renal function. In such studies, it must be kept in mind that the nephron population inaccessible to micropuncture (40% of this population(12)) could exhibit a different behavior.

One method of studying the SNGFR of deep nephrons consists of puncturing the long-looped nephrons accessible at the tip of the papilla. The primary criticism of this method stems from the fact that information from both superficial and deep nephrons cannot easily be obtained from the same kidney. This is why, during the last decade, some attempts have been made to elaborate other techniques. All these methods use ferrocyanide as glomerular indicator.

The first such technique was developed by Hanssen(13). It consisted of injecting as a pulse a small volume of concentrated sodium ferrocyanide into the
jugular vein; 10–12 sec later, the bolus was instantaneously stopped as it flowed along the proximal tubule. The kidney was immediately removed and the ferrocyanide precipitated as Prussian blue. Following the maceration of the kidney, proximal tubules were microdissected. In each tubule the distance between the glomerulus and the distal front of the precipitated bolus was assumed to be proportional to its GFR. To assess this hypothesis, the position of the distal front of the ferrocyanide was compared with the actual value of SNGFR (Table 1). It can be seen that in the rat there was good agreement between the superficial over juxtamedullary SNGFR ratio, on one hand, and the superficial over juxtamedullary front level ratio, on the other. Both of them were 0.8 in non-diuretic conditions and 1.0 after chronic salt loading. Such was not the case in the Psammomys. In non-diuretic as well as diuretic Psammomys the precipitate front level was at the same distance from the glomerulus in superficial and juxtamedullary nephrons, but the latter had SNGFR's 2.5 times higher than the superficial nephrons. Therefore, the proportionality observed in the rat between the distance of the precipitate from the glomerulus and the SNGFR seems to be due to chance.

The use by Hanssen of labeled ferrocyanide rendered the technique more valid(5). The procedure was essentially the same as in the preceding method, except that labeled 14C, rather than unlabeled ferrocyanide solution was injected as a pulse. The amount of 14C contained in each nephron could be considered directly proportional to the SNGFR. However, if the pulse is injected too rapidly, in most instances after an intrajugular injection of such a concentrated solution (Fig. 2) an immediate and significant fall in the arterial blood pressure can be observed. To prevent this fall, we modified the technique by injecting the pulse into the abdominal aorta, through a PE 10 catheter introduced into a femoral artery and threaded until its end was positioned slightly above the two renal arteries(11).

As illustrated in the right panel of Fig. 2, the injection never exerted a significant effect on the arterial blood pressure. However, because of the laminar flow in the aorta and the arteries of the kidney, the plasma concentrations of

Fig. 1. Relationship between the SNGFR and the length of the proximal tubule (left panel) or the glomerular volume (right panel) in the Psammomys. ○, Superficial nephrons, O, juxtamedullary nephrons. Solid line = regression line (from de Rouffignac et al.(21).
the $^{14}$C ferrocyanide differed from one region of the kidney to another. The uneven regional distribution of $^{14}$C ferrocyanide is illustrated in Table 2 which reports data from three pyramids taken in different regions of one kidney. Although the position of the front level precipitate for each category of nephron was approximately the same in each pyramid, the amount of $^{14}$C per tubule of each class of nephron varied from one pyramid to another. This means that the intraluminal concentration of the labeled precipitate was not the same through-

![Figure 2](image_url)

**Fig. 2.** Effect on the femoral artery pressure of an injection as a pulse (50 μl) of a 15% sodium ferrocyanide solution into the jugular vein (left panel) or into the abdominal aorta (right panel).

### TABLE 1

**Comparison Between the SNGFR and the Position of the Ferrocyanide Precipitate Along the Proximal Tubule of Superficial (S) and Juxtamedullary (JM) Nephrons**

| Rats                        | Nephrons | Single nephron GFR | Precipitate front level |
|-----------------------------|----------|--------------------|-------------------------|
|                             |          | nl/min             | S/JM                    |
|                             |          |                    | mm                      |
|                             |          |                    | S/JM                    |
| Nondiuretic                 |          |                    |                         |
| NaCl 9%                      | [10] S   | 32.7 ± 1.4         | 4.88 ± 0.46             |
| 20 μl/min $^a$               | JM       | 40.6 ± 1.9         | 6.07 ± 0.43             |
| Chronic salt loading + salt diuresis |          |                    |                         |
| NaCl 2%                      | [5] S    | 52.4 ± 3.2         | 3.84 ± 0.19             |
| 200 μl/min $^b$              | JM       | 52.7 ± 4.3         | 3.79 ± 0.25             |
| Psammomys                   |          |                    |                         |
| Nondiuretic                 |          |                    |                         |
| NaCl 9%                      | [4] S    | 8.0 ± 1.5          | 1.63 ± 0.37             |
| 10 μl/min $^c$               | JM       | 19.8 ± 4.0         | 1.63 ± 0.45             |
| Salt diuresis               |          |                    |                         |
| NaCl 4%                      | [4] S    | 7.9 ± 0.9          | 2.36 ± 0.87             |
| 37.5 μl/min $^d$             | JM       | 26.2 ± 7.3         | 2.29 ± 0.59             |

*Number of animals in parentheses.

$^a$ From Bonvalet et al.(2).

$^b$ From de Rouffignac and Bonvalet(11).

$^c$ and $^d$ From de Rouffignac et al.(21).
uneven regional distribution of $^{14}$C ferrocyanide injected as a pulse into the abdominal aorta. Example taken in one kidney of a rat undergoing a salt diuresis (NaCl 2%, 200 $\mu$l/min) and subjected to chronic salt loading (3 weeks)

| Nephrons | Precipitate front level (% proximal tubular length) | Amount of $^{14}$C per tubule i.p.m. S/JM |
|----------|---------------------------------------------------|------------------------------------------|
| Pyramid 1 | | |
| S        | 53 ± 5                                            | 10.6 ± 1.0                                | 1.12 |
| JM       | 37 ± 8                                            | 9.5 ± 1.4                                 |     |
| Pyramid 2 | | |
| S        | 51 ± 5                                            | 13.9 ± 0.9                                | 0.95 |
| JM       | 32 ± 8                                            | 14.6 ± 1.6                                |     |
| Pyramid 3 | | |
| S        | 45 ± 2                                            | 18.7 ± 1.7                                | 1.13 |
| JM       | 39 ± 4                                            | 16.6 ± 2.0                                |     |

out the kidney cortex. However, the superficial over juxtamedullary $^{14}$C radioactivity ratio was similar in each pyramid. It can be concluded therefore that if care is taken to compare only nephrons from the same pyramid, this technique gives a good approximation of the SNGFR ratio between each class of nephrons. Nevertheless, these methods allow one to obtain only relative values of SNGFR's. For this reason, we have modified the Hanssen's technique in order to measure directly absolute values of SNGFR(13).

Our modification entails the administration of a priming dose of 200 $\mu$Ci of $^{14}$C sodium ferrocyanide solution followed by the infusion at a constant rate of a sustaining dose of 10 $\mu$Ci/min. The concentration of sodium ferrocyanide in the solution was raised in order to equilibrate the plasma concentration at about 1–1.5 mM/liter, since it was demonstrated that at such plasma concentration this ion behaves like a glomerular indicator(14,15). Five to ten minutes later, 30 $\mu$I of a concentrated unlabeled ferrocyanide solution were rapidly injected as a pulse, into the aorta, just above the renal arteries. The renal pedicle was tied a few seconds after this injection. An arterial blood sample was taken and the kidney removed. Then the kidney was treated according to Hanssen's technique. Each tubule was cut at the distal front level of the unlabeled ferrocyanide precipitate. The distal part of the tubule was removed, and the radioactivity contained in the glomerulus and in the remaining part of the tubule was counted. The radioactivity contained in each nephron from the glomerulus to the distal front of the unlabeled ferrocyanide precipitate, should correspond to the $^{14}$C ferrocyanide ions filtered during the time elapsed between the injection of the unlabeled ferrocyanide, taken as zero time, and the ligature of the renal pedicle. Thus, since both the amount of $^{14}$C filtered in each nephron during a given time as well as the $^{14}$C plasma concentration were known, it was possible to calculate the absolute SNGFR value of this nephron.

Before considering the limitation of this technique, its validity to determine absolute SNGFR values must be discussed. In recent experiments, Morel, Roinel,
and Le Grimellec(16) found that in proximal as well as in distal tubule samples, F/P ferrocyanide values were identical to the simultaneously determined (F/P) inulin. This observation demonstrated directly that, in vivo, no transtubular movement of ferrocyanide occurred along the proximal tubule.

It was shown in nondoniuretic rats (Expt. I, Table 3) that the 14C ferrocyanide clearance was similar to that of tritiated inulin. However, the ferrocyanide over inulin clearance ratio was slightly lower than unity. From studies carried out by Berliner et al.(4) and Kleeman and Epstein(17), it was concluded that a constant amount of ferrocyanide was bound to proteins, and that the bound fraction became negligible when the plasma concentration reached 1.0 mM/liter. In the present technique, the infusion rate was chosen to increase the plasma ferrocyanide concentration to 1.5 mM/liter. The purpose of Expt. II reported in Table 3, was to assess the influence on the GFR of the PE 10 catheter threaded up into the aorta. The inulin clearance did not change after the insertion of such a catheter; the difference between the GFR of rats with and without a catheter was not significantly different from zero (paired t test). It must be emphasized that ferrocyanide ions are not physiologically inert, since ferrocyanide ions behave like a nonreabsorbable anion with four negative charges. In a micro puncture study carried out on the nondoniuretic rat with a ferrocyanide plasma concentration of 1.5 mM/liter, the same concentration used in our technique, Morel,

### Table 3

**Technical Verifications of the 14C Sodium Ferrocyanide Infusion Technique.**

**Experiments Carried Out in Nondoniuretic Rats (NaCl 9%, 20 µl/min)**

| I | Comparison of the 3H-inulin and 14C-Na-ferricyanide clearances |
|---|---|
| | $C_{ir}/C_{in}$ |
| | 0.988 ± 0.35 SD |

| II | Influence on the GFR of the presence of a PE 10 catheter into the abdominal aorta |
|---|---|
| | $C_{in}$ with $-C_{in}$ without |
| | $d = +0.6\%$, $n = 47$, $P > 0.01$ |

| III | Influence on the GFR of an iv infusion of a 5% Na-ferrocyanide solution |
|---|---|
| | Before infusion |
| | $C_{in}$(ml/min) |
| | 0.991 ± 0.154 |
| | (12) |
| | During infusion |
| | $C_{in}$(ml/min) |
| | 1.015 ± 0.190 |
| | (22) |

| IV | Loss of radioactivity by cutting proximal tubules |
|---|---|
| | Whole tubule (previously counted) |
| | 22.5 ± 2.7 cpm |
| | (17) |

| V | Loss of radioactivity through the walls of proximal tubule |
|---|---|
| | Radioactivity in proximal tubules dissected from a same kidney: |
| | Just after the maceration |
| | 11.8 ± 1.5 cpm |
| | (22) |
| | 1 year later |
| | 11.3 ± 2.5 cpm |
| | (17) |

Number of measurements, $n$ and figures in parentheses.
Roinel, and Le Grimellec(16) found that the composition of the distal tubular fluid was not markedly affected by the ferrocyanide infusion. As compared to controls, the (F/P) value for inulin was unchanged and the F/P value for solutes, sodium, and potassium was only slightly increased. Specifically, (F/P) sodium was 0.38 instead of 0.31 in control non-diuretic rats(18). Nevertheless, such modifications of the tubular fluid composition did not alter the GFR of the whole kidney, as demonstrated in Expt. III, Table 3. As a matter of fact, there was no significant difference between the mean value of the inulin clearance before and during the infusion of the sodium ferrocyanide solution. The fourth series of experiments presented in Table 3 were conducted to ascertain whether the open end of the proximal tubule allowed ^14C ferrocyanide to leak out in any significant amount. A number of proximal tubules were cut into several pieces and each nephron was checked to determine that the sum of the radioactivity of all the fragments did not differ from the previously counted radioactivity contained within the whole tubule. Finally, to decide whether there was a loss of radioactivity through the walls of microdissected tubules, it was verified, on the same kidney, that the labeled precipitate contained in proximal tubules of nephrons dissected just after the maceration was not different from that of nephrons dissected 1 year later (Expt. V, Table 3).

The distribution of Prussian blue precipitate within the nephron was studied in histological sections(19). In these studies, no staining of tubular cells has been found, indicating also that no transcellular diffusion of ferrocyanide occurred in vivo. However, to verify directly that there was no transcellular movement of ferrocyanide in vivo from the blood to the tubular lumen, the following experiment was performed. A monitored reduction of the arterial blood pressure by aortic constriction was performed on the left kidney of a non-diuretic rat until the GFR but not the renal blood flow ceased, and was verified by means of lis-samine green injections. Once this arterial pressure was reached, a continuous infusion of ^14C ferrocyanide solution after a priming dose was started. Four minutes later, the two kidneys were removed. The results presented in Table 4 show that the mean radioactivity contained in complete proximal tubules of the control kidney was 72 counts per minute (cpm). In the kidney presumed to be nonfiltering, although the glomerular tuft contained a significant amount of radioactivity, this value was only 0.07 cpm, that is 0.1% of the control. Inci-

| TABLE 4 |
| --- |
| **Radioactivity Contained in the Structures of the Right (Control) Kidney and the Left (Nonfiltering) Kidney of a Nondiuretic Rat** |
| **Control** | **Nonfiltering** |
| Proximal tubule | Superficial glomerulus | Proximal tubule | Superficial glomerulus |
| 72.2 ± 1.0 SE (23) | 2.00 ± 0.16 (10) | 0.07 ± 0.03 (20) | 0.67 ± 0.16 (11) |

* Number of measurements in parentheses.
dentally, these findings demonstrate that radioactivity on the peritubular surface of the nephron was negligible or was adequately removed during the histochemical procedure. Moreover, Table 4 shows that the $^{14}$C radioactivity contained in superficial glomeruli was 2.00 cpm for the control kidney and 0.67 cpm for the nonfiltering kidney. Since the $^{14}$C plasma concentration was 3.64 cpm/nl in this experiment, it can be calculated that the superficial glomeruli contained about 0.55 nl of plasma and ultrafiltrate in the control kidney and probably 0.18 nl of plasma alone in the nonfiltering kidney. These values are quite compatible with the mean volume of these glomeruli which was found to be 0.98 nl.

It could, of course, be assumed that a significant fraction of the radioactivity counted resulted from an in vivo intracellular accumulation of the label. Such an hypothesis might have been proposed on the basis of the following observation. In the rat, a positive correlation between the length of proximal tubule segment counted and the SNGFR was noticed in each kidney; the longer this segment, the higher the SNGFR as described in the right panel of Fig. 3. This correlation could presumably be explained by an in vivo accumulation of $^{14}$C in the tubular cells. In point of fact, however, the absence of such a correlation in the Psammomys kidney, as illustrated in the left panel of Fig. 3, invalidates this hypothesis.

In vitro diffusion of a small amount of ferrocyanide precipitate from the lumen to the cell during the preparation of the fragment tissues cannot be definitely excluded. As pointed out by Hanssen(20), this diffusion appears limited for the proximal tubule to the area of the brush border (cf. Fig. 4). At any rate, most of the ferrocyanide precipitate remains in the tubular lumen and such post-mortem diffusion, if any, does not invalidate our method of SNGFR determination.

As far as the accuracy of the technique is concerned, it seems difficult to give a precise figure. However, since each proximal tubule is taken with its glomerulus, this leads to an SNGFR overestimation due to the radioactivity contained within the capillary tuft. In the nondiuretic rats, the radioactivity contained in the

![Fig. 3. Front level of the unlabeled precipitate related to the SNGFR. Comparison between the Psammomys and the rat. ●, Superficial nephrons, O, juxtamedullary nephrons (from de Rouffignac et al.(13).](image-url)
Fig. 4. Photograph of proximal tubule segments showing the intraluminal localization of the ferrocyanide precipitate.

glomerulus averages 12% of the total radioactivity counted per nephron. In fact, we cannot know what percentage of the glomerular radioactivity is within Bowman's capsule. Nevertheless, it seems reasonable to suppose that this percentage was not below 50%. This, therefore, leads to a maximal overestimation of SNGFR's of about 6%. On the other hand, in this technique the proximal tubule is cut at the distal front level of the unlabeled precipitate, but not at the maximum of the precipitate concentration, since this maximum is very often not easy to detect. This maximum, in fact, could be at approximately 0.3 mm from the front. Since the mean value of the distance between the glomerulus and the front was about 6 mm in our experiments, it can be deduced that cutting at the front level leads to another probable overestimation of 5%.

Finally, the validity of the technique can be assessed by the experimental results themselves. Table 5 shows data obtained from the rat and Psammomys in our laboratory using the micropuncture and the ferrocyanide infusion technique. Each figure represents the mean value of the mean SNGFR for each animal studied. It can be seen that there was an agreement between the values given by the superficial as well as the juxtamedullary nephrons. Figure 5 sum-
marizes some results obtained in the rat during four experimental conditions when associated renin is present in the juxtaglomerular apparatus. In this figure, mean value for superficial and juxtamedullary SNGFR in each kidney was plotted against the femoral artery pressure recorded at the moment of the SNGFR determination. This figure shows the remarkable constancy of SNGFR in the 65- to 175-mm Hg range of arterial pressure. The apparent scatter of the mean SNGFR value from one animal to another could, in fact, be explained by anatomical consideration, such as differences in the length of proximal tubules and glomerular volumes.

In conclusion, we should like to state briefly the primary limitations and advantages of the ferrocyanide infusion technique as compared to the micropuncture technique. First, the ferrocyanide infusion technique does not permit repetitive experiments on the same animal. Another limitation stems from the fact that the technique cannot be used during a prolonged period of hypotension under 60 mm Hg, since in this condition, the infused labeled ferrocyanide concentrates in the proximal tubule. Thus, the labeled precipitate becomes as visible as the unlabeled one. Consequently, it becomes impossible to distinguish the labeled from unlabeled ferrocyanide precipitate. However, this technique has

| TABLE 5 | COMPARISON BETWEEN THE SNGFR MEASURED BY MICROPUNCTURE AND BY THE 14C SODIUM FERROCYNANIDE INFUSION TECHNIQUE IN SUPERFICIAL (S) AND JUXTAMEDULLARY (JM) NEPHRONS |
|-----------------|-----------------|-----------------|
| Nephrons        | Micropuncture (ml/min) | 14C-Ferrocyanide (ml/min) |
| Rat             |                  |                  |
| Nondiuretic NaCl 9°/0  | S               | 29.8 ± 2.5°      | 32.7 ± 1.4°      |
| 20 μl/min        |                  | (8)              | (10)             |
| Chronic salt loading + salt diuresis NaCl 2°/0  | S               | 50.3 ± 4.3°      | 52.4 ± 3.2°      |
| 200 μl/min       |                  | (4)              | (3)              |
| Psammomys       |                  |                  |
| Nondiuretic NaCl 9°/0  | S               | 6.2 ± 0.8°       | 8.0 ± 1.5°       |
| 10 μl/min        |                  | (10)             | (4)              |
| Salt diuresis NaCl 4°/0  | S               | 8.5 ± 1.0°       | 7.9 ± 0.9°       |
| 37.5 μl/min      |                  | (7)              | (4)              |
| JM              |                  | 22.3 ± 2.5°     | 26.2 ± 7.3°     |

° From Benesathet al.(18).  
° From Bonvalet et al.(2).  
° From Imbert et al.(23).  
° From de Rouffignac and Bonvalet(11).  
° From Morel et al.(21).  
° From de Rouffignac et al.(21).  
° From de Rouffignac and Morel(25).  
Number of animals: in parentheses.
the advantage of giving simultaneous determination of SNGFR for all categories of nephrons. Moreover, it allows one to determine a great number of SNGFR's on each kidney, and to know with accuracy the location of each microdissected tubule within the kidney cortex.

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