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QUANTITATIVE CYTOCHEMICAL METHODS FOR THE STUDY OF TUMOUR CELL POPULATIONS*

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Résumé—Les méthodes ultramicrospectrographiques générales avec enregistrement pour l'analyse cytochimique quantitative requièrent le plus souvent beaucoup de temps quand on les utilise pour travailler sur des populations cellulaires particulièrement grandes.

Plusieurs méthodes rapides sont décrites; celles-ci sont particulièrement adaptées à des recherches sur le métabolisme des nucléotides et des protéines des cellules ou des éléments cellulaires, isolés les uns des autres dans ces grandes populations.

L'appareillage comprend: (1) Un microspectrographe de haute résolution et à *scanning* rapide pour l'ultraviolet aussi bien que pour la région visible du spectre. Cet appareil convient particulièrement bien pour des mesures dans du matériel cellulaire courant Feulgen positif sur de larges séries avec une grande définition; (2) Un microinterféromètre enregistreur à haute résolution avec intégrateur; (3) Une modification de la méthode microradiographique pour la détermination du poids sec avec un appareil spécial de mesure pour des microradiogrammes des grandes populations cellulaires (L. CARLSON); (4) Un microplanimètre, spécialement indiqué pour des mesures de volume nucléolaire et nucléaire avec enregistrement électronique sur trois canaux.

L'auteur donne une série d'exemples de l'utilisation de ces instruments dans l'étude des problèmes concernant le métabolisme de l'ADN dans des populations de cellules tumorales.

THE combination of ultramicrospectrographic methods for ultra-violet and visible regions with similar procedures effective in the roentgen range (and in certain cases also in the electron microscopic range) and with micro-interferometric methods as well affords good possibilities for fairly general quantitative cytochemical work within the dimension range of the cell structures and the individual cells. The necessary technical equipment may appear relatively complex, but in routine biologic work its operation is as a rule simple and certain instruments of this type are even beginning to be available commercially. An automatic scanning high-resolution microspectrograph for ultra-violet and visible regions, modelled on the apparatus developed in our institution, for example, is produced by a German firm.

Scanning and datacomputing instruments developed within our institution and described earlier in the literature (CASPERSSON, 1955, 1956a, b, 1957; CASPERSSON *et al.*, 1955, 1957; SVENSSON, 1956; LOMAKKA, 1957) are intended to make possible

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work on a broad basis with a very wide variety of biologic material. It is essentially the following three factors which sometimes make such apparatus intended for more general applications relatively complicated.

1. The biological material is extraordinarily varied in general character, and different types of objects make different demands on the measuring instruments.

2. The optical inhomogeneities in the biologic material require both work with resolving powers better, as a rule, than 1 μ and also, for determinations of total quantities, measurements of hundreds or thousands of measuring points in each individual cell or section of a cell, which is usually most easily achieved by means of scanning measurements combined with automatic data analysis.

3. The optical conditions in the biologic preparations deviate greatly from those in true solutions, wherefore it must be possible in routine work with the aid of model systems to obtain the necessary optical constants for the data analysis.

In the case of work on limited special questions, on a particular substance or a special type of material it is frequently possible by development of special apparatus to save considerable time and instrumental investment. Since such apparatus can be standardized or calibrated with the aid of the more complete instrumental equipment, a good degree of accuracy can frequently be attained in the work with considerably simplified apparatus.

A typical example of such simplification is presented by the investigation of the cell's total content of different substances in large populations of isolated cells.

In connexion with work on composite tumour cell populations under the influence of chemical anti-cancer substances or ionizing radiation and with work on the early stages in carcinogenesis special techniques have been developed for the study of the metabolism and synthesis of deoxyribonucleic acids (DNA) in tumour material which are suitable for application in studies of other types as well. So intimate is the relation between DNA, RNA (ribonucleic acid) and the protein metabolic processes that in our investigations of biologic problems as well, no doubt, as in the majority of DNA–metabolic questions, it was desirable in conjunction with the DNA determination to obtain information concerning total nucleotides and the protein content in cell sections or total cells. The principal procedures are shown in the highly schematic diagram in Fig. 1. UMSP denotes a scanning ultramicrospectrographic procedure in the ultra-violet or visible regions. The next line represents scanning data-analysing microinterferometry; the subsequent line shows mass determination with roentgen microspectrophotography. These are the principal methods. Hereafter I shall speak, with respect to the DNA determination only of measurements of the Feulgen absorbance without discussing the chemical background of the staining reaction, of total extinction at 2650 Å and other wavelengths in ultra-violet as a basis for the evaluation of the total nucleotide content and of interferometry and roentgen methods for determination of total mass in the nucleus and cytoplasm.

For especially DNA-studies of tumour cell populations the following three situations are of greatest interest.

(1) Determination of the variability of the content of Feulgen-reacting substance per nucleus in advanced tumour cell populations of the type where appreciable disturbances in the division occur. The technical requirement in this case is only the

measurement of a sufficiently large number of nuclei so that continued measurements give reproducible histograms. The order of magnitude of the series to be measured in ascites tumours, for example, is a hundred or more cells.

(2) The search for small DNA deviations, for example, in cells from tumours of a type in which there are no numeric or morphologically-observable chromosome aberrations. This is analogous with some measuring problems during early stages

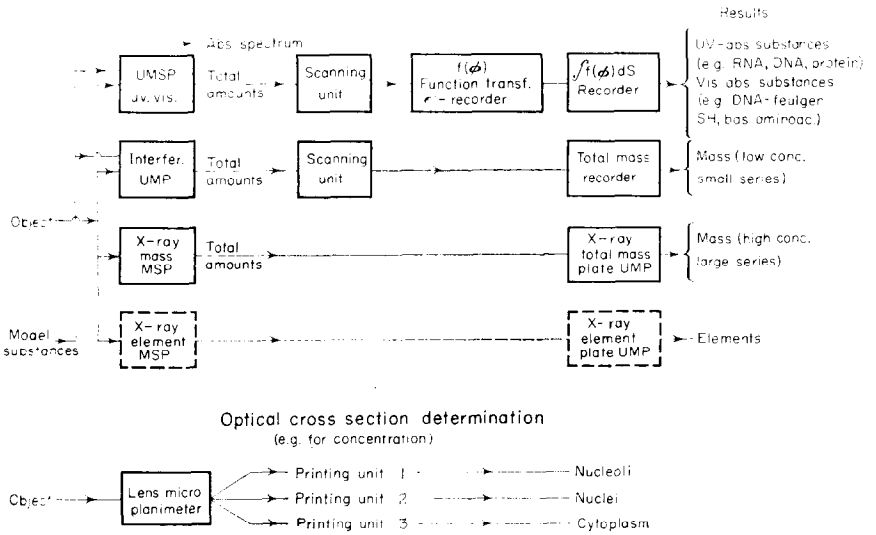

FIG. 1. Quantitative high-resolution ultramicrospectrophotometry. Arrangements for work on large cell populations, especially for determinations of DNA, RNA and total proteins.

of tumour development. This also demands large cell populations but in addition requires appreciable accuracy in the determination. It is particularly desirable to be able to carry out determinations during the different stages of mitosis, i.e. at a stage when the state of the DNA synthesis is well defined.

(3) Studies of early stages of tumour development. The most outstanding problem here is the detection of small numbers of aberrant forms in large cell populations. Technically this demands especially large series of measurements as well as comparatively great accuracy.

The large recording ultramicrospectrographs afford the required accuracy and resolution, to be sure, but for large measurement series the work is time consuming. Since it is possible, as a rule, to arrange the Feulgen staining so that the substance surrounding the nuclei remains completely unstained, the most essential requirement for recording is absent. In consequence it becomes possible greatly to increase the rate of scanning and further to save time through additional automatization of the measuring procedure. In order to make this clear Fig. 2 presents the three most important ways of measuring in optical ultramicrospectrography. At the top (1a, 1b) we see determination of the absorption course for individual spots. Below this (2a) we have the most common type of measured object: a cell or a section of a cell in which the total quantity of a particular substance is to be measured and which

is surrounded by cells or parts of cells that are not to be included in the measurement. Here registration of the individual measuring lines is indispensible The rather circumstantial measuring work in this case in the large ultramicrospectrographs can be greatly simplified with the aid of mechanical data-analysing procedures (LOMAKKA, 1957), but it is nevertheless exceedingly difficult to carry out serial measurements on this type of material. At the bottom (2b) a particularly favourable case is presented where the measured object lies on a free, non-absorbing background. There it is possible to carry the automation to a considerable degree.

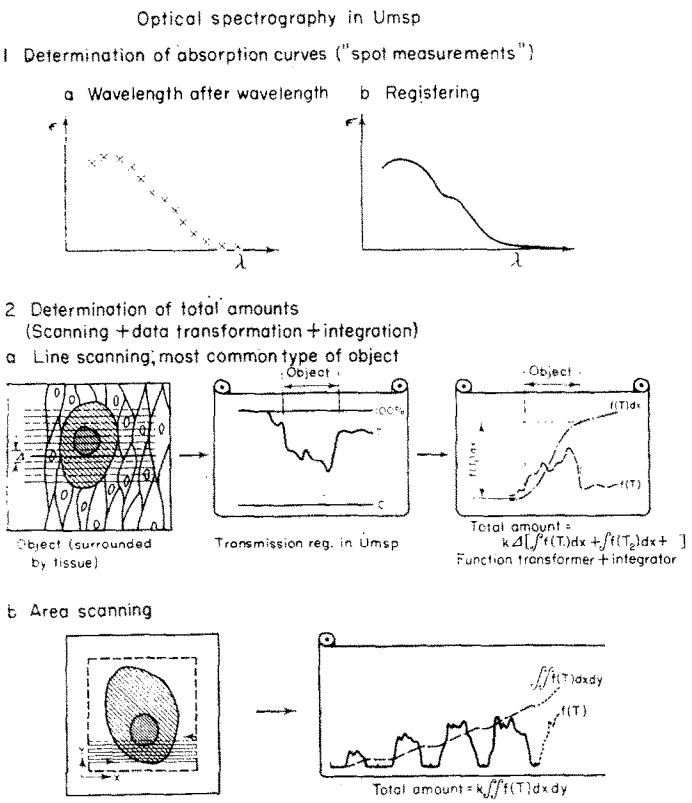

FIG. 2. The main different modes of work in optical ultramicrospectrography.

A rapid scanning ultramicrospectrograph (UMSP) intended for both visible and ultra-violet regions without resetting has been constructed along these lines (case 2b) to work with the same resolving power and approximately the same degree of accuracy as our large ultramicrospectrographs. The distance between the measuring lines in mechanical scanning is variable, and they can be adjusted to such density that the measuring spots touch when the degrees of inhomogeneity is high in the preparation, e.g. in mitoses. The optics used are the achromatic ultra-violet optics constructed by Zeiss and developed for the model they build commercially of the institution's large ultramicrospectrograph. Fig. 3 shows schematically the function of the new rapid instrument in a simplified diagram of its principles. Fig. 4 shows its

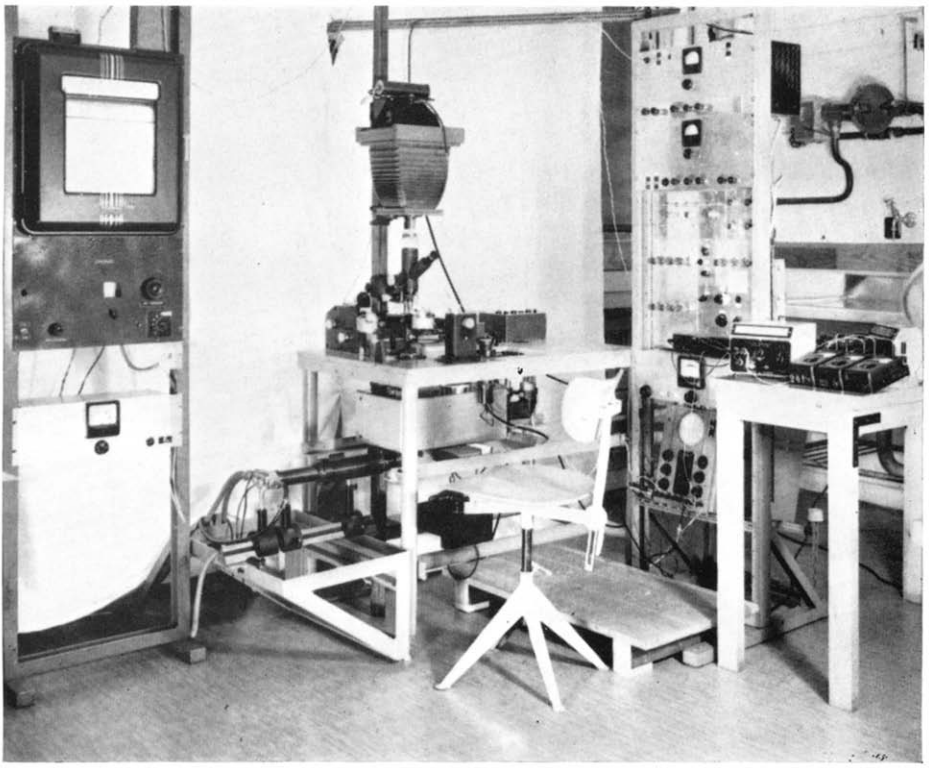

FIG. 4. The rapid ultramicrospectrophotometer.

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appearance. The scanning is mechanical, which offers very great advantages in work on many types of objects although the equipment required is considerably more elaborate than if the scanning were carried out electronically or in the optical system. The measuring time for an average mammalian cell is chosen to be about 3 sec. There is no practical advantage to be gained by further shortening this time as the interval required for moving the preparation from one measuring object to another is normally at least ten times longer. This apparatus permits convenient serial measurements in both visible, e.g. Feulgen preparations (in this case also sections), and in ultra-violet on cell series of hundreds of cells in one sequence with maximum exploitation of the resolving power of the optics. The resolving power and the scanning system are of a quality to permit even measurement of metaphase plates from mammalian material.

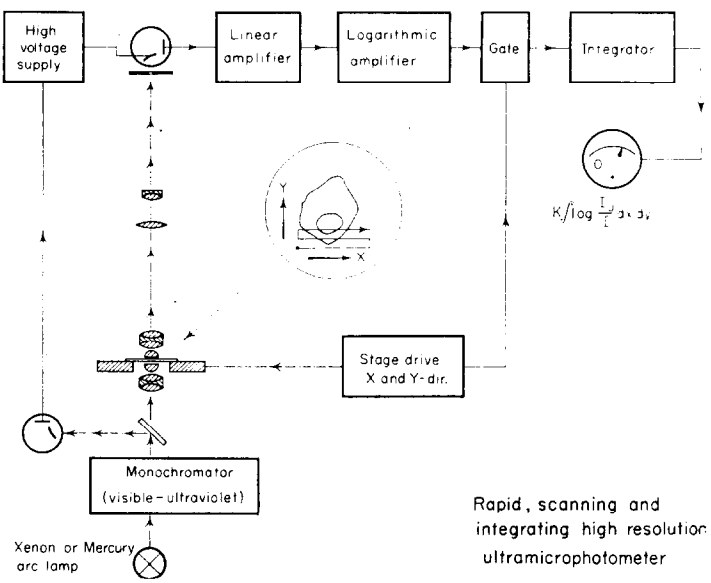

FIG. 3. Diagram of the rapid, scanning, computing ultramicrospectrophotometer for ultra-violet and visible.

The instrument is well suited to other types of measurements also on stained or self-absorbing material if it is a question of dissociated free-cell populations or objects such as isolated cell nuclei. The time saving in the work with large cell series when the automation can be carried as far as in case (2b) is so great—from ten to a hundred times as fast—that in our institution the studies have to a great extent been carried over on to just such tissues or tumour types where the cells can easily be isolated from one another, e.g. bone marrow, ascites tumours, exfoliated cells or cells isolated by mechanical or chemical means.

The measurements of the total mass of the cell, of the nucleus and, respectively, of the cytoplasm have begun to assume more and more importance in the work projects at the institution. For the majority of types of tumours it is unavoidably necessary because of the material's degree of optical inhomogeneity to work with

very small measuring spots and to carry out scanning measurements. The scanning interference microphotometric methods described earlier (SVENSSON, 1957) from the institution are inconvenient when applied to investigation of really large cell populations. Because of that a more rapid scanning microinterferometer, with a built-in electronic integrating system (CASPERSSON *et al.*, 1959) was developed and used to the advantage in population studies on different material. It works with mechanical object movement and gives directly the integral for the mass values over a measured area, affording a considerable shortening of the working time in comparison with earlier procedures.

In this instrument it is the mechanical parts that limit the speed, primarily the recording of the data for each measured point. The need for still more rapid methods was, however, felt as mass measurements have come to play an increasingly great role in the different tumour study projects in the institution. G. LOMAKKA has recently thus developed another type of scanning interference microphotometer with the same high resolution but with an electronic system substituted for the mechanical balancing and in which only the integral of the optical increment for the measured area is recorded continually (diagram in Fig. 5). This increases the

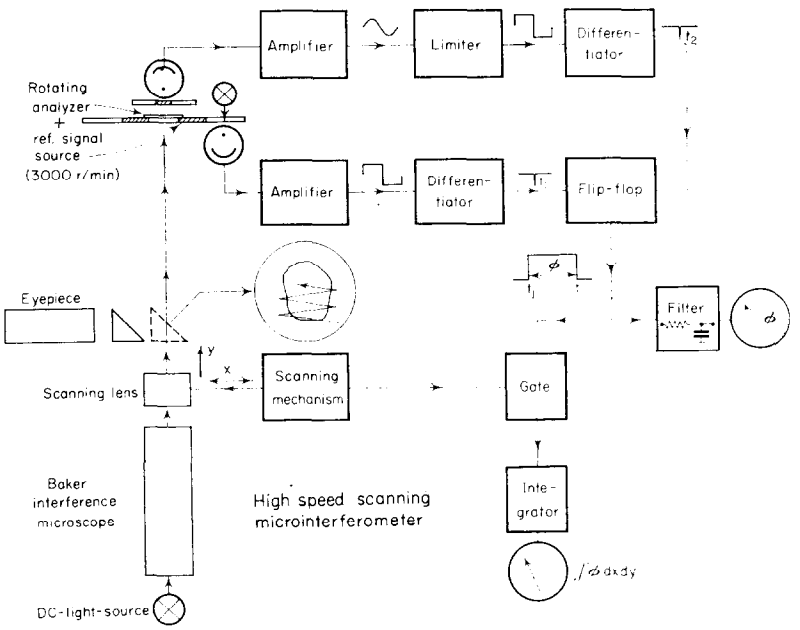

FIG. 5. Diagram of the function of the high-speed scanning microinterferometer.

speed from one to two magnitudes depending upon the type of material. This instrument is only now ready for use, and we have no experience as yet from larger measurement series. It is anticipated that it will greatly simplify the work on different types of biological material.

The determination of mass with roentgen microspectrography is primarily of importance in these problems as a means of calibrating the interference measure-

ments. The loss of time due to the necessity of working with the preparation in vacuum has been largely compensated by the variant recently developed by L. CARLSON (CASPERSSON *et al.*, 1959) where the measurement of the microradiogram can be done without scanning, which increases the speed so that the method for isolated cells has approximately the same productivity as the rapid microinterferometry as yet in routine use. When applied in work on tissue material the roentgen procedure offers great advantages over interference methods, and, generally speaking, roentgen and interference procedures for mass determinations complement each other in a highly satisfactory manner.

Finally, I should like to mention one further automation required by the aforementioned tumour-cell studies developed by a group in our institute. It is a device for measuring optical cross-sections of nucleoli and nuclei in large cell series and on the same cell material measured with instruments mentioned earlier. It is based on a principle we have described earlier (CASPERSSON *et al.*, 1953) and which is evident from Fig. 6. In carrying out the measuring the observer manipulates a

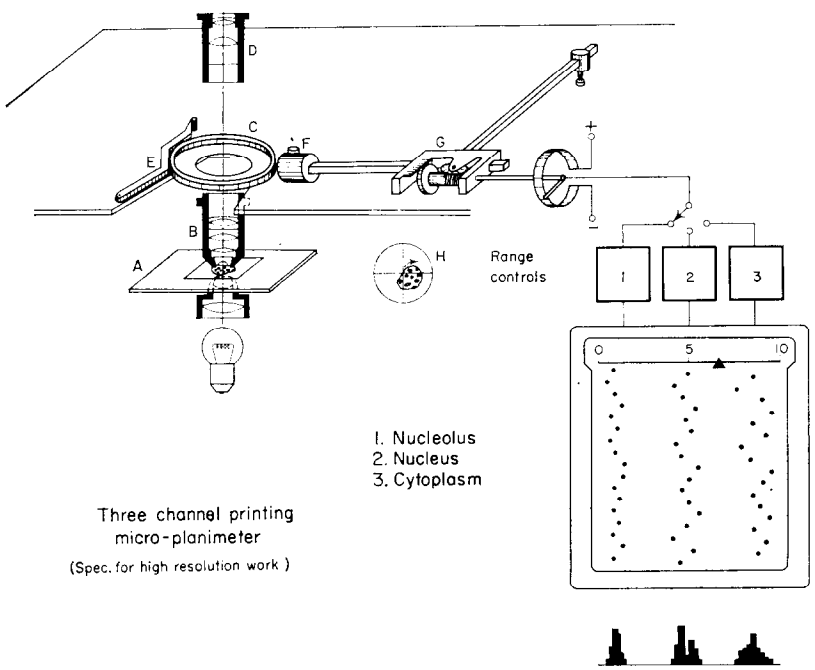

FIG. 6. Diagram of the function of the three-channel printing microplanimeter.

weak lens located in the back focal plane of a microscope objective so that crosshairs in the ocular appear to move around the structure to be measured. The movement of the lens is transferred to a planimeter system, which is coupled to an electronic recording device. As may be seen from the picture, the instrument is equipped with three recording channels. For example, the observer first measures the nucleolus, presses button 1 and the instrument records a dot in the first column. Thereafter the nucleus is measured, button 2 is depressed and a mark is recorded in

the second column. This is followed by measurement of the cytoplasm and registration in the third column. The measurements are accomplished very rapidly, and measurements on series of hundreds of cells can be quickly registered without fatiguing the observer. The instrument was developed especially for measurement of extremely small objects, primarily nucleoli, and the accuracy of measurement is limited only by the resolving power of the objective. Collocation of the histograms is easily accomplished by simple summation of the different columns of points on the recording paper as shown in the diagram. Fig. 7 shows the practical design of the instrument. The apparatus has been in use for about 4 years. In its present form it has proved to be a great time saver in routine work with large cell populations.

These possibilities of working with large populations of cells also give as an interesting by-product the possibility of making direct comparisons of the cytochemical determinations with ordinary chemical macrodeterminations, something that was formerly not possible because of the tremendous difference in the order of magnitude of their working ranges. Figs. 8 (a), (b) and (c) show as examples three

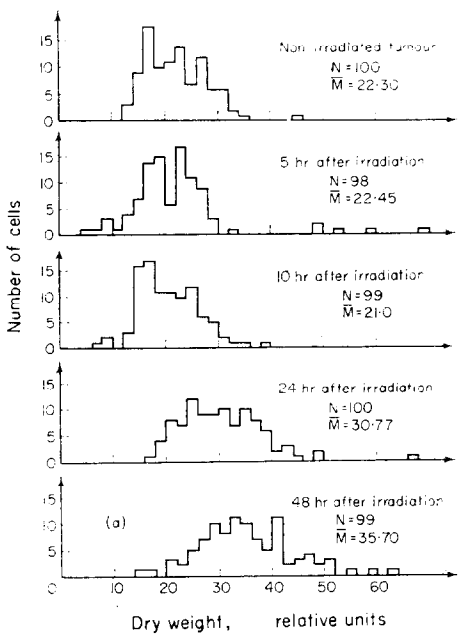

FIG. 8 (a). Frequency distribution of dry mass values of individual cells in the E/Sto tumour at various time intervals after irradiation with 1250 r. Dry mass values were measured by interference microphotometry and expressed in relative units. N = number of cells and M = mean mass value (CASPERSSON *et al.*, 1958).

series of measurements of different cell parameters in ascites cell populations during irradiation carried out with the large microspectrographic equipment, and give the general appearance of the histograms (from CASPERSSON *et al.*, 1958). With measured cell populations of the given order of magnitude the curves are completely reproducible. So much material can be obtained from one ascites tumour-bearing

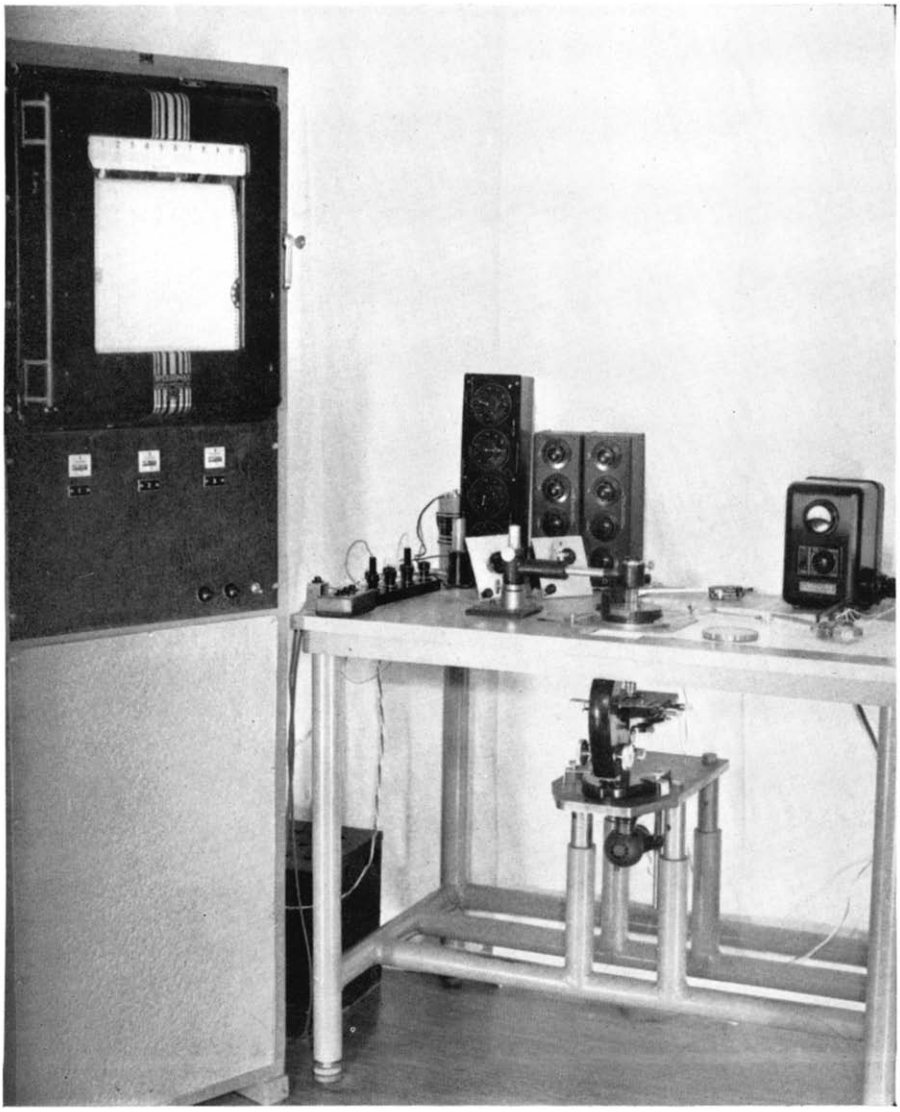

FIG. 7. The printing microplanimeter.

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mouse that even macrochemical determinations can be carried out (as a rule about 10^8 cells). Fig. 9 shows three measuring points for an average cell in the measured population. In the experiment illustrated the average absorption course for the average cell in a specific ascites population has been determined from macrochemical data on the DNA, RNA and protein content. It must fall between the two curves shown. The three measuring points are the data on this population obtained ultramicrospectrographically: as may be seen the agreement is satisfactory.

With regard to the resolving power, of course the metaphase stages represent

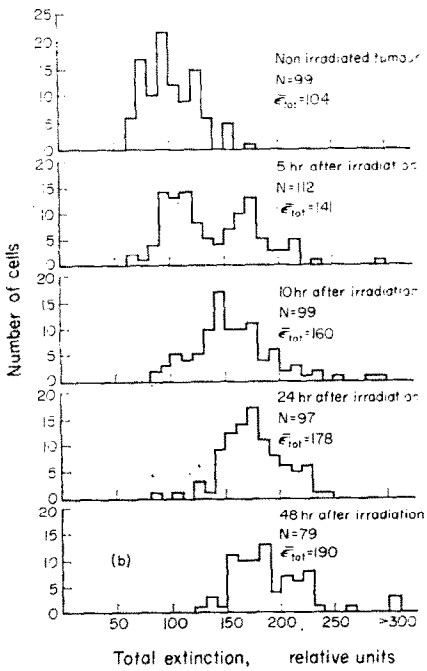

FIG. 8 (b) Frequency distribution of total extinction per cell at 2650 Å in the E/Sto tumour at various time intervals after irradiation with 1250 r. The extinctions were measured by a scanning ultra-violet microspectrograph and expressed in relative units. N = number of cells and $\bar{\epsilon}_{tot}$ = mean total extinction per cell (CASPERSSON *et al.*, 1958).

the most difficult state. The errors in these measurements have been analysed by BARKA (1959). Fig. 10 shows some of his measurements of anaphase and telophase states in ascites tumours with impaired division disturbances. Fig. 11 (from studies to be published by RINGERTZ, KLEIN and CASPERSSON) shows the typical DNA changes in ascites tumour cell populations during the development of amethopterine resistance and, respectively, the subsidence of resistance. It constitutes a good illustration of the value of being able concurrently with measurements of the resting nucleus to study the DNA content in connexion with the division when the DNA synthesis has reached its final stage.

The irradiation series from ascites material shown in Fig. 8 (c) is also an interesting example on the value of metaphase measurement. It shows that in this material with the irradiation dose administered the cell division ceases but that this is not

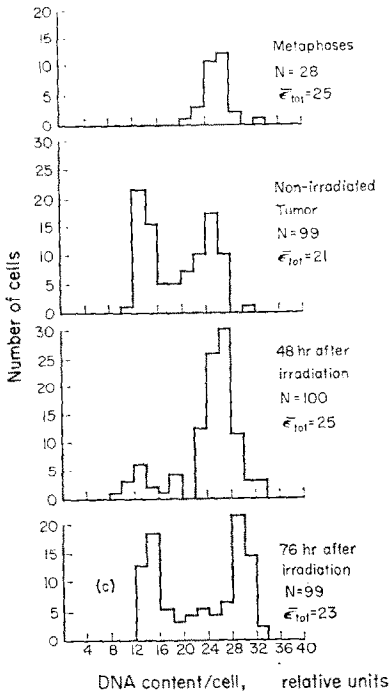

FIG. 8 (c) Frequency distribution of DNA content per cell (ELD tumour) at various time intervals after irradiation with 1250 r. The DNA content per cell was determined by measuring the total extinction per cell at 5460 Å in Feulgen-stained smears by a scanning microspectrograph (CASPERSSON *et al.*, 1958).

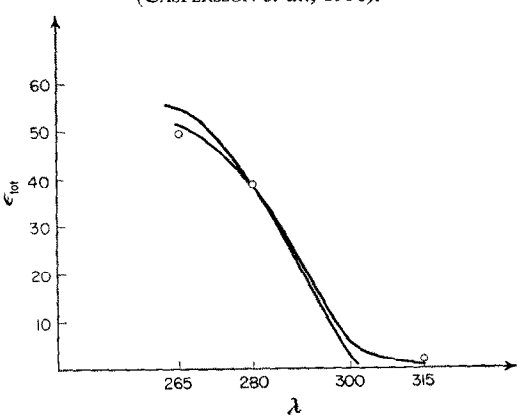

FIG. 9. Comparison between macrochemical and cytochemical determinations on same ascites tumour cell population. Continuous lines give the course of absorption (total extinction per average cell at different wavelengths in ultra-violet) calculated from macrochemical determinations on the cell population. The three points give the average total extinction per cell determined cytochemically.

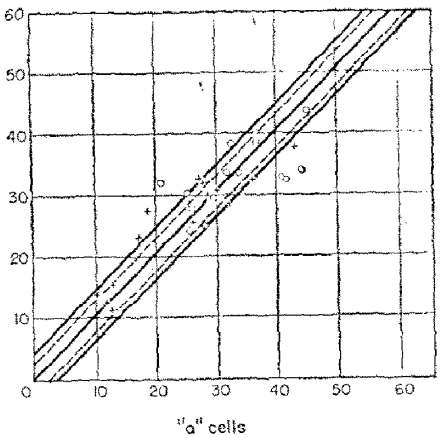

FIG. 10. Ehrlich ascites tumour. Feulgen absorbances of nuclei in anaphase and telophase. The values on the ordinate and abscissa represent the Feulgen absorbances, in arbitrary units, of daughter-cell pairs (BARKA, 1959).

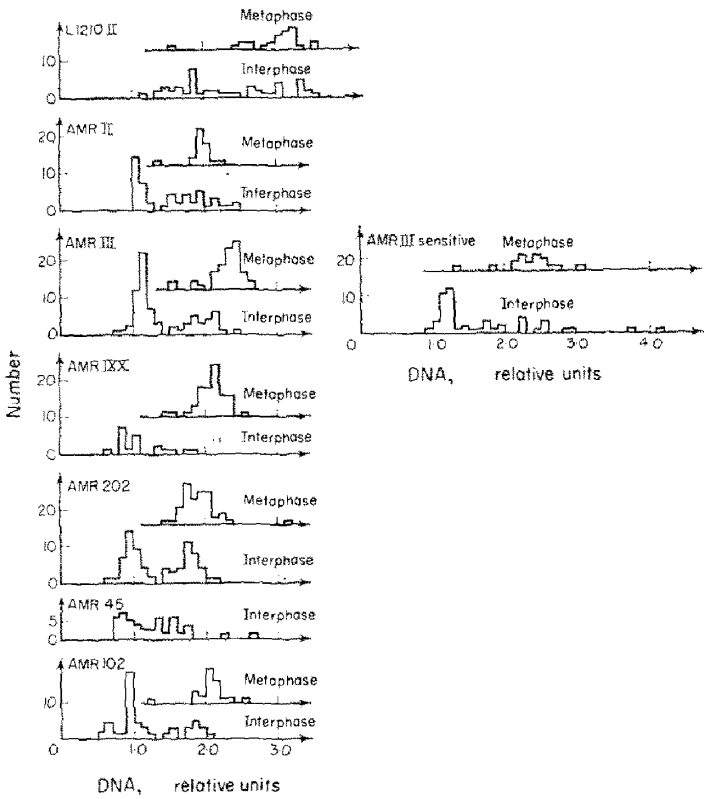

FIG. 11. Changes in the DNA-histograms of the mouse ascites lymphoma L 1210 (top curve) during the development of different amethopterine resistant strains. The strain AMR III reverted to sensitive state (AMR III *sensitive*). Note metaphase curve changes.

caused by a stop of the DNA synthesis. The DNA synthesis in each cell continues here up to the point of readiness for division—the metaphase value—but division does not follow until later when the other disturbances in the cell division apparatus have subsided.

To illustrate the advantages of the new rapid UMSP in the third of the cases mentioned in the beginning, namely the situation when one has to find small numbers of aberrant forms in normal cell populations and also to demonstrate the

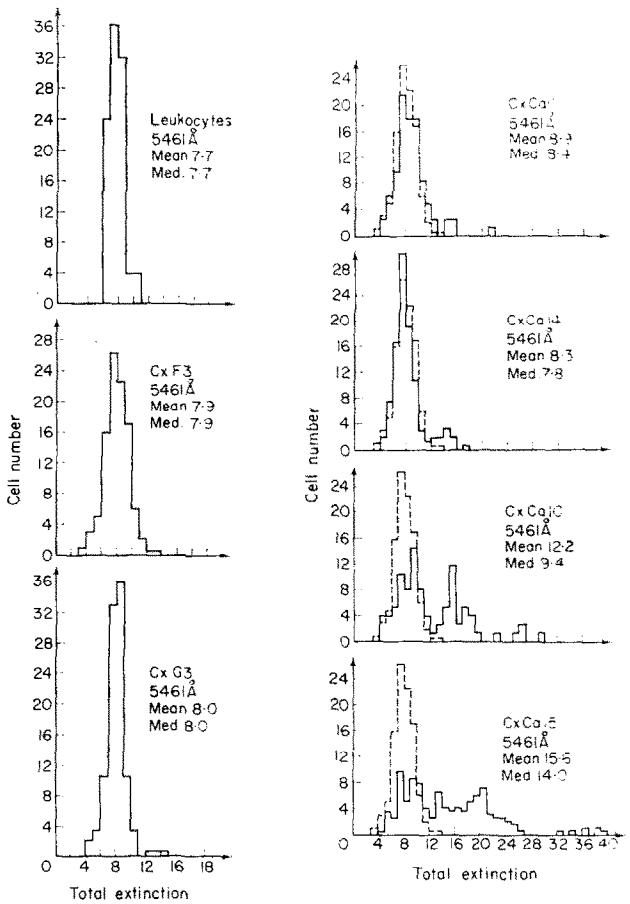

FIG. 12. Feulgen absorbance histograms of exfoliated cell material from human cervix. Left side, from the top: leukocytes, epithelial cells in follicular stage and in gravidity. Right side: the two upper curves, clinically cancer suspected, the two curves below, cancer cases.

reproducibility, I shall take an example from exfoliated cell material from carcinoma of the human cervix. Fig. 12 (left side) shows first closely coinciding maxima in three cases: leucocytes, exfoliated superficial cells from the cervix in follicular stage and cells from a pregnant patient.

The curves shown in Fig. 12 (right side) are from four cases of cervical carcinoma. The dashed curve shows the normal distribution in normal surface cells.

and the other curves show addition of cells with, primarily, increased DNA content. In this material our principal interest was directed toward the RNA and the protein components because on the basis of earlier investigations there is reason to assume that even before cytochemically or morphologically observable disturbances in the DNA component of the genoma appear during the series of processes that lead to tumour development, disturbances in RNA and the protein system of an order of magnitude technically easy to measure could be expected. A greater part, perhaps practically all the larger aberrations that appear in the DNA content in certain advanced tumours are obviously secondary to the disturbances in the division apparatus of the cell. In the aforementioned exfoliative material the RNA content per cell in advanced tumours in all cases thus far investigated was lower on the average than in the normal cells in the vicinity, but the protein content in these was even more markedly reduced.

Fig. 13 shows Feulgen measurements, 2650 Å total extinctions and mass determinations in a normal case and in a case of histologically clearly malignant growth.

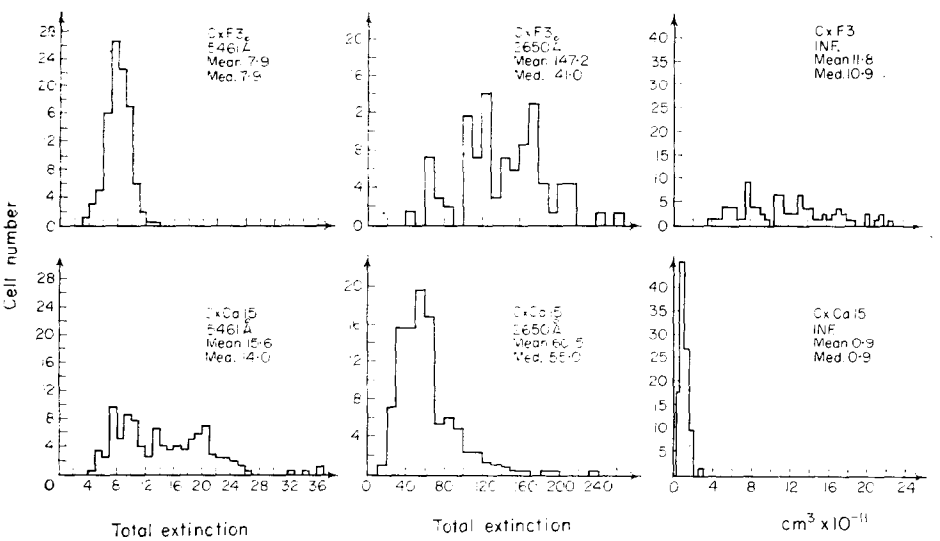

FIG. 13. Comparisons between normal cell population and tumour cell population, cf. text.

It is evident that there is a pronounced right shift of the Feulgen curve, due mainly to a secondary disturbance resulting from general cell division impairment, a left shift of the RNA curve and a very marked decrease in the mean cell protein content (the interference measurements at the extreme right). This change in the protein content that is reflected in the right curve is so great that the RNA concentrations in the tumour cells lie considerably above the normal cell's RNA concentration, on the average, although the absolute quantities of the latter often seem to exceed those of the former.

The human exfoliative material demonstrates well the possibility of working with exceedingly large cell populations and, particularly with respect to DNA, of detecting small quantities of aberrant cells in it. For more penetrating studies of

the prestages in carcinogenesis this cell material is not particularly well suited for a number of technical reasons, but it was selected in a preliminary investigation solely because of the extensive existing morphologic–pathologic experience with interpretation of the cell pictures occurring in this material. Otherwise animal material will be used. The preliminary investigations already support the assumption that pronounced disturbances occur during the tumour development in RNA and the protein system even at a stage when no clear changes in the DNA system can be demonstrated with the methods available at the present time.

I should also like to comment here that the development of microspectrophotometric methods for determinations in model systems of optical constants under the conditions encountered in the histologic and cytologic preparations (CASPERSSON, 1957, 1956a; SVENSSON, 1956; CASPERSSON *et al.*, 1959) also provides a heretofore scarcely exploited possibility for development of calibration systems for histochemical staining and reaction procedures. Numerous histochemical methods intended for quantitative work received severe criticism during the years with justification; the Feulgen method is actually a uniquely favourable special instance because of the characteristic colloid-chemical properties of DNA. The greatest difficulty in the evaluation of histochemical staining methods lies in the necessity for setting up suitable calibration systems or reference systems. Naturally, the continued biologic work on DNA metabolism must also demand constant further development of methods for cytochemical determination, e.g. for different protein substance groups. I should like only to call your attention to the fact that there are excellent possibilities that many histochemical staining reactions can be calibrated by application of the aforementioned model substance procedures, thereby making these methods utilizable for quantitative or at least semiquantitative work.

SUMMARY

The general recording ultramicrospectrographic methods for quantitative cytochemical analysis are as a rule time-consuming when applied to work on especially large cell populations. A series of rapid methods are described, methods particularly suited to investigation of nucleotide and protein metabolism in large cell populations where the cells or cell elements lie free from each other.

The instruments comprise: (1) A rapid scanning high-resolution microspectrograph for both the ultra-violet and the visible spectral region. This instrument is also especially suitable for measurements in ordinary cell material of the Feulgen absorption in large series with high resolution. (2) A recording high-resolution microinterferometer with an integrating device. (3) A modified microradiographic mass-determination method with a special measuring instrument for microradiograms of large cell populations (L. CARLSON). (4) A microplanimeter, designed especially for nucleolar and nuclear volume measurements, with electronic data recording on three channels.

A series of examples is given of the use of these instruments in the study primarily of DNA metabolism problems in tumour cell populations.

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