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Quantitative Cytochemical Methods for the Study of Cell Metabolism

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I.–*General Problems*

During the last decades biochemical research has penetrated deeply into different aspects of the cell metabolic processes. Vast fields, however, are as yet but superficially known, and there, primarily, the processes for which the inhomogeneous reaction milieu of the structural organization of the cell is a prerequisite. This group includes the majority of the synthetic processes, among them protein synthesis, the basic process of growth. Sections of certain reaction chains connected with energy metabolism which are not so markedly dependent upon structure have been possible to imitate in solutions to some extent, i.e. in structureless model systems. Only in isolated cases and to an extremely limited extent has this been possible for the processes of *cell substance formation* during growth, differentiation and different types of cell function. The heterogeneous structural organization of the cell is of decisive significance here, and the various structural elements of the cell comprise different tools that are all necessary to the course of the process. The difficulties in these fields are due principally to the fact that our knowledge of such fundamental factors as the chemical composition of the individual structural elements within the individual cell and their changes during the different phases of metabolism is as yet only in its infancy. The development of *quantitative* cytochemical procedures is indispensible to a more profound understanding of such metabolic processes. That so comparatively little has thus far been accomplished in the field is contingent in part upon the difficulty in developing dependable quantitative methods for work in this dimensional range (Fig. 1). An even more important factor, however, when it is a question of quantitative work within individual cell-structures, lies in the numerous other technical problems, all demanding resolution, which are encountered in addition to the primary, purely chemical question of method. These include, for example, the procedure of defining the element to be studied and differentiating it from its environment; the method for pretreatment of the object for study, which must not involve such damage that the results of the measurements cannot be applied to the conditions of living cell; and the determination with great accuracy of the geometric dimensions of the minute object under measurement. When it is a question of the technical manipulation of the object during the different phases of the work, a multitude of special technical problems arise. One frequently difficult detail is offered by the calibration of the chemical determination method on model systems of known composition and under the conditions prevailing in measurements in the biologic material. The methods or lines of approach that must be applied in the treatment of these technical questions will be denoted as "auxiliary methods", for the sake of perspicuity, in the

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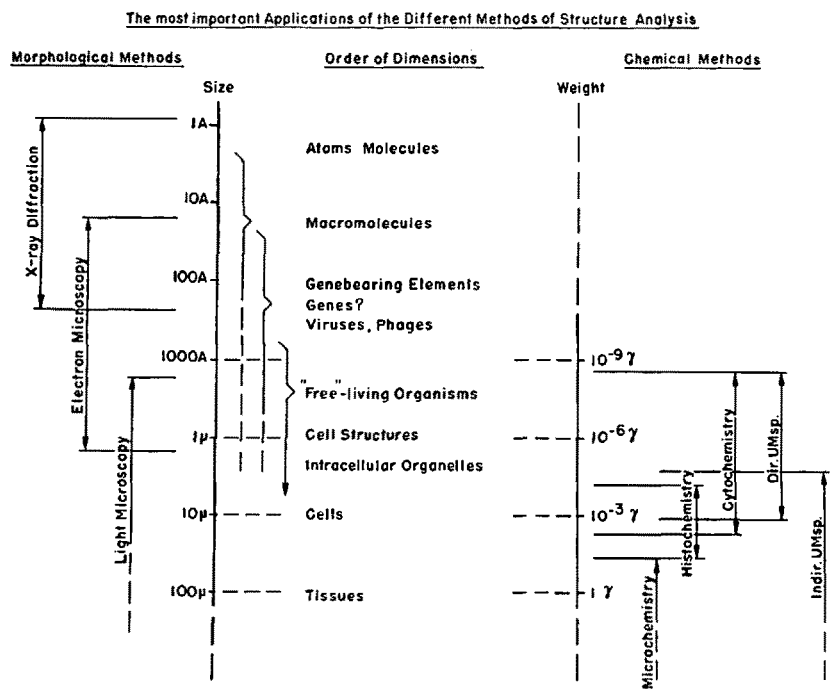


Fig. 1.–The range of application of the cytochemical methods (Dir. UMsp. = Direct ultramicrospectrography).

following discussion. Every cytochemical study aimed at *true quantitative* determination therefore becomes complex and timeconsuming. That which frequently receives little consideration, but is only too clearly evidenced by practical experience, is that the development of the basic measuring techniques represent, as a rule, only a minor portion of the necessary work and instrument investment.

For several of the conceivable cytochemical working lines, however, the necessary auxiliary methods are rather similar in character. Therefore, it might be worth while to attempt to develop more general procedures for solving the collateral technical problems in order subsequently to exploit these results for several different special cytochemical working problems. If such a more general and exacting programme is to be considered justifiable, the methods must satisfy two conditions.

The first is that the methods shall be applicable down to the dimensional region of the cell structures while fulfilling the following special conditions (denoted hereafter as "measuring conditions") for true quantitative cytochemical methods. The second is that the group of methods have sufficient breadth of scope, i.e. that they can be employed for determination of a sufficient number of substances to form a basis for the study of more comprehensive metabolic problems in order to justify the necessary developmental work involved.

The aforementioned measuring conditions which should be satisfied to the greatest extent possible are as follows, when the work is aimed primarily at average cell material from mammals and higher organisms:

(1) Elements with an order of magnitude 1 μ^2 should be accessible to study.

(2) The measuring and preparation for measurement must not damage the preparation to such an extent that the results cannot be transferred to the cell in its natural state.

(3) It shall be possible to carry out the determination with a reasonable and *known* accuracy for the special problem. This assumes (a) definite, known accuracy of measurement (dependent on the measuring instruments), and (b) definite, known accuracy in the analysis of the measured data (dependent on several factors, cf. following). Within the thus far little explored field of cytochemistry there are, to be sure, large groups of problems accessible to study with methods having only low, sometimes even very low, accuracy of measurement, this when the effects studied, e.g. differences in concentration or amounts, are of great magnitude. That the accuracy of measurement in every case be *known*, however, is an absolute prerequisite in quantitative work.

(4) It shall be possible to perform the actual measurements with reasonable speed on the perishable material, and–empirically–by the person, usually a biologist who is most familiar with it and consequently is best able to judge if the object is damaged in any way during the manipulation and, possibly, how such damage may be avoided.

Practical experience primarily from ultra-microspectrographic work with spot measuring apparatus mainly in ultraviolet in the study of a number of biologic and medical problems[1] during the last decade has led us to the *conception that ultra-microspectrography, in a broad sense, satisfies the aforementioned conditions with respect both to possible breadth of scope and to meeting the basic conditions listed herein for a cytochemical study*. Further justification for this view is presented in the following sections II 2 and 3.

Therefore, during recent years *in this institute*, which was specially equipped from the first for this particular purpose, *attempts have been made to develop general working methods on a broad basis in the field, and to find practically applicable procedures in biologic and medical work:* in the first place, for direct microspectrographic study of different metabolic problems, especially those connected with normal or pathologic growth; and, in the second place, to provide a foundation for the technically broader developments required for special types of problems. Presented in the following section, after a technical orientation covering the techniques used in earlier microspectrographic studies, is an outline of the possibilities existing for development of the working range to fulfill the conditions advanced earlier for more comprehensive developmental work: greater breadth of scope and fulfilling of the measuring conditions. The subsequent section treats the technical realization of such measurements.

II.–*The Basis of Ultra-Microspectrography*

(1) *Spot Measurements.* The actual measuring operation in ultra-microspectrography consists of determination of the absorption coefficients in wide regions of the electromagnetic spectrum of small spots within the cell, and the calculation of the absorbing substance content of these regions from the course of the absorption curve. An optical ultra-microspectrograph may be described as a combination of a spectrograph and a microscope system. The object is illuminated with monochromatic light, and the image, enlarged by the microscope, is projected into a photoelectric cell. The diaphragm of the photoelectric cell isolates the spot to be measured in the image. In certain problems the measurement may be done photographically, i.e. the object is photographed in different wavelengths under standardized conditions, and the plates are analyzed in a photographic densitometer.

[1] For survey see T. CASPERSSON, Skand. Arch. Physiol. *73*, Suppl. 8 (1936); J. Roy. Micr. Soc. *60*, 8 (1940); Naturwissensch. *28*, 514 (1940); Chromosoma *1*, 562 (1940); *Cell Growth and Cell Function* (W. W. Norton & Co., New York, 1950).

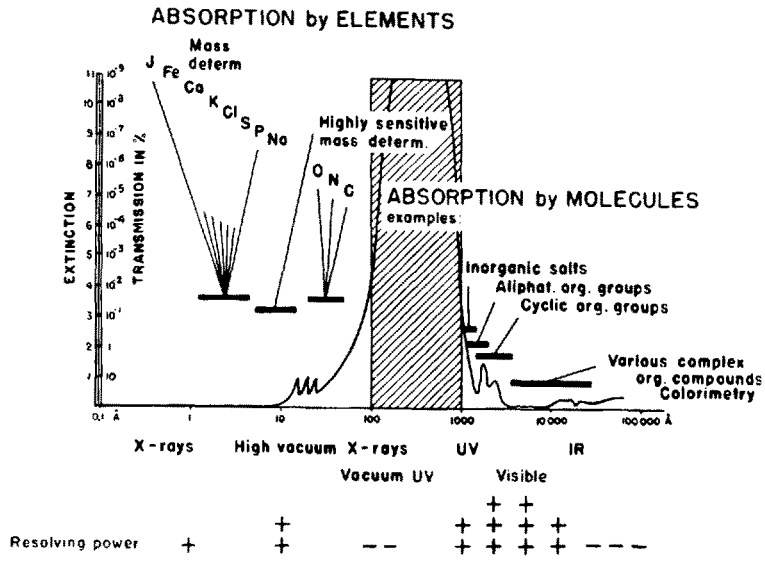


Fig. 2.–The breadth of scope of ultra-microspectrography, used as a tool for quantitative cytochemical work. The curve shows the general course of the absorption for a 5 μ layer of organic tissue.

For work in the X-ray region instruments have been developed with specially constructed X-ray tubes, permitting direct skiagraphy of the object in monochromatic X-ray radiation obtained either with the aid of suitable anticathode material in combination with filters or via monochromatization with curved crystals. Recently, the resolving power of these methods has been increased to the point where direct exploitation for ordinary cytochemical problems has become feasible. The absorption coefficient for the spot to be measured is determined by measuring the photographic high resolution plate in an optical microspectrograph.

For information on practical design and construction of microspectrographs for spot measurements reference is made to CASPERSSON[1] for instruments for the optical region. These works also contain references to earlier studies where the method is applied. About X-ray instruments see ENGSTRÖM[2], and for high resolution X-ray-procedures LINDSTRÖM[3].

(2) *Breadth of Scope.* For practical reasons in the discussion of the breadth of scope it is expedient to divide the field. By *direct* ultra-microspectrography is meant the determination of absorption coefficients of cell structures *in situ* within the cell. *Indirect* microspectrography involves measurement of absorption spectra of objects after they have been isolated with suitable procedures from the cell and possibly subsequently collected into larger quantities, which do not, however, attain the working range of ordinary spectroscopy. These are then treated with chemical agents, e.g. enzymes or colorimetric reagents (ultra-microcolorimetry). The latter methods are little used as yet, but are extremely well suited to further development and have already yielded interesting results. In the following sections principally the direct microspectrographic methods will be treated.

One distinctive feature of direct ultra-microspectrography, in comparison with ordinary macro-scale spectrography, is that the object has a given form, size and composition from the start, meaning that for the individual absorbing objects measured both the concentration and the thickness of the layer are given from the beginning. In ordinary colorimetry and spectrography the arrangements are usually so chosen that the major portion of the measurements takes place within the light transmission range from 70 to 15%, corresponding to extinction coefficients from 0·15 to 0·8, where it is easiest to measure. If a solution falls outside this range, another concentration or a different cuvette length is selected. Such adjustment is impossible in measurements made directly on the biologic object, and thus it must be possible to extend the ultra-microspectrographic work to include an extinction range of much greater breadth. Experience has demonstrated that within the optical region it is expedient to construct a routine apparatus for the extinction range from 0·05 to 1·5, but that above and below this range it is advisable to use measuring equipment designed along other lines than those followed in construction of the routine apparatus. Even in the early optical spectrographs for spot measurement, measurements were possible within this range.

The theoretical breadth of scope for ultra-microspectrographic work on average biologic material with resolving power and measuring range corresponding to that for equipment with the characteristics mentioned earlier can be easily estimated and is apparent from Figure 2. This figure shows diagrammatically the course of the absorption for a 5 μ thickness of a tissue homogenate from average mammalian tissue. The wavelength scale is logarithmic and covers the range from the X-ray region to the infrared. The resolving power for existing instruments appears below the diagram: ++ denotes that a resolution of 1 μ can be attained. This is the minimum for work on intracellular structures. In ultraviolet, visible and infrared the limit is determined for that which can be attained by the wavelength of the light. Theoretically it is impossible to record an absorption spectrum that can be analysed

[1] T. CASPERSSON, Skand. Arch. Physiol. *73*, Suppl. 8 (1936); J. Roy. Micr. Soc. *60*, 8 (1940); Chromosoma *1*, 562 (1940); Exptl. Cell Res. *1*, 595 (1950); *Cell Growth and Cell Function* (W. W. Norton & Co., New York, 1950).

[2] A. ENGSTRÖM, Acta Radiologica, Suppl. *63* (1946).

[3] B. LINDSTRÖM, Exptl. Cell Res. *6*, 537 (1954); unter publication (1954). – B. LINDSTRÖM and G. MOBERGER, Exptl. Cell Res. *6*, 540 (1954).

from a particle which is smaller than 2 to 3 times the wavelength of the light. In the visible and ultraviolet spectral regions optical systems are available which are perfected to the point that this theoretical limit can almost be attained, and the smallest measurable particle thus lies well within the desired limit. In the infrared region optics have been developed, although not with quite the same numerical aperture as in the aforementioned spectral region. Even with ideal optics, however, it is not theoretically possible in this region to attain the resolution necessary for direct microspectrography of cell details. The indirect microspectrographic procedures, however, demand lower resolution, and infrared microspectrography can be used to advantage there.

In the easily accessible part of the ultraviolet region, down to 2000 Å, a very large number of organic compounds included in the cell substance have selective absorptions which can be exploited. The best examples are provided by the nucleic acid group and the cyclic amino acids for which the absorptions per unit of mass as well as the concentrations in the biologic material are of such an order of magnitude that they are easily accessible for work with relatively simple instrumental equipment. This has also been utilized in numerous studies of the nucleotide and protein metabolism (CASPERSSON[1] with references). Among other groups of substances of biologic interest with bands in this region, and which may occur in concentrations such that the absorptions lie within the most easily accessible range for routine spectrography, we might mention sterols, unsaturated fatty acids, purines, steroid hormones, adrenaline and its precursors, thyroxine and related substances, large groups of pigments occurring in biologic material and lignine. In longwave ultraviolet and shortwave visible regions numerous substances related to chlorophyll and haemoglobin have narrow, high absorption bands. The same is true of a number of respiratory ferments and coferments. These latter substances occur in the majority of cases in such minute quantities that special methods are required in order to measure the low absorptions. Within the visible spectral region few substances have sufficiently high absorptions to fall within the working range of routine microspectrography. Primarily chlorophyll and haemoglobin and their derivatives should be mentioned in this connection. The ultra-microcolorimetric methods classified under indirect microspectrography are applicable also in this region.

Below 2000 Å the absorption of organic material increases especially rapidly. The selective absorptions within this region are principally conditional upon atom configurations which are of limited interest to biologic metabolic problems in general. Moreover, the sources of error in the measurements increase since the refractive index of the material rises especially rapidly, with the result that the absorption curves become extremely difficult to interpret. This region is indicated by oblique-line hatching in the figure. On the shortwave side of this region there are absorption discontinuities usable for ultra-microspectrography. These are conditioned by electron jumps in the inner electron orbits in the atoms, and can be utilized practically also for biological work (ENGSTRÖM[1]). They are specific for the different chemical elements and practically completely independent of the chemical linkage of these elements. This last factor is of particular importance to spectrographic work. A technique has also been evolved for a total dry substance determination of water-free biologic material in this region, based on the assumption of a certain average composition of the biologic material (ENGSTRÖM and LINDSTRÖM[2]). Through LINDSTRÖM's studies during the past few years[3] the resolving power and the sensitivity of the method for dry substance determination has been increased to the level necessary for real cytochemical work. The sensitivity of the method has been increased, so far as to make possible work with histological sections of ordinary thickness. The determination of elements was formerly carried out with low resolution, which can, however, be greatly increased along the aforementioned lines developed by LINDSTRÖM. The region for elements with atomic numbers below 8 is also accessible, although the technique is still relatively complicated (LINDSTRÖM and MOBERGER[4]).

In connection with this dry weight determination another method should also be mentioned. Interference microscopy measures optically differences in optical path in different parts of the cell material. Through this it is possible to determine dry weight on condition that there is a certain composition of the biologic material. An optical spot measuring procedure has been applied to biological objects mainly by DAVIES *et al*[5]. In principle the procedure involves conversion by aid of suitable optics of optical path differences between rays passing different parts of the preparation into differences in light transmission. Therefore, practical work with the method can also be carried out photo-electrically directly after introduction of interference optics, with the microspectrographic apparatus described[6]. Generally seen, the method gives the same results as the

[1] T. CASPERSSON, *Cell Growth and Cell Function* (W. W. Norton & Co., New York, 1950).

[1] A. ENGSTRÖM, Acta Radiologia, Suppl. *63* (1946).

[2] A. ENGSTRÖM and B. LINDSTRÖM, Biochim. Biophys. Ac. *4*, 351 (1950).

[3] B. LINDSTRÖM, Exptl. Cell Res. *6*, 537 (1954); under publication (1954). – B. LINDSTRÖM and G. MOBERGER, Exptl. Cell Res. *6*, 540 (1954).

[4] B. LINDSTRÖM and G. MOBERGER, Exptl. Cell Res. *6*, 540 (1954).

[5] H. G. DAVIES, Exptl. Cell Res. (1952). – H. G. DAVIES, J. CHAYEN, M. H. F. WILKINS, and L. F. LACOUR, in press. (1954).

[6] T. CASPERSSON, Anais da Ac. Bras. *26*, 199 (1954). – T. CASPERSSON, L. CARLSON, and G. SVENSSON, Exptl. Cell Res. *7*, 601 (1954).

previously described X-ray mass determination method and requires largely the same assumptions with respect to the average composition of the object, which gives it the same limitations in that respect. In addition, there is the difficulty due to the fact that with high concentrations the optical path difference is not always a linear function of the quantity of dissolved substance. On the other hand, the method has the very great advantage of being applicable to undehydrated preparations. Therefore, the two procedures are excellently complementary. Interference microscopy has been discussed at this place since the difficulties encountered in its application for study of biologic material, which are contingent upon its inhomogeneity, are to a great extent the same as in actual microspectrography.

Summary: Ultra-microspectrographic methods have qualifications adapting them to determination of a large number of biologically important substances with selective absorptions in the optical region. In addition, they may be exploited for certain element determinations and, under certain conditions, also for dry-weight determinations on both dried and undehydrated preparations (the latter in combination with interference microphotometry). With *indirect* microspectrographic procedure they are also utilizable for reading colorimetric reactions in ultramicro scale, extraction or enzyme digestion experiments, etc. The procedure thus has sufficient *breadth of scope* to form a basis for a relatively general cytochemical working method with appreciable range of applicability.

(3) *Measuring Conditions.* The first of the special technical conditions mentioned in section I, that should be satisfied by a quantitative cytochemical method, i.e. that the resolving power should be better than $1\,\mu^2$, has been shown to be fulfilled for spot measurements within the optical region up to shortwave infrared and in parts of the X-ray region. Interference microscopy also permits a similar resolving power. Because of the structure of the average biologic material it would be meaningless in the most common cases to work with resolutions under $0{\cdot}1\ \mu^2$. This entire dimensional range, $1{\cdot}0$ to $0{\cdot}1\ \mu^2$, is easily accessible in the most important working region, middle ultraviolet.

Condition 2, that the measuring operation and the preparation for it should not involve greater damage to the preparation than that the result might be transferred to the cell in its natural state, can be met to an appreciable extent on condition that–particularly in the ultraviolet region–the irradiation of the object during the measurement and preparation for it be kept at a minimum. If this condition is observed, moreover, ultraviolet microspectroscopy is in an unusually favourable position among cytochemical working lines in that, to a certain extent, studies are possible even *directly* on the living material. As a rule, the procedure in this is such that the actual examination is carried out on fixed material, whereafter, with especially cautious technique involving a minimum of irradiation and manipulation, the extent to which results obtained can be applied to living material, is investigated as a separate step in the work.

The pretreatment of the object to satisfy measuring condition 2 demands, as a rule, a relatively circumstantial technique. This procedure is among the most time-consuming of the different auxiliary methods, referred to earlier, for the different types of work. Particularly with respect to the work in ultraviolet especially extensive experience has been gained on widely varying types of material[1].

Condition 3, that the determination should be carried out with reasonable and *known* accuracy for the particular problem, involves two factors. The first factor (3*a* above in section I) presupposes that the actual measurement in the spectrograph takes place with definite, known accuracy, which is contingent upon the measuring instruments. This factor is easily controlled. The second factor (3*b* in section I) presupposes definite, known accuracy in the *analysis of the measured data.* This in turn presupposes principally the following factors: α) the optical characteristics of the individual object measured are known; β) the influence of certain sources of error given mainly by light refraction and scattering in the object can be controlled; γ) it can be demonstrated that the object fulfills the conditions–which are relatively complex and strict especially for the optical region–that theoretical treatment of the pathway of the beam in the optical spectrograph as shown must be fulfilled if measurement and analysis are to be possible at all[2]; and δ) the optical constants for the type of substances to be measured are known under the conditions prevailing in the object during measurement. Of these conditions the earlier-mentioned spot measurement spectrographs satisfy in the first place factors α and β, and, with certain limitations, factors γ and δ for work in ultraviolet with nucleic acids and certain proteins. For more general spectrographic working methods, however, the development of special devices in which were possible to carry out the special determinations demanded by factors γ and δ *is unavoidably necessary.* In the literature it is possible only in extremely rare cases to obtain values for the optical constants in question under the conditions prevailing in the cell. The reason is that the manipulation of model systems with the extraordinarily high concentrations and thin layers required offers very great technical difficulties. In each more comprehensive spectrographic working system it must be assumed that the observer personally

[1] T. CASPERSSON, *Cell Growth and Cell Function* (W. W. Norton & Co., New York, 1950).

[2] T. CASPERSSON, Skand. Arch. Physiol. *73*, Suppl. 8 (1936); *Cell Growth and Cell Function* (W. W. Norton & Co., New York, 1950).

performs such determinations, and, therefore, an ultra-microspectrographic set of instrumentation must also include special equipment for this purpose.

Condition 4, which demands that the measurements shall be possible to perform with reasonable speed and be sufficiently simple so that the biologist who is most familiar with the material can operate the apparatus, even with a limited previous physical-technical experience, is not fulfilled by the earlier high resolution instruments.

Summary: Even the early ultra-microspectrographic procedures fulfilled certain of the most important measuring conditions listed in the foregoing paragraphs. In order to satisfy the others it is necessary to evolve new methods. The practical development of these methods is presented in section IV below.

III.–*Area Measurement ("Scanning Measurements")*

In the foregoing chapter it has been shown that ultramicrospectrography, due to its breadth of scope and the good possibilities existing for the construction of equipment that fulfills the primary conditions of measurement, possesses qualifications for serving as a foundation for general quantitative cytochemical work on several types of cytometabolic problems.

Experience from the practical application, principally in ultraviolet, however, has shown that biologic problems in general necessitate another measuring procedure in addition to "spot measurement", i.e. "area measurement" or "scanning measurement" which is the term preferred in the continuation. This is of such great practical importance that it must be treated in detail. Spot measurements give information only on the composition of an extremely small part of the object. For broader cytochemical problems this is not sufficient; it is necessary to determine the total content of a certain absorbing substance within a certain optical *cross-section area* of the preparation, as a rule one cell, a cell nucleus or another defined part of a cell.

This "area measurement", i.e. *measurement of the total quantity of an absorbing substance within a defined region* in the preparation can be performed with spot measuring equipment by measurement of a very large number of separate spots. For measurement of a mammalian cell, for example, such a determination, photographic or photoelectric, requires, however, an enormous number of separate measuring spots because of the inhomogeneous character of the average cell material. For example, if the determination includes three wavelengths in ultraviolet, analysis of a single cell with spot measurement equipment requires about two weeks' work.

For the general biologic work, where it is usually a matter of collecting observation material not only from inhomogeneous cells but also from inhomogeneous populations of inhomogeneous cells, this circumstantiality in area measurement has meant such a fundamental limitation that *this factor has been the most essential reason why the broad potential working range offered by ultra-microspectrography has thus far been exploitable only for relatively very limited problems.*

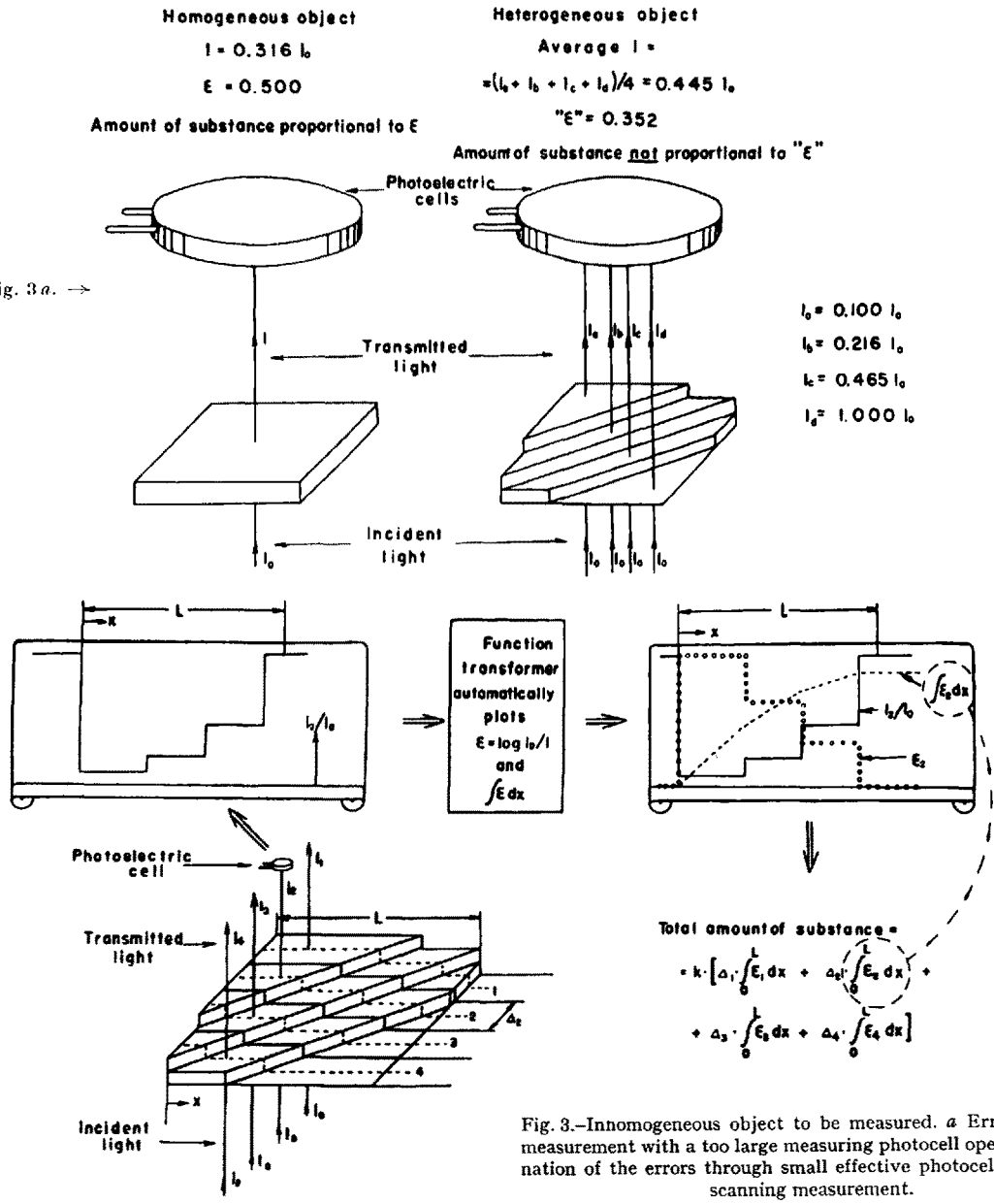
For practical work this question of area measurement, i.e. the mastery of the inhomogeneities of the material through special, very rapid measuring procedures, is a central problem of such great importance that it dominates the development of the entire instrumentation necessary for microspectrography in general. It is only through the technical advances of recent years that this question has been resolved by automatization of both the measurement (scanning) and the data analysis by means of instruments which afford more than one hundred-fold saving of time and are at the same time sufficiently simple and easily operated for routine biologic work[1].

The problems treated in this type of measuring work are illustrated by the following typical example: The quantity of nucleotides in the cytoplasm in a 5 μ thick section through a mammalian cell is to be determined. The section passes the nucleus, and the nuclear nucleotides will not be included in the determination. The cell borders on other cells on all sides and a minor area in the neighbourhood is free of substance (compare Fig. 4). Detailed measurements of the course of the absorption curve in several different spots in the cell has shown that the curves giving the logarithm of extinction plotted against the wavelength have a parallel course, meaning that the percentual composition of the different portions of the cell is the same. For reasons that are discussed in detail elsewhere, the most expedient subsequent procedure is to carry out area measurements in three wavelengths over the entire cytoplasm: one at the nucleotide absorption maximum, one at the tyrosine-tryptophane absorption region, and one at approximately 3150 Å. The two longwave measurements serve as a control that the percentual composition actually does not vary within the small portions of the cell and that certain types of measuring error have not occurred.

The only possible way to perform this measurement is to measure with high resolution an extremely large number of individual spots in the preparation, sufficient so that they surely represent together the cross-section of the entire cell. This is succeeded by recalculation of the individual transmission values of each individual spot to extinction and summation of all these separate spot extinctions over the cell area.

[1] T. CASPERSSON, Exptl. Cell Res. *1*, 595 (1950); Genetica Iberica *2*, 277 (1951); Exptl. Cell Res. *7*, 598 (1954); Verh. Anat. Ges. Münster *1954*. – T. CASPERSSON, F. JACOBSSON, and G. LOMAKKA, Exptl. Cell Res. *2*, 301 (1951). – T. CASPERSSON, F. JACOBSON, G. LOMAKKA, G. SVENSSON, and R. SÄVSTRÖM, Exptl. Cell Res. *5*, 560 (1953). – T. CASPERSSON, F. JACOBSSON, G. LOMAKKA, and G. SVENSSON, Exptl. Cell Res. *5* (1954).

The procedure has been suggested in the literature, and even carried out in many cases, of widening the aperture of the light-sensitive cell and projecting a large portion of the object or even the whole surface to be measured into the aperture of the photo-electric cell. This procedure is not permissible. Figure 3*a*, left half, presents a diagram of a model object illuminated with a wide light beam. The entire image of the extended object is projected via an optical system (not sketched in on the figure) into the aperture of a measuring photo-electric cell. *If the object lacked inhomogeneities* this procedure would be permissible, and the extinction coefficient would then be a true measure of the quantity of substance passed by the light rays. The right half of the diagram shows the same amount of substance spread inhomogenously over the same cross-section area. When measured in the same manner, it gives a value for the "extinction coefficient" which is not a measure of the total quantity of absorbing substance because the light transmission is a *logarithmic* function of the quantity of substance through which the light passes. In the case cited there is a 30% error in measurement. The case demonstrates a fairly average degree of inhomogeneity in the biologic material. It is quite usual to encounter much more inhomogeneous objects. Only in very rare cases, e.g. in the squamous epithelium of the buccal mucosa, which unfortunately is frequently selected for demonstration, are the conditions in the first mentioned case (fig. 3*a*, left) approached. The figure thus illustrates how an error originates when excessively large measuring areas are used, i.e. how too *low resolution* introduces measuring



Pig. 3*a*. →

← Fig. 3*b*.

Fig. 3.–Innomogeneous object to be measured. *a* Errors arising in measurement with a too large measuring photocell opening; *b* Elimination of the errors through small effective photocell opening and scanning measurement.

errors of great magnitude. It is especially important that this error is greatly dependent, both with respect to relative and absolute magnitude, upon the level of the absorption, which means, for example in the case cited in illustration, that its magnitude will be entirely different in the different wavelengths. If measured data in which this error is inherent are used in drawing up an absorption curve, both its *form and the absolute values of the individual extinctions will be erroneous.* The only way to avoid this source of error is to work with such high resolution and with such a large number of measuring spots that the separate inhomogeneities are spot measurements and demand an individual measuring spot in average cell material of an order between 0·1 and 1 μ^2.

The necessity of having a large number of measuring spots, each to be individually recalculated, leads naturally to the evolution of automatic scanning procedure. Figure 3*b* (and 4) shows the principle of the measuring procedure used in the spectrograph, described hereafter, which, fully elaborated with automatic data analyzer, results in a several hundred-fold increase in working speed in comparison with older procedures. The object is the same as in Figure 3. The measurement takes place through a very small measuring spot, symbolized by a small photo-electric cell (the optics are not sketched in), which is moved over the object while a recording instrument continuously records the light transmission as a function of the movement over the object. If the recorded transmission curve is converted point by point to a magnitude proportional to the quantity of substance through which the light beam has passed–in the optical spectral region, the extinction coefficient–the area under the extinction curve from the point where the moving beam enters the object to that where it leaves it on the other side, is a measure of the quantity of substance passed by the measuring beam during its passage across the object. The sum of these sums for a suitable number of scanning lines gives, multiplied by the distance between the scanning lines, the value sought for "the total extinction" for the entire area. Since, as in the example selected, there are in the practical work on biological material almost always certain parts of the scanned area (e.g. cell nucleus, surrounding tissue) which should not be included in the determination, it has proved necessary in practice always to *record each individual measuring spot* and, in the data analysis of the transmission curve to plot also the extinction curve and its integral in every detail. One effect of this condition is also that it must be possible to correlate each point on the recorded curve with the measured spot in the object with an accuracy better than 0·3 μ in the object, which puts considerable demands on the mechanical construction elements of the scanning ultra-microspectrograph.

The practical design of the scanning device is a matter of compromise. On the one hand, it is desirable to have the greatest possible rapidity in the work with retention of high resolution and accuracy of measurement, without excessive irradiation of the object. On the other, the apparatus must not be too complex or difficult to operate for use in routine biologic work in the extremely large measurement series usually encountered in this field. It is unavoidable for several reasons that each individual measuring spot shall be identifiable in the recording after the completion of the measuring operation. There must be a guarantee that no point outside the object to be measured is included, e.g. from adjacent cells or, as in

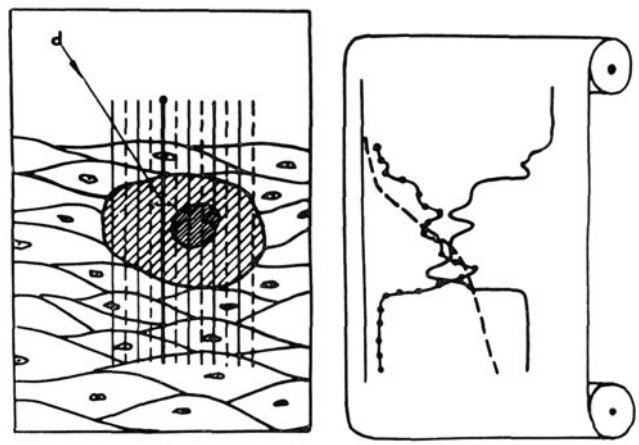


Fig. 4.–Recording scanning of ordinary object with curve analysis.

the example cited, the cell nucleus and Figure 4 represents in fact the most common type of objects encountered. In the overwhelming majority of instances the work must be carried out in several wavelengths, wherefore it is necessary, for the control of the exactitude of the work, to leave the possibility open, after the completion of the measurements, for the comparison for each individual measuring point of the absorptions in different wavelengths. A common source of error lies in the preparation changing form during the course of the measuring. In the practical design of the scanning equipment, direct two dimensional scanning was not used despite the great saving of time it would seem to allow. The effort to limit with certainty the measurement to the intended object, which is usually irregular in shape would thus become too great and time-consuming. Moreover, the necessity of being able subsequently to identify and verify data from individual measuring spots is more difficult to meet with a reasonable set up of equipment. In addition, the risk of excessive irradiation of the object is greater. The apparatus described hereafter was instead designed so that the area may be traversed by an adjustable number of scanning lines with easily adjustable distance between lines and individual

recordings are made of each scanning line. Practical experience from mammalian and biologic material during the three years the apparatus has been in constant routine use has also brought the somewhat surprising realization that, even in quite inhomogeneous cells where very high resolution is required, the *degree* of inhomogeneity is frequently so similar over large sections of the cell that only a small number of scanning lines are required per cell in order, together with the value of the optical cross-section area of the cell, in μ^2, to give the total quantity of absorbing substance.

Through the use of automatic scanning with transmission recording the gain in time in practical work is from ten to a hundred-fold, according to the nature of the material. The further desirable decrease in working time is attained through automatic data analysis. A computer has been developed for this purpose. It performs the transformations and integrates the recalculated curve, recording the results either concurrently with the performance of the measurements in the microspectrograph or as a special operation later. The latter is often more expedient in a laboratory where several persons work with the instruments. For the different fields of work mentioned earlier: optical spectrography, microphotometry of X-ray radiograms and scanning interference microphotometry, the function used in conversion differs. In the first instance it is a simple logarithmic function; in the second an empirically derived function, the density curve for the plate material; in the third case a trigonometric function. For this reason the computer was designed so that the transformation of the function may be carried out according to an arbitrarily adjustable function so that the results from all types of measuring procedure can be dealt with.

It is self-evident that the inhomogeneity problems in interference microscopy and microphotometry are of the same nature as those mentioned earlier for microspectrography. As a rule, they present still more difficulty. Because of the course of the function giving the relation between the light from the measured element and the optical pathway the risk of errors in measurement with too low resolving power, if the path differences are of certain common orders of magnitude, is greater than in absorption spectrography. The situation differs with the optical arrangement chosen. Only in occasional objects, such as the aforementioned buccal epithelial cells, erythrocytes, etc., or in objects so thick that the inhomogeneities counterbalance one another– a case that is of little practical value, however–is it possible to work with spot measurements which can be carried out visually with the ordinary interference microscope.

IV.–*Instrumentation for General Quantitative Cytochemical Work*

In order to exploit the possibilities afforded by direct microspectrography, according to section II:2, devices are necessary for automatic measurement as described in section III. These must fulfill the principal measuring conditions: Measurement with resolution better than 1 μ^2 with known accuracy, without damaging the object, and with simple, rapid and easily operated equipment. In addition, there are the aforementioned *auxiliary methods*. The most important of these serve the following purposes: Preparation of the object in a manner such that its optical characteristics after the treatment are sufficiently known so that the data obtained in a spectrograph with definite optical characteristics can be analyzed with the desired degree of accuracy. This field includes also the mechanical isolation, microdissection, ultracentrifugation, etc., and the creation of a "free field" near the site of measurement to provide a sufficiently accurate measure of the intensity of the incident light. From the discussion of scanning methods above it is evident that various types of procedures for separation of individual cells for determinations of total quantity, together with methods for standardization and control of the cutting of microtome sections are indispensable. The necessity to be able to study the same object in different wavelength regions also places special requirements on the auxiliary procedures. In such work it must be possible to relocate definite points in the object, in repeated measurements in the same object. The measurement of the dimensions of the object plays an important rôle, and for certain problems, especially in work with small objects, this has brought about the development of special methods. In order that the measured curves obtained in the microspectrograph may be broken down into their separate components, the optical characteristics of the measured object must be such that they are suitable for measurement with the technically realizable apertures in the spectrographs, and the optical characteristics of the constituent components must be known under the conditions of measurement. Both these factors require special instrumentation.

Practical Design. The scanning measuring procedure in itself demands an appreciable amount of equipment. To avoid duplication the instrumentation has been so planned that in one central instrument, *the universal microspectrograph*, the greater proportion of the more costly instrumental parts have been assembled into one unit in a manner permitting their exploitation for several different steps in the work. Moreover, the universal microspectrograph has been designed on a "building block" system so that various devices may be included as auxiliary parts, e.g. equipment for measuring especially high or low absorptions, for dichroism measurement, for measurement on model substances of the range of validity of LAMBERT-BEER's law, devices for recording the absorption dependent on the wavelength, measurements of ultra-microcolorimetry cuvettes, and the data analyzers. The high resolving power

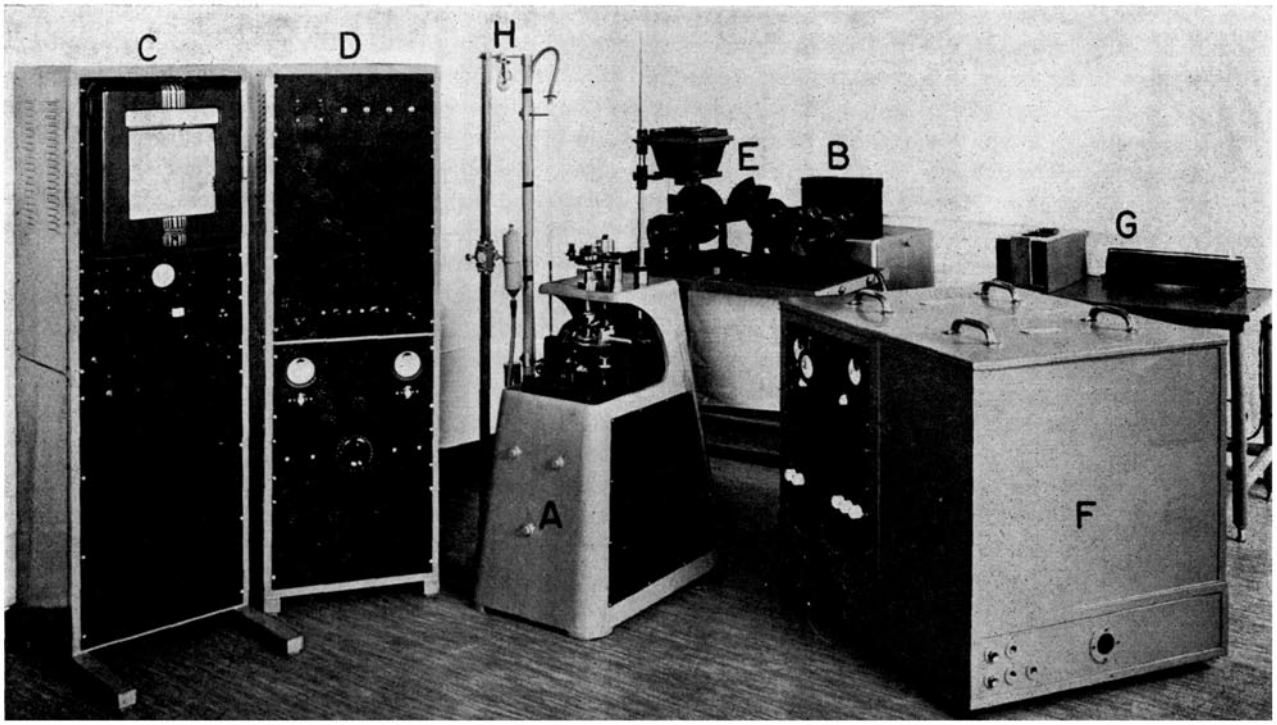


Fig. 5.–The universal ultra-microspectrograph. General view.

and the need for an extremely small measuring spot require the use of light sources with the highest possible specific light intensity and photo-electric measuring apparatus with the greatest sensitivity attainable. The necessity in both scanning and spot measurement that repeated measurements of the *same* spots in the object be possible, e.g. in different wavelengths, requires object movement with a reproducibility of 0·1 μ which presupposes extremely stable mechanical devices.

Mechanically it would be simpler, for example in scanning, to move the image instead of the object itself. For several reasons, however, it is possible to attain far greater accuracy of measurement with a movable object[1].

The main lines of design are shown in Figures 5 and 6. In the following description the letters refer to Figure 5 and the numbers to Figure 6. The central part of the optical system is assembled in very heavy cast iron framework *A*, which contains the monochromator *5* with exchangeable light sources *1–4* in its lower portion. The middle part contains the exchangeable microscope or interference microscope optics *7–11* and the device for moving the object to be measured *26, 27*. This part on which the demands for stability and precision of performance are especially high is also shown in Figure 7. The upper part of the cast iron chassis supports devices for centering the measuring beam *12*. From *A* the measuring beam and the comparison beam pass to the photo-electric cells in the photo cell housing *B*. The electronic measuring equipment is assembled in rack *C*, where the transmitted light intensity is recorded by the recording instrument *9*, and an oscilloscope *18*, serves as a balance indicator in spot measurements. At the measurement the object is moved forwards and backwards at an arbitrarily chosen rate regulated with a lever by the observer. Figures 8*a* and *b* show the principles for the two measuring methods.

The *Survey of Instrumentation* presents the entire group of equipment and the necessary auxiliary methods and instruments arranged according to the steps in a cytochemical investigation. The procedures are divided into two groups: general or earlier laboratory methods, and methods that have been newly evolved in this institute or modified especially for the cytochemical working operations[1].

(1) *Investigation of the Living Object.* Work on living material before the absorption investigation is carried out with conventional light optical procedures, ultraviolet microscopy or interference procedures. Actual measurements on living material with spectrography or interference microphotometry rarely have any meaning because of the extensive light dispersion in average cell material, the movements within the cell (both protoplasmic currents and Brownian movement), and because, as a rule, in the living cell there is not optical free projection of individual cell organelles from their environment. In optical microspectrographic work, on

[1] T. CASPERSSON, *Cell Growth and Cell Function* (W. W. Norton & Co., New York, 1950); Chromosoma *1*, 562 (1940).

[1] An universal-microspectrograph corresponding to the one described here is to be produced commercially in the near future by CARL ZEISS, Oberkochen.

the other hand, it is frequently advantageous, after the examination of fixed material is completed, to compare in individual wavelengths the distribution of the absorption between the living object and the fixed object. Photographic ultraviolet microspectrophotometry[1] is then especially suitable for several technical reasons, and its limited accuracy is not even a fundamental disadvantage.

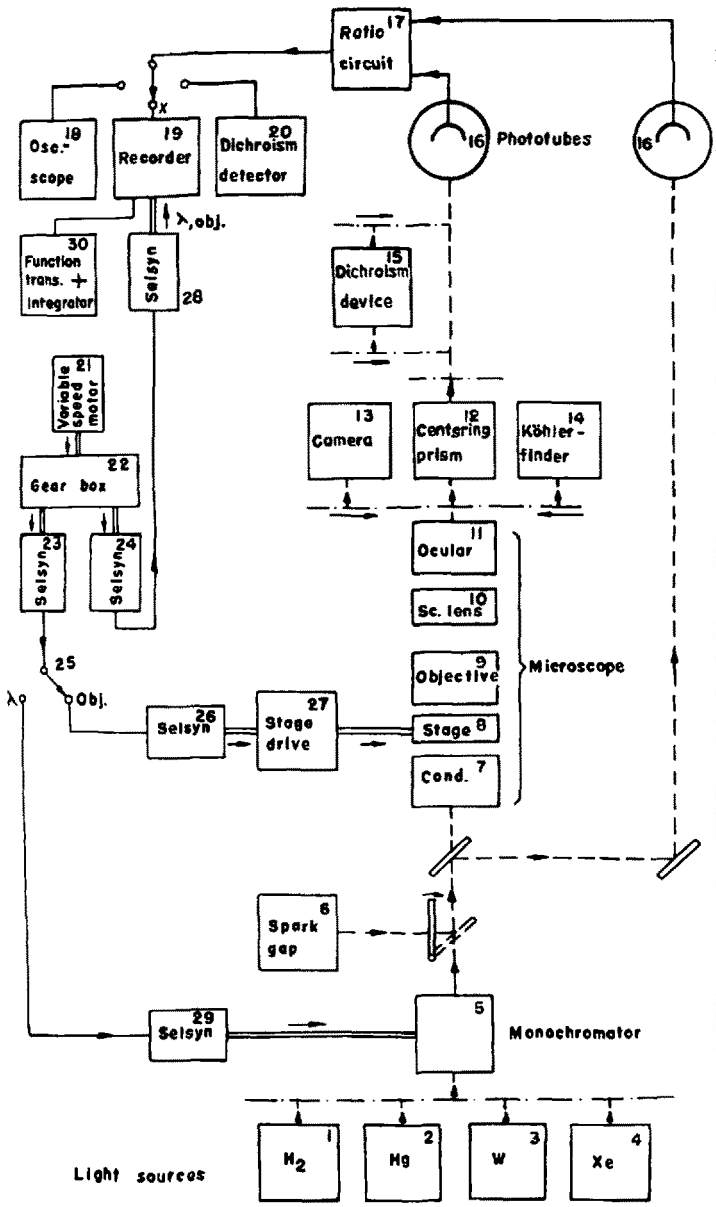


Fig. 6.–The universal ultra-microspectrograph. Block-diagram (The identifying numbers are explained in the text).

(2) *Determination of the Dimensions of the Object.* Optical cross-section determinations can be carried out on larger objects with ordinary photographic or projection-planicentric procedures. Especially for studies of the endonuclear structures, primarily the nucleolar system, and for study of changes in nuclear volume, a method is required which permits measurement to the limit of technical possibility in the visible region and on extensive series. In as much as the projection-technical, the photographic and the statistical methods are each too inexact or too circumstantial, a special microplanimeter (Fig. 9), permitting serial measurements down to the limit of the resolving power has been developed[1] for measurements in connection with microspectrography. The apparatus is simple in design and can be constructed to permit concurrent accumulation of measurements of large series of, for example, nucleoli, nuclei and cytoplasms of the same cells simultaneously.

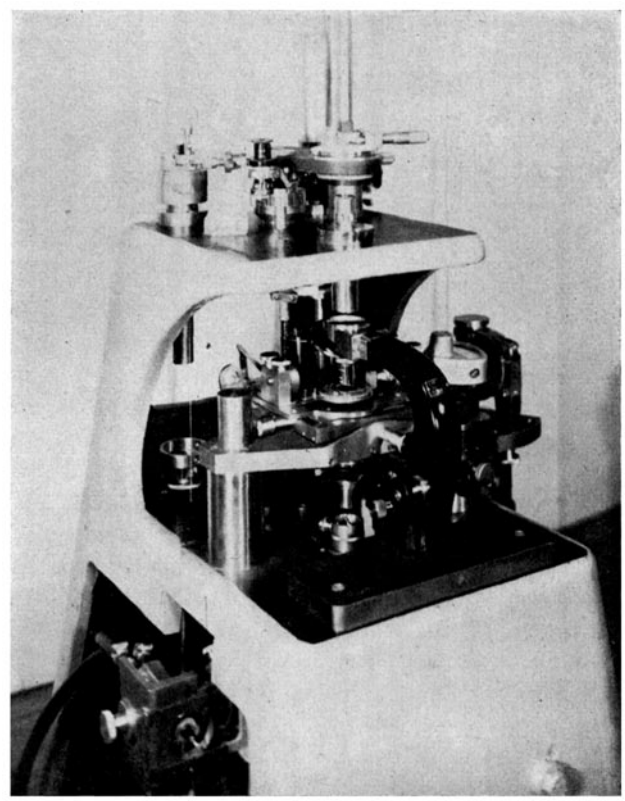


Fig. 7.–Central part of the universal spectrograph.

Determination of the thickness of the object is without great practical importance in most types of work. As the extinction is proportional to the concentration multiplied by the thickness of the layer, it is unnecessary to know the thickness of the preparation when it is a question of determination of the total quantity in whole isolated cells, for example. For sectioned preparations the distance between the slide and the cover glass can be determined with great accuracy with conventional interference or reflection methods. This distance is as a rule considerably larger than the thickness of the sectioned preparation which in average cell material cannot be determined with any method

[1] T. CASPERSSON, Skand. Arch. Physiol. *73*, Suppl. 8 (1936).

[1] T. CASPERSSON, T. FREDRIKSSON, and K. G. THORSSON, Hereditas *49*, 201 (1953).

Survey of Instrumentation for Ultra-microspectrographic Quantitative Cytochemistry

Procedures	Instruments: General	Instruments: Specially developed or adapted	Method Combinations
1. Examination of the object in living state.	Usual microscopical procedures. UV-microscopy. Phase contrast.	Photographic UV-microspectrophotometry.	
2. Determination of the dimensions of the object.	Usual microscopical procedures. Interferometrical determination of the thickness of the preparation.	Microplanimeter. Certain methods for measuring the thickness.	
3. Preparation of the object.	Usual hist. procedures. Micromanipulation	Freezing drying equipment. Methods for control of microtome sectioning.	
4. Determination of weight.	..... Interference micr. point measurement.	High vacuum X-ray spectrograph for mass determination. Interference microphotometer.	
5. Determination of biologically important elements.	.....	X-ray spectrograph for elementary analysis.	
6. Determination of molecules absorbing in the optical reg.	..... Universal umsp. for opt. spectr.	2000–2500 A 2480–3300 A 3300–IR Dichroism measurements.	Point measurement
7. Determination of compounds after special pretreatments. (Isolation, extraction or colorimetry in ultra-micro scale.) (Routine procedures in development.)		Different ranges of transmission. Different requirements of accuracy. Different measuring areas down to the limit of resolution. Indirect microspectrography. Ultra-microcolorimetry in abs. cells Ultra-microcolorimetry in "droplets"	Scanning measurement
8. Control of certain optical properties of the object.	.....	Instr. for regist. measurements of the light distribution around the object.	
9. Measurements of the optical properties of substances to be measured.	.....	"Lens cuvette". Refractometer for model subst. in ultra-micro scale.	
10. Analysis of data.	.....	Extinction calculator with integrator. General function transformer with integrator.	

other than with very low accuracy because the upper and lower surfaces of the sectioned preparation are no longer plane but rather extremely uneven after the

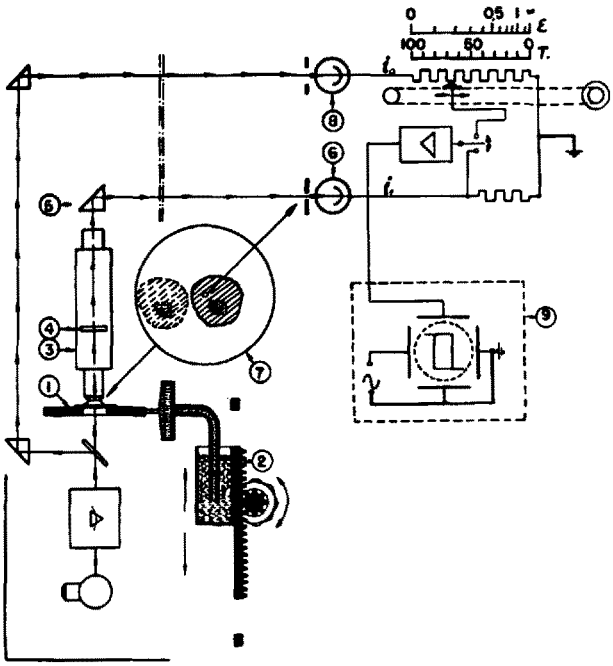


Fig. 8 *a*.

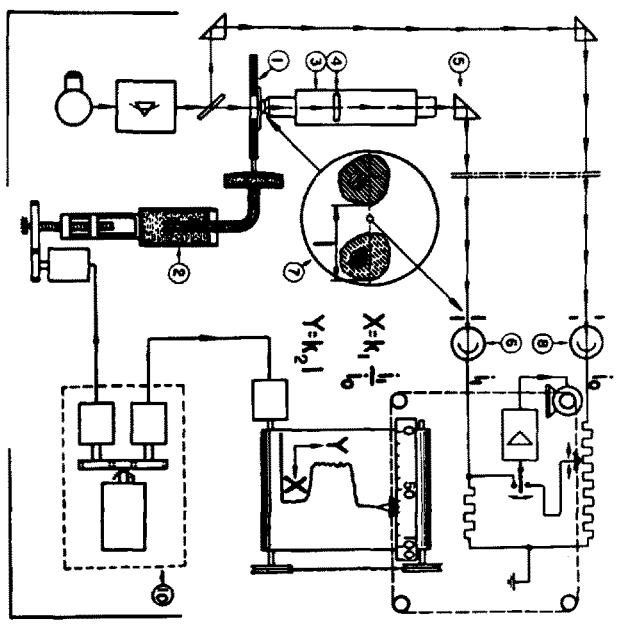


Fig. 8 *b*.

Fig. 8.–Principles of *a* spot measurement, and *b* area measurement (scanning measurement) *1* object; *2* device for moving the object along the x-axis; *3* and *5* microscope optics; *4* device for moving along the y-axis; *6* and *8* photocells; *7* field of vision in the microscope; *9* oscilloscope; balance indicator for spot measurement; *10* reversible, adjustable-speed motor with servo system for scanning measurements.

dissolution of the imbedding medium. Measurements of the distance between the upper and lower surfaces of the section with focusing or stereoscopic methods, for example, are therefore of very limited practical value. If sections are unavoidable, the method described by BAHR[1] for the preparation of sections of known thickness should be followed. He has also given a method to control the sections for even thickness. By

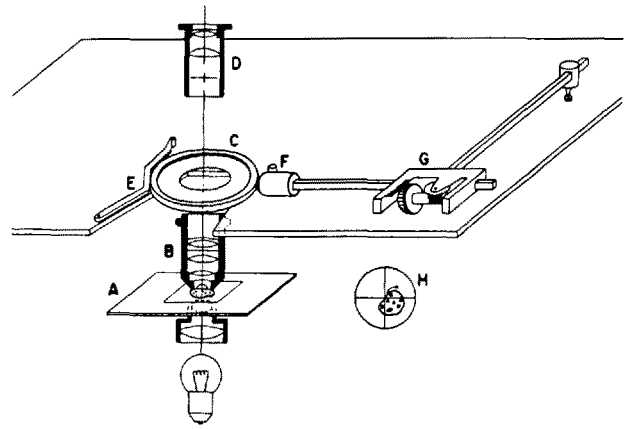


Fig. 9.–Microplanimeter, especially devised for large scale measurements approaching the limit of resolution of the microscope. *A* Object; *B* and *D* microscope optics; *C* movable long-focus lens attached to planimeter *F* and *G*; *E* device permitting reproducibility of starting points; *H* fields of vision in *D*.

working with whole cells by aid of scanning procedures in the way described in the following section, in a great many cases the difficulties in microtome sectioning can be avoided.

(3) *Pretreatment of the Object with Different Procedures for Different Purposes.* The freezing-drying-glycerine method (CASPERSSON, 1940, for details see [2]) by aid of which the main part of the biological work in the optical region has been done as yet is still by far the best method for pretreatment of material in general because of the almost complete elimination of light losses by refraction and side scattering attainable in many materials. The demands on the quality of the freezing-drying equipment are high. The best results have been obtained by modification of the apparatus of GLICK-MALMSTROM[3].

A source of error of tremendous significance is the *shrinkage due to fixation.* In the majority of cases the cytochemical study involves a comparison of the contents of a certain substance of two object regions in different functional states, etc. Ordinary fixatives frequently cause 10, 20 or 30% linear shrinkage, and it is not at all uncommon to find that cells of the same type but in different functional states show great differences in the shrinkage occurring in fixation. The most common way to avoid this difficulty is to compare the *total quantity* of absorbing substance *per cell*, i.e. *per metabolic unit*, instead of comparing *concentrations* of absorbing substances. This presupposes the possibility for rapid scanning measurements of relatively large areas in large series. *Elimination of the influence of fixation shrinkage has thus been one of the fun-*

[1] G. BAHR, Exptl. Cell Res. under publication (1954).

[2] T. CASPERSSON, *Cell Growth and Cell Function* (W. W. Norton & Co., New York, 1950).

[3] G. MOBERGER, B. LINDSTRÖM, and L. ANDERSSON, Exptl. Cell Res. *6*, 228 (1954).

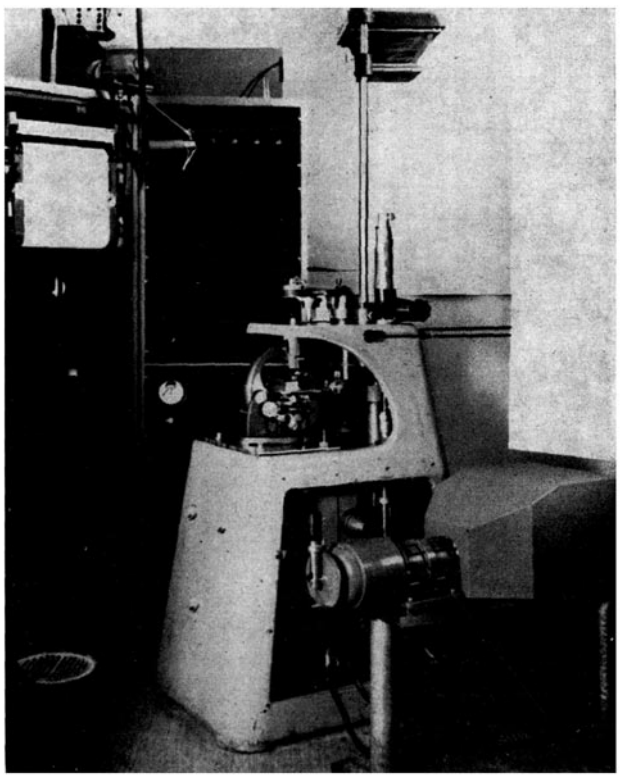


Fig. 10.–Interference microphotometry in the universal microspectrograph (see also Fig. 14).

damental reasons for the development of the rapid scanning apparatus described. This can be carried out either by cutting several sections through the same cell and measurement of the total quantity in all sections or by isolation of the cells from their environment by special procedures and measurement of the total quantity in one operation.

By the former mode of work it is of importance to note also that the *thicknesses of the sections have no influence* and thus the difficulties with microtome sectioning mentioned in the last section are avoided.

When applicable, the work on isolated cells is as a rule much more convenient than on series-sectioned material. Among the preparatory procedures the *isolation procedures* are therefore playing a role of ever increasing importance. Thus far this procedure has been employed in mechanical forms on macroscale or microscale, e.g. microdissection, fractional or gradient centrifugation, and as chemical procedures, e.g. enzymatic methods.

(4) *Dry Weight Determination.* The microradiograms are taken according to the procedure described by LINDSTRÖM[1]. They can subsequently be scanned in the universal-microspectrograph with equipment for visible light. The recording can be analyzed directly in the data analyzer. The scanning interference microphotometry is performed in the same way as the scanning transmission measurement in the visible region (Fig. 10).

[1] B. LINDSTRÖM, Exptl. Cell Res. *6*, 537 (1954); under publication.

The microscope is equipped with interference optics. In the routine work both Baker and Dyson-Cooke optics have proved satisfactory. Here also the recorded curves can be directly treated in the data analyzer. The X-ray

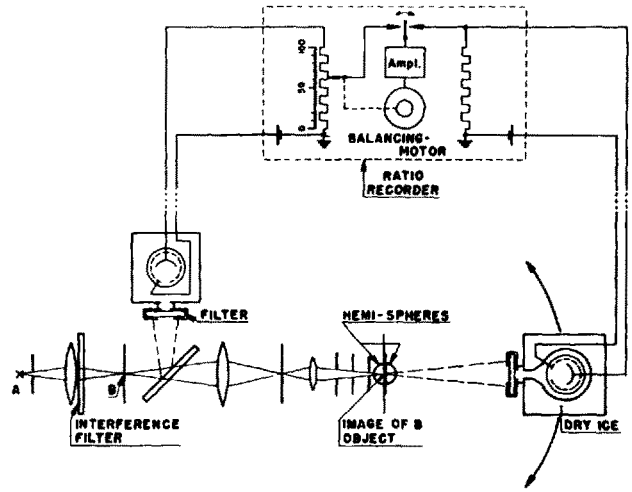


Fig. 11 *a*.

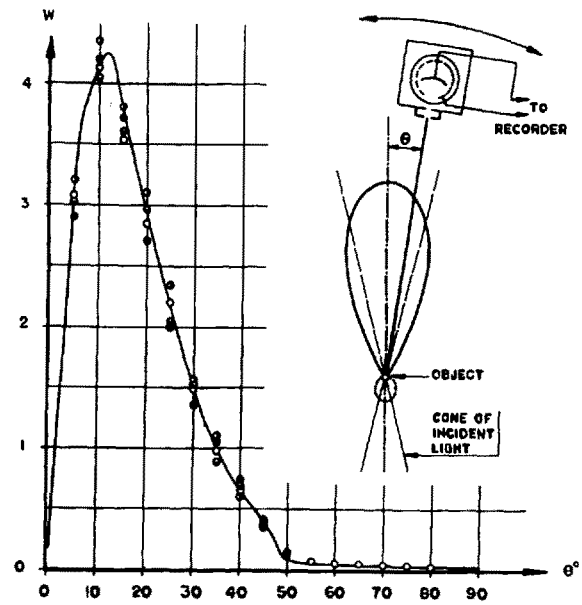


Fig. 11 *b*.

Fig. 11.–Determination of the light energy distribution about a specimen to be measured in the microspectrograph, *a* instrument, and *b* calculated curve from a model object. Points actually measured are inserted.

and the interference procedures complement each other in that the X-ray procedure gives greater accuracy over a much wider quantity range and is practically independent of the pretreatment of the object. The greatest advantage of the interference procedure lies in the fact that the preparation need not be dehydrated for measurement.

(5) *Element Determination.* Microdiagrams taken according to ENGSTRÖM's method can be treated in the universal-microspectrograph in the same manner as that given for mass determination.

(6) *Determination of Absorptions in the Optical Spectral Region.* These measurements can be carried out

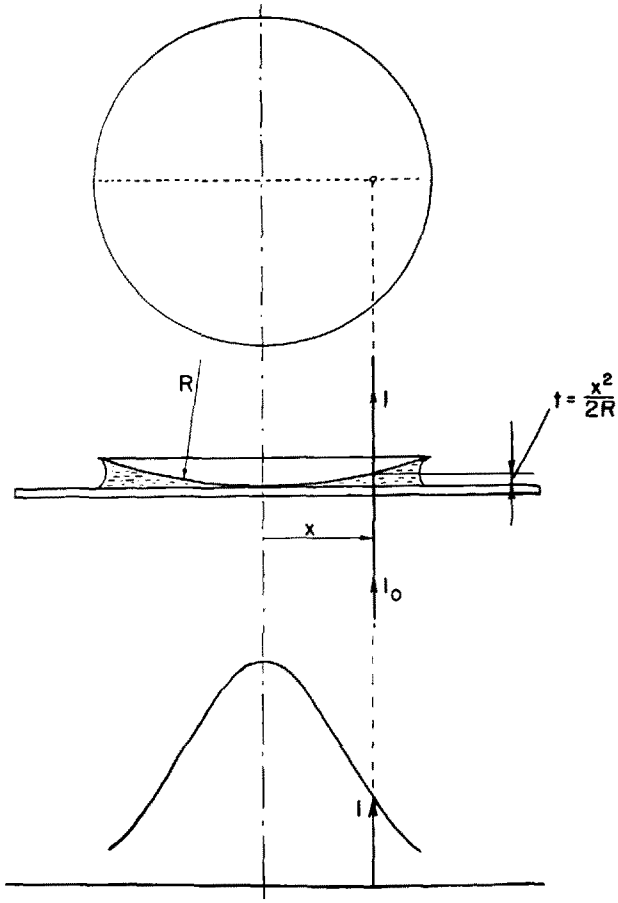


Fig. 12.–"Lens cuvette" for routine measurement in viscous solutions of deviations from LAMBERT-BEER's law at very high concentrations and at thicknesses of layers below 10 μ. The upper figure illustrates the mode of measurement with the scanning microspectrograph. The lower shows a registered curve.

directly with the standard equipment of the universal-microspectrograph as presented above. The diagram specifies a number of different accessories for specialized fields of application. Among these is also included a procedure which is not actually a spectrographic method, i.e. optical polarization measurements which can easily be performed with the apparatus. The possibility for measurement of ultraviolet dichroism is of particular importance[1]. It is used to study, for example, desoxyribonucleotide orientation in the gene-bearing material. The accessory apparatus indicated follows the principles described by RUCH[2].

[1] T. CASPERSSON, Chromosoma *1*, 562 (1940).
[2] F. RUCH, Exptl. Cell Res. *2*, 680 (1951).

(7) This section refers to the field earlier designated indirect microspectrography.

(8) *Control of Certain Optical Properties of the Object.* The light distribution about the illuminated object must be determined in order to ascertain whether the aperture of the measuring apparatus is sufficient for

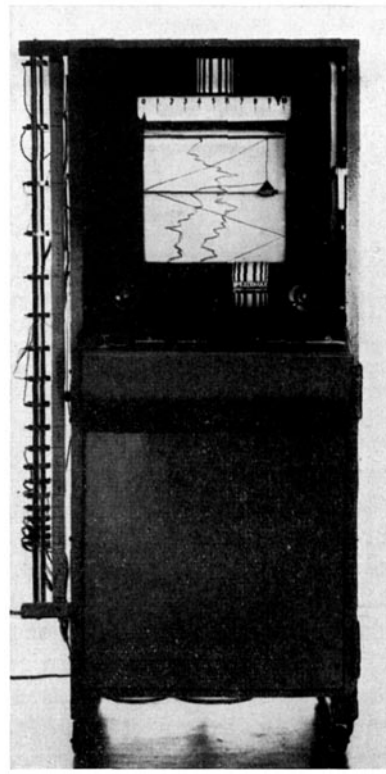


Fig. 13*a*.

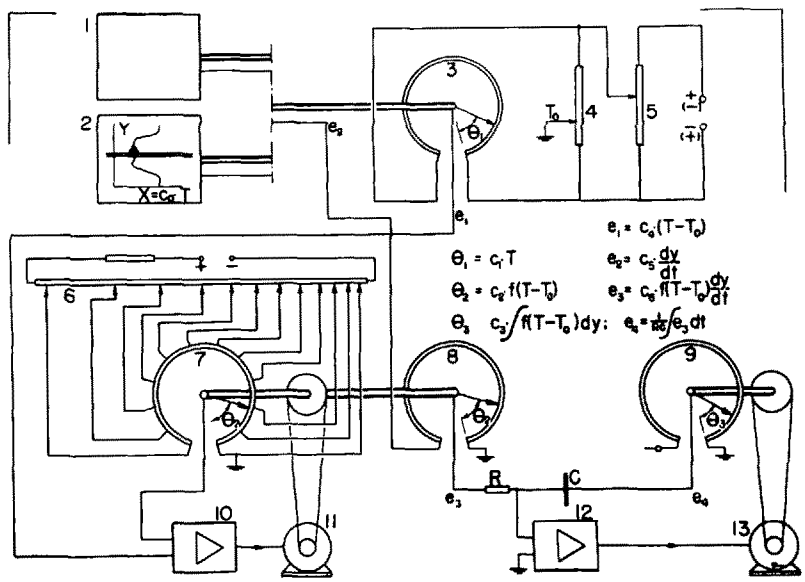


Fig. 13.–Automatic data analyzer. *a* Photograph; *b* Diagram; *1* and *2* represent the two working methods: *1* direct connection to the microspectrograph (possibly operating as an interference microphotometer), and *2* analysis of a previously recorded curve. *3*, *4*, *5*, *6*, *7*, *8*, *10*, and *11* Function transforming circuit. The function according to which the transformation is to be made is set on *6*, *9*, *12*, and *13* Integrating circuit. The split-phase motors *11* and *13* belong to the X_1–X_2 recorder (not included in the diagram) which simultaneously registers the transformed function Θ_2 and its integral Θ_3.

the measurement. SVENSSON[1] has described instrumentation for this purpose. Figure 11 demonstrates the arrangement.

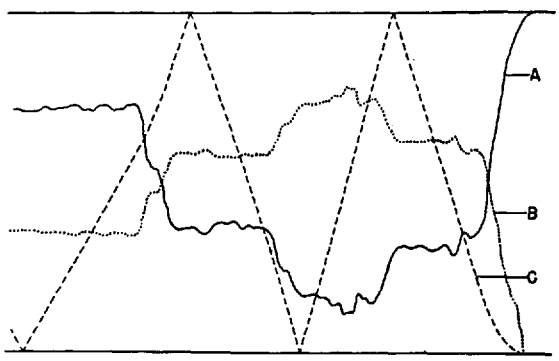


Fig. 14.–Example of analysis in the data analyzer (interference microphotometry). Object as in Figure 4. *A* Curve registered in the universal spectrograph. *B* Transformed function, proportional to the optical path length. *C* Integral of *B*, showing total amount of dry substance passed by the scanning beam (ordinate broken to increase accuracy in reading).

(9) *Measurement of the Optical Properties of the Substances in the Object.* In the literature data are rarely available on the validity of LAMBERT-BEER's law for high concentrations under the conditions prevailing in cell material. Consequently, for each special substance type to be investigated such measurement must be performed. For this purpose a special device, the "lens cuvette", has been developed (SVENSSON[2]) for the universal-microspectrophotometer (see Fig. 12). With the high extinctions and high concentrations existing in certain cases there is a risk that anomalous dispersion may occur as a course of error. A procedure for measurement of the refractive index in substances with high absorption, aimed especially at substances of importance to spectrography has also been developed by SVENSSON[3].

(10) *Automatic Data Analyzer Registering Function Transformer with Integrator.* The computer (Fig. 13) most suitable for routine spectrographic work has been designed by LOMAKKA[4]. As indicated by the arrows in the diagram, it can be connected directly to the spectrograph and plots then, simultaneously with the recording in the spectrograph of the light transmission, a curve transformed according to an arbitrary function and the integral of the curve. In many cases it is more expedient for technical reasons to perform the curve analysis after the actual measuring operation. Therefore, the analyzer was designed so that a previously recorded transmission curve could be placed in the analyzer and analyzed afterwards. Since the conversion can be carried out according to an adjustable function, the data analyzer can be used for measurements in both optical and X-ray regions and for interference measurements (Fig. 14).

As may be seen from the diagram, the equipment necessary for more general quantitative microspectrographic work is relatively complex. The basic equipment of the model of the universal-microspectrograph described above is, however, so versatile in application that in conjunction with ordinary high class histologic laboratory equipment it can be employed for work within a comparatively broad cytochemical field. It can be used directly as an ultraviolet microscope and, after insertion of suitable optics, as a phase contrast or an interference microscope. It is also suitable for the usually very comprehensive photographic or visual preliminary investigations that precede each individual measurement.

Zusammenfassung

Das heterogene, durch die strukturelle Organisation der Zelle bedingte Reaktionsmilieu ist für eine so grosse Zahl von Stoffwechselprozessen von entscheidender Bedeutung, dass die Ausarbeitung quantitativer, zytochemischer Methoden für den Grössenordnungsbereich dieser Zellstrukturen unumgänglich wurde. Die Entwicklung solcher allgemeinerer Methoden ist notwendigerweise eine instrumentell komplexe und arbeitsreiche Aufgabe. Sie kann nur in Angriff genommen werden, wenn dabei Richtlinien befolgt werden, die gewisse Erwartungen zu erfüllen versprechen sowohl in bezug auf Breite der Möglichkeiten als auch auf Genauigkeit und eine ganze Anzahl anderer Voraussetzungen.

Diese Bedingungen werden im einzelnen besprochen, und es wird gezeigt, dass die Ultramikrospektrographie ganz allgemein so viele Voraussetzungen erfüllt und einen so weiten Anwendungsbereich eröffnet, dass es berechtigt erscheint, breite Arbeitslinien auf diesem Gebiet zu entwickeln.

Eine zu diesem Zweck im Institut für Zellforschung in Stockholm entwickelte Instrumentation wird in ihren Grundzügen beschrieben[1].

[1] G. SVENSSON, Exptl. Cell Res. *6*, 529 (1954).
[2] G. SVENSSON, under publication (1954).
[3] G. SVENSSON, under publication (1954).
[4] G. LOMAKKA, Exptl. Cell Res. *7*, 603 (1954).

[1] The development of the program above described has been supported during the course of several years by the Nobel, Rockefeller, Wallenberg and Anderson Foundations, the Swedish State Natural Science Research Council and the Swedish Anticancer Society.