Odd E. Hanssen and the Hanssen method for measurement of single-nephron glomerular filtration rate

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Received 21 December 2000; accepted in final form 1 May 2001

Auckland, Knut. Odd E. Hanssen and the Hanssen method for measurement of single-nephron glomerular filtration rate. Am J Physiol Renal Physiol 281: F407–F413, 2001.—In the middle of the twentieth century, the suspicion that deep and superficial nephrons might serve different functions created a demand for measurement of single-nephron glomerular filtration rate (SNGFR). Rather unexpectedly, the answer came from Odd E. Hanssen (1917–1964), a Norwegian physician working on his own in the Department of Pathological Anatomy, University of Oslo, with minimal support and no interaction with renal physiologists. In 1963, after nearly 10 years of work, he presented the ferrocyanide method, allowing simultaneous estimates of SNGFR in a large number of nephrons in all layers of the kidney. This review first describes his early visions of the method and the elaborate and extremely time-consuming studies in mice to verify the technique. As a byproduct came valuable information on the relationship between nephron size and SNGFR, glomerular intermittency, and the emptying of the tubules on filtration stop. Hanssen died from a cerebral hemorrhage in 1964, and for several years the method seemed entirely forgotten. Fortunately, Andrew Baines took up the use of ferrocyanide in 1963–1964 while working on his thesis in Toronto, but his first publication came in 1969 from Saclay, France, in collaboration with Christian de Rouffignac. Modifications allowing determination of absolute SNGFR were worked out by de Rouffignac and by Jaime Coelho in New York. Thereafter, the “Hanssen method” spread rapidly, and in the early 1980s about 50 reports had been published from 17 laboratories in 9 countries. The distribution of SNGFR in mammals, birds, and fish was described, as well as the response to water and salt loads, vasoactive substances, hormones, varying perfusion pressure, blood loss, etc. Finally, after mentioning two recent methods inspired by the Hanssen technique but using other filtration markers, the review concludes that most of our present knowledge on SNGFR distribution and regulation has been obtained by the method developed by Hanssen 40 years ago.

ferrocyanide; kidney function

THE CONCEPT OF GLOMERULAR filtration as the initial step in urine formation dates back to the middle of the nineteenth century (10, 52), but direct evidence for a glomerular ultrafiltration had to await micropuncture and collection and analysis of tubular fluid from frogs and, later on, from small rodents, in the period from the 1920s to the 1940s (55). In the same period, the concept of clearance emerged, and establishment of substances that are freely filtered in the glomeruli and not secreted or reabsorbed in the tubules made it possible to measure total glomerular filtration rate (GFR) in the intact kidney. However, already at the beginning of the century it had been shown that the glomerular size and the length of proximal tubules were greater in deep than in superficial cortex (44). Although clearance studies done from the 1940s to the 1950s indicated a well-balanced relationship between GFR and proximal tubular functions (11), the possibility was still open for large functional heterogeneity within the nephron population. That idea was greatly stimulated by the studies of Trueta et al. (54), suggesting that both functional and pathological alterations in renal function could be attributed to reciprocal changes in blood flow in deep and superficial layers of the kidney. Another impetus for the study of heterogeneity of both blood flow and GFR came with establishment of the medullary countercurrent system for osmotic concentration of the urine (57). Together, these two studies led to a large number of measurements of intrarena!al distribution of blood flow with an everlasting search for new and better methods, with many controversial results (2, 38, 43).

An apparently straightforward method for estimating single-nephron GFR (SNGFR) distribution was to measure inulin clearance by micropuncture collection of fluid from the loops of Henle and from subcapsular tubules, and a few studies on rats suggested that the filtration in deep juxtamedullary glomeruli might be 50–100% greater than that in superficial glomeruli (37, 39). However, the method has several drawbacks. First, the puncture of Henle’s loops in rats has to be
done in immature animals, it is limited to the nephrons with the longest loops, and it cannot be done simultaneously with the puncture of superficial nephrons. Moreover, more recent studies have suggested that the higher values in the juxtaglomerular nephrons may reflect exclusion of a higher tubuloglomerular feedback tone in deep than in superficial nephrons by occlusion and puncture of the tubules (21, 49).

It was therefore a methodologically great leap forward when Odd Eiwinn Hanssen in the years 1955–1963 developed the ferrocyanide technique, which permits simultaneous estimates of filtration in a large number of glomeruli situated in all layers of the cortex. Although the “Hanssen method” became widely known and utilized, Hanssen himself remained unknown to renal physiologists, probably because he did not emerge from or ever join the physiology milieu. Before summarizing his studies, I believe it may therefore be appropriate to present a brief curriculum vitae.

Hanssen was born in 1917 in Enebakk, a rural community south of Oslo, Norway. He graduated from the University of Oslo Medical School in 1946, after a delay of a couple of years because of the events of World War II. In 1943 the Germans closed the university and deported many students to a concentration camp in Germany. Like many other students, Hanssen escaped to Sweden, where he joined a paramilitary force to participate in the liberation of Norway. After completing his medical degree in 1946, he had various brief clinical appointments in hospitals in Tromsø and Oslo. In 1948, he received a 3-yr research assignment in the Norwegian Defense Research Establishment, working in the Department of Pathological Anatomy, University of Oslo, which was chaired by Professor Leiv Kreyberg. In 1951, he attained a hospital position in the autopsy section in the same institute. He became an assistant professor at the University of Oslo in 1951 and associate professor in 1962. For the period from 1960 to 1962, he worked in the Department of Pathology, State University of New York, Downtown Medical Center, chaired by Dr. Patrick J. Fitzgerald, on a 1-yr US Public Health Service Fellowship and thereafter in a temporary position in the Electron Microscope Laboratory of Dr. Lawrence Herman. He submitted his thesis in 1961 and was awarded the degree of Doctor Medicinae (equivalent to the PhD) in 1962. Hanssen died after a cerebral hemorrhage in 1964, leaving his wife, Ingebjørg, and two teenage daughters (Fig. 1).

DEVELOPMENT OF THE FERROCYANIDE TECHNIQUE

During most of his years at Rikshospitalet in Oslo, Hanssen presumably spent much time on routine work in the Autopsy Division. He also had teaching duties, and his friends and colleagues describe him as an unusually patient and helpful teacher. He was extremely modest and self-critical, obsessed with his research, and always strove to have 100% experimental proof for his conclusions. He lived on a modest university salary and, unlike many of his colleagues, never participated in the more profitable areas of pathology.

Hanssen’s first use of ferrocyanide was in a histological study of the toxic effect of bacitracin on the kidney (25). He observed that ferrocyanide did not pass beyond protein casts in the tubules and seemed not to escape through the cells of the obstructed tubules. However, as reviewed extensively in a later article (26), this was by no means a novel technique: ferrocyanide is fairly nontoxic, dissolves easily in water, and is precipitated by ferric chloride to form water-insoluble Prussian blue. The latter had been used already from the beginning of the twentieth century to locate injected ferrocyanide in histological sections. To prevent movement of the ferrocyanide during fixation, in 1932 Gersh (23) introduced fixation by freeze-drying. By using this technique, he arrived at the conclusion that ferrocyanide was freely filtered in the glomeruli and apparently retained quantitatively in the tubules. This was supported by several other studies, leading to the concept that the amount of ferrocyanide observed in the tubules after intravenous bolus injection was an indicator for GFR. Renal physiologists entered the stage later and confirmed that, in dogs, ferrocyanide has a clearance equal to or close to that of inulin (8).

The great achievement of Hanssen was his realization that ferrocyanide could be used “to solve some of the problems regarding glomerular filtration as well as the distribution of filtrate among the nephron popula-
tion in different experimental conditions, by comparing the amount of precipitated ferrocyanide in individual nephrons" (26). Moreover, he realized that quantification of Prussian blue could not be made on histological sections, as used by Gersh (23), “except possibly by complicated and time-wasting reconstruction of serial sections” (26). Hanssen therefore decided to develop a technique that “would make the tissue suitable for microdissection (of isolated tubules) without significant loss or displacement of the ferrocyanide” (26). The answer to this demand was, in his own words:

With the present method, diffusion is prevented by freezing renal tissue excised during excretion of ferrocyanide. The frozen tissue is then treated with a cooled solution of hydrated ferric chloride in absolute ethyl alcohol. This reagent penetrates the frozen tissue and dissolves the ice. The ferrocyanide then meets a barrier of ferric chloride and is precipitated with minimal chance for diffusion. Now, maceration of the specimens with hydrochloric acid, microdissection and mounting of the nephrons may be performed with little or no disturbance of the precipitate (26).

Thereafter followed a detailed description of the procedure, leading up to microdissection of single proximal tubules after 4–5 days.

To induce rapid freezing, Hanssen chose to use mice because of their small kidneys. When renal blood flow was stopped 10–15 s after intravenous bolus injection of 0.05 ml 10% sodium ferrocyanide and the kidney was removed and frozen within 20–30 s, he found that the ferrocyanide was confined to about the first half of the proximal tubules. As demonstrated in color photos, the Prussian blue entered into the brush border and also into a few tubular cells but seemed to be retained by the basement membrane. However, because he planned to quantify the glomerular filtration by the length of Prussian blue precipitate along the proximal tubule, he had to evaluate a possible displacement of ferrocyanide in the period from circulatory arrest to complete fixation. To obtain full control of the timing, he therefore exteriorized the left kidney. Because he dismissed bilateral clearance measurement to ensure normal GFR in the exteriorized kidney, he instead measured urine flow during high osmotic diuresis and found no difference between the two kidneys (27).

Using this preparation, he then compared the tubular location of precipitated ferrocyanide in the left exteriorized kidney frozen at the moment of circulatory arrest to that in the right untouched kidney frozen 20 s-5 min after death (28). When the fixation was delayed by 5 min, the length of the Prussian blue column had increased from ~60 to 80% of the proximal tubular length, the tubules had collapsed, and the renal water content had been reduced by an average of 13%. A slight displacement (statistically significant?) was also observed after 20- to 25-s delayed fixation. He concluded that the bulk of proximal fluid was reabsorbed locally, with little displacement of precipitated ferrocyanide.

After these reassuring observations, Hanssen turned to more physiological problems, the first of which being the long-standing dispute on temporarily inactive glomeruli, following the observations of “glomerular intermittency” in frogs by Richards and Schmidt (45). Kidneys removed 10 s after ferrocyanide injection were frozen and prepared as described above, and an amazing number of 11,403 nephrons were dissected from 11 kidneys under various conditions, with or without Nembutal anesthesia, and at varying degrees of hydration. Ferrocyanide was lacking in 112 proximal tubules, but 98 of them were observed in one severely dehydrated mouse. Hanssen cautiously concluded that the frequency of inactive glomeruli, in the sense of a cessation of glomerular filtration of at least 10-s duration, is probably <3% during the conditions of everyday life (29).

In the next article (30), he compared the nephron size to glomerular filtration, estimated as the length fraction of the proximal tubules containing ferrocyanide 10 s after intravenous injection. Both tubular length and glomerular “equatorial area” in deep cortical layers were about twice that observed in superficial glomeruli, and both correlated well with their estimated filtration rate. As in the previous papers, he extensively discussed possible artifacts and technical shortcomings, concluding that “the present method should be of value in this research work when quantitative estimation of the precipitate can be performed in tubules from animals in which the experimental conditions have been more precisely controlled.”

Hanssen’s five papers published in Acta Pathologica et Microbiologica Scandinavica from 1958 to 1961 formed the basis for his thesis for the Doctor Medicinae degree, submitted in February 1961. In the preface he acknowledged technical assistance with drawing and measuring the nephrons. He performed all dissections and preparations himself. He also thanked Dr. Jean Oliver for reading the last two articles and for valuable advice. This is in fact the only information I have found for direct interaction with other “nephrologists.” Dr. Oliver, well known for his excellent studies on nephron anatomy in health and disease, was at that time retired from his position in the same department in New York where Hanssen had completed his work on the ferrocyanide method. In this connection, it may be of interest to note that Hanssen was the single author of all five papers making up his thesis. This should not be taken to indicate a lack of willingness or inability to cooperate with colleagues. Most likely, it reflects the requirement of original and independent research to qualify for a Norwegian Doctor Medicinae degree, a requirement that, as late as the 1960s, practically excluded coauthorship and even systematic guidance, at least in some departments. Today, this seems to be a preposterous way to create scientists, but sometimes it did work out. If Hanssen had joined a well-organized research team instead of working on his own, he might well have produced a respectable, multiauthored thesis in 3 or 4 years, but then we probably would not have had the ferrocyanide method.

The committee evaluating Hanssen’s thesis (32) consisted of three professors, two interns with a back-
ground in kidney studies, and one pathologist, all of whom gave extensive written statements. They had some formal complaints on language, unclear sections, overly long discussion chapters, etc., but they also agreed that Hanssen had proven himself to be a clever experimenter, one with imagination, a critical sense, and tremendous tenacity, and that his thesis represented a valuable contribution to experimental nephrology. Despite the many laudatory comments, none of these good men seemed to have realized the full potential of the method for measuring SNGFR. Nevertheless, it is interesting to note that one of them mentioned that ferrocyanide with 14C or a radioactive iron label could have been used to evaluate the loss of Prussian blue during preparation. However, when that was written in November 1961, Hanssen had already done such experiments in rats in New York, reporting the results at the 46th Annual Meeting of the Federation of American Societies for Experimental Biology in April 1962 (31). Of greater interest, he was now able to quantify the tubular content of ferrocyanide and could thereby estimate relative filtration rates in different layers of the cortex. As reported at the 2nd International Congress of Nephrology in Prague, Czechoslovakia, in August 1963, he found a loss of labeled ferrocyanide of 7% during fixation, mazeration in HCl, and softening in water, probably representing mainly extratubular ferrocyanide (33). In four rats, SNGFR in deep cortex was on average 56% higher than that in superficial cortex.

There can be no doubt that these experiments were performed in the Pathology Department at the Downtown Medical Center in New York. However, Dr. Fitzgerald has only a faint memory of a discussion on the use of radioisotopes, and neither he nor Dr. Herman has any recollection of kidney experiments. Apparently, Hanssen continued his habit of working on his own. On the other hand, both Dr. Fitzgerald and Dr. Herman clearly enjoyed having Hanssen in the department and still maintain a close relationship with the Hanssen family.

Publications on Other Topics

Hanssen’s work for the Norwegian Research Department resulted in a couple of internal reports and a brief review on toxicological problems, and in 1961 he also coauthored a case report on generalized cytomegalic inclusion disease (22). While in New York, he described, together with Dr. Herman, an electron microscopic detection of a central rodlike structure in the brush-border villi of the proximal tubule (34), apparently still a controversial observation. After his return to Oslo in 1962, he studied the ultrastructural effects of bacitracin on the kidneys and demonstrated large amounts of autophagic vacuoles within the proximal tubular epithelium, whereas the glomeruli remained normal. Because of his untimely death, the results were not published until several years later (40).

APPLICATIONS AND MODIFICATIONS OF THE HANSSEN METHOD

As mentioned above, Hanssen died in 1964. His wife tells that he had plans to exploit the Hanssen method after returning to Oslo in 1962, but there is no evidence of the extent to which the plans were realized. More surprising, even if many renal physiologists were interested in intrarenal heterogeneity, nobody seemed to have noticed this remarkable methodological progress during the following 6 years. One reason may be that few physiologists had discovered his publications in Acta Pathologica et Microbiologica Scandinavica. On the other hand, the three pages published from the 2nd International Congress of Nephrology in Prague in 1963 (33) should have had a reasonably good chance to be noticed. At any rate, one might in fact have feared that the method could have passed into oblivion forever.

Fortunately, the use of ferrocyanide was taken up by Andrew Baines for his PhD Thesis at the University of Toronto in 1963–1965, as cited by Baines et al. (6) in 1968. In a recent letter to me, Baines speculates that his first use of ferrocyanide might have been proposed by Oliver. As mentioned above, Oliver had also been consulted by Hanssen in 1961 during his stay in New York, and for all we know, Oliver may even have been instrumental in Hanssen’s adoption of radiolabled ferrocyanide. Subsequently, in the laboratory of Carl Gottschalk in Chapel Hill, North Carolina, stimulated by discussions with Gottschalk, Oliver, and Paul Lysac, Baines carried out most of the research that appeared in 1969 (4), estimating relative single-nephron filtration from the length of tubular ferrocyanide. In Francois Morel’s laboratory in Saclay, France (1967–1968), Baines collaborated with Christian de Rouffignac to complete the experiments on rats and add additional studies on psammomys, using [14C]ferrocyanide to obtain more direct estimates of relative SNGFR (4). This study, published in 1969, was the first to use 14C-labeled ferrocyanide after Hanssen’s reports in 1962–1963 (31, 33) and seems to contain the first reference to those studies.

Apparently, Jaime B. Coelho and collaborators (17) at Columbia University were next out, publishing their first paper in 1971. Coelho had been in Gottschalk’s laboratory and may have learned about the method there.

Both of these groups modified the original Hanssen technique to provide not only relative but also absolute SNGFR, which obviously required recording of ferrocyanide concentration in arterial plasma. That was elegantly solved by de Rouffignac et al. (48) as follows. A steady plasma concentration of [14C]ferrocyanide was provided by constant intravenous infusion. Then, a bolus of unlabeled ferrocyanide was injected into the renal artery, followed, after 10 s, by clamping of the renal pedicle. After fixation, precipitation of Prussian blue, and dissection of proximal tubules, the radioactivity was determined in the tubular segment proximal to the blue-stained precipitate of unlabeled ferrocya-
nide. SNGFR could then be calculated by dividing this amount by the product of plasma concentration and time (10 s). The approach of Coelho et al. (16) was more direct: [14C]ferrocyanide was given intravenously as a constant infusion for 12 s. During the same period, arterial blood was drawn at a constant rate by a syringe pump up to the moment of clamping of the renal pedicle, directly providing the desired time-integrated arterial plasma concentration. (Under the name “artificial reference sample,” this procedure was later widely used for estimating blood flow by microspheres). An alleged advantage compared with the method of de Rouffignac was that distal tubular segments could be used to correct for extratubular activity.

Thorough methodological tests were made both at Saclay and in New York, in essence confirming, but also extending, Hanssen’s original observations (4, 5, 17, 42, 47).

During the following 15 years, the original Hanssen technique or its modifications were adopted by 17 groups in 9 countries, resulting in approximately 50 articles published before 1985. The publications of Hanssen himself have been quoted in nearly 180 papers.

The distribution of GFR has been mapped out in several mammalian species: rat, psammomys, rabbit, dog, and sheep (For reference, see Refs. 1, 4, 7, 14, 46, 47). In immature animals, the superficial-to-juxtamedullary SNGFR ratio, S/JM, is low, usually in the 0.2–0.5 range, and increases to 0.6–0.9 during maturation, reflecting continued growth of superficial glomeruli (1, 3, 20). In Brattleboro diabetes insipidus rats, the SNGFR in deep cortex is lower than in normal animals but may be restored to normal level by vasopressin treatment from week 2 (53).

As summarized in an excellent review by Dantzler (18), single-nephron filtration rates have also been mapped out in fish and birds, i.e., in vertebrates with much greater zonal variation of glomerular size and filtration rate than that in mammals. Later, the Hanssen method was also used in dogfish (small shark) by Brown and Green (12). The main experimental focus has been on glomerular intermittency vs. regulation of SNGFR induced by salt or water loads, as well as the response to vasotocin and adrenaline.

Most studies on the effect of various experimental interventions in mammals have been made in rats, and the general impression is that of proportional or close to proportional alteration in deep and superficial filtration rates. Thus unaltered S/JM has been reported at varying renal arterial pressure (9, 35), renoprival hypotrophy (14), water deprivation (36), and during hemorrhagic hypotension in anesthetized (13) and unanesthetized rats (15). A relative reduction in deep SNGFR was observed after release of chronic, but not of acute, ureteral obstruction (56). Somewhat unexpectedly, Pitressin (Parke-Davis) was found to increase SNGFR in juxtamedullary nephrons in hereditary diabetes insipidus rats (19). Many studies were made to test the hypothesis that the natriuresis induced by acute or chronic extracellular volume expansion might result from a relative increase of filtration in superficial glomeruli (24). In reviewing 14 different studies, Lameire et al. (41) found that about half of the studies were confirmative, whereas the other half showed unaltered S/JM ratios. The discrepancy has been attributed to the use of rats of different age groups or strains, or to the use of different modifications of the Hanssen method (3, 16, 46). At any rate, some inherent limitations of the method should not be overlooked. Whatever modification is used, one obtains only one single clearance period of 10- to 15-s duration, which necessarily gives a large scatter of absolute SNGFR. Moreover, the dissection of 5–20 proximal tubules from each zone is not only time-consuming but may give different nephron populations in different hands. Thus in the inner cortex it is difficult to distinguish true juxtamedullary glomeruli from those of the cortical type. As a rule, ratios between deep and superficial glomeruli will be more reliable than absolute values. Nevertheless, the statistics in most studies suggest that a change in the S/JM ratio has to exceed at least 10% to become detectable.

The use of the Hanssen method ended in the 1980s, possibly because at that time the most interesting problems had been addressed, but more likely this occurred because topics other than nephron heterogeneity had caught the interest of renal physiologists.

HANSSEN-INSPIRED METHODS NOT BASED ON FERROCYANIDE

Two more recent methods were clearly inspired by Hanssen’s technique. The first, developed by Ulfendahl’s group in Uppsala, was even referred to as a “modified Hanssen technique” (21, 49). They measured the uptake of 51Cr-EDTA in tissue samples from outer, middle, and inner cortex 10 s after intravenous bolus injection. An arterial reference sample was used for subtracting tracer content in renal interstitium and plasma and for calculating zonal GFR per gram tissue. Average SNGFR may then be obtained by counting the number of glomeruli in each tissue sample, providing information on many more glomeruli than is practically obtainable by dissection of single nephrons.

Another method developed in our group in Bergen also measures GFR per gram tissue but is based on a different principle, namely, collecting the filtration tracer, radiolabeled aprotinin (Ap), in the tubular cells rather than in the tubular lumen (50, 51). Ap, with a molecular weight of 6,500, is filtered without any steric restriction, is completely endocytosed by the proximal tubular cells close to the filtering glomerulus, and remains there for 15–20 min before appreciable amounts of breakdown products begin to be lost to peritubular plasma. By successive bolus injections of 125I-Ap and 131I-Ap, it is therefore possible to obtain two clearance periods of several minutes duration. Thus local GFR may be estimated in a control period and then, in the same tissue samples, after some acute experimental intervention such as pressure change, drug infusion, etc.
Interesting results have been obtained with both of these methods, but we must still admit that most of what we know about the distribution of glomerular filtration has been obtained by use of the ferrocyanide technique developed by Hanssen 40 years ago. His method is a truly impressive example of cross-fertilization between pathological anatomy and experimental physiology.

For personal information, I am greatly indebted to Odd Hanssen’s widow, Ingebjorg Hanssen, and her two daughters and to Hanssen’s closest friend and colleague, Professor L. Asmund Frølich, who also contributed valuable comments about this manuscript. Christian de Rouffignac and Andrew Baines are acknowledged for helping to disclose the relationship of extraluminal tubular deposition of ferrocyanide to peritubular perfusion rate in cortical and medullary nephron segments of the rat kidney. *Circ Res* 29: 21–28, 1971.


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