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USE OF FORMALIN FIXED, PARAFFIN EMBEDDED TUMOURS FOR ESTIMATION OF CELLULAR DNA CONTENT AND S-PHASE FRACTION BY STATIC CYTOFLUOROMETRY

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Abstract

Sections with a thickness of 50 μm from 34 formalin fixed, and paraffin embedded tumours were deparaffinized and hydrated and the cells were disaggregated with pepsin. The cells were stained with Hoechst 33258 and a rapid method for static fluorometry was used. DNA ploidy and the fraction of cells in S-phase were estimated and compared with results obtained with freshly prepared cells. The coefficients of variation for the tumour stemlines for both the fresh and the embedded material were approximately 6 per cent. There was a good correlation between DNA ploidy determined from fresh and embedded material, respectively; there was also a close correlation between the fractions of S-phase cells estimated from fresh and embedded tumours. It was also possible to cut out small histologically defined lesions, such as carcinoma in situ of the breast from thick paraffin sections and obtain DNA histograms with high resolution.

The cellular DNA content reflects the biologic behaviour of many tumour types (1, 2, 6, 8).

Minor ploidy deviations can be detected in fresh or frozen tumours with the use of flow cytometry (15). Rapid procedures for static cytofluorometry are also described (4). Fresh, frozen, or smeared cells are seldom available when small histologically well defined lesions or tumours removed long ago are to be studied. In such cases formalin fixed, paraffin embedded material often has to be employed. Formerly we used thin (4–14 μm) sections stained with Feulgen-pararosaniline. Tissues with closely lying or overlapping nuclei were, however, difficult and time-consuming to analyse, and determination of cellular DNA content was sometimes difficult (3).

TAKAMATSU et coll. (13) devised a method for DNA analysis using carnoy fixed paraffin embedded material.

HEDLEY et coll. (10) described a method using pepsin to prepare 30 μm sections of formalin fixed, paraffin embedded tumours for flow cytometry.

In the present investigation HEDLEY's method (10) was used for disaggregation of cells, and nuclear DNA content was determined by a rapid procedure for static cytofluorometry (4) after Hoechst 33258 staining. With this procedure interest could be focused upon morphologically identifiable cells and damaged nuclei and cell aggregates could be excluded. It allowed a rough estimation of the proportion of cells with a DNA content corresponding to the S-phase of the cell cycle.

Material and Methods

Thirty-four tumours (Table 1) were delivered to our laboratory within 30 min after removal. The fresh tumours were cut through and their surfaces curetted. The material obtained was suspended in a

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polyionic buffer solution (pH 7.4) (90 mmol/l NaCl, 30 mmol/l sodium gluconate, 6 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 30 mmol/l sodium acetate, 25% ethanol by volume). Frozen trout erythrocytes were added as internal marker cells. The suspensions were washed twice by centrifugation at 500 × g. The cells were suspended in 0.1 mol/l phosphate buffer (pH 7.4) containing 1 mg per ml of RNAase (Sigma), and incubated under agitation at 37°C for 30 min. The cells were resuspended in Carbowax (20 g polyethylene glycol (MW 1 300–1 600) in 1 000 ml 50% ethanol) and briefly dispersed with the aid of a syringe before centrifugation at 100 g for 10 min onto microscopic slides using a Cytospin 2 (Shandon, Southern Instr., Runcorn, England). The specimens were briefly air dried and fixed in a freshly prepared mixture of methanol/formaldehyde (35% w/v) /glacial acetic acid (85/10/5 by volume) for 60 min followed by washing in 0.1 mol/l phosphate buffer (pH 7.0) for 30 min. The specimens were stained in 1 μg Hoechst 33 258/ml in 0.4 mol/l NaCl, 0.1 mol/l phosphate buffer pH 7.0. The specimens were mounted in the staining solution, and the cover glasses were sealed with Kaiser's glycerolgelatin (Merck, Darmstadt, West Germany).

A block was cut from the curetted tumour and formalin fixed for 12 to 24 hours, dehydrated, cleared in xylene, and embedded in Paraplast. A 50 μm thick section was cut from each paraplast block followed by a 4 μm section stained with haematoxylin and eosin for histologic confirmation. The 50 μm sections were deparaffinized in xylene for 2×60 min, hydrated by passing through an ethanol series (100%, 95%, 70%, and 50%, 10 min each), washed twice in distilled water, resuspended in 1 ml of 0.5% pepsin (Sigma) in 0.9% NaCl adjusted to pH 2.0 with 2N HCl, and incubated with intermittent 'Vortex' mixing at 37°C for 30 min. Residues visible to the naked eye were removed and the suspension was filtered through a 41 μm nylon mesh and washed twice in 0.9% NaCl. Finally, the suspension was centrifuged onto microscope slides and fixed and stained as described above.

Four aneuploid breast carcinomas with abundant lymphocytes in the stroma were selected for the study of reproducibility. In each case 3 consecutive 50 μm paraffin sections were made. The 3 sections were processed on different occasions as described above. In each case the 3 sections resulted in identical histograms.

Two breast carcinomas, one with large and the other with small tumour cell nuclei, were used to study the effect of the section thickness. Sections with a thickness of 15, 30, 50 and 100 μm were cut out from these tumours and processed as described above.

Table 1

Histopathologic diagnosis of the tumours analysed

Diagnosis	Code
Ductal breast carcinoma	BC 1, 3–7, 9–15, 17–22
Lobular breast carcinoma	BC 2, 8
Medullary breast carcinoma	BC 16
Renal carcinoma	RN 1
Ovarian carcinoma (grade 3)	OV 1-2
Malignant Lymphoma (low-grade)	LY 1–3
Thyroid 'toxic' adenoma	TH 1
Thyroid papillary carcinoma	TH 2, 3
Uterine cervical, squamous cell carcinoma	CX 1, 2
Parotid gland, mixed tumour	PA 1

Table 2

Coefficient of variation (CV) of the GO/1 tumour cell peak (2 breast carcinomas; A and B) after pepsinization of paraffin sections of different thicknesses. For comparison the CV values obtained from freshly prepared material of the same tumours are also shown

Thickness of section (μm)	Coefficient of variation-GO/1 tumour cell peak (per cent)	
	Tumour A	Tumour B
15	16	7
25	8	5
50	4	4
100	4	4
Freshly prepared cells	3.5	5

Four 2- to 4-year-old paraffin embedded specimens of mammary carcinomas with both invasive and intraductal carcinoma in situ and a specimen from a uterine-cervical conization with flat condyloma and carcinoma in situ were also investigated. Pieces (2–5 mm) of invasive carcinoma, carcinoma in situ or flat condyloma were cut out from 50 μm paraffin sections—with the aid of a stained 4 μm section—under stereomicroscopy.

Cytofluorometry was performed with a Leitz MPV 3 cytophotometer, a Luxor ABC 800 microcomputer (Luxor, Motala, Sweden), and the FLOWRA computer program as previously de-

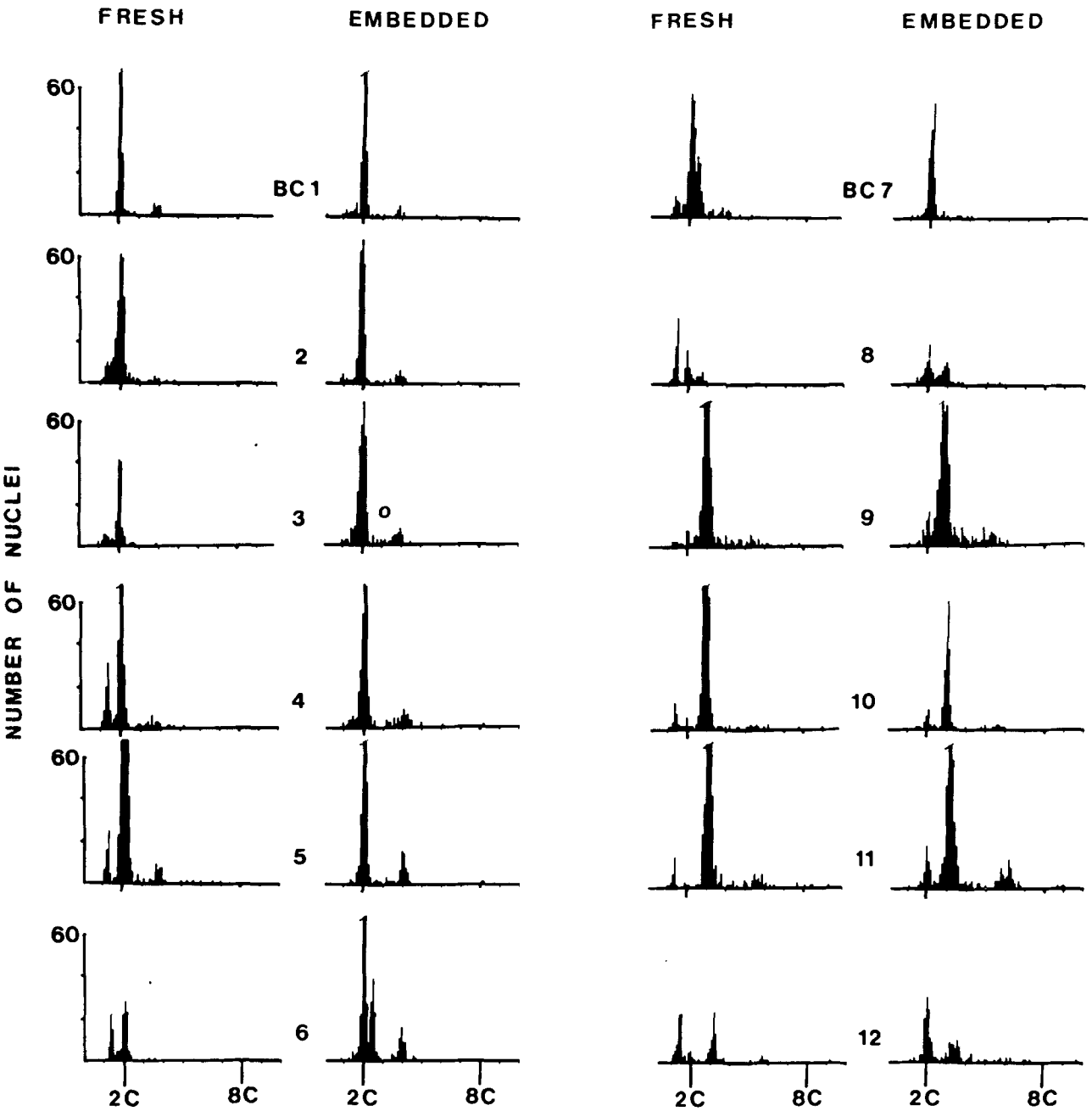

Fig. 1. DNA histograms for all 34 tumours obtained from both fresh and embedded material. The histologic diagnoses are shown in Table 1. In some specimens (=0) no reference cells were identified, and ploidy could not be determined.

scribed (4). The cells were identified in phase contrast to enable the rejection of damaged nuclei and cell clumps. All other cells were analysed. Non-tumour cells such as lymphocytes, plasma cells, and granulocytes were also measured, and the results stored separately in the computer.

Calculation of data. The DNA ploidy of the tumour stemline was determined in relation to the mean fluorescence of the reference cells. These were lymphocytes, plasma cells, granulocytes, or, in the case of fresh material trout erythrocytes (trout erythrocytes show 68% of the DNA fluorescence of human diploid cells stained with Hoechst 33258). The coefficient of variation for the tumour stemline and the proportion of cells in the S-phase were calculated by the computer. Assuming a rectilinear distribution of S-phase cells, the number of bars between the GO/1 and G2M peaks was multiplied by

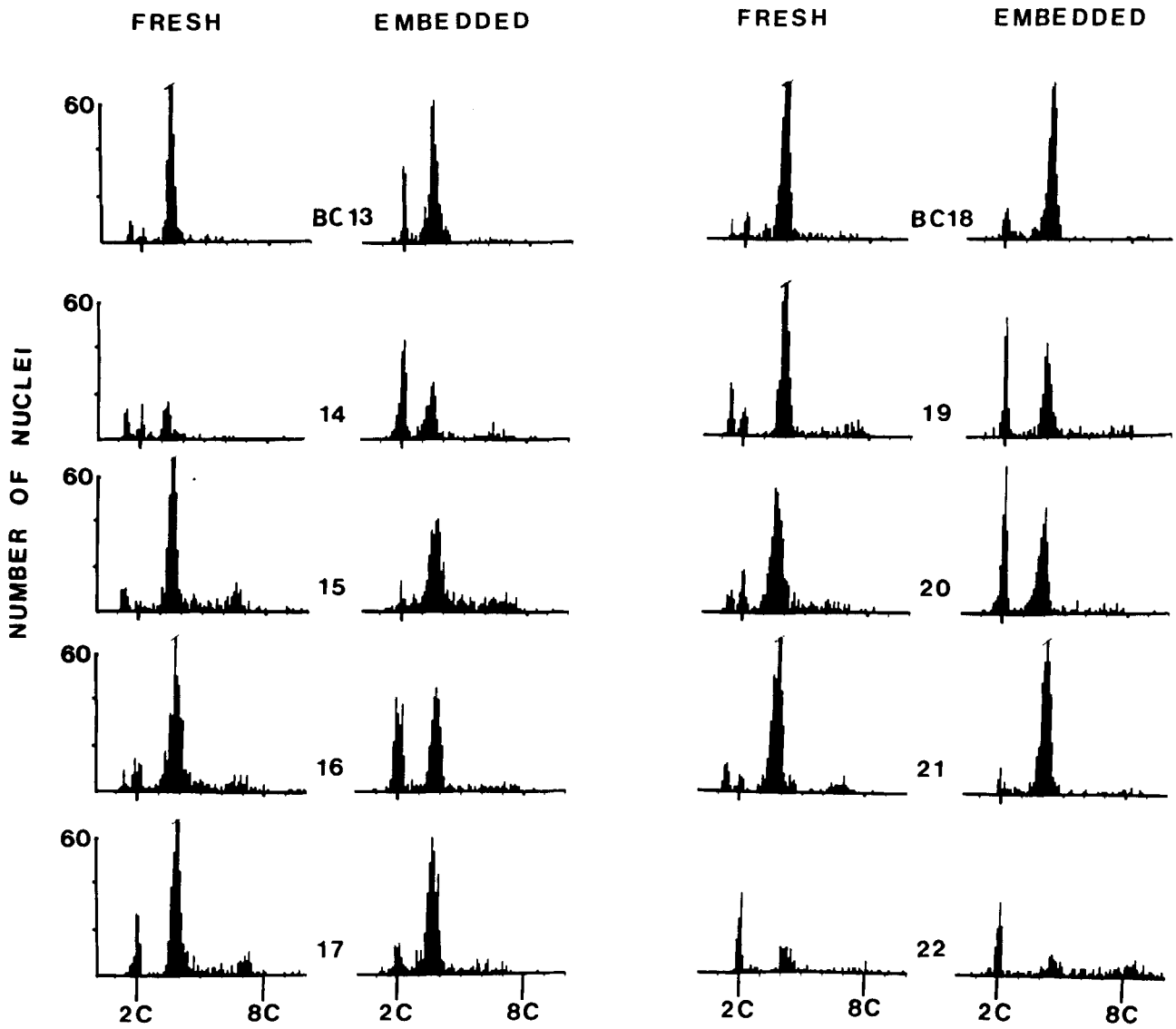

Fig. 1

the mean number of registrations in the bars interactively selected in the S-phase region of the histogram.

Results

Optimum section thickness. Paraffin sections obtained from 2 breast carcinomas and with a thickness of 15, 30, 50 and 100 μm were pepsinized and stained. The smallest coefficients of variation were seen with preparations from 50 μm and 100 μm paraffin sections (Table 2). With thinner sections a slighly higher variation and a slope to the left of the peaks was obtained. Thus 50 μm sections gave peaks with low coefficients of variation even with breast carcinomas with large nuclei (Table 2). Sections thicker than 50 μm could also be used, but they were more difficult to handle especially when specific areas had to be cut out from the sections.

DNA ploidy determination. About 350 (110–600) cells were measured. The mean coefficient of variation for the tumour stemline was 5.7 per cent (3.5–8.4) with fresh and 6.2 per cent (4.1–10.5) with embedded tumours. In 22 of the 34 cases the coefficint of variation was less with the measurements from the freshly prepared tumours but in 10 cases the opposite was true. In all fresh specimens from breast carcinomas the DNA ploidy could be determined using internal lymphocytes or added trout erythrocytes, but in 3 of the 12 tumours of other types this proved impossible as too few trout erythrocytes had been added or no identifiable internal reference cells were present (Fig. 1). With the speci-

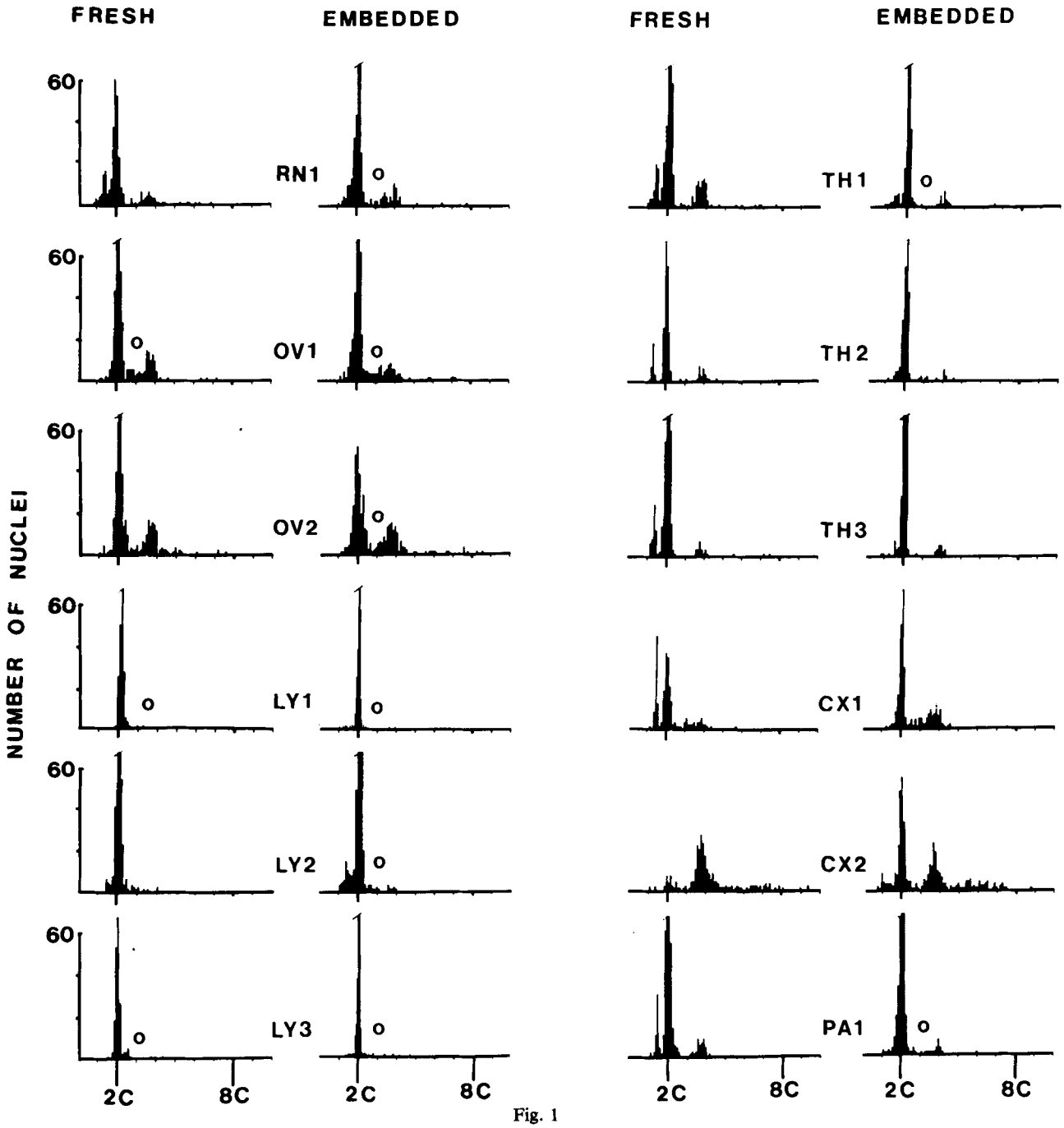

Fig. 1

mens from the embedded tumours the DNA ploidy was determined in 21 of 22 breast carcinomas and 4 of 12 other tumours using internal reference cells (lymphocytes, plasma cells, or granulocytes) (Fig. 1). The comparison between DNA ploidy determination of 18 DNA aneuploid fresh or embedded tumours showed a close correlation (Fig. 2). In one embedded breast carcinoma (BC 6) an additional peak at 2.3 C was not detected in the histogram from the freshly prepared material, in which only 110 cells were measured, while it was detected in 210 cells from the embedded specimen. On the other hand an additional peak at 2.2°C seen in the freshly prepared specimen (BC 7) was not detected in the corresponding embedded material.

Since some tumours were devoid of internal reference cells, we tried to add lymphocytes from embedded lymph nodes. Sections from the axillary

lymph nodes and the primary tumour were prepared and stained at the same time. This was done with 5 breast carcinomas with DNA aneuploid stemlines and abundant internal lymphocytes. The cell suspension from the lymph nodes was centrifuged onto the same slides but separately from the cells of the primary tumour; the staining and preparation were thus identical. In 4 of these specimens no significant difference in mean fluorescence intensity emerged between lymphocytes from the lymph node and from the tumour, while in the fifth there was a significant difference of more than 5 per cent. The method of adding lymphocytes from the axillary nodes as reference cells for DNA ploidy determination is thus not reliable.

S-phase estimations. In 27 tumours more than 150 cells were analysed in both freshly prepared and embedded specimens and the proportion of cells in S-phase was determined. There was a close correlation between the results obtained with the 2 types of material (Fig. 3). The similarities between the DNA histograms obtained with freshly prepared and embedded specimens are also obvious from Fig. 1.

Small lesions. All the small lesions analysed yielded enough cells for the production of DNA histograms. When the intraductal carcinoma in situ was compared with the invasive carcinoma from the same breast the histograms were almost identical. With the uterine cervix, a histogram obtained from the flat condyloma showed a DNA diploid cell population (lymphocytes were identified in the specimen), and the carcinoma in situ showed a DNA aneuploid population (Fig. 4).

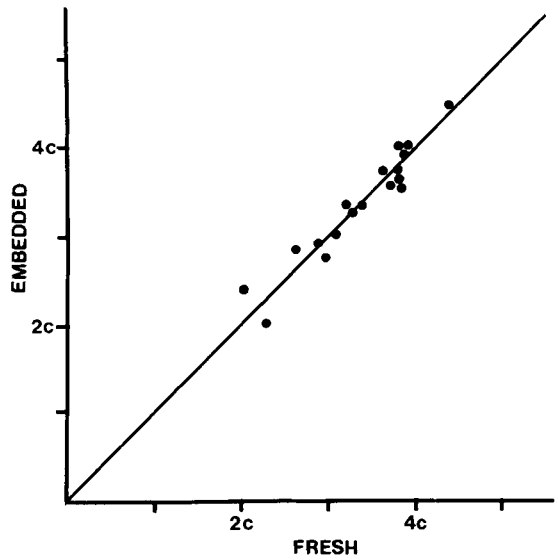

Fig. 2. Diagram showing relation between ploidy determined in embedded and fresh material from 18 tumours. Line of equality is drawn (r = 0.903).

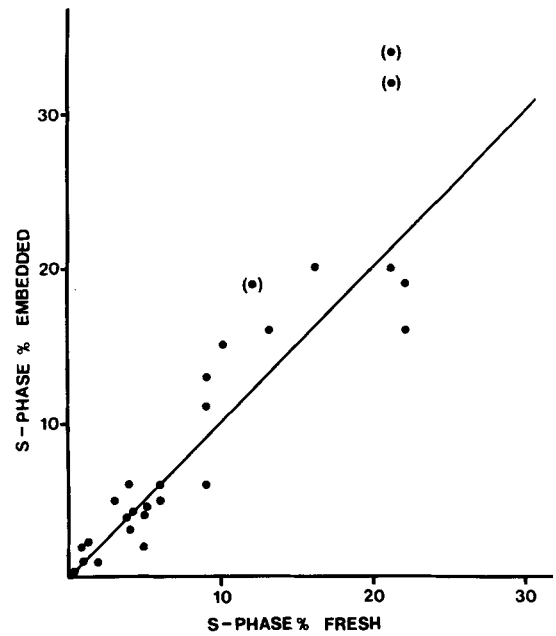

Fig. 3. Diagram showing relation between estimated S-phase in embedded and fresh material from 27 tumours. Line of equality is drawn (r = 0.919). Cases with statistically significantly different S-phase values indicated by parantheses.

Discussion

Retrospective analyses of the clinical importance of cellular DNA content require reliable data from old paraffin embedded tumours. Important work towards this goal was done by HEDLEY et coll. (10) and FRIEDLANDER et coll. (9). We tried their method, which employs flow cytometry, but were embarrassed by the background noise caused by sectioned nuclei and cell clumps. To learn more about the method and to enable the exclusion of damaged nuclei and cell clumps we chose instead to use static cytometry (4). This method gave reproducible results. Further, unstained slides could be stored for more than one year and stained specimens for at least 1 month without deterioration of the results.

Because we had previously used Hoechst 33258 (4) we continued to use this stain, which proved to be as good as the DAPI stain used by HEDLEY et coll. (10).

Our ploidy and S-phase analyses of fresh and embedded material yielded almost identical results. Estimation of the S-phase fraction determined with

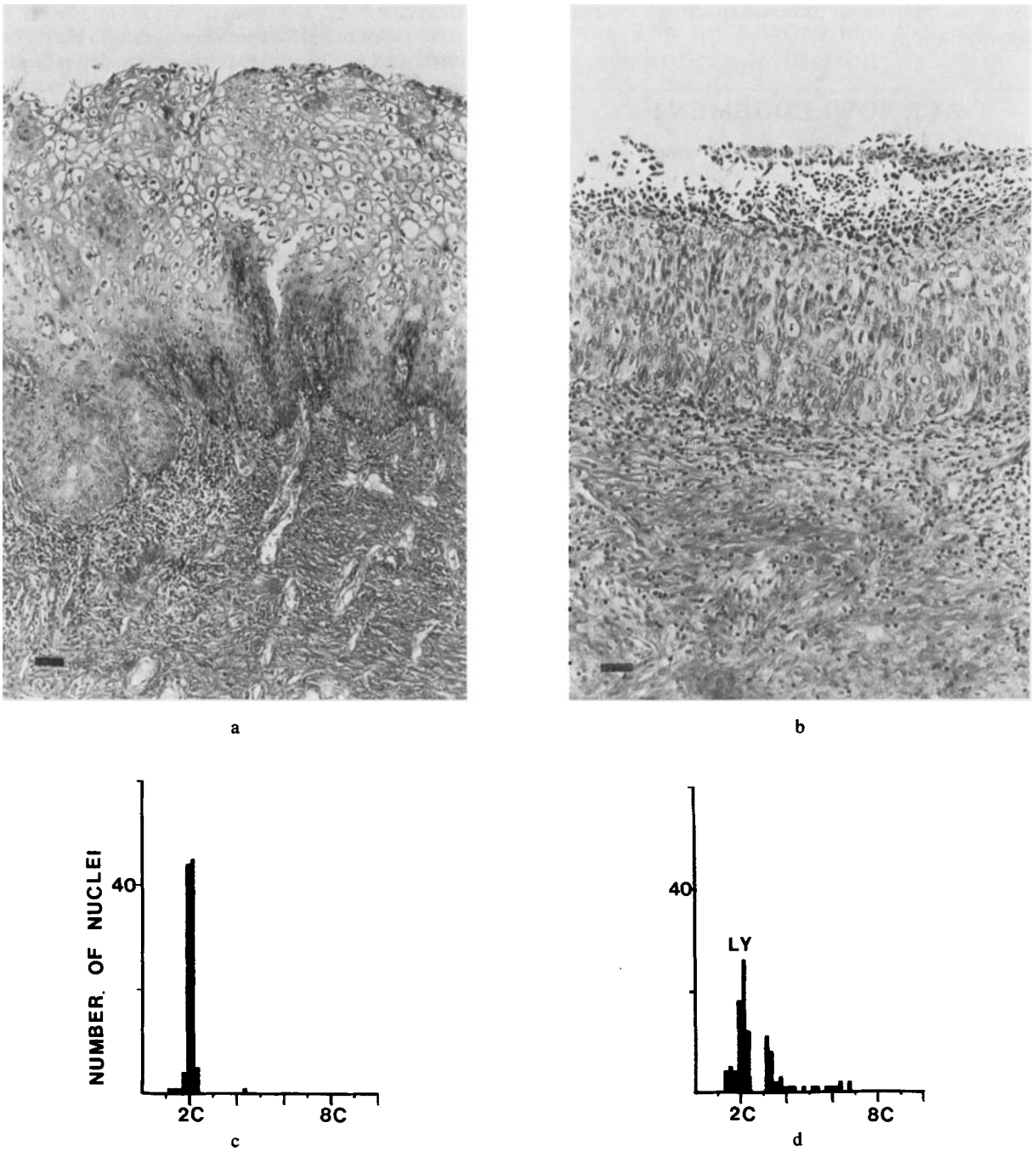

Fig. 4. Histologic images and corresponding DNA histograms obtained from small (2–5 mm) areas of a conization specimen with flat condyloma and carcinoma in situ—the material was cut out from 50 μm thick paraffin sections using stereomicroscopy and routinely stained 4 μm sections. LY = lymphocytes. The left-hand photomicrograph and histogram show the flat condyloma and those on the right the carcinoma in situ. (Bar indicates 50 μm.)

different methods appears to be a very important prognostic indicator for several types of tumour, as illustrated by our previous investigations (5, 12) and by those of BRAYLAN et coll. (7), MEYER et coll. (11) and TRIBUKAIT (14). Because paraffin embedded material can be used it is now possible to investigate the importance of the S-phase after very long follow-up periods.

The method also allows the study of small, histologically well defined lesions such as carcinoma in situ, and gives better resolutions than Feulgen stained sections. It should also make possible the

study of the very small tumours disclosed by mammography.

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