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Preliminary investigations of a correlation between electron energy loss and morphometric analyses on ultrathin cryosections from normal and neoplastic gastric tissues*

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Summary. Electron energy loss spectroscopy (EELS) has been used to measure the ratios of C, N, O, P and Ca in ultrathin cryosections from normal and neoplastic gastric tissues. First results show a correlation between the EELS, and morphometric data in cells from these tissues. We have found that ultrathin, freeze-dried cryosections, with an average thickness of up to 75 nm, are stable enough for EELS-analysis in a 200 KV electron microscope with an adapted Gatan-EELS-Spectrometer.

Key words: EELS – Morphometric analysis – Cryo-sections – Gastric carcinoma

Electron energy loss spectroscopy (EELS) has become an invaluable technique for the rapid qualitative microanalysis of light elements (Krivanek et al. 1982; Bourdillon et al. 1983; Ottensmeyer 1984).

In order to analyse the components of biological material with the electron microscope, estimation of the C:N:O:P:Ca ratios is interesting for some applications. For such investigations, application of the EELS technique is advantageous for a rapid analysis of light elements ($n \leqq 30$), offering a very low detection limit (2×10^{-21} g) (Ottensmeyer 1984).

Variations in these relationships in several substructures within cells from normal and neoplastic tissues may give useful data in the future, concerning the malignant or benign behaviour of such tissues.

A first screening on ultrathin cryosections of different samples from normal and neoplastic gastric tissues has shown that several ultrastructural

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morphometric data correlate with the data from EELS-analyses. Cryosections were used since plastic embedded material shows high loss of polar proteins and phospholipids, (Younossi-Hartenstein 1985).

The exact knowledge of the ultrastructural morphometric relationships in normal and neoplastic gastric tissues (see Wolf et al. 1985c) allows a precise identification of the sample cell type and provides the basis for such studies. The data presented in this paper show the advantages of such techniques. However, the investigation of more samples and a correlation with clinical data will be necessary before the practical advantages of this method can be evaluated.

The method, after the necessary standardisation, is a fast one (operation times as low as 30 min can be achieved). Thus widespread use of the method could allow additional criteria for distinguishing between benign and malignant tissue behaviour.

Materials and methods

For this study, biopsies from 5 normal stomachs, 3 with chronic atrophic gastritis with intestinal metaplasia and 5 neoplastic cases (tubular adenocarcinoma G2) were investigated. The material was diagnosed in the Pathology Institute of our University. Tissues were briefly (30 min) fixed in cold 2.5% glutaraldehyde in 150 mM cacodylate buffer at pH 7.4, washed in the same buffer and incubated for cryo-protection in 1.1 M saccharose in 100 mM cacodylate buffer, pH 7.4 by applying a semiautomatic system (Wolf et al. 1983c). The following specimen preparation has been carried out using the published methods of Tokuyasu (1980), Griffiths et al. (1984) and Christensen et al. (1985) without application of methyl-cellulose. By using a self-constructed apparatus for rapid freezing in freon R 22 of surgically gained samples, the formation of ice crystals was prevented (Wolf 1984b and Wolf et al. 1985b). Frozen samples were sectioned with selected glass knives according to Griffiths et al. (1984) and Christensen et al. (1985) with a Reichert-Jung Ultracut E-FC4-System with modified temperature control (Wolf 1984b) or a cryonova ultramicrotome from LKB. The temperature of the specimen was $-120°$ C, that of the knife being $-100°$ C. Since, normally larger sample section areas are required to ensure sectioning of the actual tumour tissue, the samples were not trimmed but cut carefully over the whole width using the manually operated advance system.

Estimation of section thickness was performed as described by Griffiths et al. (1984) and Hama (1982). Morphometric investigations were carried out using a newly self-developed interactive morphometric system for cytological and histological studies on biological samples (Wolf 1983b; 1984a). Methods and results concerning morphometric investigations on normal and neoplastic gastric tissues have been published elsewhere (Wolf 1985c).

EELS-investigations were carried out using a Gatan-607-EELS-spectrometer adapted to a Jeol 200 CX transmission electron microscope operated at 200 KV. Spectra were accumulated and evaluated with a Tracor Nothern multi channel analyser system. The data were recorded with a collection semi angle of 7.85 mrad and a recording time of 100 s.

Morphometrically identified cells from the various samples were placed over the entrance aperture of the detector and the electron beam focussed onto the region to be analysed (nucleus or cytoplasm).

Due to the circular geometry of the detector aperture, only nuclei with a more or less isodiametric section profile were measured. The nuclei and their corresponding cytoplasm were analysed using the same electron beam diameter.

Results

On the basis of earlier ultrastructural, morphometric investigations on normal, and different stages of tumorous, gastric tissues (Wolf et al. 1985a),

we have selected sample specific cells on large ultrathin cryosections of the material to be investigated (Fig. 1–2). Fig. 3–6 show representative electron micrographs of ultrathin cryosections for the analysed cell types. The choice of cells was achieved using the afore mentioned, computer-morphometry system, utilizing the morphometric criteria already published elsewhere (Wolf et al. 1985b and 1985c). Table 1 shows data from ultrastructural morphometric relationships comparing the ratios of the subcellular distribution of C, N, O, Ca and P.

The ratios of the carbon and nitrogen concentration between the nucleus and the cytoplasm exhibit no differences. The variability in the oxygen values is too high to allow determination of significant differences.

However, the calcium and phosphorus relationship for nucleus and cytoplasm showed distinct changes between the normal and the corresponding neoplastic tissues in the samples investigated.

Due to variations in the section thickness of the ultrathin cryosections on the one hand, as well as differences in the physiological condition of the material investigated on the other, absolute element concentrations were not determined. Instead, the relative values from two morphologically, well – characterized, structural regions were calculated. When comparing the data for the whole cell or, for example, the cytoplasm alone, no significant differences were found between cells from normal tissues (mucoid neck cells) and tumour cells (in the case of chronic atrophic gastritis with intestinal metaplasia: the cells lying between the goblet and paneth cells were used). Further, the analysis of smaller sample areas also lead to uncertain results, necessitating more measurements per cell and cell type. However, characteristic differences can be seen in Table 1.

The ultrathin cryosections (area e.g. up to 2 μm^2) were surprisingly stable in the electron beam, both with or without support films on the object grids, and even when applying a narrow beam (0.5 μm) at higher beam currents (up to 100 nA at 200 KV).

Discussion

The data reported here only indicate the possible applications of the techniques involved. For this reason, discussion of these data is tentative and the work cannot give satisfactory practical results as yet. However, the data show the great advantage of morphometric investigations for further experiments. The exact knowledge of stereological data can help to identify certain characteristic cells or cell structures for elemental analysis or other experimental procedures eg. ultra-immunohistochemistry. Morphometry can either accelerate such investigations, or strengthen the data through comparison with the corresponding structural data.

Investigations of breast tumours in our laboratory using EELS-analysis of ultrathin cryosections of cells of defined ultrastructural properties also shows that characteristic changes in the elemental ratios can occur. Fully reliable data is, however, not available owing to the insufficient sample quantity.

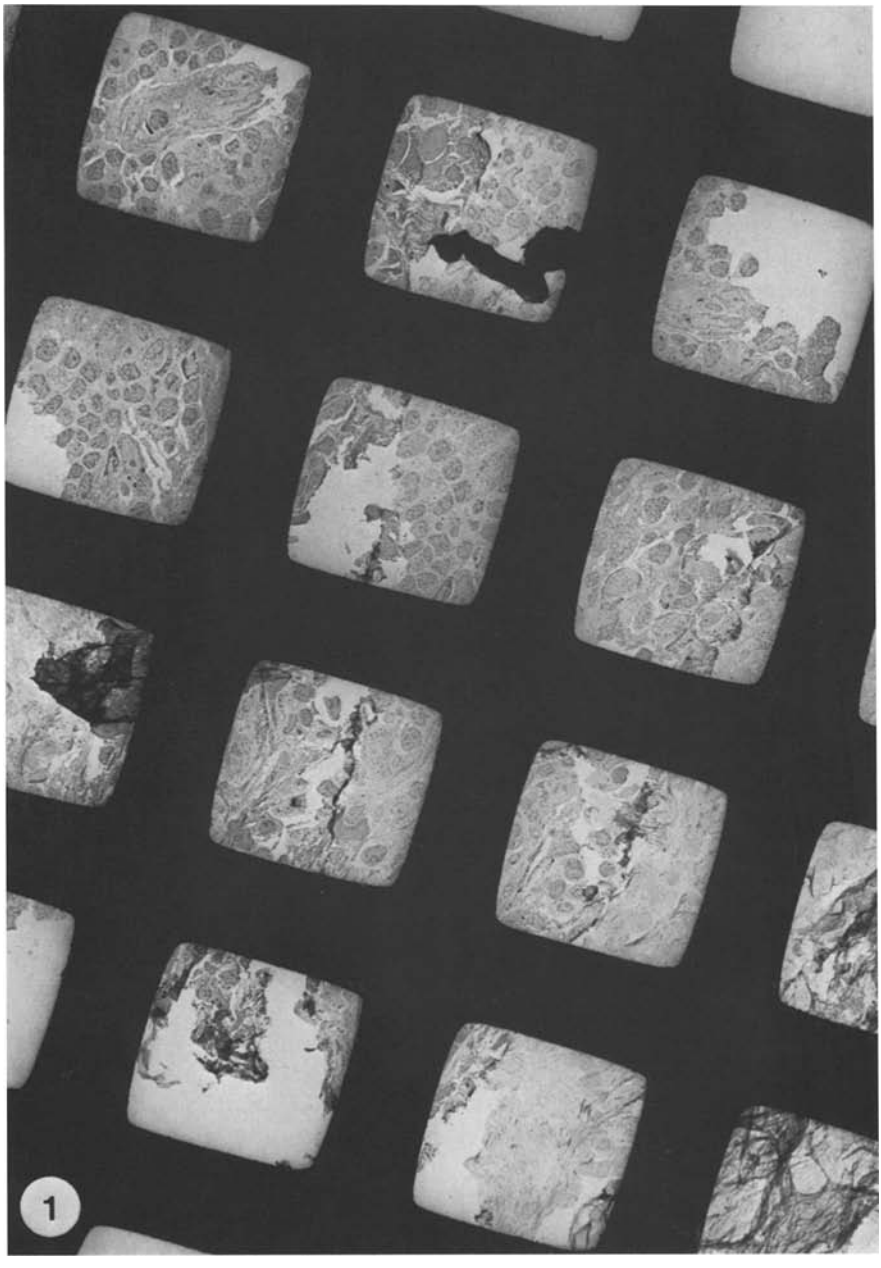

Fig. 1. Low power magnification of a large ultrathin cryosection of a tubular ardenocarcinoma (G2) ×480

Fig. 2. Higher magnification of Fig. 1 ×1,200

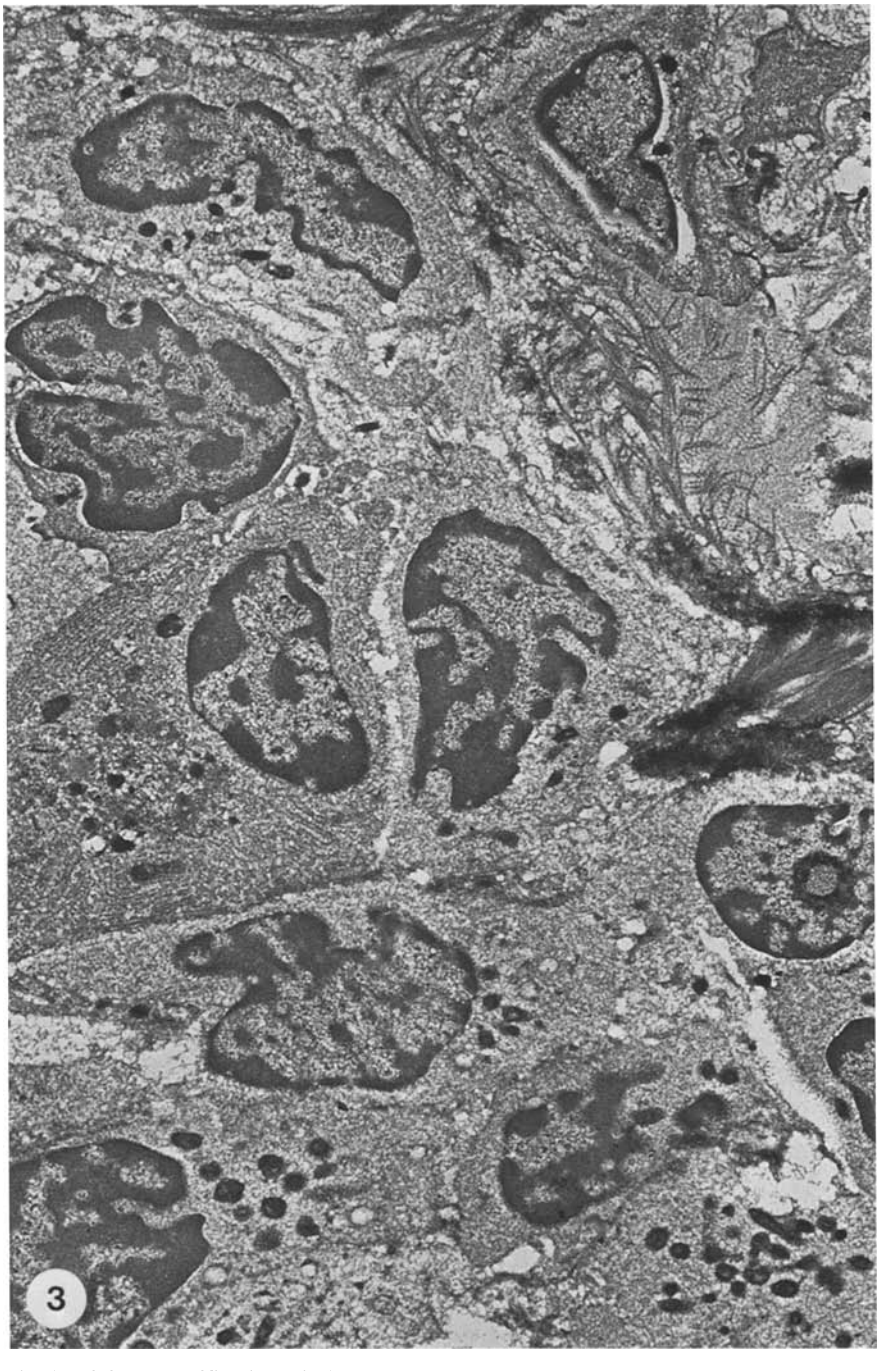

Fig. 3. Higher magnification of Fig. 2 × 7,500

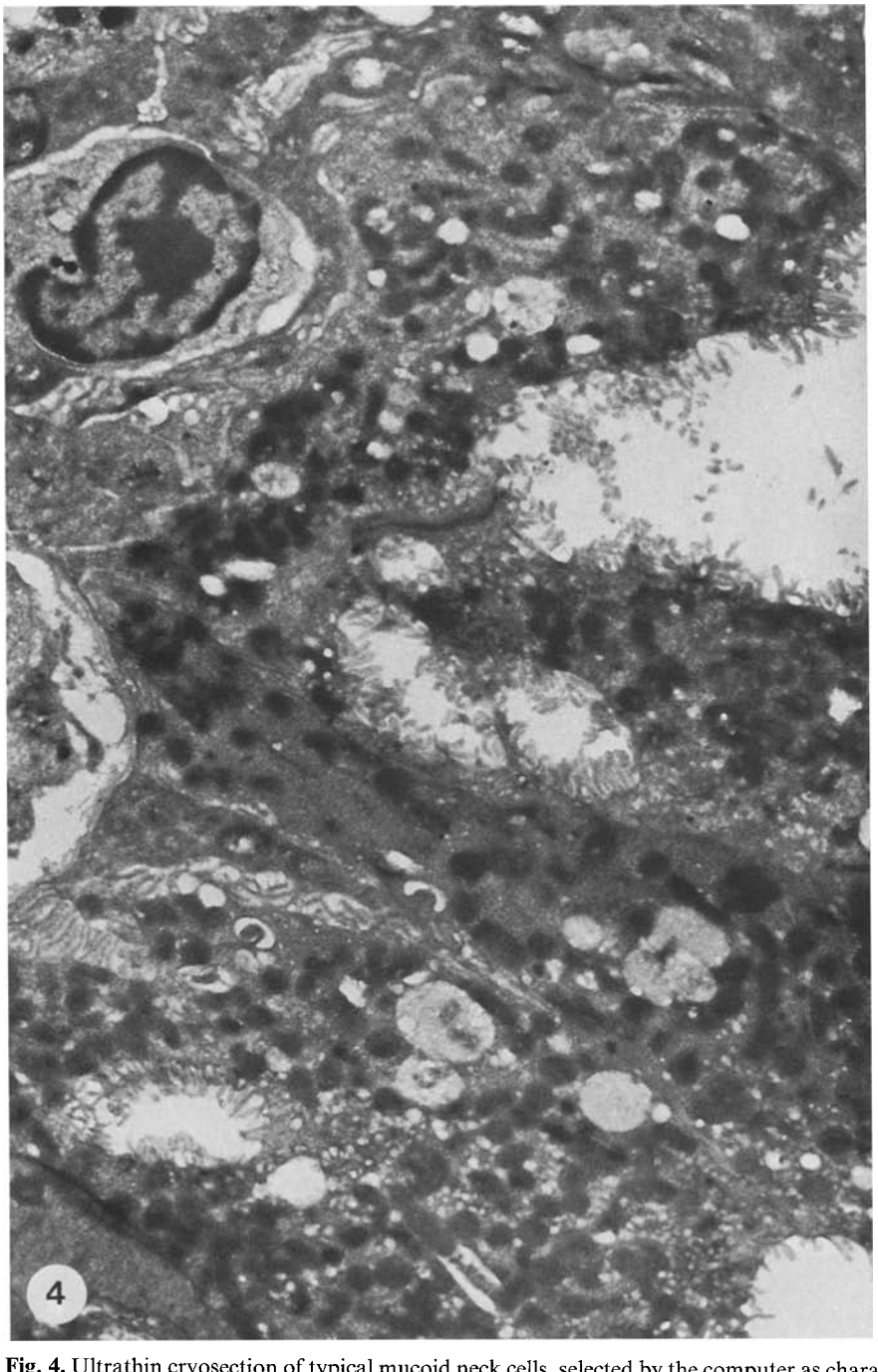

Fig. 4. Ultrathin cryosection of typical mucoid neck cells, selected by the computer as characteristic. × 7,200

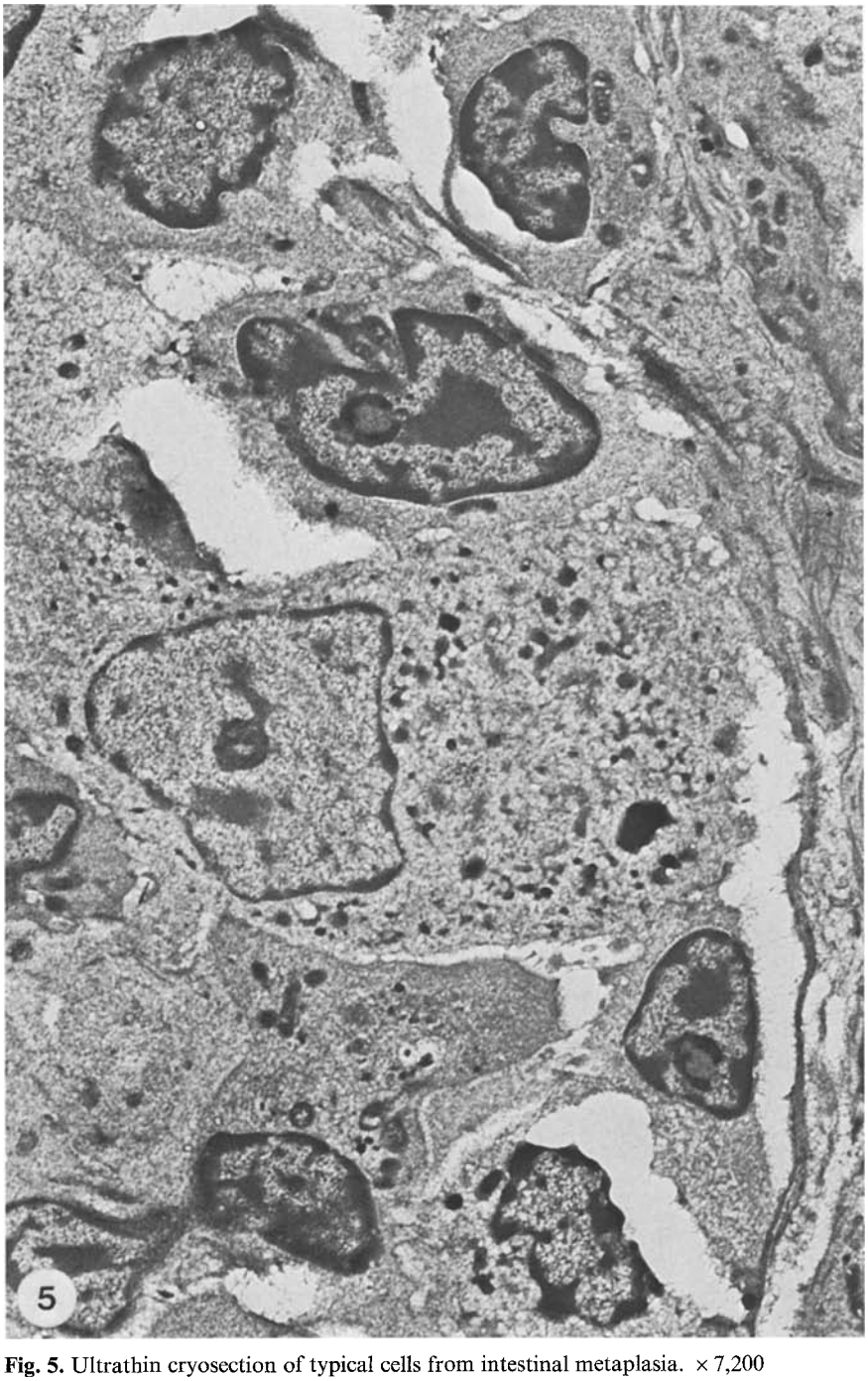

Fig. 5. Ultrathin cryosection of typical cells from intestinal metaplasia. × 7,200

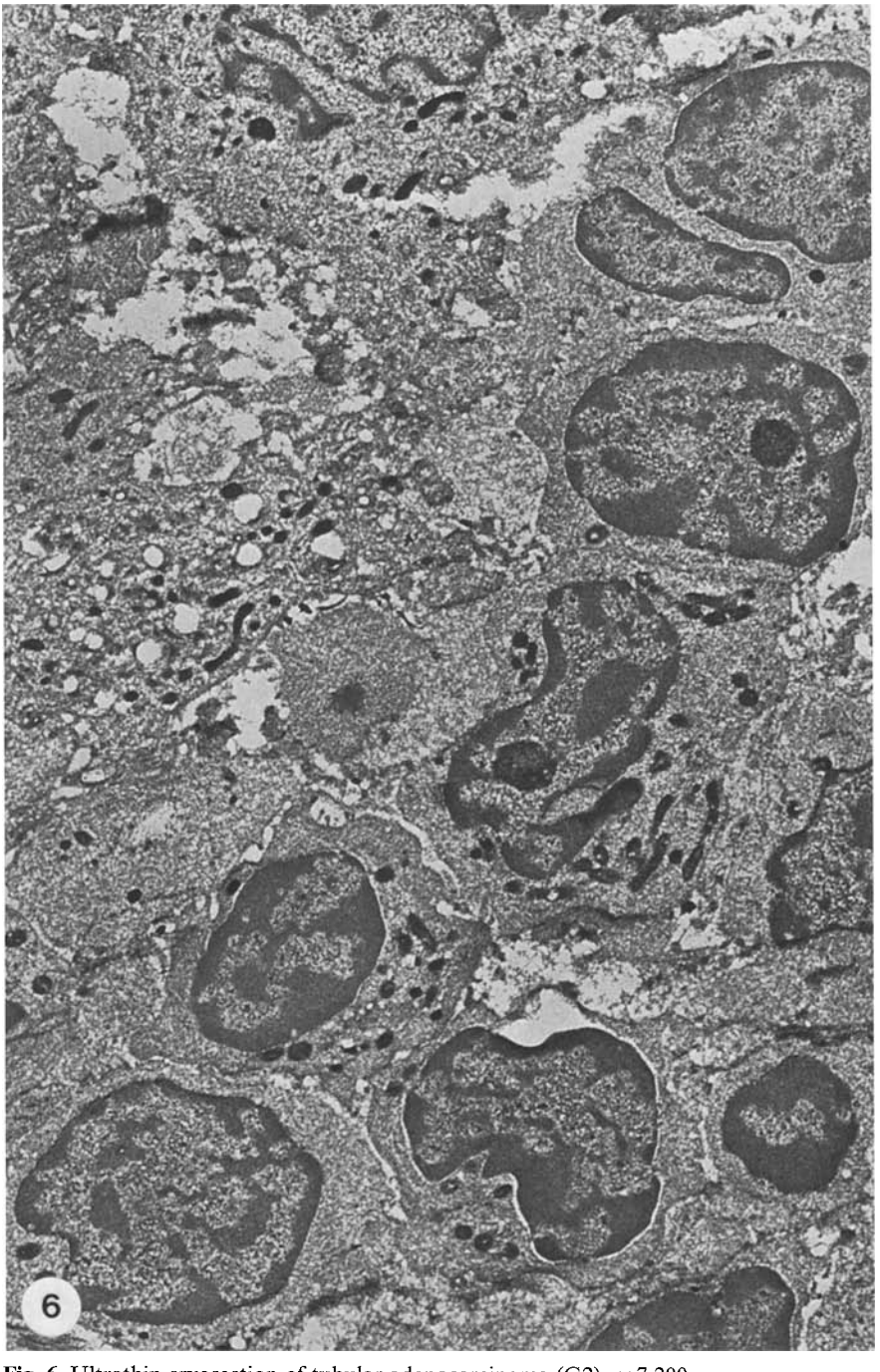

Fig. 6. Ultrathin cryosection of tubular adenocarcinoma (G2). × 7,200

Table 1. Ratios of cell parameters and element concentrations for mucoid neck cells, cells from chronic atrophic gastritis with intestinal metaplasia, and tubular adenocarcinomas G2

	Mucoid neck cells	Chronic atrophic gastritis with intestinal metaplasia	Tubular adeno-carcinoma G2
	$n=5$	$n=3$	$n=5$
Number of cells	85	70	108
V_n/V_c	0.28 ± 0.03[a]	0.29 ± 0.04	0.60 ± 0.05
S_c/V_c ($\mu m^2/\mu m^3$)	0.80 ± 0.09	1.50 ± 0.31	1.10 ± 0.10
S_n/V_n ($\mu m^2/\mu m^3$)	0.99 ± 0.05	1.83 ± 0.49	1.25 ± 0.10
$\%[C]_n/\%[C]_p$	0.90 ± 0.05	0.90 ± 0.04	1.02 ± 0.081
$\%[N]_n/\%[N]_p$	1.20 ± 0.40	1.20 ± 0.04	1.15 ± 0.45
$\%[O]_n/\%[O]_p$	1.33 ± 0.45	1.20 ± 0.24	0.82 ± 0.20
$\%[Ca]_n/\%[Ca]_p$	1.00 ± 0.20	0.60 ± 0.18	0.50 ± 0.10
$\%[P]_n/\%[P]_p$	1.75 ± 0.20	1.60 ± 0.40	0.25 ± 0.40

V_n = Volume-nucleus, V_c = Volume-cell, S_c = Surface area-cell, S_n = Surface area-nucleus
$\%[\ ]_n$ = percentage $[\ ]_{nucleus}$, $\%[\ ]_p$ = percentage $[\ ]_{plasma}$

[a] Standard deviation

In contrast to EDX-investigations (Roomans 1981) the EELS-technique is more sensitive and allows a rapid determination of light elements on ultrathin cryosections (detection limit EDX: $\sim 10^{-18}$ g in comparison to EELS $\sim 10^{-21}$ g). In contrast with publications from other authors applying EDX- or EPMA-techniques, (see for example Somlyo et al. 1985) we have not presented absolute data for the elemental values. Such data are very dependent on the preparation conditions of the specimen and on the section thickness.

Contrast margination of the nucleoplasm in tumour cells has often been described (Fisher 1976; de Harven 1966), but the data reported here show no significant loss in the overall nuclear mass, whereby calcium and phosphorous play an insignificant role.

Although we have standardized the sample preparation by applying semi-automatic techniques (Wolf et al. 1983c), the reproducibility of section thickness smaller than 50 nm is very limited with present day cryo-ultramicrotomes.

For this reason, but in another context (i.e. morphometric analyses on membrane surfaces, see Wolf et al. 1983d), we have already begun experiments in an attempt to improve the reproducibility of section thickness for such ultrathin cryosections (Wolf et al. 1983a).

A recent development in this direction utilizes the sample it self as the advance sensor in the cryo-ultramicrotome, i.e. the factor determining the degree of advancement of the sample is the distance between the sample and the cutting edge of the knife (Wolf 1984c).

However, for the present we have limited our data to comparing numerical ratios of the calculated elemental concentrations between two well – characterized subcellular compartments. In this way, electron energy loss spectroscopy can be used to analyse ultrathin cryosections of biological material, irrespective of the present drawbacks in non-reproducible section thickness and variations in the physiological condition of samples.

For such quantitative analyses, the maximal section thickness is dependent on the electron acceleration voltage and it's stability. Our investigations have shown that quantitative analyses, using the above-mentioned systems, allow a maximal section thickness of about 75 nm at 100 KV, and at 200 KV a thickness of nearly 100 nm is tolerable (see also Reimer 1984).

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