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Contents

Studies on the Pathogenesis and Morphogenesis of Glomerulonephrosis

Application of a Newly Developed Morphometric Method*

J. TORHORST**

With 20 Figures

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** Dedicated in gratitude to Professor H. U. ZOLLINGER, M.D. on his 60th birthday.

1. Introduction: Definition and Aetiology of Glomerulonephrosis

Glomerulonephrosis is defined as a primary non-inflammatory change of the glomerulus referable to a derangement of permeability (dysoria) (ZOLLINGER, 1966 Lit.). It may be differentiated from the various forms of glomerulonephritis by the absence of marked proliferation of local cells. Nor is an increased number of neutrophils, typical of acute diffuse glomerulonephritis, found at any stage of glomerulonephrosis. Another important distinction is that, in contrast to glomerulonephritis, no immune complexes are demonstrable at the peripheral basement membrane in either a linear or granular form. In the various forms of glomerulonephrosis however, immunoglobulins and complement have been found in the mesangium. The part played by these deposits is still obscure. Glomerulonephrosis is characterized

Table 1. Etiology of Glomerulonephrosis[a]

Clinical diseases	Experimental models
General infectious diseases	Extrarenal foci of inflammation
Amyloidosis	Amyloidosis
Diabetes mellitus	Diabetes mellitus
Gout	
Myxedema	
Liver diseases	Liver damage
Hemolysis	
Myolysis	
Malformation of the heart with cyanosis	
Hypertension	Hypertension
Ischemia	Ischemia
Renal vein thrombosis	Renal vein thrombosis
X-irradiation	X-irradiation
Burns	
Mercury-poisoning	
Tumors	
	Aminonucleoside nephrosis
	Antigen-antibody-complexes
	Protein load
	Synthetic polysaccharids load
	Uran-nitrat-poisoning

[a] For Literature review see: STRAUSS and WELT (1971); MOSTOFI and SMITH (1966); ZOLLINGER (1966); DALTON and HAGUENAU (1967); BECKER (1968); HAMBURGER and coworkers (1968); ROUILLER and MULLER (1969); BARIETY and coworkers (1970).

by a morphological change involving an increase in the intercellular substance in the mesangium and a mild proliferation of local cells. The first of these is due to the deposition of material which either originates from the capillary blood or is synthesized by the glomerular cells. Plasma components, alien substances or even antigen-antibody complexes may reach the mesangium via the blood stream. Aetiologically the morphological pattern of glomerulonephrosis can be produced by a multiplicity of clinical syndromes and experimental models (Table 1).

The aim of the present study is to characterize the morphology of glomerulonephrosis with reference to examples from clinical and experimental pathology. At the same time an attempt is made to draw the line separating it from glomerulonephritis with the aid of morphological criteria. To this end a *morphometric method* has been developed for the evaluation of kidney tissue. This method can be applied both to whole kidneys and also, with certain qualifications, to renal biopsies.

2. Development of a Morphometric Method for the Stereological Evalution of Renal Glomeruli in the Light Microscope*

2.1. Prerequisites for the Application of Morphometric Methods for the Evaluation of Renal Glomeruli

Conclusions about three-dimensional conditions can be based on morphometric studies only if the structures are largely homogeneous in their distribution. This homogeneity cannot be automatically assumed in the study of glomeruli because of the anatomical features of kidney tissue.

The renal cortex—the only zone in which glomeruli are found—is not a homogeneous band. On the contrary it is interrupted by aglomerular medullary rays. These rays are broader in the juxtamedullary than in the subcapsular zone of the cortex. The tissue inhomogeneity thus arising is compensated for by determining the proportion constituted by the medullary rays and applying correction factors derived therefrom (cf. Sec. 3.4.2).

A certain degree of inhomogeneity is also due to the fact that a narrow subcapsular zone (cortex corticis) contains fewer glomeruli than the rest of the renal cortex. The glomeruli are disposed round interlobar arteries, thus forming discrete areas of blood supply in the cortex, and between these areas there are additional zones containing few glomeruli. These inhomogeneities are possible sources of error and they must be rectified by choosing a measuring field of sufficient size and a large number of fields.

* I should like to express my thanks to Prof. H. P. Rohr, M. D. for his introduction into the problems of morphometry and for his encouragement and support.

The differences between the subcapsular and juxtamedullary cortical zone noted by various authors (FETTERMAN *et al.*, 1965; ZOLNAI and PALKOVITS, 1965; ELIAS and HENNIG, 1967; PARKER, 1967) are another anatomical cause of inhomogeneity. Like these authors we have found no essential differences between these two zones in subjects over 30 years of age (cf. Sec. 3.5.2).

In determining intraglomerular parameters account must also be taken of various factors capable of falsifying the results of measurement. This holds good in particular of Bowman's capsule. Alterations in this part of the glomerulus may, for example, be responsible for a change in the nuclear count merely being simulated.

If these conditions are observed, it is possible to make a morphometric study of the renal glomeruli, for the requisite homogeneity will have been ensured. Investigations by SØRENSEN (1972) show that the number of glomerular nuclei and the relative mesangial volume have a distribution which is very close to normal. This again is a statistical condition that must be fulfilled before sterological studies can be made of biological material.

2.2. Preparation of Material

Kidneys and kidney sections are selected on the principle that focal lesions such as are to be found in virtually every kidney (small vascular or pyelonephritic scars, cysts, etc.) are not taken into consideration. Since most of the material studied is obtained at autopsy particular attention must be paid to autolytic changes. The time elapsing between death and autopsy should not exceed 20 hours. The nuclei of the tubular epithelium must be preserved. Subjects with the diseases listed in Table 2 are precluded as a source of kidneys for the control group. In comparative studies of different forms of glomerulonephrosis, groups should be formed—as far as casuistic study allows—in which the glomerulonephrosis is referable to a single factor.

Table 2. Diagnoses and morphological findings which were excluded in control kidneys

Macroscopy	Histology	Clinical and anatomo-pathological diagnoses
Scars	Scars	Diabetes mellitus
Stenosing arterio-sclerosis	Arteriosclerosis	Amyloidosis
	Arteriolosclerosis	Cirrhosis of the liver
Pyelonephritis	Pyelonephritis	Tumors
Hydronephrosis		General infectious disease
	Glomerulonephritis	Icterus
	Amyloidosis	Hypertension
	Advanced autolysis	Rheumatic diseases
	Artifacts of sectionning	Gout
		X-irradiation
		Nephropathy of pregnancy
		Oxalosis
		Chronic treatment with corti-costeroids.

Table 3. Change of kidney volume (cm^3) after fixation in 4% formaldehyde

Kidney	Fresh tissue		Fixation 24 hours		Fixation 5–15 days	
	whole kidney	kidney cortex	whole kidney	kidney cortex	whole kidney	kidney cortex
1	105	2.0	110	2.4	110	2.2
2	130	2.0	140	2.4	140	2.3
3	135	2.25	140	2.3	140	2.4
4	140	1.70	145	2.0	145	1.9
5	210	2.50	210	2.7	210	2.7
6	100	1.00	110	1.3	105	1.3
7	115	2.00	125	2.2	125	2.3
8	140	1.90	150	2.0	145	2.0
9	70	2.00	80	2.2	80	2.1
10	70	1.30	75	1.3	70	1.4
Mean	121.5	1.87	128.5	2.08	117.0	2.06
Standard-deviation	40.6	0.44	38.8	0.46	26.8	0.44

The kidneys are sectioned longitudinally and fixed in 1000 ml 4% formalin for 24–48 hours. Sometimes the material is kept in formalin for longer periods. Since the tissue volume may vary with the different fixation times, studies have been made of the change of kidney volume as a function of the fixation time.

Table 3 shows that after 24 hours the volume of the whole kidney has increased by 5–6% of its fresh volume. If fixation is continued longer, there is a negligible degree of shrinkage. Calculations of the amount of swelling occurring in cortical tissue yield an increase in volume of 10–11%, which is independent of the fixation time.

Dehydration in alcohols of increasing strength results in tissue shrinkage which amounted in the 8 excised specimens of kidney studied to 8% of the volume after formalin fixation for 9 months (decrease from 1.537 ml to 1.418 ml). Thus dehydration restores the tissue roughly to its fresh volume. Investigations by BAHR *et al.* (1957) show that kidney tissue sustains no further substantial change of volume by subsequent embedment in methyl methacrylate. It may therefore be assumed that the volumetric characteristics of the renal tissue studied are not substantially changed by processing. On the other hand these authors state that the tissue shrinks on paraffin embedment by 20–30% of the value obtained after dehydration. Hence the results obtained from material processed in this manner are comparable with ours only to a limited extent (IIDAKA *et al.*, 1968; SØRENSEN, 1972).

After formalin fixation the longitudinally sectioned halves of kidney are each cut into 16 slices 3–5 mm thick (Fig. 1). Ten of these 32 slices are chosen at random, no two adjacent pieces being used. Care is also taken in making the selection that there are no scars in the cortical specimens and that the capsule and boundary between medulla and cortex are clearly demarcated.

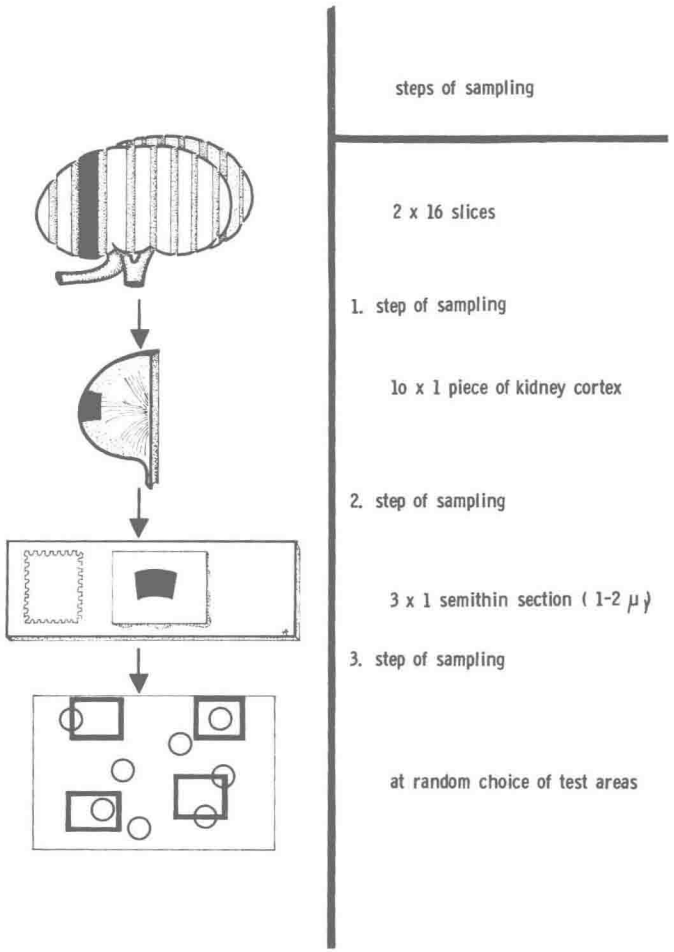

Fig. 1. Stages in sampling for the morphometric evaluation of renal glomeruli

Of these 10 pieces of cortex any 5 are chosen at random and embedded in methyl methacrylate. Embedment must be performed in such a way that the capsule and the medullo-cortical boundary are clearly discernible. Sections 1–2 μ thick are then made from all 5 blocks. The thickness of the section is checked from time to time in the microscope by focusing the two surface planes. The three most suitable blocks are used for morphometric evaluation. A particular watch is kept for sectioning artefacts and cicatrization. Two different stains must be used for counting procedures: methenamine silver staining is suitable for evaluating most of the parameters. The mesangium in particular can be clearly visualized with its aid (Fig. 2).

Only the nuclei cannot be evaluated. For this purpose chromotrope aniline blue staining is used since a particularly good nuclear contrast can be obtained with it even in thin sections (Fig. 2). WEHNER's team (1968a, b) performed

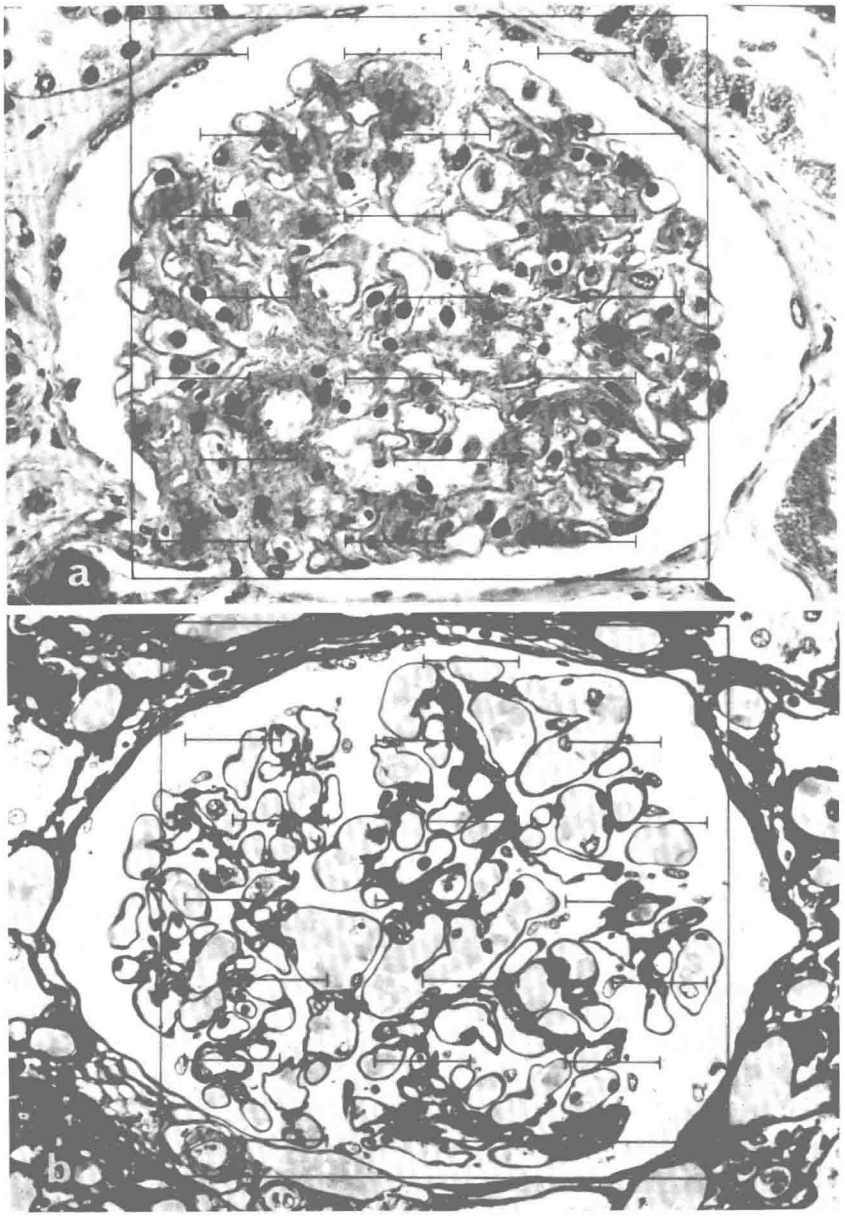

Fig. 2a and b. Multipurpose test screen according to WEIBEL. a Chromotrope aniline blue staining to visualize nuclei. b Methenamine silver staining to visualize the mesangium and basement membrane. ×460

the nuclear count on sections stained with methenamine silver and thus obtained substantially smaller numbers than all other teams studying the kidney by morphometric or histometric methods.

2.3. Morphometric Evaluation Technique for Obtaining the Primary Counts

Precisely defined random sampling is essential for selecting the test field in the histological section in morphometric studies. An important point in this connection is that once the field distance has been chosen it should be consistently maintained. The sampling stage microscope of WILD, Instruments Inc., Heerbrugg, Switzerland, was used for this purpose. Constant field displacement on this microscope is ensured by an electronic control. The sections are examined at four different magnifications ($\times 45$, $\times 150$, $\times 750$, $\times 1500$). The measuring field is shifted about and the cortex scanned uniformly from the capsule to the medullo-cortical boundary (Fig. 3). The field distance is altered at the various magnifications so that always roughly the same area of cortex is evaluated.

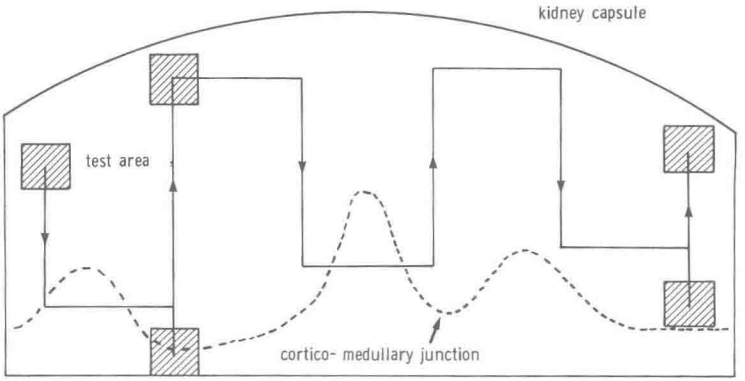

Fig. 3. Choice of test field in the renal cortex for morphometric analysis

The test field consists of the multipurpose test screen (Fig. 2) which was developed by WEIBEL (1963) and enables 3 different basic values to be determined. 1. number of compartments transsected (glomeruli, nuclei), 2. hits scored by test points on specific compartments (e.g. mesangium, capillaries, Bowman's capsule), 3. intersections of test lines with certain surfaces (glomeruli, nuclei, capillaries). Primary and secondary parameters are derived from these primary counts (Table 4) with the aid of correction factors and these parameters allow estimates to be made of particle numbers, volumes and surfaces in three dimensions (cf. Sec. 2.4.1). In our studies a total of 16 primary parameters has been determined on each section. From these, 9 secondary parameters are derived. The symbols for the various parameters are summarized in Table 5.

For the reasons adduced in the foregoing the size of the random sample necessary for correcting the anatomic causes of inhomogeneity must be determined for the individual parameters. The number of samplings within the individual sections is increased until the standard error is about 10%

Table 4. Synopsis of magnifications, test screens, colorations and of primary measurements

Magnification	45 ×	150 ×	750 ×	750 ×	1 500 ×
Number of points per multipurpose test screen	42	42	42	42	100
Coloration of the section	*Meth*-S[a]	*Meth*-S[a]	*Meth*-S[a]	*CAB*[b]	*CAB*[b]
Parameters for point counting[c]	$P_{P_{ex3}}$	P_{PG_a} P_{PG_b} $P_{P_{ex10}}$	$P_{P_{mes}}$ $P_{P_{kap}}$ $P_{P_{bow}}$ $P_{PG_{mes}}$	$P_{PG(NANG)}$	P_{PNG} $P_{PG(PPNG)}$
Parameters for intersection-point counting[c]		I_{LG}			
Parameters for particle counting[c]	N_{F3} N_{AG}	N_{F10}		N_{ANG}	

[a] Methenamin-silver stain.
[b] Chromotrop-anilin-blue stain.
[c] Meaning of symbols see table 5.

(WEIBEL, 1963). Table 6 contains details of the sample size within a section ascertained in this manner.

Comparison of the 3 sections selected at random from a kidney gives an indication of the homogeneity of the changes within the kidney. It has been found in this connection that the deviation lies within ± 10 to 20% of the mean value even for pathologically altered kidneys, particularly for the diagnostically important intraglomerular parameters.

About 380 measuring fields have been evaluated per kidney. For this a practised investigator needs about 5 hours. The present morphometric analysis is based on 39 kidneys obtained at autopsy, 2 operation specimens and 5 open kidney biopsies. In addition 27 rat kidneys have been morphometrically evaluated.

2.4. Determination of Sterological Parameters

2.4.1. Formulae for Calculating Primary and Secondary Parameters

Volumetric Densities

The volumetric density of a tissue compartment (V_{V_i}) is determined by the Equ. (1)

$$V_{V_i} = \frac{P_i}{P_T}, \tag{1}$$

where

P_i Number of points per tissue compartment,
P_T Total number of points evaluated.

Table 5a–c. Meaning of symbols

Table 5a. Primary counting

N_{F3}	Test fields at 45-fold magnification
$P_{P_{ex3}}$	Hits on kidney medulla at 45-fold magnification
N_{AG}	Glomerular sections per test area
N_{F10}	Test fields at 150-fold magnification
P_{PG}	Hits on glomerular sections
I_{LG}	Intersections points of test lines with Bowman's capsule
$P_{P_{ex10}}$	Hits on kidney medulla at 150-fold magnification
$P_{PG(mes)}$	Hits on glomerular sections in determination of $P_{P_{mes}}$, $P_{P_{kap}}$, and $P_{P_{bow}}$
$P_{P_{mes}}$	Hits on mesangial area
$P_{P_{kap}}$	Hits on glomerular capillary area
$P_{P_{bow}}$	Hits on Bowman's space area
$P_{PG(NANG)}$	Hits on glomerular sections in determination of N_{ANG}
N_{ANG}	Nuclear sections per test area
$P_{PG(PPNG)}$	Hits on glomerular sections in determination of P_{PNG}
P_{PNG}	Hits on nuclear sections

Table 5b. Primary parameters

$N_{AG_{eff}}$	Glomerular sections per test area of kidney cortex
N_{VG}	Numerical density of glomeruli (per mm³ of kidney cortex)
V_{VG}	Volume density of glomeruli in kidney cortex (per cent of unit volume)
S_{VG}	Surface density of glomeruli in kidney cortex (cm² per mm³ of kidney cortex)
$N_{ANG_{eff}}$	Nuclear sections per test area of glomerulus
N_{VNG}	Numerical density of nuclei (per mm³ of glomerulus)
V_{VNG}	Volume density of nuclei in glomerulus (per cent of unit volume)
$V_{V_{mes}}$	Volume density of mesangium in glomerulus
$V_{V_{kap}}$	Volume density of capillaries in glomerulus
$V_{V_{bow}}$	Volume density of Bowman's space in glomerulus

Table 5c. Secondary parameters

$\frac{VVG}{SVG} \times 6 = d$	= mean glomerular diameter [mm]
$\sqrt[3]{\frac{VVG \times 6}{\pi \times NVG}} = d$	= mean glomerular diameter [mm]
$\frac{\pi}{6} \times d^3$	= mean glomerular volume [mm³]
$\frac{VVG}{NVG}$	= mean glomerular volume [mm³]
$\frac{VVNG}{NVNG}$	= mean nuclear volume [mm³]
$\frac{VVG}{NVG} \times NVNG$	= number of nuclei per single glomerulus

Table 6. Size of sample for determination of different parameters

Parameter[a]	Size of sample per section	Standard error in % of the mean value
N_{VG}	8 test fields	8
V_{VG}, S_{VG}	3000 hits on kidney tissue	9
$V_{V_{mes}}, V_{V_{kap}}, V_{V_{bow}}$	1000 hits on glomerular sections	8
N_{VNG}	300 hits on glomerular sections	7
V_{VNG}	1000 hits on glomerular sections	10

[a] Meaning of symbols see table 5b.

To determine the volumetric density of the glomeruli in the cortex (V_{VG})* or the volumetric density of the glomerular nuclei within the glomerulus (V_{VNG}) we employ the general formula (1) in the form of the following equations:

$$V_{VG} = \frac{P_{PG}}{N_{F10} \times 42 - P_{Pex\,10}} \tag{1a}$$

and

$$VVNG = \frac{P_{PNG}}{P_{PG\,(PPNG)}} \times H_1. \tag{1b}$$

The denominator of Eq. (1a) is composed of the total number of evaluated points ($N_{F10} \times 42$) minus the points falling on medullary areas (P_{Pex10}).

The constant H_1 in Eq. (1b) is the correction factor for the error due to the section thickness (HOLMES effect). For more precise details of the correction factors cf. Sec. 2.4.2. In determining V_{VG} the HOLMES effect can be neglected, for with a ratio of approx. 1:100 between section thickness and glomerular diameter it is not significant.

Numerical Densities

The numerical density of a structure (N_{V_i}) is determined by the Equ. (2) (WEIBEL *et al.*, 1969):

$$N_{V_i} = \frac{1}{\beta} \times \sqrt{\frac{N^3_{A_i}}{V_{V_i}}} \times K \tag{2}$$

when

N_{A_i} Number of structure profiles within the unit area,
V_{V_i} Volumetric density of the structure,
β Shape factor,
K Correction factor for size distribution of the structures.

In determining the numerical density of the glomeruli in the renal cortex (N_{VG}) or the numerical density of the glomerular nuclei within the glomerulus

* For sterological symbols cf. Table 5.

(N_{VNG}) we use the general formula (2) in the form of Eqs. (2a) and (2b).

$$N_{VG} = m_1 \times \sqrt{\frac{N^3_{AG_{\text{eff}}}}{V_{VG}}}, \tag{2a}$$

number of glomeruli per cu. mm. of cortex

$$N_{VNG} = m_2 \times \sqrt{\frac{N^3_{ANG_{\text{eff}}}}{V_{VNG}}}. \tag{2b}$$

number of glomerular nuclei per cu. mm. of glomerulus

The parameters $N_{AG_{eff}}$ and $N_{ANG_{eff}}$ relate to a unit area of cortex or glomerulus respectively corresponding to the area of a multipurpose test screen with 42 points at a magnification of $\times 45$ (N_{AG}) or $\times 750$ (N_{ANG}).

$$N_{AG_{\text{eff}}} = \frac{N_{AG}}{N_{F3} \times 42 - P_{P_{\text{ex 3}}}} \times 42 \tag{2a$_1$}$$

$$= N_{AG} \times \frac{1}{N_{F3} - 1/42 \times P_{P_{\text{ex 3}}}},$$

$$N_{ANG_{\text{eff}}} = \frac{N_{ANG}}{P_{PG\ (NANG)}} \times 42. \tag{2b$_1$}$$

The denominator of Equ. (2a$_1$) is composed of the total number of points evaluated ($N_{F3} \times 42$) minus points which fall on medullary areas ($P_{P_{ex3}}$). In this way we obtain the evaluated cortical area when counting the number of glomeruli.

The constants m_1 and m_2 contain various factors:

$$m_1 = K_1 \times 1/\beta_1 \times 1/(EV)_3 \times \sqrt{\not{p}_1^3}, \tag{2a$_2$}$$

$$m_2 = K_2 \times 1/\beta_2 \times 1/(EV)_{50} \times \sqrt{H_2^3} \times \sqrt{\not{p}_2^3}, \tag{2b$_2$}$$

where

K_1 and K_2	Factors for size distribution of the glomeruli and glomerular nuclei respectively,
β_1 and β_2	Shape factors for the glomeruli and glomerular nuclei respectively,
$\not{p}_1$ and $\not{p}_2$	Correction factors for small profiles not taken into consideration for technical reasons,
H_2	Correction factor for the section thickness (HOLMES effect),
$(EV)_3$	Unit volume at $\times 45$ magnification,
$(EV)_{50}$	Unit volume at $\times 750$ magnification.

Surface Density

To determine the surface density of a particle contour (S_Vi) we apply Equ. (3) (WEIBEL *et al.*, 1969):

$$S_{V_i} = \frac{2\,I_i}{L_T}, \tag{3}$$