Virchows Arch [Pathol Anat] (1984) 403:117–125

Virchows Archiv A

Karyometric investigations on urinary bladder carcinoma, correlated to histopathological grading*

Kerstin Helander, Per-Åke Hofer, and Göran Holmberg
Departments of Pathology and Urology, University of Umeå, S-90187 Umeå, Sweden

Summary. The aim of this study is to provide karyometric data which may be of value in the grading of urinary bladder tumours. For this purpose 27 biopsies were studied: four from normal bladder mucosae, eleven from grade I tumours, six from grade II tumours and another six from grade III tumours, according to a I-IV scale. After standardized fixation and plastic embedding, semithin sections were used for light microscopic stereology.

Mean profile areas and mean volume densities of the nuclei tended to be higher in the more malignant cases. The nuclear volume densities were significantly higher in grade II than in grade I. The most important finding relates to the large nuclear profiles (>90 μm^2), which were found almost exclusively in grade III tumours. Simple measurements of nuclear size can thus provide objective data to aid in the diagnostic procedure.

Key words: Urinary bladder carcinoma – Stereology – Karyometry – Morphometry – Light microscopy

Introduction

The incidence of urinary bladder carcinoma in humans is increasing (National Board of Health and Welfare 1979). It is currently responsible for 5% of the neoplasms of the male and 2% of the female population. Successful treatment depends on an early diagnosis with correct staging and grading of the tumour, but experience has demonstrated considerable difficulties in grading this type of malignancy. After re-examining material from

* Supported by grants from the Medical Faculty, Lions Research Foundation at the Department of Oncology and Nephrology Research Foundation, Umeå University, Umeå, Sweden
Valuable advice was given by the teachers at the Sixth Course in Morphometry and Stereology at Saint-Aubin, France 1982

Offprint requests to: K. Helander at the above address

107 biopsies Busch et al. (1977) arrived at a different grading at their second examination of the microscopic slides in about 20% of the cases.

Morphometric studies have previously been carried out on urinary bladder carcinoma with the aim of obtaining an objective and reliable grading (De Sanctis et al. 1982; Ooms et al. 1983). However, these studies have been hampered by the lack of standardized histotechnological methods and by the use of relatively thick paraffin sections, which may produce errors in the morphometric results. The advent of new techniques for light microscopy with embedding in plastic, and thin sectioning (0.5–3 μm) have greatly improved the accuracy in morphometry (cf. Helander 1983). Moreover, the comprehensive methods for the treatment of various stereological problems, which have been compiled in recent years (cf Weibel 1979), have facilitated quantitative morphological studies.

The aim of the present study is to provide detailed karyometric data on urinary bladder carcinoma. In particular we wanted to investigate whether the different grades of carcinoma could be distinguished on the basis of results of karyometry.

Material and methods

The material consisted of 30 biopsies from patients (20% women) with suspected or verified carcinoma of the urinary bladder. The mean age of the women was 65 (range 53–79) and of the men also 65 (range 45–82). None of the patients had been treated with radiotherapy or cytostatic drugs.

The biopsies were taken during cystoscopy with isotonic glycine as the irrigating medium. The tissue specimens were fixed without delay at room temperature in a solution consisting of 2% formaldehyde and 3% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.4, modified from Karnovsky (1965). After a short rinse in the buffer the biopsies were dehydrated in rising concentrations of ethanol, infiltrated and finally embedded in glycol methacrylate.

The blocks were sectioned in a JB4 microtome with the thickness feed set at 1.5 μm. The sections were floated briefly on water, picked up on glass slides and stained in haematoxylin-eosin.

Grading of tumours

The tumours were graded according to the system described by Bergkvist et al. (1965). Thus, each specimen was classified within one of the four groups, according to the severity of the deviation of the cellular pattern from that of normal bladder mucosa. Three of the biopsies were excluded: in one case the biopsy contained very few urothelial cells; in another case the mucosa was mechanically damaged, and in the third case the diagnosis was in doubt. The grading of the biopsies and the morphometric investigations were carried out independently.

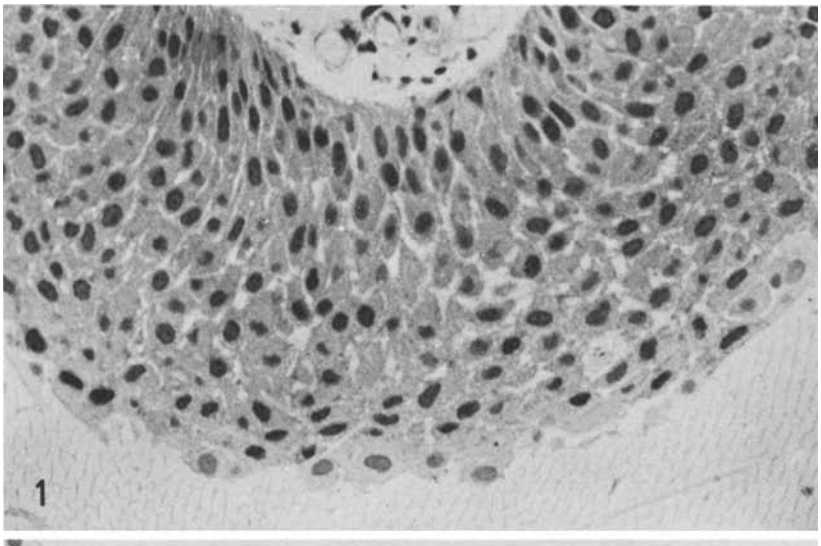
Morphometric methods

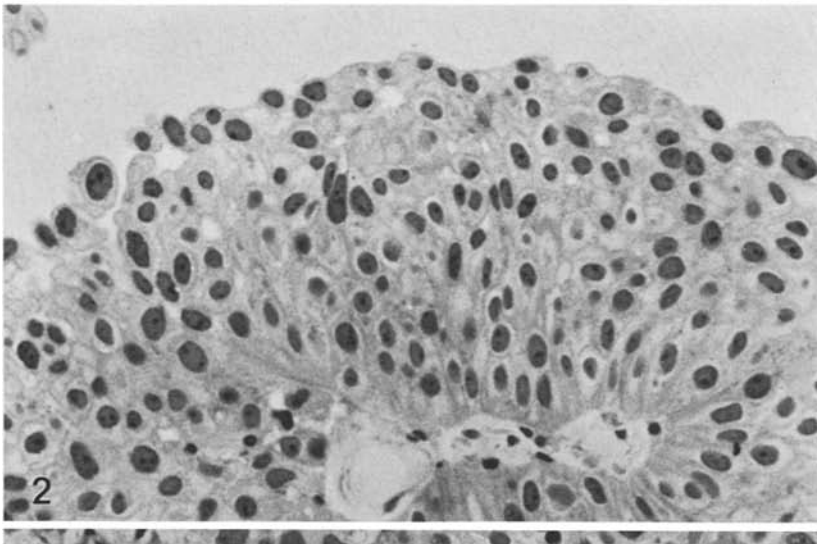
Nuclear profile areas were measured on a Hipad digitizing tablet (Bausch & Lomb) connected to a Luxor ABC80 microcomputer. The microscope was equipped with a ×100 oil immersion

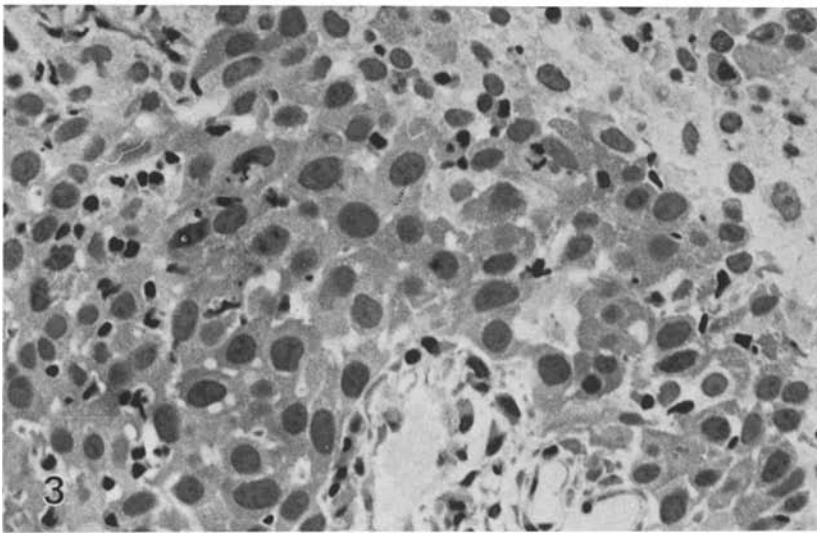
Fig. 1. Micrograph of urinary bladder carcinoma, grade I (×300)

Fig. 2. Micrograph of urinary bladder carcinoma, grade II (×300)

Fig. 3. Micrograph of urinary bladder carcinoma, grade III (×300)


1


2


3

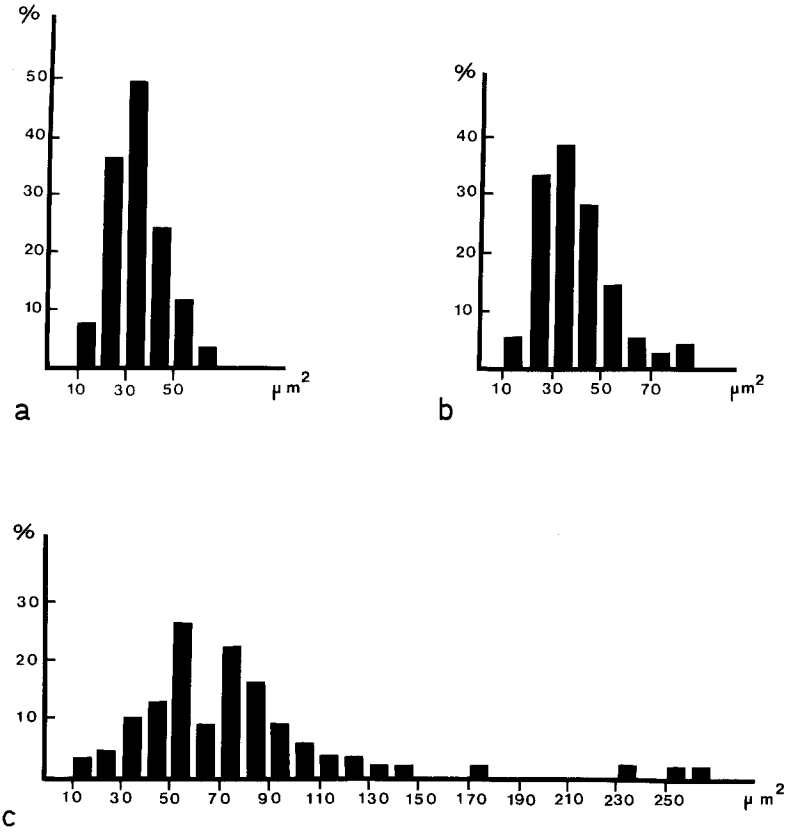


Fig. 4a–c. Histogram showing the size distribution of the nuclear profile areas in three cases of urinary bladder carcinoma. The same cases are illustrated in Figs. 1–3. **a** grade I; **b** grade II; **c** grade III

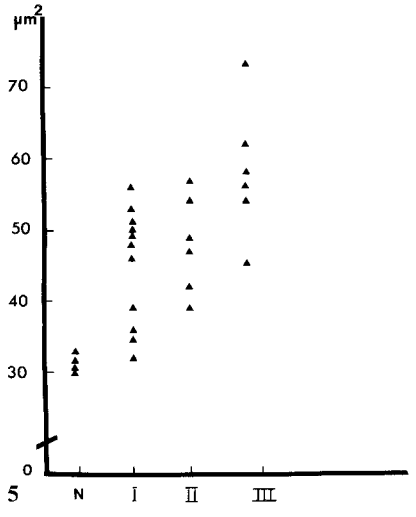
lens and a tracing device. In each biopsy 150 nuclear profiles were measured. In those cases where the basement membrane was retained, measurements were performed systematically in all levels of the mucosa except for the most superficial layer. When no basement layer was visible, the measurements were carried out in randomly chosen areas.

Nuclear volume densities (i.e. the proportion of tissue volume – except for vessels – occupied by nuclei) were estimated in the light microscope using a ×40 objective lens and a square grid inserted into the eyepiece. In each tumour about 600 test points were counted; this corresponded to an area of 0.08 mm^2.

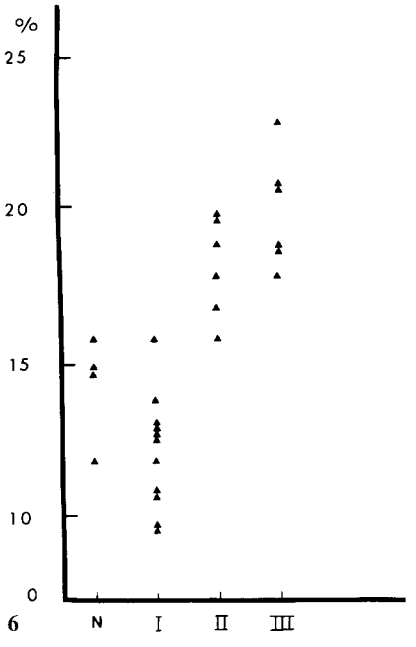
The numerical density of the nuclei (i.e. the number of urothelial nuclei per mm^3 of tissue) was calculated according to the method of Weibel and Gomez (Weibel 1979):

$$N_V = \frac{K}{\beta} \sqrt{\frac{N_{An}^3}{V_{Vn}}}$$

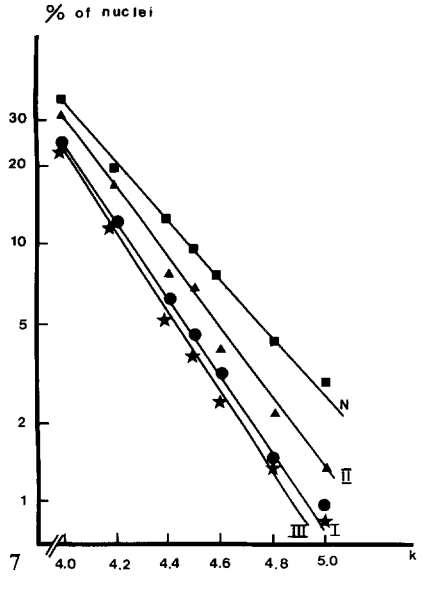
where N_{An} is the number of nuclear profiles per mm^2, and V_{Vn} is the volume density of the nuclei. The size distribution factor K was calculated from the S.D. and mean values of the nuclear profile areas. The form factor β was obtained from the measurements of contour indices. No correction factor for section thickness was included.



5



6



7

Fig. 5. Histogram showing the mean values of the nuclear profiles areas in each of the 27 biopsies. *N*, normal epithelium; I, II, III, malignancy grading

Fig. 6. Histogram showing the mean values of the nuclear volume densities in each of the 27 biopsies. *N*, normal epithelium; I, II, III, malignancy grading

Fig. 7. Semilogarithmic diagram showing the cumulative frequency distributions of contour indices, k, for normal (*N*) and cancerous epithelium (grades I–III). In general, nuclei from normal tissue display higher k values, indicating more elliptical nuclei, than in the cancerous epithelium

The contour index k is expressed by:

$$k = \frac{\text{Perimeter}}{\sqrt{\text{Area}}}$$

The value of k is 3.54 for a circle; higher values are obtained for elliptical profiles and for profiles with irregular perimeters (Meijer et al. 1980). For further discussion of these methods see Weibel (1979).

Table 1. Nuclear profiles exceeding 60 μm², 70 μm², 80 μm² and 90 μm² in normal and cancerous urinary bladder epithelium. Mean values given in per cent of the total number of nuclei (range in parenthesis)

	>60 μm²	>70 μm²	>80 μm²	>90 μm²	No of biopsies
N	0	0	0	0	4
I	15 (1–37)	5 (0–15)	1 (0–4)	1 (0–1)	11
II	21 (7–43)	9 (1–20)	4 (0–7)	2 (0–5)	6
III	39 (17–56)	25 (7–51)	15 (3–34)	9 (2–21)	6

Table 2. Numerical densities of nuclei in normal and cancerous urinary bladder epithelium (nuclei $\times 10^3$ per mm³)

	Mean	Range	No of biopsies
N	486	392–624	4
I	350	190–531	11
II	424	262–529	6
III	450	323–612	6

Table 3. Contour indices for nuclei in normal and cancerous urinary bladder epithelium

	Mean	SD	No of biopsies
N	4.00	0.12	4
I	3.94	0.12	11
II	3.97	0.05	6
III	3.90	0.05	6

Results

Grading of the biopsies

With regard to the epithelium four of the biopsies were graded as normal. The remaining biopsies were classified as urothelial cancers: eleven were of grade I, six of grade II and six of grade III. Micrographs of typical cases are seen in Figs. 1–3.

Morphometric findings

Nuclear profile area measurements demonstrated higher mean values in the more malignant cases, but the differences were not statistically significant (Figs. 4 and 5). However, further analyses of the data showed the absence of large nuclei from normal epithelium. The proportion of large nuclei increased with increasing grade of malignancy (Table 1). Thus, nuclear profiles >90 μm² were almost exclusively found in grade III tumours; these profiles were about 3 times larger than the mean nuclear profiles of normal biopsies.

Mean nuclear volume densitites were significantly higher in grade II than in grade I. There was considerable overlapping between grade I and normal epithelium, and also between grades II and III (Fig. 6).

Numerical densities of nuclei showed higher mean values for the higher malignancies (Table 2). However, somewhat surprisingly, the highest values were seen in normal epithelium. This is presumably related to the relatively small size of the normal nuclei in combination with a relatively high nuclear volume density.

Mean contour indices, k, showed no significant differences between the various grades (Tables 3). However, high k values (indicating relatively elliptical nuclei) were more frequent in the normal epithelium than in the tumours (Fig. 7).

Discussion

Preparative procedures such as fixation, dehydration, embedding and sectioning of biological tissues all result in morphological changes. The magnitude of the swelling, shrinkage or distortion of the tissues depends on the choice of methods, and for this reason it is important to use strictly standardized procedures. One consequence of this is that more attention should be paid to the relative changes of stereological data with increasing degrees of malignancy than to the absolute stereological values. These might vary considerably when other preparative methods are employed, which should be borne in mind when comparing our data with those of other investigators.

When using stereological methods some methodological errors must be considered. One such error relates to the thickness of the histological sections: a thick section will contain more nuclear profiles than a thin one. This problem has long been recognized (Holmes 1927), and various mathematical formulas have been constructed to correct for this error (cf. Weibel 1979). A related problem is that of overlapping nuclei, which has been mathematically analyzed by Würthner et al. (1972). In order to correct for these methodological errors it is necessary to know the section thickness; in paraffin sections which are usually about 5 μm thick this is not easily measured and, moreover, we have previously demonstrated variations in the thickness of the individual section of up to 40% (Helander 1983). In the present material of $1^1/_2$ μm thick glycol methacrylate sections the thickness variations were less than 0.3 μm, and, because of the thinness of the sections, there were no overlapping nuclei. Thus, this method minimizes the morphometric problems which are encountered with paraffin sections.

Over the years the histological grading of urothelial tumours has been the cause of some concern among pathologists. Broders' four grade system (1922) is still used by many pathologists, but slightly different systems have been proposed by Ash (1940), Mostofi (1960) and Bergkvist et al. (1965). A more recent grading system is the WHO classification (1973), which is basically similar to that of Bergkvist et al. and de Voogt et al. (1977). One

obvious reason for the interest in accurate grading of the tumours is the well-known correlation between prognosis and grading and between staging and grading (cf. Maltry 1971); the two latter will decide the choice of treatment. Thus, tumours of high grade – which usually are invasive – are treated with radiotherapy or radical surgery. The low grade tumours are generally not invasive, and here transurethral surgery is the primary choice of treatment.

Against this background we have tried to find additional –and more objective – criteria which could be used to aid the diagnosis.

The prognostic implications of our data cannot yet be evaluated. They will be dealt with when the long-term (5 and 10 year) results of the treatment have been assessed.

The mean *nuclear profile areas* cannot alone be used to distinguish between the various grades of malignancy. However, there was a clear tendency towards larger value in the more malignant grades. This is in agreement with recent findings in 12 paraffin embedded biopsies by Bjelkenkrantz et al. (1982), who reported slightly larger nuclear profiles than in the present study (the figures given in their table should be divided by four). This can be explained by the use of different histological techniques. Using Broders' classification de Sanctis et al. (1982), carried out similar measurements in 35 biopsies.

They reported increased mean nuclear profile areas in the more malignant cases, but their figures are about twice as high as ours; no explanation can be given for this discrepancy.

Further analyses of our histograms demonstrated that large nuclear profile areas (>60 μm^2) were absent from normal epithelia and scarce in grade I tumours. Nuclear profiles exceeding 90 μm^2 (i.e. about 3 times larger than the average for normal epithelium) were almost exclusively found in grade III tumors (Table 1); this feature could be of use in the diagnostic procedure.

Volume density measurements proved to be a valuable tool when trying to distinguish between grades I and II, but no significant difference was found between grades II and III or between normal epithelium and grade I. Boon et al. (1981) have described a similar difference between grades I and II in urothelial cells of voided urine.

The numerical density of nuclei is positively correlated to nuclear volume density and negatively correlated to the nuclear profile area. Considering the large range of sizes of nuclear profile areas it is not astonishing that there is only a slight relationship between numerical density of nuclei and tumor grade.

The measurement of the *contour indices* (k) is a prerequisite for the calculations of the numerical densities. The results indicate that the normal nuclei were more elliptical than the malignant ones.

It is concluded that in general the nuclei in our material were larger in the more malignant tumours, and that very large nuclei are indicative of high malignancy. Volume density measurements can distinguish between grades I and II.

Valuable diagnostic information is obtained by cytofluorometry and cy-

tophotometry, but these methods require expensive equipment and cannot be carried out in routine laboratories. In contrast, morphometry is readily accessible: an eye-piece micrometer and a simple calculator is the only extra equipment needed.

References

Ash JE (1940) Epithelial tumours of the bladder. J Urol 44:135–145

Bergkvist A, Ljungqvist A, Moberger G (1965) Classification of bladder tumours based on the cellular pattern. Acta Chir Scand 130:371–378

Bjelkenkrantz K, Herder A, Gröntoft O & Stål O (1982) Cytophotometric characterization of the WHO grades of transitional cell neoplasms. Pathol Res Practice 174:68–77

Boon ME, Kurver PHJ, Baak JPA & Ooms ECM (1981) Morphometric differences between urothelial cells in voided urine of patients with grade I and grade II bladder tumours. J Clin Pathol 34:612–615

Broders AC (1922). Epithelioma of the genito-urinary organs. Ann Surg 75:574–604

Busch C, Engberg A, Norlén BJ & Stenkvist B (1977) Malignancy grading of epithelial bladder tumours. Scan J Urol Nephrol 11:143–148

De Sanctis PN, Tannenbaum M, Tannenbaum S & Olsson C (1982) Morphologic quantitation of nuclear size in various grades of transitional cell carcinoma of urinary bladder. Urology 20:196–199

Helander K (1983) Thickness variations in individual paraffin and glycol methacrylate sections. J Microscopy 132:223–227

Holmes A (1927) Petrographic Methods and Calculations. Murby, London

Karnovsky MI (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27:137A–138

Maltry E (1971) Benign and Malignant Tumors of the Urinary Bladder. Medical Examination Publishing Company. New York

Meijer CJLM, van der Loo EM, van Vloten WA, van der Velde EA, Schefter E, Cornelisse CJ (1980) Early diagnosis of mycosis fungoides and Sezary's syndrome by morphometric analysis of lymphoid cells in the skin. Cancer 45:2864–2871

Mostofi FK, Sobin LH, Torloni H (1973) Histological typing of urinary bladder tumours. Internal Histological classification of tumours No 10, WHO, Geneva

National Board of Health and Welfare (1979) The cancer registry. Cancer Incidence in Sweden. Socialstyrelsen, Stockholm

Ooms ECM, Essed E, Kurver P, Veldhuizen RW, Alons CL, Boon ME (1983) Morphometric grading of bladder tumours in comparison with histologic grading by pathologists. Human Pathol 14:144–150

de Voogt HJ, Rathert P, Beyer-Boon ME (1977) Urinary Cytology Phase Contrast Microscopy and Analysis of Stained Smears. Springer Berlin Heidelberg New York

Weibel ER (1979) Stereological methods. Practical Methods for Biological Morphometry, vol 1. Academic Press. London

World Health Organization, Histological typing of urinary bladder tumours. International histological classification of tumours. Geneva, 1973

Würthner K, Sachs H, Bahnsen J (1972) Zum Problem der Kernschnitte bei der Zytophotometrie an histologischen Präparaten. Histochemie 32:261–270

Accepted January 20, 1984