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A VARIANT CYTOPHOTOMETRIC TECHNIQUE AND ITS APPLICATION TO THE STUDY OF ERYTHROPOIESIS THROUGH MEASUREMENT OF TOTAL DNA AND HEMOGLOBIN IN SINGLE ERYTHROID CELLS

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Publication Date
1964-05-01
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Berkeley, California
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Lawrence Richards Adams
(Ph. D. Thesis)

May 29, 1964
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Lawrence Richards Adams

Lawrence Radiation Laboratory
Donner Laboratory of Biophysics and Medical Physics
University of California
Berkeley, California

May 29, 1964

ABSTRACT

A photographic-photometric method for cytophotometry is described. Solution of the distributional error of cytophotometry is implicit in the method. Other cytophotometric errors have been variously minimized. The method is applied to the study of single-cell amounts of hemoglobin and Feulgen-stained DNA in rat erythrocyte precursors and erythrocytes. The data so obtained allow plotting a novel cytophotometric hemogram. Comparison of the hemograms, and other experimental parameters, for normal and severely bled rats suggests that the stimulus of severe bleeding causes the premature release from the marrow of erythrocyte precursors. This finding is discussed in context with recent findings and models of erythropoietic development reported in the literature of experimental hematology.
I. INTRODUCTION

The work described here consists of the development and demonstration of a technique for measuring the absorbing constituents in single tissue cells. The method is photomicrographic, and depends on the linearity of a certain region of the photographic characteristic curve plotted in an unusual way. This linear function translates, point by point, the concentration of absorbing cellular constituents into transmittance of the developed photographic film. Photometric measurement of the light passed by an entire cell image then provides a measure of the total mass of absorbing substance.

Though the method is applicable to all radiation that can be registered photographically, it was developed in this Laboratory for measurement of cellular constituents with absorbances in the visible spectrum. A slight modification of the conventional Feulgen-DNA staining technique allows measurement of relative quantities of DNA in a population of cells. Hemoglobin, by virtue of its strong absorption in the violet region of the spectrum, and because of the fortuitous optimum absorbance by quantities of it normally present in erythroid cells, is particularly suited to the method.

Because the solution of the so-called "distributional error problem" of microspectrophotometry is implicit in the method, it has proved useful to apply the method to determination of total cellular hemoglobin in the immature erythroid cells of the bone marrow. In these cells the distribution of the measurable constituents is nonuniform. Conventional microspectrophotometric hemoglobin determinations are therefore quite difficult and have been rare.

In the analysis of a complex population, such as one of erythroid cells, a single parameter (e.g., hemoglobin content) is not sufficient to rank the cells in a meaningful way. In lieu of correlating the cellular hemoglobin data with morphological observations, cells were ranked in terms of hemoglobin content and nuclear content of DNA. These data allowed plotting a novel cytophotometric hemogram.
The cytophotometric method described here has been designed and tested in accordance with criteria and objectives set forth in the microspectrophotometric literature. Therefore, a portion of this thesis is devoted to the theoretical and technical aspects of the instrumental problem.

To evaluate the validity of the method and the hemogram, I tested the effect of severe bleeding in rats. A notable effect was shown in the hemogram.

An understanding of the implications and utility of the hemogram is facilitated by acquaintance with the concepts and knowledge of hematology. Therefore one section of this thesis is devoted to a review of the pertinent aspects of this field. On the basis of concepts and theories from the recent hematological literature, a simple model to explain the experimental results of this study is proposed. This model stresses the importance of "growth pressure" in accounting for changes that occur as the result of erythropoietic stimulation.
II. MICROSCOPETROPHOTOMETRY

Microspectrophotometry is absorption spectrophotometry on a micro scale and is made possible by construction of a spectrophotometer around a microscope. The specimen, rather than being contained in a cuvette -- with flat, parallel windows, and traversed by a beam of collimated light, as would be the case in macrospectrophotometry -- is placed upon a microscope slide and positioned between the objective and the condenser of a compound microscope. The microspectrophotometric specimen is usually a single biological cell, or a portion of one. The major problems of microspectrophotometry are related (a) to biological cells not having flat confines to their absorbing constituents (b) to the physical inability of the microscope to form an image with collimated light, and (c) to the microscopically and submicroscopically granular nature of protoplasm. In addition, some technical problems arise because of difficulty in obtaining pure monochromatic light.

If the problems of microspectrophotometry can be solved, or understood sufficiently that corrections for microspectrophotometric errors can be made, it is evident that spectrophotometric analyses of single cells, or parts of cells, can be a powerful tool in studies for which either quantities of naturally or artificially colored substances are parameters, or spectral data indicate the nature of the absorbing materials. Although this particular study is quantitative, it has proved considerably more convenient to state measured quantities in relative rather than in absolute terms.

It is possible to make microspectrophotometric measurements on either living or fixed cells. Although the low granularity of a living cell makes it a very favorable specimen, keeping cells alive but immobile on the microscope slide is difficult. Also, the use of living cells does not lend itself at all to studies in which a population of cells covers many microscope fields of view that must be rephotographed under different conditions. For these reasons preparations of fixed cells are used in this study.
A. Basis of Microspectrophotometry

1. Techniques

The initial development and application of microspectrophotometry is attributable to Caspersson, who studied cellular proteins and nucleic acids by means of their ultraviolet absorption spectra (Caspersson, 1936). The continuing work of Caspersson and his associates has been reviewed elsewhere (see Caspersson, 1950; Caspersson et al., 1960).

Caspersson's original microspectrophotometers, and all subsequent microspectrophotometers, are in essence spectrophotometers assembled around a compound microscope. Many of the problems of microspectrophotometry relate to the nature of microscope optics. The other components of microspectrophotometric systems are: the light source, which in Caspersson's studies generally consisted of a Köhler rotating spark gap; a monochromator or other device for excluding unwanted wavelengths; and a means for registering the light intensity in the image plane of the microscope.

Both photoelectric and photographic techniques for image registration have been developed by Caspersson's group. Relatively recent developments in electronics have engendered a large number of refinements of photoelectric microspectrophotometers in various laboratories (Caspersson et al., 1960; Wagener and Grand, 1963; Deeley et al., 1954; Deeley, 1960; Mendelsohn, 1961; Freed et al., 1962). Photographic-image registration remains the simpler means of light registration, though subject to the vagaries of the photographic process. Both photographic and photoelectric processes require that a reference measurement be taken at some point(s) in the image plane unoccupied by an image. This is necessary because (a) available light sources do not burn at constant intensity, (b) equipment associated with photoelectric devices tends to drift, or (c) photographic results are subject to the variabilities of the photographic process.

For the interpretation of microspectrophotometric data, it is necessary to know the dependence of film blackening or of photometer output on the intensity of light in the image plane. One standard method
entails the use of a photoelectric cell to find a null between the intensity of the measuring beam and that of a reference beam attenuated by a calibrated attenuator (see Walker, 1958). In photographic-image registration, emulsion calibration can be done with a stepped attenuator placed near to the plane of the photographic material. Either a rotating-sector wheel or a neutral density wedge can be used, but each has its disadvantages for emulsion calibration. Step wedges are bound to vary in their attenuating power as a function of wavelength. Sector wheels produce intermittent light. The photographic emulsion does not necessarily respond in the same way to intermittent light as to constant light of the same average intensity (the "intermittency" effect, see Mees, 1954). These properties of attenuators, however, are completely understood, and appropriate allowances can be made for them.

2. Law of Exponential Absorption

According to the law of exponential absorption\(^\text{a}\) of light, the fraction \(I/I_0\) of light unabsorbed after traversing an absorbing medium depends on the absorption coefficient \(k\) of the medium for light of the wavelength used, and the mass per unit area \(m/a\) of the absorbing constituent in the medium, as follows:

\[
\log \left(\frac{I}{I_0}\right) = -km/a.
\]

The quantity \(\log (I_0/I)\) is known as the absorbance \(A\), thus

\[A = km/a.\]

Validity of the exponential law requires (a) that monochromatic light be used, or that the absorption coefficient be independent of wavelength over the range of wavelengths used, and (b) that the absorbing material be uniformly distributed over the cross section of the measuring beam.

\(^{\text{a}}\)See discussion of Bougher's law (Jenkins and White, 1950, p. 197). The law of exponential absorption is variously known as Beer's law, Bougher's or Lambert's law, or the Beer-Lambert law, depending on the dimensions in which the absorption coefficient is stated.
Note that the product concentration \( X \) path length is dimensionally the same as mass per unit area. When the exponential absorption law is applied to various concentrations of absorbing substance under the condition of fixed path length, there is another condition, namely (c) that the absorbing molecular entities not interact, as concentration is increased, in such a manner that their electronic energy levels are altered.

The exponential nature of the absorption process, then, requires determination of the logarithm of the photoelectrically measured quantity \( \frac{I}{I_0} \) in order to determine the absorbing mass. A photoelectric microspectrophotometer that measures \( \frac{I}{I_0} \) is therefore best suited to measure absorbing mass only in specimen subareas small enough to be considered homogeneous. Automatic photoelectric microspectrophotometers have been constructed that scan the specimen with a small measuring beam, and that integrate over the specimen area a quantity (proportional to absorbance) that corresponds to the logarithm of the photocell output (Caspersson et al., 1960; Wagener and Grand, 1963; Deeley, 1960; Freed et al., 1962). Such instruments, though technically complicated, are capable of measuring total cellular quantities in the presence of distributional irregularities of the absorbing substances.

3. Photographic Registration

In the photographic method of Caspersson (1936, 1950), the transmittance of a photomicrograph of a biological cell is interpreted by means of the calibration curve of the photographic material to indicate the amount and distribution of absorbing materials within the cell. Thorell (1947) introduced the practice of calibrating individually each photomicrograph by means of a rotating sector wheel positioned very near to the plane of the photographic plate. Thus each developed plate shows an image of the sector wheel at the edge of the exposed portion, and the photographic characteristic curve can be obtained for each plate by comparison of the photometrically determined plate transmittance for each sector-wheel step with the known absorbance value for the step. The transmittance \( t \) of the developed photographic emulsion is defined as

\[
t = \frac{I'}{I_0'},
\]
the primes being used to indicate that these intensities I relate to the measuring beam of the photometer. The symbol \( I_0' \) is the intensity of the measuring beam after it passes through the clear unexposed portion of the emulsion. To avoid confusion, the term absorbance \( A \) will be used to refer only to the specimen or the sector wheel.

The reader may be familiar with the term photographic density \( D \) and the convention of describing the photographic characteristic in terms of \( D \) and \( \log E \), \( E \) representing photographic exposure (Mees, 1954) and \( D \) being defined as \( \log I_0'/I' = -\log t \). \( \log E \) and \( A \) are related by an additive constant and a multiplicative factor of \(-1\). This point is a matter of definition and is further discussed in Sec. II. C. 1.a. It is convenient in photographic microspectrophotometry to plot the photographic characteristic curve as represented in Fig. 1(b). This manner of plotting may seem at first a bit unusual and arbitrary but it serves a useful purpose. The conventional manner of describing the photographic characteristic is shown in Fig. 1(a). On both plots, increasing ordinate represents increasing film blackening, but different units are employed. A point of importance is that there is a straight-line portion on both curves. However, the straight-line portion of the transmittance-absorbance plot [the \( t-A \) curve, Fig. 1(b)] corresponds to a narrow region in the "toe" of the \( D \log E \) plot [Fig. 1(a)] as indicated by dotted lines on the figure. The presence of a straight-line portion, or "constant gamma" portion of the \( D \log E \) curve, has long been known for most photographic materials (Mees, 1954). But the existence of a region of linearity on the \( t-A \) plot is not so well known. It was known to Caspersson (1950, p. 71) and is reflected in the published photographic data of Thorell (1947). Sondhaus (1958) used it to simplify his calculations on the total hemoglobin content of red-blood cells. Figure 1(c) is a sketch of a microphotometer tracing of a photographic plate such as those used by Sondhaus to record red-blood-cell images. This idealized densitometer trace represents a diametric slice of a red-cell image beside the trace of the sector-wheel image. If the microphotometer measures transmittance \( t \), the \( t-A \) curve is obtained by plotting the height of each sector-step trace against the absorbance of the corresponding sector step.
Fig. 1. Graphical illustrations for text. Curves (a) and (b) illustrate two ways of describing the photographic characteristic. (a) Typical $D \log E$ curve. The meaning of $\gamma (\gamma = \tan \theta)$, which describes photographic contrast, is illustrated. (b) Typical $t-A$ curve. (c) Typical photometer tracing of erythrocyte and sector-step images.
No matter what the shape of the t-A curve, the total absorbing mass of a cell could be determined by (a) dividing the cell image into an arbitrary number of subareas, (b) measuring the transmittance of each subarea, and, using the t-A curve and the exponential absorption law, (c) computing the absorbing mass in each subarea, hence the total absorbing mass. (Each subarea should correspond to a known area in the specimen plane of the microscope -- this area must be small with respect to irregularities of distribution of the absorbing material.)

More simply, the absorbances for all subareas could be averaged, and the average absorbance multiplied by the area occupied by the cell on the microscope slide, then divided by the absorption coefficient to yield the total cellular absorbing mass.

If the cell (like an erythrocyte,) has radial symmetry, and photomicrographic exposure has been made so that all portions of the cell image lie within the linear range of the t-A curve, then the task of computing the total absorbing mass is simplified in two respects: First, the t-A curve can be replaced by a linear analytic function; and second, a microphotometer tracing of the radius of the cell image can describe the transmittances of all points within the cell image. Thus the tedium of point-by-point measurements and conversions can be replaced by a simple calculation. The approach of Sondhaus (1958) is based on these simplifications, plus the circumstance that in many cases the tracings of erythrocyte images were flat-topped, which allowed calculations on the basis of a cylindrical rather than a radial geometry. In the method used by Sondhaus, unlike the method described here (see Secs. II.C and D, IV.C. 5-8) it is necessary that the area occupied by each cell on the microscope slide be accurately known.

4. Linear Photographic Characteristic

The basis and the utility of the linear portion of the t-A curve have been obscure. Though some investigators have used it to simplify the photographic interpretation of microspectrophotometric data, few seem to have appreciated that through the linear region the t-A characteristic performs a most useful function transform. Specifically, it transforms the concentration (mass per unit area, or optical-path length
times mass per volume) of absorbing material to a photographic transmittance \( t \) that, for any subarea of specimen, is linearly related to this concentration.

The method described here depends on the ability of a simple optical device to additively integrate the transmittances of the array of infinitesimal subareas of the photographic image of the specimen, and thus to obtain a meter reading proportional to the total mass of absorbing material in the specimen.

The ability of photographic emulsions to serve this purpose was first described by Carlson (1957) at the Karolinska Institute, Stockholm, Sweden. He proposed that a procedure similar to the one described here be used to evaluate soft x-ray microradiograms for the determination of total cellular dry mass.

Another approach, identical in principle, has been developed for the quantitative interpretation of electron micrographs of specimens such as erythrocytes and isolated mitochondria (Zeitler and Bahr, 1962; Bahr and Zeitler, 1962a; Bahr and Zeitler, 1962b).

This integrating method, which I will call the photographic-photometric method, apparently was not previously proposed as a means of interpreting microspectrophotometric measurements. In this thesis, the photographic-photometric method is developed for quantitative evaluation of photomicrographs obtained with visible light, though it is obviously applicable to photographic methods in general. Though there are only a limited number of cytologic pigments that absorb visible light (e.g., respiratory, photosynthetic, and visual pigments), there are many cytologic substances to which optical density can be added with cytologic stains. The Feulgen-DNA reaction has been particularly useful in this respect (Sec. II.F), and, together with the Soret-band absorption of hemoglobin, constitutes the cytologic basis for the particular method developed here. Visible-light microspectrophotometry has been referred to as cytophotometry, and this convention is adopted here.
5. Distributional Error

An unfortunate basic difference between a microspectrophotometer and a spectrophotometer is that the spectrophotometer contains the sample in a cuvette with parallel optical flats for sides whereas the microscopical specimen, usually a single biological cell, is contained in its own membrane. The distribution of absorbing material within the cell is typically far from uniform. Such nonuniformity is the basis for the so-called distributional error of microspectrophotometry.

Though the other errors inherent in microspectrophotometry are discussed in Sec. II. B, the distributional error is discussed now because much of the effort in microspectrophotometric research has been devoted to various solutions of the distributional-error problem. This problem has been frequently discussed in the microspectrophotometric literature (Walker, 1958; Pollister and Ornstein, 1959) and has been set forth in mathematical form by Glick (1953).

The nature of distributional error can be visualized by considering a uniform optical filter one unit square and assuming that it passes one-half of the light incident upon it. This square filter is placed so as to fill a uniformly illuminated square aperture of the same size. A photometer that measures the light passed by the aperture indicates a transmittance of 0.50 for the filter. If the filter is now cut into four identical squares, and these squares are superimposed in the aperture so as to occlude one-fourth of the aperture area, the photometer indicates $3/4(1) + 1/4(0.50)^4$ or 0.766 as the apparent transmittance of the filter. If the amount of absorbing material in the filter were calculated by applying the law of exponential absorption, the calculated quantity would obviously be erroneously low when the filter was distributed nonuniformly in the aperture.

The situation is similar to that of a biological cell in the specimen plane of the microscope. Aggregations or nonuniformities of absorbing materials lead to low estimates of quantity when microspectrophotometric measurements are made with a photoelectric cell in the image plane of the microscope.
In some cases it has been possible to represent a cell or cell nucleus as a uniformly absorbing body with a simple geometry. Studies by Caspersson and Engström (quoted by Thorell, 1947) demonstrated that photoelectric measurements taken through a certain central portion of a spherical cell could reliably measure concentrations of absorbing material, if the refractive index of the medium surrounding the cell were sufficiently closely matched to the refractive index of the cell. A large number of measurements on Feulgen-stained nuclei have been interpreted on the basis of the "plug method" (Leuchtenberger and Leuchtenberger, 1960). This method entails determining the absorbance of a central cylindrical plug through a nucleus and calculating the total nuclear absorbance with the assumption that the nucleus can be described as a sphere (Leuchtenberger, 1958).

Unfixed cells may be flattened on the microscope slide by the mechanical "crushing-condenser" method of Davies et al. (1954). This method reduces distributional error due to irregularities in the shape of the specimen, but cannot be expected to avoid distributional error due to particulate aggregation of absorbing materials.

Ornstein (1952) and Patau (1952) independently suggested a method of correcting the distributional error in Feulgen-DNA cytophotometry. By this method, known as the "two wavelength method," one photoelectric transmittance measurement is taken through an entire Feulgen-stained nucleus at a wavelength near the Feulgen-DNA absorption peak, and another similar measurement is made at a wavelength at which the absorption coefficient is half of that at the first wavelength. A correction factor computed from the two measured transmittances in effect compares these transmittances with those that would be expected in the absence of distributional error, and allows determination of the transmittance that would be found in the absence of distributional error. This transmittance is related to the total absorbing mass by the exponential absorption law. Tables of the correction factor have been published (Patau, 1952; Mendelsohn, 1958a). The two-wavelength method has recently been automated by Mendelsohn (1961).
Ornstein (1952) also suggested a photographic method for interpreting cytophotometric measurements subject to distributional error. By the law of exponential absorption, the amount of silver in developed photographic emulsion is proportional to the photographic density D. Thus in the straight, or constant-gamma, portion of the photographic D log E curve [Fig. 1(a)], the amount of silver in any portion of the emulsion is linear with log E. Since log E and specimen absorbance A are directly related (Secs. II. A. 3 and II. C. 1. a) and since A is proportional to the absorbing mass in any subarea of the specimen, the amount of silver in any subarea of the developed emulsion is related linearly to the absorbing mass in the corresponding subarea of the specimen. If photographic exposures are made with careful attention to the limits of D log E linearity, a point-by-point correspondence can be achieved between the absorbing mass of the specimen and the amount of silver in the photographic emulsion; thus quantitative analysis of the silver in the specimen image can provide a measure of the absorbing mass of the specimen. Inexplicably, Ornstein suggested that the silver analysis be done on disks cut from a photographic print of the photomicrographic negative. Disks from the negative would do as well inasmuch as the quantity desired is the difference between the amount of silver in a disk containing the cell image and the amount in a disk of the same size representing the background illumination in the specimen plane of the microscope. (A direct positive rendition of the negative, achieved by reversal processing, could also be used.) In preparation of a print from the photomicrographic negative, the bounds of D log E linearity for both positive and negative material must be carefully observed.

Ornstein later suggested that the photographic material to be analyzed be developed with a color-coupling developer so that analysis of the amount of dye leached out of the disks could replace quantitative analysis for silver (Pollister and Ornstein, 1959).

A combination of Ornstein's two-wavelength method and his photographic method has been devised and evaluated by Mendelsohn (1957, 1958b). In this combination method, a photomicrograph of the specimen is printed on positive photographic film, the positive image being
obtained by use of a color-coupling developer. (In this process the silver does not remain in the emulsion.) Then the amount of dye in the emulsion is evaluated by the two-wavelength method. This combination method may be adaptable to microspectrophotometric measurements of cellular constituents whose absorption curves are not amenable to the two-wavelength method either (a) because of spectral interference due to other substances of (b) because the 2-to-1 relationship of absorption coefficients cannot be obtained.

A number of so-called "flying-spot" microspectrophotometers have been constructed (see discussion at the end of Sec. II.A.2). These specifically seek to correct the distributional error, and do so by electronically computing the absorbance at each point along the flying-spot scan. The electronic parameter corresponding to absorbance is generally integrated electronically (by charging a capacitor). Thus these devices can measure total cellular absorbing mass in the presence of distributional irregularity of the absorbing material.

6. Some Microspectrophotometric Applications and Findings

Microspectrophotometric studies are already quite numerous, so reference is made here to several recent discussions and reviews (Pollister and Ornstein, 1959; Leuchtenberger, 1958; Walker, 1958). Some of the more significant studies are discussed briefly below.

The original definitive ultraviolet microspectrophotometric studies by Caspersson (1936) on the cellular localization of nucleic acids and proteins demonstrated the association of nucleic acids with chromosomes, indicated the functional relationship between the cellular substructures, anticipated the DNA doubling and halving cycle that occurs during cellular growth and division, and explained in terms of chromosome composition the striations that are seen in suitably prepared dipteran salivary gland chromosomes. Caspersson and Schultz (1951) proposed that cytoplasmic regions rich in pentose nucleic acids (RNA) are the sites of intracellular protein synthesis in secreting and actively growing tissues.
Thorell's (1947) microspectrophotometric studies of marrow cells (and also of dentine and tumor cells) clearly indicated the "functional precedence" of cytoplasmic nucleic acid synthesis with respect to protein synthesis during cell differentiation and growth. His results for erythroid cell development are discussed in detail in Sec. III. A. 3.

Cytophotometry resulted from a combination of cytochemical techniques and the microspectrophotometric methods of Caspersson (Pollister and Ris, 1947). The utility of Feulgen's reagent for cytophotometric demonstration of DNA has been clearly shown by a number of workers (see Sec. II. F). The application of cytophotometry has confirmed the "principle of DNA constancy" (Sec. II. F) and demonstrated that DNA synthesis takes place in interphase (Swift, 1950).

The use of Feulgen-DNA cytophotometry, particularly by Leuchtenberger, has yielded a wealth of information regarding the normal constancy of DNA within the cell nuclei of a given organism, and regarding variations that occur under pathological conditions such as surgical stress, tumors, virus infections, and infertility (Leuchtenberger and Leuchtenberger, 1960; Leuchtenberger, 1958). Some interesting possible exceptions to the principle of DNA constancy are reviewed by Leuchtenberger (1958).

The applicability of Feulgen-DNA cytophotometry as a "cytotaxonomic tool" has been pointed out by Hughes-Schrader (1953) who studied DNA content in several species of Mantidae, and found that DNA content is a useful parameter in evaluating the evolutionary relationships among species whose karyotypes are not analyzable by methods of comparative cytology.

Various other cytochemical reagents have been investigated for possible cytophotometric use (Pollister and Ornstein, 1959; Kasten, 1960; Shugar, 1962; Mendelsohn, 1957; Rasch and Swift, 1960). Only the Feulgen-DNA reaction, however, has found widespread use in biological studies.

Certain technical problems in ultraviolet microspectrophotometry have confined most of the recent studies to methodology (see Walker, 1958). There is evidence that even the Caspersson laboratory has
accepted the practicality of Feulgen-DNA cytophotometry when the need for DNA measurements on cell nuclei arises (Caspersson et al., 1960).

B. Microspectrophotometric Error

The problem of distributional error is considered in Sec. II. A. 5, and is discussed in detail in Sec. II. C. For the purpose of discussion, the remaining potential sources of error in microspectrophotometry can be divided into two groups. The first considered are those errors that have their origin either in the light source or in the monochromator. The second group considered have their origin between the microscope-substage-condenser diaphragm and the photographic emulsion. The discussion of this section is confined to generalities; evaluation and correction of errors in the system used is specifically discussed in Sec. II. E. A third group of potential errors must also be considered in this study (see Secs. II. D. 1 and 2); these are related to the photographic process and the photometric interpretation of the photomicrographic film strips.

1. Light Source and Monochromator

The law of exponential absorption applies strictly only to monochromatic light. Monochromators by nature pass a finite band of wavelengths and are subject to scattering and reflections of light at their inner surfaces. Such scattered and reflected light (known as stray light) may be directed to the specimen along with the selected band of wavelengths. The quantity and quality of stray light may be a function of the wavelength band selected and the nature of the lamp used. Stray light can be reduced by the appropriate placement of baffles within the monochromators and by the use of optical filters.

The finite bandpass of the monochromator, on the other hand, can be reduced only by compromise. Reduction of the bandpass, by closing of the entrance and exit slits of the monochromator, reduces the total amount of light passed. Photographic registration on fine-grained emulsions requires much higher light intensity than that required for other means of registration, and a narrowing of the slits can raise the
required exposure time to several minutes. When a spectral line source, such as a mercury lamp, is used, good spectral purity can be obtained by closing the slits down to the line of choice so far that adjacent lines are barely excluded. However, for reasons given in Sec. II. E. 1, a ribbon-filament tungsten lamp was chosen for this study. Such a light source provides a continuous spectrum at the plane of the monochromator-exit slit. If the slits are opened to admit more light, the bandpass is extended and spectral purity is reduced. The absorption coefficient of most substances is wavelength dependent. For any discrete wavelength, absorption should be an exponential function of the mass of absorbing material. But absorption of a group of wavelengths is described by the sum of a number of exponential functions, which is not in itself exponential. Fortunately, if the bandpass is small enough, or if the variation of absorption coefficient with wavelength is small, the law of exponential absorption can be demonstrated to hold, at least through a certain absorbance range (Sec. II. E. 2 and Appendix D.)

2. Microscope and Specimen

a. Condenser glare. Poor optical corrections of the microscope-substage condenser can be an important source of error in microspectrophotometry, so the origin and nature of this error need be considered in detail.

Under Köhler illumination (Shillaber, 1944) an image of the light source is formed at the diaphragm of the substage condenser, and an image of the lamp-condenser diaphragm is seen parfocal with the specimen. The substitution of a monochromator for the lamp dictates that the exit and entrance slits of the monochromator be parfocal with the light source and the substage-condenser diaphragm, and that a surface of the dispersing element be parfocal with the specimen and with the monochromator-condenser diaphragm. When the substage condenser is properly focused, it should direct onto the specimen a cone of light with a vertex angle $\alpha$. If the substage condenser is not properly corrected for spherical aberration, bundles of rays will come from the peripheral concentric zones of the condenser optics whose vertices
occur in planes other than the specimen plane. If the specimen is small, as an isolated cell or cell nucleus would be, these improperly directed peripheral rays can bypass the specimen but still be collected by the objective. In the objective image plane, light arriving by such a path corresponds to out-of-focus images of illuminated optically empty points, above or (typically) below the specimen.

Microscope optics corrected for spherical aberration (and for coma, which is a nonaxial aberration similar to spherical aberration) are referred to as aplanatic. The aplanatic aperture of inexpensive condensers is small, though such condensers are usable for ordinary purposes at higher apertures. For microspectrophotometry, however, it is essential that the condenser be used at an aperture for which its optical behavior is aplanatic.

A good test for aplanatism of the substage condenser, and thus for the glare inevitable in its absence, is examination of the image of the monochromator (or lamp) condenser diaphragm in the specimen plane. A substage condenser that suffers from spherical aberration cannot image this sharply, whereas a well-corrected condenser images it as a sharply defined disk on a dark background.

Any light that bypasses the specimen, but that arrives in the image plane within the area of the image, spuriously reduces the apparent absorbance of the specimen. In photomicrography this is objectionable because it reduces the contrast of the photograph. In microspectrophotometry it is intolerable because it leads to erroneously low values for the measured absorbance. Some microspectrophotometrists make it a practice to stop down the condenser very radically (Leuchtenberger, 1958; Pollister and Ornstein, 1959). In addition to reducing condenser glare, this practice also reduces biconical error (see Sec. II. B. 2. c). Unfortunately, condenser aperture affects the effective numerical aperture of the microscopical system; high resolution cannot be obtained with low condenser aperture (Shillaber, 1944). [Pollister and Ornstein (1959) contend that microscopical resolution at minimum condenser aperture is half of that obtained when the condenser aperture is matched to the objective aperture. It is uncertain, however, whether
their hypothetical "pure amplitude object" could be resolved at all when a collimated beam of light is supplied by the condenser.

b. Light scattering and microscopical resolution. A rationale for using a small substage condenser aperture has been developed on yet another basis. Caspersson (1936, 1950) considered the interaction of light with the specimen. According to his analysis, light is both absorbed and scattered by the specimen. The amount of scattering depends both on the size of the colloidal constituents of the specimen, and on the degree of refractive-index mismatch between them and the medium in which the specimen is immersed. Smaller colloidal particles (down through unresolvable sizes) divert the incident light through larger angles than do larger particles. Consider the biconical locus of light rays between the condenser and the objective with its apex at the specimen: If the collecting aperture of the objective subtends a greater angle than does the illuminating aperture, it is evident that a certain amount of light scattered out of the biconical locus can be collected by the objective. A well-corrected objective places this light at the appropriate point in the specimen image. Thus such scattered light is not lost, and the absorbance registered is true absorbance to the extent that scattered light is brought back to the image. Caspersson's analysis indicated that approximately 95% of the scattered light can be collected when an objective of numerical aperture (N. A.) greater than 0.85 is used in conjunction with a condenser of one-third to one-half this value, and when the refractive index of the immersant is closely matched to that of the specimen substance.

On the basis of the parallel application of the Abbé theory of microscopical resolution and of light-scattering theory (Mie theory), Caspersson (1936, 1950) concluded that spectrophotometric measurements are possible on particles having sizes as small as several times the wavelength of the light used.

The latter conclusion has been challenged by Wilkins (1950), who considered cases for opaque specimens of various widths in the sub-micron range. Wilkins pointed out that Caspersson's use of Abbé theory pertains to very small condenser apertures, and that the image quality
can be considerably improved by using larger condenser apertures. Such improvement results from lessening the extension of the diffraction fringes into the image. However, Wilkins also notes that images of objects of finite depth are subject to considerable distortion due to out-of-focus effects, and also that lens aberrations tend to remove light from the central Airy disk. Wilkins' arguments are largely quantitative, but suggest that Caspersson's specimen-size criterion can be relaxed somewhat, a hazard being that out-of-focus effects become more serious.

c. **Biconical error.** Consideration of the nature of the specimen is essential for evaluation of microspectrophotometric error. In the properly adjusted microscope, the specimen lies at the vertex of the biconical light pattern of vertex angle $\alpha$, subtended by the condenser and the objective. If the specimen is considered to be a slab, the most oblique rays will have a path length of $d/\cos \alpha$, for which $d$ is the shortest optical path length through the specimen. The biconical error is defined as either the difference or ratio between the absorbance that would be obtained with perfectly collimated light, and that obtained with the conical bundle of rays. The mathematical statement of this problem is transcendental and solution requires numerical integration. The solution has been given in graphical form by Blout et al. (1950) and adapted to microspectrophotometry by Walker (1956). Caspersson (1936) has shown that biconical error is not important for spherical specimens. A living cell in the specimen plane is probably best represented by a sphere whereas a fixed cell can conceivably be represented as a cylindrical section. In some cases, microscopic examination of a fixed cell may reveal that it is more nearly an aggregate of globular bodies than a uniformly absorbing slab. The distribution and nature of absorbing material depends on the preparation of the specimen, and no generalizations can be made except to say that it is hard to find a simple geometrical description of the distribution of cellular contents.
d. Refractive-index differences. The refractive-index gradients within the living cell are quite gradual (Caspersson, 1950) and only small refractive-index discontinuities usually occur between the cell and its surrounding medium (exception: plant cells whose cell walls and vacuoles cause appreciable scattering and reflection of light). Because cells are typically large with respect to the wavelength of light, scattering and reflections of light that occur at the cell boundaries can be considered in terms of geometrical optics. Calculation of light losses due to these causes is straightforward, and has been carried through by Caspersson (1936).

That the substance of fixed protoplasm is granular comes as no surprise to anyone who has made observations with the phase or dark-field microscope. If the scattering particles are large enough, the refractive-index mismatch between them and the immersing medium surrounding them is small enough, and the collecting aperture of the objective is large enough, then essentially all of the light scattered by the specimen will be imaged with the specimen image (Sec. II. B. 2. b). My observations on fixed cells indicate that the protoplasm condenses into aggregates or globules of high refractive index. Some of these are undoubtedly below resolvability and would lead to serious light losses if their refractive index could not be matched. According to Caspersson's application of the Rayleigh and Mie light-scattering formulations, particles that are small with respect to the wavelength of light tend, as their size decreases, to scatter light 180° away from the collection aperture of the objective (Caspersson, 1936, 1950). The refractive index of fixed protoplasm has been given as 1.53 to 1.57, according to the manner of fixation (Pollister and Ornstein, 1959). In the preparation of fixed specimens for ultraviolet photomicrography, Caspersson (1950) has found that light-scattering losses can be considerably reduced by immersing the specimen in redistilled glycerin; this tends to swell the fixed proteins at the immersant-protoplasm interfaces, thus reducing the severity of refractive-index discontinuities.

One may wonder if the immersant actually penetrates to the interior of a fixed cell, and if so, that it is correct that fixed protoplasm consists of an aggregate of globules. Both of these questions can be
resolved by examining fixed specimens with both phase and dark-field microscopes. Both of these microscopes produce an image only where there is a deflection of image-forming light rays (Barer, 1959). If the immersant were chosen for slight refractive-index mismatch, and either the immersant failed to penetrate to the cell interior or the fixed cell were a uniform body of homogeneous (nonparticulate) material, then the cell edges only would be visible. That this is not the case, at least for the erythroid cells used in this study, can be readily determined by observation. The cell image in either phase or dark field shows contrast throughout and has a brilliant granular texture.

A conceptually difficult problem occurs as the result of the knowledge that refractive index necessarily goes through a discontinuity in the region of an absorption band (Jenkins and White, 1950, p. 466). This phenomenon is known as anomalous dispersion, and has been discussed in the context of microspectrophotometry by Scott (1952). If the absorbing substances of a cell are aggregated into particles small enough to lead to scatter losses, it follows from the concept of anomalous dispersion that no choice of immersant refractive index can allow determination of true absorptive losses in the absence of scattering losses. It seems, however, very reasonable to assume that the pigments, or chromophores, of cytophotometric interest are not free in the fixed cell, but are contained in a matrix of protein and thus cannot be in contact with the immersant. If this is true, the problem of matching the refractive index of the chromophores can be sidestepped. Since the scattering due to the anomalously high refractive index of the chromophore at the absorption band would be a function of the refractive-index mismatch between the chromophore and the protein surrounding it, no amount of refractive-index adjustment of the immersant could eliminate the scatter component of the absorption.

The scatter component of absorption is not a serious problem in a study such as this one where only relative measurements of absorbing mass are attempted. Choice of a large collecting aperture (objective aperture) can be expected to hold scatter losses to low levels. If the physical-dispersion state of the fixed protoplasm does not change from
one cell to another, the amount of scattered light lost from the collecting aperture should be a function of the collecting aperture only. In other words, it does not matter whether the measured absorbance is true absorbance, or merely has a scatter-loss component. Both components will be measured, and compared from cell to cell. Scatter loss due to refractive mismatch between immersant and protein can be almost totally eliminated.

The above treatment applies to substances that exhibit absorption bands in the visible spectrum and rests on the fortuitous fact that, with the exception of biological and cytochemical pigments of interest, cells are optically empty with respect to visible light. In the ultraviolet region, the problem is much more complicated. Proteins and nucleic acids are the materials of major interest in ultraviolet microspectrophotometry. They exist in close association in the cell, and their absorption bands overlap one another. According to the concept of anomalous dispersion, the refractive index of each must undergo radical fluctuations at its own absorption band. The scattering due to the refractive mismatch between these two substances, each undergoing refractive-index anomalies through the absorption band of the other, is all but impossible to cope with theoretically. The complications added by uncertainties about the size of the scattering entities, and the refractive-index mismatches between the two absorbing substances and the immersant, compound the problem of understanding scatter losses in ultraviolet microspectrophotometry, making it a very formidable one indeed.

e. **Exponential absorption at high concentration.** The assumption of submersion of the chromophoric elements in a protein matrix, if true, has another fortunate aspect. It is known that, at high and increasing concentrations, chromophores in solution may interact in such a way that their mutual electronic energy levels are altered. This leads to a change in the absorption spectrum that invalidates the law of exponential absorption (see discussion of Beer's law in Pollister and Ornstein, 1959). But chromophores imbedded in a protein matrix are
held separated from one another by the matrix, and there should be no concentration so high that the exponential absorption law fails because of interaction between the chromophores. The maximum concentration of the chromophores should be limited by the dry volume of the fixed protoplasm. Even if there were interaction, the degree of it should be uniform throughout the protoplasm of a given cell, and throughout an experimental population of cells subjected to the same preparation procedure.

f. Glare, lenticular flare, and reflections. Lenticular flare (under the formidable pseudonym of Schwarzschild-Villager effect) has received a good deal of attention in the microspectrophotometric literature. Naora (1955) has thoroughly reviewed the subject and enlarged it and, in the opinion of some, overcomplicated it (Vendrely and Vendrely, 1956). A recent article presents new insights and analytical approaches (Howling and Fitzgerald, 1959). Rather than attempt to review this complicated literature, I shall endeavor to present an intuitive approach to the problem, and later to show that error from this source has been held to low limits.

If a bright object such as the sun is photographed on a dark field, the negative will show a spurious density in the region of the image. This effect, first described in connection with astronomy by Schwarzschild and Villager (1906), now bears their name and is attributable to multiple reflections at the glass-air interfaces of lens systems. The effect can be minimized, at certain selected wavelengths, by the use of optical coatings. An effect of similar origin can be demonstrated in a photographic dark room by setting up an enlarger to project an image, and viewing the enlarging lens from a point off the light path. The lens appears to be self-luminous.

Substage condenser glare has been purposely discussed in another context (Sec. II. B. 2. a). Although lenticular flare can originate in the condenser, I feel that flare from this source is not important, and that glare originating from the spherical aberration of the condenser is a much more serious problem.
Consider an opaque disk, perhaps 10 \( \mu \) in diameter, on a brilliant self-luminous background, in the specimen position of the microscope. (This makes it possible to consider the situation in the top section of the microscope without yet introducing the complexity of the condenser.) To make the situation analogous with Köhler illumination, assume that the self-luminous background is a disk smaller than the field of the microscope objective. Some of the light that enters the objective is reflected back and forth in the air-spaced glasses of the objective, and some may be scattered by dust, or bubbles and imperfections in the optical glasses. This could be confirmed by looking obliquely down the microscope barrel so that the image-forming beam\(^*\) does not strike the eye; the back lens of the objective would appear as a luminous disk. In the image plane of the objective there is an image of the opaque disk. There is no image-forming light in this image, but the image is irradiated with flare light from the objective. (Actually, aberrations of the objective, and diffraction effects at the margin of the specimen, would introduce some light to the image, but these effects are not important for the analogy.) At this point in the development, the situation is analogous to that for the astronomical camera for which the Schwarzschild-Villager effect was first described. There is also an analogy to photography with hand cameras. If a minute light detector were placed in the image plane, it would record flare light in the image of the disk, and also it would record less light in the (luminous) background portion of the image than would be expected in the absence of flare. If it is assumed that flare light that does not arrive in the image plane directly is lost in the baffles of the microscope barrel, flare light should decrease with distance between the objective and the image according to the inverse square law. The total intensity of the image-forming beam does not decrease.

\(^*\) The term "beam" as used here denotes a bundle of light rays, which may be collimated, converging, or diverging. A more proper term for a noncollimated bundle of rays is "pencil" (Schillaber, 1944).
Consider now the effect of projecting this image onto a photographic plate or film by means of the microscope ocular. The ocular projects a very much out-of-focus image of the back lens of the objective onto the photographic plate. A very small portion of the flare light from the objective is converted to ocular flare light, and a small portion of the image-forming beam is converted to ocular flare light. If the system of baffles in the microscope barrel is inadequate, light reflected from the inside of the barrel will also illuminate the ocular, and a portion of this illumination will be converted to ocular flare. Therefore the image of the opaque disk in the ocular focal plane (the film plane) will be illuminated by ocular flare light plus the very much out-of-focus image of the flare-illuminated objective back lens.

An order-of-magnitude calculation shows that only the flare light primarily generated in the ocular is of importance. Assume that the objective transmits 70% and converts 30% to flare. Assume also that the area of the ocular-lens element closest to the objective is 3 cm$^2$ and that the distance between objective and ocular is 14 cm. If the objective flare light is lost isotropically over a solid angle of $4\pi$, only $3 \times 30\% / 4\pi (14)^2$ or 0.04% of the objective flare light can enter the ocular at all. (This implies that no light reflected from the microscope barrel reaches the ocular -- in practice this can easily be checked by looking down the microscope barrel with the ocular removed.)

Oculars are relatively simple lens systems, and are therefore more efficient in the transmission of light than are objectives. Flare light should decrease with distance from the ocular according to the inverse square law, but, again, there is no inverse-square attenuation of the total intensity of the image-forming beam. Assume that 20% of the image-forming light is converted to flare light by the ocular, and that the ocular-to-film distance is 12 cm. (Such is roughly the case in this study.) Assume also that the image (formed by the ocular) of the illuminated background has an area of 15 cm$^2$ and is illuminated with an intensity of 0.8 unit per cm$^2$. Thus the total amount of flare light generated at the ocular is 0.2$\times$15 units. If this light is dispersed isotropically over a solid angle of $4\pi$, the intensity at 12 cm is
0.2 \times 15/4\pi (12)^2 \text{ or about } 0.2\% \text{ of the background intensity. It has been reported that flare (or glare, which as a term has been variously used to include flare) can actually be reduced to this extent through careful attention to photomicroscopy parameters such as alignment, diaphragm settings, and specimen preparation (Leuchtenberger, 1958; Swift and Rasch, 1956; Pollister and Ornstein, 1959).}

Note that the Schwarzschild-Villager effect, as originally reported, refers to a local redistribution of intensities near the image rather than to a uniform distribution of flare light over the entire field. An effect that leads to the same result as the Schwarzschild-Villager effect can be found in the diffusion of image-forming light through the photographic emulsion (Mees, 1954, p. 1000).

The degree of ocular flare can be lessened by reduction of the size of the illuminated background in the specimen plane. Extraneous illumination originating in reflections from the sides of the microscope barrel and bellows or tube of the camera can generally be completely eliminated by proper choice of diaphragm settings. By looking down the microscope barrel with the ocular removed, one can usually find aperture diaphragm (substage-condenser diaphragm) settings that will cause illumination of the inside of the barrel. But by moderately closing down the aperture diaphragm, these reflections can be eliminated. Similarly, too large an opening of the field diaphragm (the monochromator-condenser diaphragm in this study) leads to illumination of the inside of the camera mount. Reflection glare of this origin can be eliminated by closing down the field diaphragm so that the film only is illuminated.

The analysis so far has not included discussion of condenser flare. I choose to use the term condenser glare for non-image-forming light arising in the condenser. This seems desirable in order to distinguish condenser effects from the flare effects discussed above. Spherical aberration of the condenser can lead to flooding of the image with light that does not pass through the specimen at all (Sec. II. B. 2. a). Such light corresponds to slightly out-of-focus images of illuminated, optically empty points above and below the specimen. But also, light
scattered in the optical components of the condenser might lead to similar out-of-focus images. Because of the very short focal length of the objective, such images should be so far out of focus -- that is, they should be distributed over such a large solid angle by the objective -- that they should lead to no more trouble than does objective flare, as considered above.

The analysis has led to another more significant problem. If slightly out-of-focus images of points above and below the specimen lead to glare, any light-scattering loci in the vicinity of the specimen must also produce glare. Two potentially troublesome possibilities are apparent. One lies in optical inhomogeneities in the specimen immersant, such as refractive-index discontinuities and particulate matter, and the other lies in refractive-index mismatch between the specimen-immersant and the slide or the cover glass. The fixed dry cells have a measured refractive index of 1.545 (Sec. II.E.6), but crown glass has a refractive index of 1.515. If the cells are matched the glass is not, and some error is expected from this source. Multiple reflections between the slide and the cover glass can be expected to lead primarily to light loss, with some deterioration of image quality. Irregularities in the glass, and particulate matter in the immersant, present a more serious potential problem, but one that can be overcome by care and cleanliness in the preparation of the specimen.

The several sources of flare and glare are difficult to evaluate individually. However, it is shown by means of measurements that flare light can be held down to about 1% in the system used in this study (Sec. II.E.4).

C. A Solution of the Distributional Error Problem

As previously discussed, the crux of the method developed here is the peculiar property of photographic materials whereby through a discrete range, the transmittance of the developed emulsion is linear with the log of photographic exposure. Where this linear relationship is valid, the photographic film can perform a very useful function transform: It can transform the absorbance of a cytophotometric
specimen to film transmittance, on a point-by-point basis. Thus photometric measurement of the light passed by the photographic image can provide a measure of the total mass of absorbing material in the specimen.

In this section, this linear photographic characteristic is discussed in detail. A simple model is devised to explain why it should exist, and the results of tests on a number of photographic materials and development procedures are given. On the basis of these tests, a photographic film and a standard development procedure are chosen for the best and most reproducible rendition of the linear characteristic.

In addition, it is demonstrated mathematically that the use of the linear photographic characteristic does indeed make possible the determination of total cellular quantities by the photographic-photometric method.

1. Linear Region of the Transmittance-Absorbance Curve

a. Discussion. The published data of Thorell (1947) and Sondhaus (1958) indicate the approximate limits of t-A linearity to be \( t = 0.30 \) and \( t = 0.75 \) for "process" photographic plates. The corresponding absorbance range on their plots is 0.4 to 0.5 absorbance units long. As the basis for the following discussion, a typical t-A curve is shown in Fig. 2.

The points on the curve indicate the \( t \) values that might be obtained from a rotating sector wheel having steps whose absorbance values differ by 0.1 absorbance units. By definition absorbance \( A \), as used here, is related to photographic exposure \( E \) as follows:

\[
A = \log E_0 - \log E.
\]

in which \( E_0 \) is the exposure corresponding to \( A = 0 \). Both \( A \) and \( E \) are measures of the light transmitted either by the specimen or by a sector wheel used for film calibration (sensitometry). Thus, if \( \log E \) is increasing to the right, \( A \) is increasing to the left. The blackening of the film increases on the ordinate, and thus the sense of the conventional \( D \ log \ E \) curve is preserved, though the units of the ordinate are quite different.
Fig. 2. A typical photographic transmittance-absorbance (t-A) curve. This curve is similar to that of Fig. 1(b), except that it represents a photographic exposure chosen to place all points on the low t end of the curve in the linear region of the t-A characteristic.
Why might this region of the \( t-A \) curve be linear? Ultimately, our acceptance of the linearity is based on experimental observation but there is a way of intuitively understanding why it may exist.

Assume that, at low exposure, the probability that a photon exposes a silver-halide grain in the emulsion is not affected by previous exposure. Assume also that we are dealing with a uniform population of silver grains, each of which may be exposed by interaction with a single photon. Then the number \( N \) of exposed (e.g., developable) silver halide grains per unit area of film is

\[
N = pE.
\]

The constant \( p \) relates exposure \( E \) to the number of photons falling on a unit area of emulsion, and also contains a term for the probability of interaction between photons and silver halide grains. Assume also that the transmittance \( t \) of the developed film is related to the number of developed silver grains per unit area of film by the exponential law

\[
\ln 1/t = 2.3 \log 1/t = qN = qpE.
\]

The absorption coefficient \( q \) is expressible as the geometric cross section of a developed silver grain. By taking logarithms and rearranging, we obtain

\[
\log \log 1/t = \log (qp/2.3) + \log E.
\]

Carlson (1957) has pointed out that, through a limited range, \( \log \log 1/t \) is linear with \(-t\), which allows writing,

\[
t = r - b(\log \log 1/t)
\]

where \( r \) and \( b \) are constants. Therefore,

\[
t = \left[ r - b(\log qp/2.3 + \log E_0) \right] + bA,
\]

or, if the bracketed constants are expressed as one constant, \( a \),

\[
t = a + bA.
\]

By plotting, one can verify that linearity between \( \log \log 1/t \) and \(-t\) holds over a \( t \) range of 0.25 to 0.60, with a slope \( b \) of 0.82. It would be surprising to find that the slope of an actual \( t-A \) curve did not vary
with the emulsion type and the development conditions. In Sec. II. C. 1. b it is shown that the slope \( b \) generally has a value greater than one, and that \( b \) depends on development conditions. Thus it may be concluded that the above model is too simple. An exhaustive analysis of the \( t-A \) curve is not attempted here. It is considered sufficient to make sensitometric measurements to determine \( b \) and to establish the limits of \( t-A \) linearity for each strip of film used for experimental measurements.

b. Testing photographic films for the optimum \( t-A \) characteristic. I made sensitometric exposures by two methods. In the first method, a calibrated 21-step neutral-density step tablet (Eastman Kodak) was "contact printed" on a strip of 35-mm film with a commercial xenon-flash sensitometer. The density or absorbance difference between the steps is about 0.15. In the second method, the sector-wheel sensitometer described in Sec. II. D. 1.a was used. After development, the transmittance of the film strips was evaluated with the Jarrell-Ash microphotometer. The commercial sensitometer was used initially when a large number of films and development procedures needed evaluation, but films that seemed promising were carefully evaluated with the rotating-sector unit by means of monochromatic light.

Tests were run on fast, medium-speed, and slow panchromatic 35-mm films. Graininess was objectionable for all but the slowest films, though a favorable \( t-A \) characteristic could usually be obtained. The \( t-A \) characteristic for four slow films is shown in Appendix A. Eastman Kodak High Contrast Copy film was chosen because of satisfactory performance and availability.

During development, photographic contrast, or "gamma" [see Fig. 1(a)], approaches a limiting value asymptotically. Long development times allow precise control of both degree and uniformity of development through careful routine agitation. Considerable experience indicated that the most uniform and reproducible development was possible with slow-acting developers. All developing was done at constant temperature in Nikor (Nikor Products Company, West Springfield, Mass.) film-developing tanks. Trials indicated that end-to-end
uniformity of development of the film strips could be achieved by gentle inversion of the tank 10 or 12 times during development. Agfa Rodinal was chosen for development because it acts slowly and because it allows convenient control over the degree of development through variation of dilution.

Figure 3 shows the t-A curves obtained for High Contrast Copy film with various dilutions of, and times of development in, Rodinal. One of the Eastman Kodak developing formulas recommended for this film is D-72, but, as the figure shows, the absorbance range of linearity with this developer is not as long as with Rodinal.

Standard development conditions selected were as follows: Two exposed 36-frame strips of 35-mm High Contrast Copy film are wound on Nikor reels and placed in a two-reel Nikor developing tank. The tank is allowed to equilibrate thermally with the water in a 20°C water bath. The tank is filled to overflowing with 1% Rodinal solution at 20°C, agitated for 15 sec, and rapped several times on a table top to free the emulsion of adhering air bubbles. The tank is replaced in the water bath and development proceeds for 10 min. At the end of each minute the tank is gently inverted in the water bath and rotated approximately a quarter turn. Following development the developer solution is discarded, and an acid short-stop solution (at 20°C) allowed to remain in the tank for 30 sec; any commercial photographic fixing solution can be used, preferably at 20°C. Fixing is followed by washing, wetting with any commercial wetting agent, and dust-free air drying.

2. Mathematical Demonstration of the Validity of Photometric Integration

It is shown in Sec. II. C.1. b that a region of linearity in the t-A curve can be obtained by use of suitable photographic material and development procedure. In this linear region the t-A characteristic has been expressed analytically as

\[ t = a + bA. \]

By the law of exponential absorption (Sec II. A. 2)

\[ A = k(m/a), \]
Fig. 3. The t-A curve for High-Contrast Copy film under various developmental conditions. These curves represent actual sensitometric measurements. Under the conditions of development chosen (curve d, see text), the t-A characteristic is linear from $t < 0.3$ to $t > 0.7$. The corresponding absorbance range is approximately 0.4. Curve a, 1:100 Rodinal, 20 min; 9-sec exposure; Curve b, 1:1 D-72, 6 min; 3-sec exposure; Curve c, 1:50 Rodinal, 10 min; 9-sec exposure; Curve d, 1:100 Rodinal, 10 min; 9-sec exposure.
k being the absorption coefficient and m/a the mass per unit area of absorbing material.

Let us now determine average transmittance \( T \) over an area \( F \) of developed film exposed so that \( t \) is everywhere linear with \( A \); assume a cell image within this area. For a film whose \( t-A \) linearity extended to \( t = 0.30 \), this would mean that the exposure \( E_0 \) had been chosen to give a film background transmittance of 0.30 or higher (see Fig. 2).

The absorbance of a cellular subarea, which is sufficiently small so that the absorbing material can be considered uniformly distributed over it, is designated as \( A_c \), and the absorbance of a similar area of the background is designated as \( A_{bg} \).

It would be possible to determine the average transmittance \( T_{c+bg} \) of the area \( F \) containing the cell image by making \( n \) photoelectric measurements of the transmittances \( t \) of subareas \( a \) of the photographic film, where \( n = F/a \). This operation measures the average transmittance of the cell image plus that of the background underneath as well as adjacent to the cell image:

\[
T_{c+bg} = \frac{1}{n} \sum_{i=1}^{i=n=F/a} \left[ a_i + b(A_{c+bg})_i \right] = a + \frac{b}{n} \sum A_c + \frac{b}{n} \sum A_{bg}
\]

This equation implies that \( A_{c+bg} \) is equal to \( A_c + A_{bg} \), which follows from the logarithmic dependence of \( A \) on the specimen transmittance, namely

\[
A_{c+bg} = \log \left( \frac{1}{t_{c+bg}} \right) = \log \left( \frac{1}{t_c} \cdot \frac{1}{t_{bg}} \right) = \log \left( \frac{1}{t_{bg}} \right) + \log \left( \frac{1}{t_{bg}} \right) = A_c + A_{bg}.
\]

The necessity of assigning a transmittance or absorbance to each subarea of the background results from the possibility that the microscope-field illumination may be nonuniform.

If an identical photograph could be obtained of the same area \( F \) of the pattern of microscope-field illumination, without the cell in the field, the average transmittance of the background could be obtained:
\[
T_{\text{bg}} = \frac{1}{n} \sum_{i=1}^{i=n=F/a} \left[ a_i + bA_{bg} \right] a + \frac{b}{n} \sum A_{bg_i}.
\]

The difference between the average transmittance of $F$ with the cell image and that without it is

\[
\Delta T = T_{c+bg} - T_{bg} = \frac{b}{n} \sum A_{ci}.
\]

The law of exponential absorption is now applied to this difference, yielding

\[
\Delta T = \frac{bk}{n} \sum \left( \frac{m_i}{a_i} \right) = \frac{bk}{n} \sum m_i \frac{n}{F} = \frac{bk}{F} M
\]

where $M$ is the total mass of the absorbing constituent.

The term $n$ for the number of divisions of the area $F$ has dropped out. Therefore it may be concluded that the measurement can just as well be made with one photocell reading of the transmittance of area $F$ as with $n$ readings of areas $F/n$. The area $F$ is a constant of the system. In practice, $F$ corresponds to an aperture (in the photometer system) that excludes all of the background portion of the photographic image except the small portion containing the cell image. The numerical value of $F$ is the physical area of the aperture divided by the square of the magnification of the system (microscope magnification times photometer magnification). (The projected area of the cell is not a parameter of measurement.)

A compromise has been necessary in determining the value $T_{bg}$. Nonuniformity of microscopical illumination, inherent in the equipment available, prevents the practical assumption of uniform background. The effect of background variation under the cell image is difficult to assess because such variation is masked by the cell image. One way
of correcting for such variation would be to photograph the background with no cells in the field. Provided that conditions of exposure and development were identical for this frame of film, and for the frames on which cells were photographed, it would be possible, with a device for accurately and reproducibly positioning the frames in the photometer, to measure \( T_{bg} \) corresponding to the illumination pattern under any cell. Such a procedure was attempted but abandoned because of the difficulty in obtaining identical consecutive exposures. I attempted to use an integrating light meter to close the shutter after a certain time-integrated quantity of light had been collected by a photocell that viewed the microscope illumination. Perfection of the integrating light meter poses a problem for the future; it did not seem possible in the time allocated for this work. Integrating light meters immediately adaptable to this use are available commercially.

The method adopted for background estimation is as follows. Photometer readings are taken of two diametrically opposite background areas adjacent to each cell image, and the average of these is used as the estimate of \( T_{bg} \). The error generated by this procedure is probably the largest error in the method as used for this study.

D. Photomicrographic System and Integrating Device

This section deals with assembly and use of the system for cytological photographic-photometric measurements. The operations described here are sensitometry, photometry, and photomicrography. It is desirable to discuss the errors of sensitometry in this section along with description of the sensitometric operation. The sources of error in photometry and photomicrography are considered in Sec. II.E.

1. Sensitometry

As used here, the term sensitometry refers to the determination of the characteristic curve of a photographic film. In the present study, photographic materials are described by the t-A curve (see Figs. 1 and 2). The photographic film is subjected to a series of exposures of different known intensities, is developed according to a standard procedure, and the film transmittance corresponding to each
exposure is measured. The device used for making the sensitometric exposures is called a sensitometer. The procedure for standard development is described in Sec. II. C. 2. b. In the testing of films, sensitometric exposures were made with white light, and the Jarrell-Ash microphotometer was used to measure film transmittance. But when the slope $b$ of the $t-A$ curve is to be used as a parameter in the determination of cellular quantities, it is desirable that sensitometric exposures be made with the photomicrographic illumination and that measurements of film transmittance be made with the same device used to measure cell-image transmittances. If the conditions of exposure and development are standard and reproducible, and the film strips are cut from the same 100-ft roll (as in this study), one determination of the $t-A$ curve should suffice for each 100-ft roll of film. However, because of the possibility of uncontrollable irregularities in the photomicrographic procedure, each film strip (i.e., each 36-frame roll of reloaded 35-mm film) was evaluated sensitometrically.

a. Sensitometer. The device chosen for regulating the sensitometric exposures was a sector wheel. Using a drafting machine I made this by carefully laying out ten steps with incremental absorbance values of 0.10 on 1/16 in. black Bakelite stock, and then shaping the wheel with a jeweler's coping saw and watchmaker's files (see Plate I). A 10X dissecting microscope increased accuracy and reduced eyestrain.

The sector wheel was mounted to the shaft of a 12-volt dc motor, and enclosed in a housing of Bakelite (see Plate I). Exakta (Ihagee, Dresden, Germany) lens-mount flanges were attached to the housing in such a way that the sector-wheel unit could be fastened to the front of Exakta camera body and to the back of an Ihagee Versal unit. The motor is powered with a variable-output 6-volt power supply. All the inner surfaces of the sector-wheel unit were painted flat black. This unit was designed to allow the sector wheel to be swung in and out of the light path.

The entire sensitometric unit, consisting of Exakta body, sector-wheel unit, and Versal, is clamped on the bench, paraxial with the monochromator exit beam. The only dimensional requirement is that
Plate 1. Detail of sensitometer. (a) The side of the sector-wheel unit that attaches to the front of the camera. (b) The side of the sector-wheel unit that attaches to the back of the Versal bellows unit. (c) Detail of the sector wheel. (d) The sector-wheel unit attached to the rear of the Versal bellows unit. The camera (not shown) would fasten to the rear of the sector-wheel unit to complete the sensitometer.
the height of the slit image at the camera focal plane be longer than the greater dimension of the rectangular focal-plane stop of the camera. In this study, the distance between the monochromator condenser and the camera focal plane was 70 inches. To limit reflections in the Versal, a narrow rectangular aperture was inserted at the front of the unit. With this arrangement, when the sector wheel is interposed in the beam, it defines eight shadow images of sector steps across the camera focal plane, plus an open, or background, step.

In practice, the sensitometric unit is aligned with the monochromator after the monochromator is aligned with the microscope. For sensitometry, the microscope is swung out of the beam by means of the pivot between the microscope base and the microscope stand, and the monochromator condenser is focused to throw a sharp image of the exit slit onto the camera focal plane. The monochromator-condenser diaphragm was adjusted to provide the proper amount of light to the sensitometer as follows: The diaphragm is viewed through the microscope is set to correspond to a predetermined number of divisions on an ocular micrometer (see also Sec.IV.C. 5).

Because reciprocity-law failure can lead to differences in the photographic characteristic at different exposure rates (Mees, 1954, p. 199), the sensitometric exposures were always chosen to be of duration equal to the corresponding photomicrographic exposures. To avoid the possibility of intermittency effect (Mees, 1954, p. 213; Thorell, 1947) the speed of the rotating sector was set so that each exposure was interrupted several hundred times. At some sector speeds, an "optical-beat" due to synchrony between the sector frequency and the 60-cycle ac fluctuations of the illumination could be observed in the camera focal plane. The sector speed was chosen carefully to avoid this effect.

b. Uniformity of sector-wheel illumination. It was Caspersson's experience that the image of the Köhler illumination pattern could not be made sufficiently uniform to serve as an illumination source for sensitometric exposure in ultraviolet microspectrophotometry with the rotating-spark source (Caspersson, 1950). Thorell (1947), on the other
hand, was able to achieve illumination flat enough that the Köhler illumination pattern in the camera focal plane could serve for sector-wheel illumination. With the equipment available to me, I found that the pattern of illumination at the focal plane of the camera (as used for photomicrography) could not be made sufficiently uniform for sensitometric exposures. Sensitometric curves could be obtained in this way, but it was necessary to correct each step for the variation in background intensity between the steps. The correction procedure was approximate and laborious, and was not felt to be sufficiently accurate for characterization of the t-A curve to the desired 1 or 2% accuracy.

It is for this reason that the means of sensitometry described above was used. The variation of intensity along the slit image can be made negligible by placing the sensitometer far enough from the monochromator. Furthermore, the illumination pattern can be monitored for flatness by swinging out the sector wheel and making a test exposure. Such a test exposure is routinely made with each sensitometric exposure, and the flatness of the slit image confirmed by transmittance measurements.

Because there is wavelength variation across the monochromator exit slit, and because only the center of the slit is used for sensitometric exposure, the light used for sensitometry is spectrally purer than that used for photomicrography. Although there may be considerable variation of the photographic characteristic (the D log E curve or t-A curve—see Fig. 1) with wavelength throughout the visible spectrum (Mees, 1954, p. 191), it is unlikely that appreciable variation exists in the wavelength interval corresponding to the monochromator bandpass (13 μm half width).

c. Flare originating between specimen and focal plane. Ideally, the sector wheel, or calibration standard, should be placed in the specimen plane. In that way, flare generated in the upper portion of the microscope would affect the sector-wheel image and the specimen image identically, and no flare correction would need be considered. Technically, such a placement is extremely difficult because of the minuteness of the calibration standard required, and furthermore, the problem of nonuniformity of illumination again appears.
If the flare can be held low, its effect on the t-A curve, obtained by the method described, can be demonstrated to be quite small. A simple calculation (Appendix B) based on the discussion of Sec. II. B. 2 g, shows the following: If $b'$ is the slope that would be measured if the sector wheel were in the specimen plane, and $b$ is the slope obtained with the sector wheel mounted close to the plane of the film, then

$$\frac{b'}{b} = \frac{b}{\Delta t} \log \left[ \frac{1 + f}{4 \left( \frac{\Delta t}{b} \right) + f} \right]$$

If $b$ were 1.2, the flare $f$ were 0.01, and the limits of t-A linearity on the transmittance axis were 0.25 and 0.75 (making $\Delta t = 0.5$), the correction factor $b'/b$ would be 0.984 and the slope $b'$ would be 1.18. This correction corresponds to a slight horizontal shift and a slight rotation of the linear portion of the t-A curve. When a similar procedure was applied graphically, it did not affect the linearity noticeably.

If absolute quantitative determinations were to be made, it would be desirable to use the slope $b'$ as opposed to $b$. Such a usage would imply that $f$ were known accurately for each set of measurements; this is not necessarily true in this study. The use of $b$ provides adequate reproducibility from experiment to experiment.

2. **Integrating photometer**
   a. **Design and construction.** The criteria for the design and construction of the integrating photometer are:
      1. A beam of white light is to be directed through the film in such a way that the beam is collected by an objective that projects an image of the film;
      2. It must be possible to define a photometric area $F$ in the film plane by means of an aperture in the system;
      3. There should be provision to accurately position the cell images in this area;
      4. The light falling within the image of the photometric area $F$ must be directed to a photocell that is uniformly sensitive to light passing through all portions of $F$. 
Certain advantages of construction would be gained were it not necessary that an image be formed. But because the cell images are quite small, and because it is necessary to position them accurately with respect to the photometric area, it is important to have a magnified image to facilitate adjustments.

Two photometric systems were considered. The first was assembled from a photographic enlarger and a commercially obtained photometer. The second involved modification of the use of the Jarrell-Ash microphotometer, but was found to be invalid for a reason discussed in Appendix C.

The enlarger photometer was assembled from a photographic enlarger (Beseler Model 23C) and a photometer (Photovolt Model 514 M) (Photovolt Corporation, 95 Madison Ave., New York, N.Y.). The only fabrications necessary were (a) an adjustable film carriage and (b) a box to house the probe containing the photodiode of the photovolt unit and to provide a surface on which to view the image.

The film carriage was constructed from an inexpensive mechanical stage for a microscope and from the film carrier for a 35-mm film-strip projector. As constructed, the film carriage is less than 1 in. thick and fits well in the position in the enlarger normally occupied by the film holder. It allows micrometer-controlled movement of the film in two mutually perpendicular horizontal directions. To advance, the film is pulled through against lightly loaded friction retainers that grip its perforated edge.

The unit containing the photocell probe is a plywood box 12 in. by 18 in. by 4 in. (see Fig. 4). In the middle of the top surface is a round opening, which accepts the low-power elements of an achromatic microscope condenser. A recess just above this lens accepts any of a series of aperture plates consisting of round holes cut in 2-in. squares of 1/16-in. aluminum stock. The lens focuses all of the light admitted by aperture to a point on a piece of opal glass. This glass is attached to the opening that admits light to the photodiode of the Photovolt unit. This arrangement has the advantage that the variations of sensitivity expected from point to point on the cathode of a phototube do not affect the
Fig. 4. Schematic diagram of light-integrating unit. Cell images are focused on the white cardboard matte. The image of a particular cell to be measured is centered in the opening of the aperture plate by means of the micrometer controls of the film carriage (not shown). The lens system, consisting of the low-power elements of an achromatic microscope condenser, brings the light passed by the aperture to a point on the opal glass disk. (Note that the point of light on the disk is actually the image of the enlarging objective.) A constant portion of the light from the opal glass falls upon the photodiode of the photometer (Photovolt Model 514M).
accuracy of the measurements. The phototube "sees" only the illuminated point on the opal glass, and the geometric pattern of illumination of the photocathode does not depend at all on the route by which the light arrived at this glass.

There are several possibilities in the placement of the aperture that determines F. The above method is certainly the simplest. Another possibility is the placement of the aperture in the plane of the film. However, the depth of field in the film plane is quite short and placement of the aperture here either scratches the film or provides an unsharp image of the aperture. In assembling another photometer, I would employ the following arrangement, as suggested by Carlson (1962), and also used in the apparatus of Zeitler and Bahr (1962): A separate optical system of good quality would project an image of the aperture onto the film, and the rest of the system would be essentially as described. This arrangement would avoid the small degree of lenticular flare inherent in the system actually used (See Sec. II. E. 7).

b. Photometry. To use the enlarger photometer, a film strip, with sector images and photomicrographs of microscope fields containing many cells, is placed in the film carrier. The magnification of the enlarger is adjusted to an arbitrary reproducible magnification by means of a fiducial mark on the upright member carrying the enlarging head. The Photovolt unit is balanced to zero dark current as indicated by the galvanometer. The enlarger lamp is connected to the 117-volt line through a Sola voltage-regulating transformer. The enlarger is focused, and magnification is rechecked by measuring the distance between the images of points on the film. Carefully placed pinpricks in the emulsion serve well for this.

First, the sector-step images are read. An aperture, whose diameter is no greater than the width of the projected sector-step images, is placed over the photocell lens (the lens that focuses the light to a point on the opal glass). By means of the variable diaphragm of the enlarger objective, the illumination is adjusted so that the galvanometer indicates full scale (1.00) on a portion of clear film adjacent to the frame on which the measurements are to be made. Fine adjustment is made
with the sensitivity adjustment of the photovolt unit. The transmittance values for the sector-step images are then measured and plotted against the absorbance values of the sector steps. The t-A curve so obtained yields the parameter b and indicates the transmittance range in which measurements can be made.

After readjustment of the controls to provide a full-scale galvanometer reading on an area of film adjacent to a frame showing cell images, measurements of the cell images are commenced. Apertures are selected so that the projected cell images are barely contained within them. Each time the aperture plate is changed, the controls must be readjusted to provide a full-scale galvanometer reading on clear film between the frames. The background for each cell image is read at two diametrically opposite points adjacent to the cell image. These readings are averaged to estimate the background under the cell.

The product Mk (dimensions, absorbance X area) is calculated for each cell by means of the relationship (from Sec. II. C. 2)

\[
Mk = (T_{c+bg} - T_{bg}) (F/b)
\]

If F is expressed as the area of the aperture-plate opening divided by the square of the linear magnification of the system (enlarger plus microscope), and if k is known, the absorbing mass per cell can be obtained by dividing the value Mk by the absorption coefficient.

3. Photomicrographic Procedure
   a. Photomicrography. The photomicrographic arrangement for this study consists of a Bausch and Lomb 250-mm grating monochromator with an achromatic quartz-fluorite condenser, and Bausch and Lomb microscope components as follows: a research-microscope stand fitted with a 61 X, N. A. 1.4 apochromatic objective, an N. A. 1.4 achromatic condenser, and a 10X compensating ocular. The 35-mm film is carried in an Exakta camera body mounted to the microscope barrel by means of an Ihagee microscope adapter (Model 1). Focusing and field finding is done with an Ihagee Magnear viewfinder with bisected clear glass and a 10 X magnifier. Light is provided either by a 100-watt AH-4 mercury lamp (General Electric) or a 108-watt tungsten-ribbon filament lamp (General Electric). The AH-4 lamp was not used
in the latter part of the study for reasons given in Sec. II. E. 1. Sensitometric exposures are made with the separately mounted sensitometer described in Sec. II. D. 1. a. The camera body may be used interchangeably on the microscope and sensitometer. All rolls of film for a given experiment are obtained from the same 100-ft roll.

Vibration of the photomicrographic apparatus is minimized as follows: The camera is mounted rigidly to the barrel of the microscope. The microscope is fixed to a shock-mounted and critically damped 50-pound steel slab. Exposure is controlled by a solenoid-operated leaf shutter mounted near the exit aperture of the monochromator. The camera shutter is also operated by a solenoid control; it is opened several seconds before the exposure-controlling shutter to allow vibrations in the microscope to settle down before the exposure is made.

b. Alignment. The optical components are adjusted to achieve Köhler illumination (Shillaber, 1944). (See also Sec. II. B. 2. a). The alignment procedure consists of:

1. An image of the lamp filament (or arc) is focused and centered on the entrance slit of the monochromator.

2. The monochromator is placed at a certain distance from the microscope (determined as discussed below), all optics removed from the microscope, a pinhole eyepiece inserted in the ocular position, and the first-surface mirror below the condenser used to center the monochromator exit lens on an axis defined by the pinhole and the lower part of the microscope barrel.

3. The monochromator lamp is turned on, the slits almost closed, the exit Hartmann diaphragm (a "V"-shaped wedge in the plane of the slit) almost closed, and the monochromator body pivoted around the exit lens to project a trapezoidal image of the exit slit onto a ground glass that now replaces the pinhole eyepiece at the ocular position. (Focusing of the monochromator condenser is necessary to bring this image to a sharp focus on the ground glass.) This image is then centered with respect to the microscope barrel.

4. Because of the difficulty in accurately pivoting the monochromator around the exit lens, it is necessary to go back and repeat steps (2) and
(3) in sequence until no further adjustments are required. (This procedure places the monochromator lamp on the optical axis of the microscope.)

(5) The substage condenser is placed in its mount (in the microscope used, there is no provision for condenser centering), and alignment of the condenser to the optical axis of the microscope is checked. This is done by closing down the condenser diaphragm then using a telescope at the ocular position to check the centering of the closed monochromator-condenser diaphragm within the substage condenser diaphragm.

(6) The objective lens in a centering mount is put in place and approximately centered. Then, with the telescope at the ocular position, the monochromator-condenser lens is focused to give a sharp image of the exit slit, parfocal to the substage diaphragm.

(7) Alignment is completed by replacing the ocular, focusing to obtain an image of the monochromator-condenser diaphragm, and then using the objective centering screws to center this diaphragm in the field of view.

In the experiments done in this study, it is necessary to set the substage diaphragm reproducibly. The substage diaphragm is set to an N. A. of 0.65 by comparing the aperture of a dry objective with this value to the aperture of the substage diaphragm. This comparison is made with the telescope. The substage diaphragm is closed until it just begins to cut into the periphery of the disk of light at the rear of the objective lens, as focused in the telescope.

The choice of the distance between the monochromator and the microscope is dictated by several circumstances. First, the pattern of illumination of the grating, viewed in the specimen plane of the microscope, was overall quite nonuniform. Second, there were local irregularities produced by defective rulings, unremovable dirt in the system, scratches on the grating and on the monochromator spherical mirror. Last (and surprising), the exit beam of light does not pass directly through the monochromator condenser--its path is slightly oblique. I was not able to correct this situation by means of several realignments of the monochromator optics. If the image of the grating in the specimen plane were made large by placing the monochromator
close to the microscope, the overall nonuniformity of illumination was not evident inasmuch as only the central portion of the grating image could be seen. However, the small irregularities were quite apparent. Conversely, the use of the monochromator far from the microscope de-emphasized the small irregularities, but the overall nonuniformity was apparent.

A distance more towards the latter extreme was chosen because it is relatively simple to correct for a gradually changing pattern of illumination. The monochromator-to-microscope distance selected for this study was such that the monochromator-condenser diaphragm setting, which barely excludes the grating edges, defines an illumination field, at the camera focal plane, that has a diameter slightly smaller than the diagonal of the rectangular field stop of the camera. Under this condition, the distance between the monochromator condenser and the optical axis of the microscope is 24 inches.

The procedure of using such a short distance between the film plane and the ocular, as is provided by the Ihagee microscope adapter, is not the most advisable. A distance of 10 in. between the ocular and the film plane is considered the shortest that can be accommodated by the spherical corrections of the microscope optics (Shillaber, 1944, p. 294). In this study, the problem of vibration was overriding; a mount to hold the camera at a greater distance would have required external support and thus would have rendered quite formidable the task of shock mounting the assembly.

E. Evaluation of the Cytophotometric Method

This section describes measurements and calculations relevant to the cytophotometric method described in the previous section. The intent is to evaluate this method and the instruments and devices involved, and to determine the optimum conditions of use.
1. Choosing the Light Source

Monochromatic light is desirable when measurements are to be made on the basis of the law of exponential absorption. Because monochromators necessarily admit a band of wavelengths, there is the possibility of error when continuous-spectrum light sources are used.

Mercury lamps were tried, and, though they produce illumination of discrete wavelength, they were eliminated as a possibility for the following reasons: (1) the mercury line at 405 μm, although within the oxyhemoglobin Soret band, is sufficiently far from the absorption maximum for oxyhemoglobin (412 μm) that optimal absorbance is not obtained; (2) an adjacent mercury line at about 408 μm is too close to be eliminated with the monochromator; (3) it was impossible to control the time variation of the lamp intensity and thus to make accurate sequential photographic exposures; and (4) movement of the arc between the electrodes causes a time variation in the pattern of illumination both on the grating (the image of which appears in the specimen plane) and at the exit slit (the image of which is used for sensitometric exposures).

Use of a tungsten-band lamp, on the other hand, provides both a fairly uniform illumination pattern at the exit slit and a nonfluctuating pattern across the grating. Furthermore, there is little time variation in intensity when a 250-watt Sola voltage-regulating transformer is interposed between the 117-volt line and the lamp transformer. (It was found that there was a considerable voltage drop in the leads between the 6-volt lamp transformer and the lamp; with the lamp unit used as obtained, 6 volts at the output side of the transformer corresponded to less than 5 volts across the lamp terminals. All wiring on the secondary side of the transformer was replaced with #13 gauge heat-proof wire. If the lamp contacts are periodically cleaned, the voltage drop can be held low and constant. It was found desirable to monitor the lamp current with an ammeter.)

In order to achieve sufficient illumination for focusing and short photographic exposures at 412 μm, it was necessary to burn the lamp at lamp-terminal voltages of slightly over 6 volts, corresponding to currents of 16 to 18 amperes. This amount of power gave too much
illumination for Feulgen photomicrography, so power was reduced in that case by controlling the voltage to the lamp-transformer primary with a 200-watt Variac.

2. Bandpass and Stray Light

The combination of a continuous-spectrum light source with a monochromator can provide illumination that is near monochromatic, but microspectrophotometric error is implicit in any departure from strict spectral purity. To achieve the full use of the condenser aperture, the monochromator beam must be wide enough to fill this aperture. Also (as discussed in Sec. II. D. 3), the image of the grating must be of a certain size in the specimen plane, this size being determined by the requirement that the image be not so large that its fine structure is accentuated nor so small that its overall nonuniformity of illumination is conspicuous. These two conditions impose restraints that determine the monochromator-to-microscope distance and, in addition, determine the slit-width setting of the monochromator. Recalling that the image of the slits is focused in the substage aperture, I find it evident that there is a certain slit width that will just allow the aperture to be filled. Opening the slits further decreases the resolution of the monochromator, that is, increases the bandwidth, without producing any improvement in microscope resolution. Wavelength of light varies from one side of the slit to the other, but under the condition of Köhler illumination, wavelength distribution is identical from one point in the grating image to another. The wavelength distribution of the light in any part of the grating image depends on the following: (1) the intensity-wavelength characteristic of the monochromator lamp, (2) the bandpass of any stray-light-reducing filter placed in the illumination system, (3) the width of the entrance slit of the monochromator, and (4) the size and shape of the substage diaphragm of the microscope (note that this diaphragm is effectively the exit slit of the monochromator when the image of the exit slit upon it is broader than the aperture diameter). The distribution of wavelengths, thus shaped, is affected by the specimen according to the absorption characteristic of the specimen substance.
This situation varies considerably from the ideal situation in which the specimen is illuminated with a discrete wavelength of light. Though failure of the absorption law is often attributable to aggregation of molecular-absorbing entities at high concentration (Ornstein and Pollister, 1959), spectral impurity of illumination is probably as important a cause (see discussion of waveband error, Niemi, 1958).

The bandpass of the monochromator at 412 μm is calculated in Appendix D on the basis of an assumed lamp-color temperature of 3000° K. Further, the effective absorption coefficient is estimated on the basis of a hemoglobin-absorption curve taken from the literature. The estimated absorption characteristic follows an exponential law through the range of absorbance over which measurements are to be made. The effective absorption coefficient could be used to convert the measured cellular absorbances to total cellular hemoglobin. But because absolute quantities are not relevant to the interpretation of the experimental results, and because I was not able to actually measure the bandpass characteristic or to determine the absorption spectrum for hemoglobin in dried fixed cells, this conversion is not made in the presentation of the data.

A similar calculation was not done for the absorbance of the Feulgen-DNA complex for several reasons. First, the lamp is run at reduced power for this determination, and it is difficult to estimate the color temperature. Second, the absorption peak of the Feulgen-DNA complex is much flatter than the Soret peak, so if hemoglobin absorption follows an exponential law through the range of measurement, then Feulgen-DNA absorption should do the same. The stoichiometry of the Feulgen reaction is affected by the time of hydrolysis (Sec. II. F), and other factors such as the conditions of mounting and storage of the stained specimens (Kasten et al., 1962). These effects preclude the application of Feulgen cytophotometry to measurement of absolute quantity.

The origin of stray light in the monochromator is complex (Sec. II. B. 1) and depends on the particular monochromator. One source is reflection from structural surfaces. Perhaps the most serious
source with the instrument used is the layer of tarnish that inevitably forms with time over the reflective surface of the grating. Light scattered from this tarnish is not dispersed, but an image of the tarnish is formed by the optics of the system, parfocal with the monochromatic grating image. Another stray-light source, which inspection of the instrument revealed to be important, was a layer of tarnish that formed over the spherical mirror shortly after it was realuminized (and overcoated). In the Bausch and Lomb monochromator, the spherical mirror is used twice: once to project the incoming light onto the grating, and again to project the light from the grating onto the exit slit. The mirror is near-parfocal with the grating. Thus an image of the mirror tarnish, which is illuminated directly by light from the entrance slit, is included by the optics with the grating image. Stray light from tarnish should be constant, but the amount of reflective stray light would probably depend on wavelength. Light is reflected, for instance, from the exit-slit hardware back to the spherical mirror and grating.

A correction could be made for wavelength-setting-independent stray light if its amount were known. Such a correction would be similar in principle to that given by Walker (1958), but would need adaptation to the present method so that it would take a form similar to the flare correction given in Sec. II.D.1.c. But sophisticated measurement of stray light turned out to be a formidable instrumental problem and was not attempted in view of the questionable assumption of wavelength-setting independence required for applying the stray-light correction, and in view of the continuous deterioration of the mirrored surfaces.

Instead, the following analysis indicated that the use of optical filters could hold stray light down to limits suitably low with respect to the overall precision of measurement.

Green light at the Feulgen absorption peak is abundantly produced by the tungsten-band lamp; thus little error is expected from monochromator stray light at this wavelength. Sondhaus (1958), who used the identical monochromator, studied the stray light adjacent to the yellow mercury doublet and found it to be essentially unmeasurable with an instrument capable of responding to less than 1% of the peak
intensity. Such error as exists at the wavelength used for Feulgen measurements may be tolerated because only the mode (prereplicative, replicative, or postreplicative) of DNA synthesis need be known, there being no attempt to measure absolute quantities of DNA.

The wavelengths near the violet end of the spectrum, on the other hand, are produced at low efficiency by the tungsten-ribbon-filament lamp, and the inside of the monochromator is flooded with light of longer wavelengths. Thus stray light is troublesome in the measurement of hemoglobin. But the longer wavelengths can be blocked by placement of an optical filter in front of the entrance slit. The filter selected, a Dow-Corning C. S. 7-59, admits light between 290 and 500 nm with a broad peak between 340 and 400 nm. Transmittance at 412 nm is about 70%. Visualization with a hand spectroscope of the light passed by the combination of this filter with the monochromator, set to a wavelength of 412 nm, indicates that essentially all light of longer wavelength is removed. This test is a very good one because of the poor response of the eye to violet compared with its high efficiency for light of longer wavelength. In order to protect the C. S. 7-59 filter against heat damage, an infrared-bar filter, C. S. 1-69, is placed in front of the C. S. 7-59 filter.

3. Choosing the Wavelengths

In the foregoing discussion the wavelengths for hemoglobin and Feulgen-DNA measurement are referred to as 412 nm and 555 nm, respectively. In this section the means of choosing the wavelengths is described. It should be evident from the discussion under Secs. II. E. 1 and II. E. 2 that the bandpass of the light source can be estimated but that it is not known. Therefore an empirical procedure was used to determine the most appropriate wavelengths. This consisted simply of running an absorption spectrum for typical cells through the wavelength region of interest in each case, and choosing the wavelengths where the absorption was greatest. This was done photomicrographically. Each photomicrograph at each wavelength was individually calibrated sensitometrically. The monochromator-slit-width setting for both curves was 2 mm.
The photomicrographs for the hemoglobin-absorption curve were read by the microphotometer method outlined in Appendix C. The measurements are subject to the errors therein described. Each point on the curve, shown in Fig. 5(a), represents the average of the total cellular absorbances of five fixed reticulocytes immersed in a medium of refractive index (r. i.) 1.545. There was no concise peak in the curve of Fig. 5(a). Referring also to published curves (Lemberg and Legge, 1949, and Heilmeyer, 1943) I have taken the absorption maximum for hemoglobin (oxyhemoglobin) as 412 mp.

The Feulgen-DNA absorption curve was determined by microphotometer measurements of photographic negatives and is shown in Fig. 5(b). Because this experiment had the additional purpose of confirming that the Feulgen-DNA absorbance range could be made smaller than the range of t-A curve linearity (by means of the method described in Sec. II. F), the cell images were scanned diametrically with the minimum slit-height setting of the microdensitometer, and the maximum absorbances were plotted. Each point on the curve represents the average value for four Feulgen-stained erythroblast nuclei immersed in a medium of r. i. 1.545. The absorption peak found at 555 mp occurs at a shorter wavelength than reported elsewhere (Kasten, 1956). The reason for this difference is not clear, though the width of the bandpass and the procedure of under-hydrolysis (Sec. II. F) are suspect. It is quite reasonable, however, to set the monochromator for the absorption peak measured under the actual conditions of use.

4. Aperture Settings, Glare, and Flare

According to reasoning that is classical in the microspectrophotometric field (Sec. II. B. 2. b), the combination of large collecting aperture (objective aperture) and small illuminating aperture (condenser aperture) should minimize error owing to light being scattered out of the collecting aperture by diffracting particles and refractive-index discontinuities in the specimen. The objective used, a 1.4 N. A. apochromat, has a collection angle big enough that, by Caspersson's criteria, use of an illuminating aperture of N. A. 0.5 to 0.7 would not result in objectionable scattering loss and would provide good resolution.
Fig. 5. Hemoglobin and Feulgen-DNA absorption vs wavelength. (a) Hemoglobin curve constructed from measurements taken on a series of photomicrographs of five fixed reticulocytes. (b) Feulgen-DNA curve constructed from measurements on a series of photomicrographs of four Feulgen-stained nuclei (4.5-min hydrolysis at 50°C). For both curves, the monochromator was set to a bandpass of 13 μm.
It turned out that such a choice of illuminating aperture was also consistent with conditions for the lowest measured glare and/or flare, as follows:

The photomicrographic components were set up as described in Sec. II. D. 3, but with a 5× micrometer ocular rather than the 10× ocular. Both condenser and N. A. 1.4 objective were oiled (oil r. i. 1.515) to a slide and cover slip enclosing a suspension of graphite particles in immersion oil. The effects of wavelength (546 μm and 405 μm Hg lines), field-diaphragm aperture, and substage-condenser aperture, on the apparent transmittance of an irregularly shaped graphite particle in immersion oil of r. i. 1515 were studied. The apparent transmittances were read with the Photovolt Model 514 M photometer. A range-expanding switch on this unit allows fractions of 1% of full scale to be read. The probe of the photometer was placed to receive the light from a 2-mm-diameter opening in a mask placed over the rectangular-focal-plane aperture of the Exakta camera (mounted to the microscope barrel), the back of the camera having been removed. The position of the 2-mm opening was indicated by a pencilled circle on the ground glass of an Exakta waist-level finder inserted in the camera. Thus it was possible to locate a graphite particle by means of the reflex viewing feature of the camera, and then open the shutter and take a photometer reading down to a fraction of 1% transmittance.

The diagram (Fig. 6) indicating the results of this experiment needs some explanation. The diameter of the field diaphragm opening is represented on the abscissa, the figures referring to the diameter (in cm) of the field diaphragm image as seen on the ground glass of the camera viewfinder. The field-diaphragm opening corresponding to 2 cm on the abscissa was almost identical with the one used in photomicrography. The ordinate represents the apparent percent transmittance of a graphite particle, the photometer is set (or corrected) to 100% for each point. In each case the 100 percent setting was made with the graphite particle moved out of the field. Each curve on Fig. 6 represents a set of four such points at a different aperture diaphragm setting. The smaller condenser (aperture) diaphragm settings were
Fig. 6. Apparent transmittance of a graphite particle. The three curves, representing three different substage condenser apertures, at each of two wavelengths, show the change in the apparent transmittance of a graphite particle as the field diaphragm is closed. Numbers on the curves refer to condenser apertures. At the diaphragm settings used, namely condenser aperture $= 0.65$ and the field diaphragm set to correspond to 2 cm on the abscissa, apparent transmittance of the particle is slightly over 1%.
obtained by using the telescope to compare the size of the condenser diaphragm image with the size of the back lens of a reference objective of appropriate aperture. The image of the graphite particle just occluded the 2-mm camera focal-plane aperture.

The results shown in Fig. 6 may be stated as follows: (a) Flare (and/or glare) increases rapidly as the field diaphragm is opened; (b) it is lower at condenser N. A. 0.65 than at 0.40; (c) at 405 μ it is lower at 0.65 than at the wide-open setting, though at 546 the wide-open setting gives slightly lower flare than the 0.65 setting; and (d) flare (and/or glare) with the settings used for photomicrography is slightly over 1%. (The point for 1 cm on the N. A. 0.4 curve at 546 μ is assumed to be erroneously low.)

It is difficult, however, to generalize these results to measurements on cells that have absorbances of less than 0.4 and that have been matched by the r. i. of the medium in which they are immersed. The total opacity and abrupt boundaries of the graphite particle represent an extreme situation. Flare error in photomicrography of cells may actually be less than 1%.

The experiment may be interpreted to indicate that some of the glare noted at small aperture diaphragm settings may have its origin in diffraction effects at the boundaries of the specimen. (At low aperture the diffraction patterns generated at the boundary of the specimen would tend to spread to the center of the specimen.) That the error was reduced at 546 μ by opening the aperture diaphragm from 0.65, but increased slightly at 405 μ, may testify to the better optical corrections of the condenser in the middle of the visible spectrum.

One more remark is in order here. The tests above were performed with a great deal of attention to cleanliness in the preparation of the slide, and care in placing the immersing fluids. It was found that any dirt, or any bubbles occurring between the condenser and the objective, increased glare markedly.
5. Biconical Error and Depth of Field

As discussed in Sec. II. E. 4., the numerical aperture of the condenser was set at 0.65. The fairly wide cone of light at this setting may lead to cytophotometric error through increase of the mean-path length of light through the specimen, according to the reasoning of Sec. II. B.2. c. Furthermore, a large aperture corresponds to a small depth of field. Fortunately it is possible to assign numerical values to the maximum error that might result from these causes.

From the chart given by Blout et al. (1950), it may be found that the biconical error, expressed on a transmittance basis, for a numerical aperture of 0.65 is less than 2% when an immersion medium of refractive index of 1.515 is assumed. Recall from the discussion of Sec. II. B. 2. c that this error is evaluated with the assumption that the specimen can be geometrically approximated with a slab and that the error would be smaller for a sphere-like specimen, or, by inference, for a specimen consisting of a number of sphere-like objects. Thus the biconical error determined for a slab specimen sets an upper limit on the actual error from this cause. Biconical error, then, in the photomicrographic arrangement used, may lead to a measured transmittance that is as much as 2% too low.

The depth of field is a function of the numerical aperture of the system and can be determined by the application of geometrical optics. If it is assumed that the condenser aperture of 0.65 controls the system aperture, then the depth of field [from the expression given by Shillaber (1944)] is 1.8 μ at a wavelength of 555 mμ. (It can be seen that this assumption is somewhat erroneous for it would lead to the conclusion that the effective aperture of the phase microscope is determined by the phase annulus.) No measurement of the thickness of the fixed cells used in this study has been attempted, but it is a reasonable assumption that a cell with a live diameter of 10μ dries to a slab about 1 or 2 μ thick.

The field of focus for the apochromatic objective used with the compensating ocular is far from flat. Some early trials indicated that the photomicrographic arrangement afforded well-focused cell images...
throughout most of the field of the 35-mm camera. The manner of correcting for distributional error, of course, requires that the information about the distribution of absorbing materials be accurately recorded on the photographic film. The final and most conclusive test for adequate field flatness and depth of focus was the examination of enlargements of the photomicrographs. Enlargements of photomicrographs of the same field by both Soret band and Feulgen-DNA photometry are shown in Plate II. These photomicrographs are representative of most of the negatives used for quantitative analysis; through a series of trials I found it possible to reproduce focus, using the fine adjustment control of the microscope, to within ±0.15 μ, whereas an error of 1 μ in either direction was required to degrade appreciably the definition of the image.

What is the effect of an error in focusing on the measured quantity? To determine this by experiment, it was necessary to prepare a series of photomicrographs and to evaluate these quantitatively by the methods of Secs. II. D. and IV. C. 5. A fixed-bone-marrow smear was immersed in a medium of r. i. 1.545, covered with a cover glass, and oiled to objective and condenser. Photomicrographs were taken at 405 μ at a number of consecutive focus settings. As indicated above, it was possible to reproduce the in-focus setting to within 0.15 μ. Figure 7 gives the results for measurements on three reticulocytes.

Of interest in connection with the above experiment is the recent report of Latimer and Robinson (1963). These authors state that so-called "phase contrast effects" make apparent specimen absorbance a function of focus error, implying that such effects may be so serious as to make microphotometric quantitative assay impossible (Latimer and Robinson, 1963).

With the equipment and method tested here, I estimate that a focus error of ±1 μ could lead to an error of about 5% in the measured quantity of hemoglobin. However, focus is reproducible to within ±0.15 μ. Latimer and Robinson may have used faulty optics or too low a numerical aperture.

A similar analysis of focus error has been given by Niemi (1958) based on the method of Ponder and Barreto (1955).
Plate 2. Comparison of hemoglobin and Feulgen photomicrographs. (a) Photomicrograph of a fixed rat-marrow smear made with the monochromator set to 412 mμ. (b) Same field photographed after Feulgen staining with the monochromator set to 555 mμ. These photomicrographs should be compared with field 1(a) on Plate 3. Note that nonabsorbing cellular elements have disappeared as the result of optimum-refractive-index matching with an immersant of r. i. 1.545. These illustrations were prepared from negatives made for quantitative interpretation in connection with an experiment in progress at the time of writing.
Fig. 7. Variation of measured quantity with focus error. Photomicrographs were taken at wavelength 405 mμ. Each of the curves represents measurements on a series of photomicrographs of a single reticulocyte taken at varying degrees out of focus. Each point represents three measurements on the same cell image. The error limits are given as $\left[\sqrt{\sum \delta^2/n-1}\right]$ where $n = 3$ and $\delta$ is the deviation of the individual measurements from the mean of the three measurements.
6. **Refractive-Index Matching**

A series of immersing fluids was prepared by mixing Squibb mineral oil (refractive index 1.483) and 1-chloronapthalene (tech.) (r.i. 1.639). First, two stock solutions were prepared, one of r.i. 1.530 and the other of r.i. 1.575. Refractive index was assumed to be linear with volume fraction. Fine adjustment was made after measuring the refractive index (corrected to 20° C) with a Zeiss Abbe refractometer. Then, by means of two burettes and the assumption of linearity in the relationship between volume fraction and refractive index, a series of solutions covering the r.i. range 1.535 to 1.570 by 0.005-unit increments was prepared. These were checked for r.i. at 20° C with the refractometer. The general procedure followed was as given by Kaiser and Parrish (1939).

The following procedure was repeated several times with both the Tiyoda phase microscope at 40 X 10 power, and a Zeiss phase microscope with fluorite phase optics at comparable power. In both cases a green filter was used. A specimen of fixed smeared marrow cells was immersed in the first fluid, and a photomicrograph taken on copy film. Following two rinses in xylene, this was repeated with the next r.i. fluid with the same cells in the field. This procedure was repeated until the entire refractive-index range had been covered. Prints were made and examined. The results were as follows:

Nonhemoglobinized cells were matched best, that is, showed smallest phase contrast, at r.i. 1.545, whereas hemoglobinized cells were matched optimally (though their images did not disappear completely) at a slightly higher refractive index (1.555 to 1.560). Since only relative measurements of cellular hemoglobin are desired for this study, and because anomalous dispersion would make it impossible to match hemoglobin at absorption bands, I decided to match the nonhemoglobinized cells and to accept the possibility that some scatter loss is included with measured hemoglobin absorption. (For complete discussion, see Sec. II. B. 2. d.) The cleaning up of the background due to nonerythroid cells when smears are immersed in medium of r.i. 1.545 is very notable; for the most part, nonerythroid cells are completely invisible even when an extreme of photographic contrast is achieved (see Plate II).
Since I had the immersion fluids, I proceeded to determine the effect of refractive-index mismatch on measured quantity. To do this, photomicrographs were taken in the Soret band (405 m\(\mu\)) and interpreted according to the procedure described in Secs. II. D and IV. C. 5. The procedure for changing the fluids was the same as described above in connection with the phase-microscope determination of best-refractive-index match. The results are given in Fig. 8. Each point represents the average of measurements on six photomicrographs of the same reticulocyte. The reticulocyte with the greatest amount of hemoglobin showed a minimum apparent absorbance at r. i. 1.555 to 1.560, this being consistent with the observation that refractive index is best matched for hemoglobinized cells by such an immersant. The general indication is that small errors in refractive-index matching do not lead to much variation in the observed quantity.

A further result of studies with refractive-index fluids is that erythroid cells may be picked out of a population at a glance by reference to phase photomicrographs made from marrow-cell smears immersed in a fluid of proper refractive index (see Plate III). For the optics used (Tiyoda 20× phase), Cargille oil (Type A, r. i. 1.515), rendered the white blood cells and their precursors visible, and showed the nuclei light in a cytoplasm darker than background. The cytoplasm of erythroid cells and of one class of granulocyte was definitely very dark. Comparison of such phase photomicrographs with quantitative measurements on photomicrographs of the same fields (taken at wavelength 412 m\(\mu\)), shows that these phase micrographs reliably demonstrate erythroid cells containing less than 10% of the mature amount of hemoglobin.

7. Photometric Integrator

This device, described in Sec. II. D. 2, is subject to some of the same sources of error as the photomicrographic arrangement. The most important of these sources are lenticular flare in the objective, and unevenness in the pattern of illumination. It is also important that the photocell be equally responsive to all portions of the field that it
Fig. 8. Variation of measured quantity (of hemoglobin) with refractive index of immersant. Two reticulocytes of differing hemoglobin content are represented. Refractive index of the immersant was changed by increments of 0.005, as shown on the abscissa. Each point represents the average of measurements on six similar photomicrographic images. Error limits were determined in the same manner as in Fig. 7.
Plate 3. Phase photomicrograph of a fixed marrow smear. This phase photomicrograph was made with 20X phase, a 10X ocular, and a cell immersant of refractive index 1.515. The field designated 1(a) is the same as that shown on Plate 2. Note that nucleated cells have a characteristic appearance, and that the cells which contain hemoglobin show up as quite dark in the phase photomicrograph [cf Plate 2(a) and (b)].
reads, that the readings of the galvanometer can be unerroneously interpreted in transmittance units, and that the entire apparatus be free of electrical drift.

But perhaps the first matter to consider is the possible degradation in resolution introduced by the use of photographic film and the photographic enlarger. If the distributional error is to be corrected, information about the distribution of absorbing materials must not be lost during the procedure. Assume that the microscope can resolve objects larger than 0.4 μ, which size corresponds roughly to 2.5×10^3 lines/mm. The microscope magnification (specimen to film) in the photomicrographic arrangement was determined (by photographing a Bausch and Lomb stage micrometer) to be 218 X; thus the film should be capable of recording (2.5×10^3/218) or about 11 lines per mm. The film used, High Contrast Copy film, is stated by Eastman Kodak Company to have a resolving power in excess of 150 lines/mm. Thus there is an order of magnitude of safety in the photographic resolving power. The enlarging objective, at an aperture of f/8, has a theoretical resolving power of about 170 lines/mm and tests have shown that it in fact resolves more than 58 lines per mm (the limit of the reticle used for testing) in the central portion of the field used for measurement. Thus there is roughly an order of magnitude of safety in the resolving power of the enlarging objective. I conclude that the photographic step does not lead to an appreciable degradation of the image quality.

Lenticular flare in the enlarger is analyzed as follows: The enlarger objective is illuminated with light directed by the enlarger condenser through the film. If the enlarger is properly adjusted, an image of the lamp appears at the back of the objective, and some of this light is collected by the objective. However some light may be reflected from the lens board or scattered from the film, thus illuminating the inside of the bellows unit. Light from the bellows may find its way to the objective. It is important to realize, however, that scattered light which enters the objective directly will be imaged at the image point corresponding to the point where the scattering or reflection occurred. The objective is optically coated and may be expected to convert perhaps 10% of the light incident on it to flare light.
Most of the light incident on the objective is image-forming light, though the small portion of it arising from internal reflections is not. The enlarger is used to produce a magnification of 6X with a 50-mm objective, which corresponds to a lens-to-image distance of 35 cm. If the flare light is dispersed over a solid angle of $4\pi$, it is reduced by a factor of $6.5 \times 10^{-5}$ at 35 cm. The image-forming light is spread over an area of $6^2 \times 2.4 \times 3.6$ (36 times the area of the 35-mm film frame) or 310 cm$^2$; so it is reduced by a factor of $3.2 \times 10^{-3}$. When flare light arising from internal reflections is neglected, the relative proportion of flare light in the image plane should be about $(0.1 \times 6.5 \times 10^{-5}) / (0.9 \times 3.2 \times 10^{-3})$ or 0.23%. To allow for the possible inaccuracy of the assumptions, it can be said that flare, by the above analysis, should lead to an error of less than 1%.

This has been checked as follows: The enlarger photometer system was set up as described in Sec. II.D.2 with a 1/2-in. diameter aperture over the photocell lens. Small disks were cut from opaque paper of such size that when the disks were glued to photographic film, their projected image was just barely larger than the photocell aperture. One disk was glued to a strip of clear film that had been fixed but not exposed. The other was glued to heavily fogged film. The photometer was zeroed and set to 100% on either the clear or fogged film, then the image of the disk was brought over the photocell aperture. In these measurements, the field was defined by the rectangular frame-sized aperture of the film carrier. In both instances, measured flare—that is, the apparent transmittance of the opaque disks—was approximately 1/2% regardless of whether clear or fogged film was used.

Demonstration of illumination pattern evenness and the spatial nondependence of photocell response was equally straightforward. In the first place, the pattern of illumination supplied to the film by the enlarger condenser is considerably larger than the 35-mm film frame. (The illumination pattern is limited or masked by the rectangular-field aperture of the film carrier.) The cell images, at the photomicrographic magnification used, are 1 or 2 mm in diameter. Measurements are made with the cell image centered with respect to the condenser.
light pattern. It is reasonable to assume that the small central portion of the light pattern behind the cell image is uniform, and that the image of this pattern is also uniform. But uniformity of photocell sensitivity with respect to various points, or subareas, of the photocell lens aperture may be doubted. Uniformity of photocell response across this aperture was tested as follows:

The enlarger photometer was set up as above, and an opaque card with a centrally located pinhole opening was placed in the film carrier. By means of the micrometer controls of the film carrier, the pinhole image was placed successively at equal intervals across the photocell lens, there being no aperture plate in place at this time. The galvanometer was read for each placement. The results are given in Fig. 9.

The diameter of the largest aperture used (5/8 in.) is indicated on the figure. At the magnification used, this aperture can easily accommodate the image of a cell of 10-μ diameter. The photocell response over this diameter is uniform to within ±1%.

Another way of testing the photocell response is to measure the transmittance of apertures of known size. Such measurements were made, with the photometer set to 100% for the 7/8-in. aperture. The results are shown in Fig. 10.

Linearity of photocell response was tested as follows: A 2-in.² neutral density filter, value 0.1 density unit, was cut into smaller squares (greater than 1/2 in. on a side). The enlarger photometer was set to read 100% through the 1/2-in. aperture. Then the filter segments were stacked one on the other over the aperture, and the expected readings compared with the measured readings. Results are shown in Fig. 11. In several identical experiments, both positive and negative departures from linearity were noted, and these departures varied with the order and manner in which the various filter segments were stacked. The points shown are (a) the worst ones obtained and (b) better ones obtained after restacking the filters. I conclude that the photometer response is linear within the bounds of error of the testing method. The reason for the testing error no doubt lies in variation of filter density.
Fig. 9. Pinhole test of photocell response uniformity. The image of a pinhole in opaque paper was moved, stepwise, diametrically across the aperture of the photocell lens. Sensitivity of the photometer was adjusted to maximum. Uniformity of response throughout the 5/8-in. aperture is excellent, and larger apertures are usable though with some sacrifice of response uniformity.
Fig. 10. Apparent transmittance of photocell apertures of various sizes. The photometer was set so that the galvanometer indicated full scale for the amount of light passing through the 7/8-in. aperture. Then the various aperture plates were inserted and the apparent transmittance for each aperture recorded. The slight deviation from nonlinearity is expected on the basis of the departure from uniformity of response, for large apertures, as shown in Fig. 9.
Fig. 11. Test of photometer linearity with neutral density filters. Neutral density 0.1 filters cut from the same sheet were stacked on the aperture plate to achieve the absorbances indicated on the abscissa. The points (●) show the photometer response when the filters were stacked in random order, and the triangles (▲) show the response when the same filters were stacked in a sequence chosen to optimize linearity.
The apparatus was subject to electrical drift. But this could be controlled to an extent by use of a Sola voltage-regulating transformer on the enlarger lamp, and by a warmup period for the enlarger lamp and the photometer unit for at least one-half hour prior to use. It was found necessary to warm up the photometer with the selector switch in the "on" rather than the "warm up" position. As an added precaution against drift, frequent checks of the zero and 100% setting were made during use.

F. Adaptation of the Feulgen Reaction

The applicability of the Feulgen-DNA reaction for the quantitative determination of DNA in solutions was reported by Widström in 1928 and by Caspersson in 1932 (Leuchtenberger, 1958). These and other workers noted that the presence of proteins in the DNA solution leads to interference with the development of the characteristic complex between the Feulgen Schiff reagent* and the aldehydic groups of hydrolyzed DNA. This observation may have delayed the eventual application of the Feulgen reaction for cytophotometric determination of DNA.

However, circa 1950 it was shown in many laboratories (e.g., those of Pollister, Swift, Ris, and Mirski) that cytophotometric determination of the amount of color developed in Feulgen-stained nuclei could provide a reliable indication of the nuclear quantity of DNA. At that time biochemical studies had indicated that DNA in the tissues of a given organism occurred in quantities corresponding to integral amounts of a quantity C characteristic of the organism; thus if a 1C amount of DNA was found in a gamete, a 2C amount would be found in the somatic tissues of a diploid organism and an nC amount in tissues where polyploidy occurred. This result was confirmed by Feulgen cytophotometry and thus the "principle of DNA constancy" was established (Vendrely and Vendrely, 1956; Leuchtenberger, 1958).

* The Feulgen reagent is a specially bleached solution of basic fuchsin—see Appendix E.
Further studies of the Feulgen-DNA reaction established the validity of its applicability for quantitative determinations of this nature. Sibatani and Naora studied the possible failure of the law of exponential absorption by extracting and separately measuring the quantities of DNA and fuchsin combined in isolated nuclei stained by the Feulgen technique. They found no difference in the relative quantity of fuchsin and DNA thus combined in liver or thymus nuclei, in spite of a marked difference in the protein/DNA ratios in these two types of nuclei (Sibatani and Naora, 1953; Sibatani, 1954). Another indication that the protein/DNA ratio does not affect the quantitative applicability of the Feulgen reaction was given by the following study: Schrader and Leuchtenberger (1950) showed that in the pentatomid Arvelius there are three sorts of spermatocytes that differ considerably in volume (200, 400, and 1600 \( \mu^3 \)) and protein content, but the Feulgen measurement of DNA per nucleus yielded exactly the same result in each case. Correlation of measurements of ultraviolet absorption with measurements of the absorption by the Feulgen-DNA complex in the same portion of cell has also served to demonstrate the validity of the Feulgen reaction for quantitative measurements (Walker and Richards, 1957; Barka and Dallner, 1959).

Some other DNA-specific reagents have been evaluated for possible use in cytophotometric determinations (Shugar, 1962; Pollister and Ornstein, 1959). One of these is methyl green (Kurnick, 1955; Swift, 1955). Gallocyanin-chrome alum has been proposed as a specific stain for both DNA and RNA (see Shugar, 1962); Mendelsohn (1957) has thoroughly evaluated its applicability as a cytophotometric reagent for RNA. The proportionality between DNA in situ and the histones associated with it allows determination of ploidy in some cases by means of the fast green stainability of histones (Alfert and Geschwind, 1953). Kasten (1960) has recently reported the results of a general study of Schiff reagents and evaluated them in terms of possible cytochemical applications. But as yet no DNA-specific cytochemical reagent other than Feulgen's reagent has found a place in practical cytophotometric measurements.
Leuchtenberger and Leuchtenberger (1960) have published the collected results of 10 years of work in Feulgen-DNA cytophotometry; measurements were reported on over 75,000 individual cells, and integral modal DNA values are the rule. An initial variant observation by Pasteels and Lison has been discredited (Alfert and Swift, 1953). However, reports of nonintegral DNA contents continue to appear (e.g., Hale and Wilson, 1961).

In spite of the usefulness of the Feulgen-DNA reaction, its stoichiometry, in the strict chemical sense, has never been established and may well be doubted. To prepare a cytologically fixed biological sample for staining with the Feulgen reagent, the sample is customarily hydrolized in 1N HCl at 60° C for a period of time that depends on the nature of the specimen and the manner of its fixation. The hydrolysis has the effect of breaking up the tight structure of nuclear DNA and exposing the aldehydic groups of the deoxyribose sugar units so that they may undergo a Schiff reaction with the bleached dye, thus restoring the color of the dye and fixing it at the site of reaction. For a complete discussion of the Feulgen-DNA reaction, which is still not completely understood, refer to recent articles (Kasten, 1960; Pearse, 1960).

It has been demonstrated that the amount of DNA -- as measured by ultraviolet-light absorption -- decreases steadily during hydrolysis of the sample, and thus has decreased considerably by the time the Feulgen stainability has reached its peak; Feulgen stainability disappears completely after prolonged hydrolysis as does the ultraviolet-light absorption (Di Stefano, 1948). It seems obvious, then, that such a reaction cannot be considered stoichiometric except in a most conditional sense.

The results of Feulgen-DNA cytophotometry are therefore customarily stated in arbitrary units. Only when the 1C or 2C amounts are defined by gross biochemical analysis can the cytophotometric measurements be stated in absolute units.

In staining the rat-marrow cells by the usual Feulgen procedure (Pearse, 1960), I found that the maximum absorbance of the nuclei could easily reach 2 absorbance units (1% transmittance) at the Feulgen
absorption peak. But the linear portion of the t-A curve is only about 0.4 absorbance unit long. Radically changing the method to allow measurement of absorbances of up to 2 units would have led to trouble from glare, which probably occurs to the extent of about 1% in the photomicrographic system. The other alternative was to reduce the absorbance and use the same method utilized for hemoglobin measurement. Reduction in the absorbance could be accomplished in two ways. One way would involve setting the monochromator to a wavelength off the Feulgen-DNA absorption peak. Though this possibility was not thoroughly investigated, the wide bandpass of the monochromator as used could easily lead to failure of the exponential absorption law (see Sec. II. E. 2). The other way of reducing the absorption of the Feulgen-DNA complex was to understain, which could be conveniently done by reducing the degree of hydrolysis.

Though the degree of hydrolysis for optimally intense Feulgen staining has been thoroughly investigated (see Vendrely and Vendrely, 1956), the conditions for a given degree of understain must be chosen on the basis of the nature of the material studied. Some initial trial and error indicated that the proper degree of staining could be obtained by hydrolyzing for several minutes at reduced temperature. A more quantitative determination was then made.

An air-dried smear of normal marrow was selected. This had been first fixed for 5 min in methanol, then fixed for 15 min in chloroform-Carnoy's (see Appendix E). The chloroform-containing fixative was chosen to avoid spurious absorbance due to the Feulgen-plasmal reaction (Pearse, 1960) sometimes caused by cytoplasmic lipids. The fixed slide was put through the Feulgen-staining procedure given in Appendix E, with the following modification: The smear was lowered stepwise into the hydrolysis bath (1N HCl at 50±0.5° C) so that eight zones of the slide each received different intervals of hydrolysis, the intervals differing by 1 min.

Photomicrography was performed on the stained slide according to the procedure described in Secs. II. D. 3 and IV.C. 5 and 7. I selected photographic images of nuclei and scanned them diametrically, using
the smallest possible slit settings of the Jarrell-Ash microphotometer. From the microphotometer trace and a sensitometric calibration curve, a maximum absorbance was found for each nucleus. The results (Fig. 12) define a hydrolysis curve. Figure 13 shows the same data, except that the absorbance for each nucleus is weighted by the ratio

\[ \frac{\text{diameter of nucleus in microns}}{6.9} \]

This correction is based on the approximation of the shape of the nuclei by cylindrical sections, and it corrects the maximum absorbance in each case to that which might be expected if each nucleus were a cylindrical section of 6.9 \( \mu \) diameter. The figures indicate that a minimum in the hydrolysis curve occurs at about 4.5 min, and that the maximum absorbances obtained by this degree of hydrolysis are between 0.19 and 0.28. Such absorbance can be well accommodated by the 0.4 absorbance-unit linear range of the t-A curve. Correction of the maximum nuclear absorbance for variation in nuclear size (Fig. 13) leads to the expected variation of nuclear contents over a range of about a factor of two. That the correction was required to show this variation indicates that a nucleus with a larger DNA content tends to occupy a proportionately larger area on the slide. There is also an indication that there may be two or more sorts of DNA with different hydrolysis characteristics; thus the observed hydrolysis curve (Fig. 12) could result from the superposition of the two curves shown below.

That both (or all) components are in fact DNA rather than other Feulgen-reacting species is indicated by the absence of any perceptible density in the cytoplasm of the stained cells. No attempt has been made to follow up this observation.
Fig. 12. Variation of maximum nuclear absorbance with hydrolysis time. Each of the eight histograms represents, for the hydrolysis time given, the distribution of nuclei with respect to maximum absorbance. The shape of the hydrolysis curve, indicated by the dashed line, can be estimated from these histograms.
Fig. 13. Variation of maximum nuclear absorbance with hydrolysis time -- corrected for variation in nuclear size. These histograms differ from those of Fig. 12 only in that the maximum absorbance per nucleus in each case has been corrected to that which would be expected if all nuclei had a diameter of 6.9 μ (see text).
I chose for Feulgen cytophotometry the hydrolysis time corresponding to the minimum absorbance because, in this region of the curve, small accidental variations in the Feulgen technique should have the least effect on the density developed. Furthermore, it is desirable that the maximum density be kept as low as possible in order to avoid the possibility of an occasional compact nucleus having an absorbance in excess of 0.4.
III. ERYTHROPOIESIS

Prior to discussion of the application of the previously described cytophotometric technique to a problem of current interest in hematology (Sec. IV), it is desirable to review here the pertinent hematological literature. This section is divided into two parts. In the first part it is sought to construct an adequate background in the fields of classical and modern hematology for consideration of some of the newer techniques, findings, and theories discussed in the second part. In turn, an appreciation of the material expounded in the second part seems basic to the design and interpretation of hematological experimentation (such as is described in Sec. IV).

A. Introduction to Erythropoiesis

1. The Erythroid Sequence According to Morphological Criteria

Nearly a century ago formation of blood cells in bone marrow was observed by Neumann and Bizzozero, and mitosis observed in marrow by Flemming; shortly thereafter correlation of changes of the peripheral blood to changes in the marrow was commenced by Naegeli and Schilling (Downey, 1938). These, and most subsequent observations on the role of the marrow in blood-cell formation, result from the application of the compound microscope and of cytological techniques for rendering visible the substances of biological cells. Until the recent development of phase optics by Zernike (1935), morphological examination was limited to cytologically fixed specimens. Even the phase microscopist was faced with the limitation of not being able to follow a single cell or synchronized cohort of cells through development in situ. Only very recently has it proved possible to demonstrate maturation of erythroid marrow cells in tissue culture (Astaldi, 1960; Jacobsen and Doyle, 1962, Sec. VIII).

Thus present knowledge of the sequence of events and changes that occur during erythropoiesis is derived mainly from observations on cells separated from live marrow. In marrow morphology, erythroid cells are ranked according to their precedence, but in order to distinguish between mother cell and daughter cell the morphologist has
had to rely on characteristics such as affinity for cytological stains, degree of nuclear stippling, presence or absence of nucleoli, nuclear size, cell size, and cytoplasmic inclusions. Though morphological designations of erythroid cells ostensibly correlate with interdivisional stages, the number of divisions remains a matter of uncertainty (Maximow and Bloom, 1957, p. 98).

The first cell in the erythroid development sequence is by definition the stem cell, but very little else is clear about this cell. The following is summarized from Wintrobe (1961). According to the monophyletic school, the lymphocyte of lymphatic tissue is identical to the primitive blood cell—it is thus totipotential, giving rise under proper stimulation to any other type of blood cell, whether erythrocyte or leukocyte, granulocyte or monocyte. Neo-unitarians maintain that the lymphocyte has the potential for such change, but changes only under abnormal environmental conditions. Proponents of the polyphyletic theory have described a number of "blast cells" that are said to be precursors of the various completely differentiated blood cells. According to the number of "blast" cells recognized, the polyphyletists have been distinguished as dualists, trialists, and "complete" polyphyletists.

When discussion is limited to the erythroid-development sequence, a conception of the current status of erythroid morphology can be gained from the complexity of morphological nomenclature.

Thorell (1947) chose to describe the erythroid sequence in the following terms:

- reticular cell → proerythroblast (or stem cell)
- → basophilic erythroblast → polychromatic erythroblast
- → orthochromatic erythroblast → reticulocyte → erythrocyte.

Maximow and Bloom (1957) retain the same basic terminology, referring, however, to the first cell in the sequence as a hemocytoblast and suggesting that the proerythroblast is an intermediate cell. They designate Thorell's orthochromatic erythroblast as a normoblast.

The various synonyms for the erythroid developmental stages are given by Whitby and Britton (1963): The stem cell is known also as lymphoidocyte, hemohistioblast, haematogone, and rubrioblast; the
proerythroblast as pronormoblast, macroblast, erythrogone, megaloblast, lymphoid haemoblast, and prorubricyte; the basophilic erythroblast as early normoblast, basophilic normoblast, early erythroblast, macroblast, type A normoblast, and rubricyte; the polychromatic erythroblast as intermediate normoblast, polychromatic normoblast, late erythroblast, type B normoblast, and rubricyte; the orthochromatic erythroblast as late normoblast, orthochromatic normoblast, normoblast, Type C normoblast, and metarubricyte. The terms reticulocyte and erythrocyte are consistent in all nomenclatures, though erythrocytes may also be called normocytes.

The rubri-nomenclature above is attributable to the Committee for the Clarification of the Nomenclature and Diseases of the Blood and Blood-Forming Organs. Their recommendations for a consistent nomenclature were published (Osgood, 1949) but have not been generally accepted.

Mature erythrocytes have been conventionally divided into three sizes: macrocytes or megalocytes, normocytes, and microcytes. It has sometimes been assumed that the megalocytes and the normocytes stem from separate developmental lines. Thus in some representations (e.g., Maximow and Bloom, 1957) of the erythroid developmental sequence, two series of blast cells are given—megaloblasts and normoblasts. Further, erythrocytes may be classified by their degree of hemoglobinization according to the adjective nomenclature hypo-, normo-, and hyperchromic. Any standard textbook on hematology (e.g., Wintrobe, 1961) may be consulted for the terminology relative to abnormal inclusions and shape variations in erythrocytes and the meaning of these to the clinician.

Blood-cell morphology rests on the reactability of the cellular constituents with basic and acid dyes, and on morphological characteristics. The nucleic acids and other acid substances are thus said to be basophilic. A basophilic erythroblast is one whose cytoplasm takes a basic stain. Accumulation of hemoglobin (acidophilic in its staining reaction) in the basophilic erythroblast leads first to a polychromatic staining reaction. Subsequently the disappearance of cytoplasmic nucleic
acids in the hemoglobinized erythroblast results in the orthochromatic
reaction, that is, a stain affinity due to hemoglobin alone. Maturation
of the erythroblast to reticulocyte occurs by extrusion of the nucleus
(Bond et al., 1962). Thereafter the cell may contain a characteristic
network of cytoplasmic elements demonstrable by vital staining and is
known, by this criterion, as a reticulocyte. Lajtha (1962) believes that
the orthochromatic erythroblast is the result of atypical maturation
and that it will mature directly to an erythrocyte, skipping the reticu-
locyte stage.

2. Distribution of Blood-Forming Tissue

The mode of development and distribution of blood-forming tissues
is quite similar for all mammals. Thus the description given by
Wintrobe (Wintrobe, 1961), and summarized below, for embryological
development of erythropoietic function in the human can be considered
typical.

Embryonic blood cells are first formed in the numerous blood
islands of the yolk sac. Until the hepatic period of hematopoiesis,
which commences in the embryo of 5 to 7 mm, most blood cells are
formed outside of the embryo. Blood cells formed in the liver during
hepatic hematopoiesis are thought by most investigators to be derived
from undifferentiated polyvalent mesenchyme, which has spread out
between the liver cells. Erythropoiesis occurs transiently in the spleen
between the second and fifth month of fetal life, and also occurs briefly
in the thymus. The myeloid period of hematopoiesis begins in approxi-
mately the fifth month, concurrent with the establishment of placental
circulation. During this period the marrow assumes first the function
of erythropoiesis, and later all hematopoietic function. The reticular
cells are reduced to a very scant reticular stroma, which nevertheless
remains throughout life with all of its potentialities intact.

According to Maximow and Bloom (1957), red (hematopoietic)
marrow is found in the normal human adult in the vertabrae, the ribs,
the sternum, the diploe of the bones of the skull, and in the proximal
epiphyseal of the femur and the humerus. Bone marrow forms 2 to 3%
of the body weight.
In the human, the distribution of marrow at birth is quite different from that in adulthood (Mitchell-Nelson, 1948); the marrow cavities of the long bones as well as of the flat bones are crowded with active hematopoietic tissue. The extensive distribution of marrow, and the megaloblastic polycythemia of the newborn both suggest intense erythropoietic activity necessitated by the transition from the low tissue-oxygen requirement in the fetal environment to the relatively much higher oxygen requirement in infant life. The only potential reservoir for more hematopoietic foci in the infant lies in the extramedullary blood-building sites in the liver, the spleen, and the lymph nodes; these organs, in times of need, quickly revert to their fetal activities, become hematopoietic, and even visibly and palpably enlarge.

A good deal of the experimental work in erythropoiesis is done with laboratory animals. Therefore it is necessary to understand the similarities and differences between large and small animals before generalizing results of experimentation with small animals. Informal advice I obtained from Dr. D. C. Van Dyke (private communication) indicates that many of the observed differences in experimental responses may be understood in terms of the ability of the erythropoietic marrow to expand function in order to meet crisis.

Van Dyke et al. (1964) studied erythropoietic marrow distribution in man, rabbit, and rat by means of radioiron. Sites of uptake were determined by means of the whole-body counter by the recently developed positron scintillation camera (Anger, 1963), and by well-type scintillation counting of the individual bones of rats. Scintillation photographs indicated more disperse distribution of erythroid marrow in the skeleton of the rabbit and the rat than in man. In the normal rabbit very little activity registered in the distal parts of the shanks of the tibiae of the forearms. The normal rat seemed intermediate between rabbit and man, having the erythroid marrow concentrated in the pelvis and proximal portion of the legs, with relatively little in the distal portions of the legs or tail. A positron photograph of a rat bled severely over an interval of 6 days was not distinguishable from that of the control. In the rabbit, however, severe anemia, produced by seven consecutive daily treatments with phenylhydrazine, led to extension of erythropoietic
function to the distal portion of the shanks of humeri and the femora. In rats stimulated with erythropoietin (13 standard A units per day for 14 days prior to Fe$^{59}$ injection) and assayed by well-counting of the individual bones and organs, there was no significant increase in total marrow volume and no change in distribution of marrow within the skeleton.

In man, by whole-body counting of Fe$^{59}$ incorporation by the marrow, the above study demonstrated extension of the marrow to unusual sites only after prolonged and severe bleeding of many years' duration. On the other hand, in a patient whose acute hemolytic anemia led to a rate of erythropoiesis 13 times normal, only minimal extension of the marrow was demonstrated. (Dr. Van Dyke has told me that a new study has demonstrated marked marrow extension after an interval of only a few months.)

An inference from this study is that the human adult has considerable potential for expanding marrow function by increasing the concentration of erythroid elements in normal erythropoietic loci, or by peripheral extension of the erythroid marrow. Small experimental animals, on the other hand, appear deficient in the ability to expand marrow function through extension or expansion of erythroid marrow. In this respect, small animals are similar to the newborn human as discussed above.

3. Functional Relationship of Nucleic Acids and Proteins during Erythropoiesis

Much of the present knowledge of the interrelationships of the cytologically observable cellular constituents in developing blood cells stems from the studies of Thorell (1947), based on the techniques of Caspersson (1936, 1950). Though Thorell's original study treated both erythropoiesis and leukopoiesis, and touched on nucleic-acid-protein patterns in cancer and dentine cells, only erythropoietic aspects will be discussed here.

Thorell (1947) used the Köhler rotating-spark gap, a monochromator and filters, and monochromatic quartz optics to study ultraviolet light absorption by freshly isolated human marrow cells suspended in
saline solution. Ultraviolet-light was registered on photographic plates calibrated individually at the various wavelengths used. Spark spectra from Mg, Cd, and Ni provided eight spectral lines between 244 and 310 mμ. Microphotometry of the developed plates provided eight-point absorption spectra for small regions of cytoplasm. The absorbance at 275 mμ provided a measure of the protein concentration, and that at 257 a measure of nucleic acid concentration. Because the absorbance of each of these constituents is appreciable at the wavelength used to measure the other, determination of the nucleic acid and protein concentrations required the solution of a system of two equations in two unknowns. This method of calculation could be used because measurements were made only at points sufficiently far from the edge of the cell image that light loss due to reflections at the cell edge were avoided.

Morphological examination indicated that the earlier erythroid cells were larger than the later ones, thus that size could be used as an indication of relative cell age, hence morphological type. By plotting the ratio of the absorbance at 257 mμ to that at 275 mμ plus various derivative quantities against cell size, Thorell showed that the concentration of cytoplasmic nucleic acids fell off quite sharply during the earliest stages of development.

Thorell's measurements of hemoglobin were made with violet light from a mercury lamp and with apparatus slightly different from that described above. The image of the cell could be moved about on an aperture in front of a photomultiplier tube by means of micrometer adjustments on a 45-degree prism placed just above the microscope ocular. In this way a subarea of the specimen cell could be selected, and the optical transmittance through it measured. A series of such measurements provided a profile of the hemoglobin concentration across a diameter of the cell. Nonspecific light loss was estimated from the apparent absorbance at a longer wavelength (496 mμ). From such measurements the total amount of hemoglobin could be determined and the hemoglobin concentration estimated. The output of the photomultiplier was determined to be linear with the intensity of light by means of calibration against a rotating sector wheel of known absorbance values.
Coordination of the information obtained with violet and with ultraviolet light allowed the following, now classical, generalization (Thorell, 1947): "... the endocellular synthesis of hemoglobin does not start before the ribose polynucleotide metabolism (associated with the new formation of cellular protein-substances) is finished -- the latter process being measurable as a decrease of the cytoplasmic ribose polynucleotide concentration from 5% to less than 0.5%. After this maturation-phase, the main synthesis of hemoglobin begins, and the cellular content rises rapidly from an amount less than $2 \times 10^{-6}$ μg up to $25 \times 10^{-6}$ μg, the final value being $28 \times 10^{-6}$ μg." A further finding was that in the series of developmental stages considered, only the proerythroblast has a discernible nucleolus.

In two cases of pernicious anemia, Thorell saw large erythroid cells with atypically high hemoglobin complements but with normal amounts of cytoplasmic nucleic acids. In a heavily bled rabbit, he found abnormally low amounts of hemoglobin in erythroid cells that were relatively mature as judged by the criteria of small size and low cytoplasmic nucleic acid concentration.

In a later paper, Lagerlöf, Thorell, and Åkerman (1956) showed--by a combination of microinterferometry with the methods described above--that the synthesis of protein, as indicated by cytoplasmic dry mass, preceded the appearance of cytoplasmic hemoglobin.

4. Biochemistry of Hemoglobin Synthesis

Considerably more is known about the details of hemoglobin synthesis than is relevant to this presentation; the following is only a brief summary. Because the latter stages of erythroid-cell metabolism are directed almost entirely to hemoglobin production, erythroid cells have constituted fortuitous subjects for the study of protein synthesis. Much of the following discussion has been taken from three general references (Wintrobe, 1961; Bothwell and Finch, 1962; Harris, 1963).

The last step of hemoglobin synthesis is the compounding of ferrous iron, protoporphyrin, and the basic protein, globin, under the mediation of the heme synthetase system. The metabolic requirements
of this enzyme system are known (Harris, 1963, p. 56), and it is thought to be localized in the mitochondria (Bothwell and Finch, 1962, p. 223).

The initial step in the biosynthesis of protoporphyrin is the condensation of succinate and glycine. This reaction requires the mediation of coenzyme A, pyridoxal phosphate, and other factors. The product is α-amino β-keto adipic acid, which becomes δ-amino levulinic acid by loss of carbon dioxide. Under the influence of a dehydrase system found in erythroid cells and in other tissues and not necessarily associated with the particulate cytoplasmic fractions, two molecules of δ-amino levulinic acid react to form the substituted monopyrrole, porphyrobilinogen. Excretion of large amounts of this substance in the urine is of diagnostic importance in some acquired and hereditary defects of heme synthesis.

An enzyme system has been characterized that catalyzes the reaction of four molecules of porphyrobilinogen to form specific isomers of the tetrapyrrolic ring compound uroporphyrinogen. Through successive removal of carboxyl groups from side chains, uroporphyrinogen is transformed to coproporphyrinogen, which by further decarboxylation and by dehydrogenation is converted to protoporphyrin.

Some 6 to 8 grams of globin are synthesized daily by the human adult. The structure of globin is well understood: Its molecular weight is close to 68,000, and it is composed of 574 amino acid residues of 19 different kinds. These residues are arranged in 2 alpha chains, of 141 amino acid residues each, and 2 beta chains of 146 amino acid residues each. In the intact molecule a heme radical is associated with each of the polypeptide chains. (See Wintrobe, 1961, p. 158.)

According to the present understanding of protein synthesis, the information determining the sequence of amino acids on the peptide chains proceeds from the nuclear DNA to the ribosomes in the form of messenger RNA. Small transfer RNA molecules specific for the various amino acids form energy-requiring complexes with them, and position them in the proper sequence along the ribosomal RNA templates by means of complementary base configuration between transfer and template RNA. Information as to composition and sequence of the peptides is thus directly under genetic control. Genetic defects of hemoglobin
synthesis, such as sickle-cell anemia and thalassemia, have been studied extensively by the various techniques of experimental biology and medicine.

Unlike iron, which is almost completely conserved (see Sec. III. A.5), and unlike the porphyrin moiety of heme, which is excreted as bile pigment (see Sec. III. B. 1. a), globin at the end of the 100- to 120-day life span of the erythrocyte is catabolized and returned to the body stores as constituent amino acids wherefrom constituents may be metabolized or reutilized according to systemic requirements.

Nutritional protein deficiencies surprisingly do not generally lead to anemia, presumably either because infection on nutritional factors such as vitamin B₁₂ or folic acid become limiting before the body exhausts its capacity to mobilize endogenous protein sources.

5. Iron Metabolism

A representative estimation of the allocation of body iron in a 70 kg man is given in Table I (Harris, 1963, p. 32).

Table I. Allocation of iron in body of 70-kg man.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Total iron in body (g)</th>
<th>Iron factor (g/g)</th>
<th>Total iron in compound (g)</th>
<th>Percent of total body iron</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>650.0</td>
<td>0.0034</td>
<td>2.21</td>
<td>64.0</td>
<td>O₂ transport</td>
</tr>
<tr>
<td>Peripheral blood</td>
<td>25.0</td>
<td>0.0034</td>
<td>0.09</td>
<td>2.5</td>
<td>O₂ transport</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>40.0</td>
<td>0.0034</td>
<td>0.14</td>
<td>4.0</td>
<td>O₂ transport and &quot;storage&quot;</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>4.0</td>
<td>0.00043</td>
<td>0.0034</td>
<td>0.097</td>
<td>O₂ utilization</td>
</tr>
<tr>
<td>Cytochrome</td>
<td>5.0</td>
<td>0.009</td>
<td>0.0045</td>
<td>0.13</td>
<td>H₂O₂ destruction</td>
</tr>
<tr>
<td>Catalase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>H₂O₂ destruction</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Storage iron</td>
<td>2.0</td>
<td>0.23</td>
<td>0.46</td>
<td>13.0</td>
<td>Fe storage</td>
</tr>
<tr>
<td>Ferritin</td>
<td>1.5</td>
<td>0.37</td>
<td>0.56</td>
<td>16.0</td>
<td>Fe storage</td>
</tr>
<tr>
<td>Hemosiderin</td>
<td>6.5</td>
<td>0.0004</td>
<td>0.004</td>
<td>0.12</td>
<td>Fe transport</td>
</tr>
<tr>
<td>Transport iron</td>
<td>6.5</td>
<td>0.0004</td>
<td>0.004</td>
<td>0.12</td>
<td>Fe transport</td>
</tr>
<tr>
<td>TOTAL IRON</td>
<td>3.47</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Dietary ferric iron, mostly in organic complexes, is reduced in the upper gut to the ferrous state and perhaps 10% of ingested iron is absorbed by the mucosal cells of the intestinal epithelium. (Harris, 1963, p. 34). Mucosal cells can accumulate large quantities of ferritin, a complex of iron with a specific protein, apoferritin. (Harris, 1963, p. 48). Ferritin may be involved in the kinetics of intestinal-iron absorption (Granick, 1956), but more likely has a passive role in absorption, and an active-transport process may be involved (Harris, 1963, p. 36). It is also likely that the movement of iron through the mucosal cell may depend only on the degree of plasma-iron saturation (see discussion below). Once inside the body, iron is generally believed to be transported in the blood stream, though a recent study has indicated some involvement of the lymphatic circulation (Reizenstein et al., 1960).

In the blood the essentially insoluble ferric iron is complexed to transferrin, a $\beta_4$-globulin of mol wt 90,000. Normally 100 ml of human plasma has sufficient transferrin to bind about 300 $\mu$g of iron, whereas the normal serum-iron level is 100 $\mu$g per 100 ml (Wintrobe, 1961, p. 147).

According to Mitchell et al. (1960), transferrin may exist in two phases: one circulating in the plasma, and the other at cell sites where iron is being absorbed, utilized, and stored. The evidence for this view is the reduction of serum-iron-binding capacity following oral administration of inorganic iron. Iron is preferentially transferred from transferrin to immature erythroid cells, the erythroblasts having the highest uptake (Paoletti, 1957; Paterson et al., 1952). A number of in vitro studies suggest the following mechanism (see Harris, 1963, p. 55). Iron-saturated transferrin is preferentially bound to young red cells with an affinity four or five times that of iron-free transferrin. Thus unladen transferrin may be released after the capture of iron by competitive binding sites on the cell surface, but, since cell-bound unladen transferrin is in equilibrium with unladen, unbound transferrin, the likelihood of release is conditioned by the concentration of unladen plasma transferrin. Thus maximum transfer of iron to the cells should occur at a low level of relative saturation of plasma iron-binding capacity. At high saturation, nonspecific unloading of iron occurs.
Once bound to specific binding sites at the cell surface, the iron is not elutable by washing or treatment with chemical chelating agents (Harris, 1963, p. 55). The binding sites can be largely destroyed by trypsin (Jandl et al., 1959). The half-time of transit across the membrane has been shown to be about 8 min (Allen and Jandl, 1960). Inside the cell there appears to be another iron-carrier protein between the cell membrane and hemoglobin or ferritin (Greenough et al., 1962). Recent studies by Mazur et al. (1962) indicate that it may be necessary that iron proceed to heme by way of ferritin. Another report describes involvement in cytoplasmic-iron transfer of a protein fraction with electrophoretic mobility different from that of ferritin (Salera et al., 1961). Since heme synthesis is blocked by lead, but uptake of iron by the specific receptors is not, the indication is that iron uptake is not dependent on heme synthesis (Harris, 1963, p. 55). Electron micrographs indicate abnormal concentrations of ferritin iron inside immature cells affected by lead poisoning (Bessis and Bretón-Gorius, 1962).

An alternative mechanism for the intake of iron by immature erythroid cells has been suggested by Bessis et al. (1962), who observed in electron micrographs pinocytotic transfer of ferritin from reticular cells to young erythroblasts aggregated around them. The groups of cells, or erythroblastic islands as they are called, appear to be arranged in the marrow as the units of erythropoietic development, with the youngest cells closest to a feeder "reticular" cell and the more mature cells at the periphery. Bessis designates the process of pinocytotic transfer of ferritin a "ropheocytosis." Sondhaus and Thorell (1960) have studied immature salamander erythroid cells by microspectrophotometry. They found that the youngest cells contain considerable ferritin, which decreases as hemoglobinization proceeds during maturation. Bothwell and Finch (1962, p. 227) suggest that nonheme iron normally represents only a small fraction of the cellular content; the amount of ferritin formed, they suggest, depends on the amount of iron available to the cell.
When radioiron complexed to transferrin is intravenously administered to an experimental subject, the iron is cleared from the plasma in an approximately exponential manner. Scintillation counters (fitted with collimators), which indicate the distribution of iron through the body, show that the iron is first taken up by the plasma and then moves rapidly to the marrow (Pollycove and Mortimer, 1961). The plasma iron turnover (PIT) may be calculated from the half-time of disappearance of the iron from plasma. At some later time quantities of radioiron reappear in the peripheral blood, incorporated into the hemoglobin of circulating red cells. The clearance and re-emergence curves provide both clinical and experimental information. Considerable experimental information supports the conclusion that most of the injected iron appears as hemoglobin and relatively little is relegated to long-term iron stores (Harris, 1963, p. 41). Stores of nonheme iron represent a significant portion of the systemic iron (see Table I), yet surprisingly, injected iron does not readily equilibrate with these stores. A similar situation holds in the case of the reuse of hemoglobin iron—it is preferentially used for new hemoglobin synthesis. Harris (1963, p. 47) suggested that this phenomenon is attributable to the peculiar properties of the transferrin-iron complex and its interaction with developing erythroid cells. Wintrobe (1961, p. 148) suggested that reduction in the quantity of blood transferrin, as the result of sequestration of iron-laden transferrin at the membranes of erythroid cells, may be a mechanism for the regulation of absorption in the intestine.

Pollycove and Mortimer (1961) have demonstrated that radioiron clearance is not a simple exponential process, but has a slow exponential component that appears after the initially rapid exponential clearance has reduced serum iron to relatively low levels. The long-lived component they attribute to a feedback from a labile pool of iron presumably reversibly associated with the cell membrane of immature erythroid cells.

Under certain conditions [e.g., hemachromatosis (Pollycove and Mortimer, 1961)], the content in the stores may become abnormally high, and feedback of iron from long-term stores may become appreciable relative to feedback from labile pools. Normally, however, it
requires about a year for administered tracer iron to equilibrate with the long-term iron stores (Finch, 1959).

The long-term iron stores consist of ferritin and hemosiderin distributed throughout various sites in the body. Hemosiderin, once considered to be a degradation product of heme, is now known to differ from ferritin mainly (a) in being present in granules large enough to be visible in the optical microscope (b) in having a larger proportion of iron (Wintrobe, 1961, p. 149), and (c) perhaps in containing other substances such as porphyrin pigments (Harris, 1963, p. 51). The characteristic structure of ferritin allows it to be readily identified in electron micrographs (Bessis and Bretón-Gorius, 1962). Systemically, stored iron is divided about equally between ferritin and hemosiderin (see Table I). It is found in mucosal cells of the gut, in the liver, spleen, marrow reticuloendothelial cells, and in developing erythroid cells.

Once absorbed, iron is almost completely conserved. Wintrobe (1961, p. 149) estimates losses as follows: About 1 mg per day is lost from the dermal surfaces (desquamation), fecal loss is 0.13 to 0.5 mg per day, and menstruation may cause an average loss of another 0.5 to 1.0 mg per day. Information on perspiration loss is equivocal.

It is known that ferritin granules may appear in young circulating reticulocytes, but that these granules are removed rapidly from the reticulocytes by the spleen (Crosby and Sheehy, 1960). Harris (1963, p. 54) believes that it is also possible that reticuloendothelial cells in the marrow may remove excess ferritin from erythroblasts by a process opposite to the rophoeocytosis of Bessis.

Some complicating aspects of iron metabolism remain. Recall from Table I that there are several other heme-containing systems, and these to some extent must be involved in iron kinetics. Furthermore, there is in the marrow some synthesis of hemoglobin that will not appear in the circulating red cells, and the amount of such hemoglobin formed is considerably increased with intense erythropoietic activity (Sec. III. B. 1. a). Though the prophyrin from such unused hemoglobin is metabolically degraded and excreted, the iron is conserved and is subject to reutilization. An appreciation of these phenomena is necessary for the interpretation of ferrokinetic data (Pollycove
and Mortimer, 1961; Alpen et al., 1962). It is possible that the
erythropoiesis of Bessis (see Bessis and Bretón-Goriús; 1962) relates
to a physiologic mechanism for the reutilization of iron from erythroid
cells that die and are phagocytized in the marrow; in this connection
it has been shown that immature erythroid cells readily assimilate
iron from transferrin in vitro, whereas reticular cells have only limited
ability to do so (Bothwell and Finch, 1962, p. 225). Furthermore, it
has been shown that iron absorbed from the plasma can quantitatively
account for hemoglobin synthesis (Pollycove and Mortimer, 1961).

Both in vitro studies on reticulocytes and studies on cell-free
extracts of reticulocytes have demonstrated that reticulocytes have the
ability to take up heme and globin precursors and inorganic iron, and
to utilize these in the synthesis of hemoglobin (Harris, 1963, pp. 67 and
68). The more immature the reticulocyte, the greater its ability for
iron incorporation. Later discussion indicates that strong erythro­
poietic stimulation may lead to the early release of immature cells
from the marrow. These factors must also be considered in the evalua­
tion of ferrokinetic data.

6. Factors Affecting Erythropoiesis

The maturing erythroid cell must sustain itself metabolically,
undergo cell division, equip itself for hemoglobin synthesis, and
synthesize hemoglobin. Interference with any of these processes may
be evident in a specific way.

Though the evidence is yet to be discussed, a failure of synchrony
between cell division and hemoglobin synthesis is expected to produce
characteristic signs. This asynchrony may result either from impair­
ment of nuclear replication or of hemoglobin synthesis. If cell
division proceeds relatively faster than hemoglobinization, microcytic
or hypochromic cells should result; if it proceeds relatively slower,
heavily hemoglobinized cells at any stage may be expected. Inability
of the cell to sustain itself metabolically will of course lead to cell
death.
According to the findings of Thorell, cytoplasmic nucleic acids are required for hemoglobin formation, and the cytoplasmic quantities of such nucleic acids are related to the rate, per cell, of hemoglobin synthesis (Thorell, 1947). That quantities of cellular constituents halve at cell division would seem an inescapable conclusion, but one that has been frequently ignored in the interpretation of data on erythroid cellular growth. Therefore, in the following discussion and development, this conclusion is taken as a premise, but critically evaluated.

The amount of marrow space available for erythropoietic function is discussed in Sec. III. A. 2, and although it is not specifically investigated in this study, it constitutes an important parameter in the interpretation of experimental results.

It is of interest to review some of the common clinical manifestations of specific deficiencies of metabolic substances involved in erythropoiesis. Unless otherwise indicated, the following discussion is summarized from Wintrobe (1961).

Hemoglobin production can be impaired by derangement of heme synthesis, by unavailability of iron at the sites of hemoglobin synthesis, or by failure of globin synthesis.

a. **Heme synthesis.** The commonest failure of heme formation is due to the lack of dietary iron. Such lack leads to deficiency of stored iron, to increase in serum-iron-binding capacity, to low-serum iron, to microcytosis, and/or to hypochromocytosis. Vitamin B₆ (pyridoxal, etc.) is required for the condensation of succinate and glycine, and deficiency of it leads to microcytic hypochromic anemia. Pantothenic acid, a constituent of coenzyme A, is also needed for this condensation, but pantothenic acid deficiency leads to symptoms other than anemia. Copper is a constituent of δ-aminolevulinic acid dehydrase (Iodice et al., 1958) and deficiency of it leads to hypochromic, microcytic anemias in cattle.

b. **Protein synthesis.** Protein synthesis is dependent on the cell's complement of cytoplasmic nucleic acids. Because vitamin deficiencies that lead to impairment of nucleic acid formation lead to macrocytosis
and hyperchromia (see discussion below)—conditions indicating a higher degree of impairment of nuclear DNA replication than of capability for cytoplasmic protein synthesis—it may be concluded that the erythroid cell "feels" the shortage of DNA precursors before the availability of cytoplasmic RNA precursors becomes limiting. Mild or moderate anemias may be produced in experimental animals by diets deficient in amino acids. A number of syndromes that have been described result from genetic defects of globin synthesis, e.g., thalassemia and sickle-cell anemia. Red cells containing abnormal globins frequently have shortened life spans and may not function well in their oxygen-carrying role. Thus these conditions may lead to overtaxation of the erythropoietic system.

c. Iron incorporation. As discussed in Sec. III. A. 5, iron makes its way to the sites of hemoglobin synthesis by a complicated route; a block or failure along any leg of this route can impair hemoglobin synthesis. Iron lack in diet, failure of intestinal absorption, atransferrinemias, poisoning as with lead, congenital lack of the heme synthetase system, and other anomalies have been described. Impairment of erythropoiesis through a defect in nucleic acid synthesis is evident in pernicious anemia, a once-fatal affliction that is now treated, usually with complete success, by administration of vitamin $B_{12}$. The structure of this vitamin is now known (Hodgkin et al., 1955); it contains cobalt and can be produced commercially by biosynthesis. Red-blood cells in pernicious anemia are megalocytic and generally hyperchromic. Deficiency of this vitamin is conditioned by failure of alimentary production of "intrinsic factor," which is now thought to be a mucoprotein. Though normally produced in the lower gut by the intestinal flora, vitamin $B_{12}$ may be absorbed only in the upper gut and in the presence of "intrinsic factor."

There are a number of megaloblastic anemias. In some cases these respond to folic acid administration. Megaloblastic anemias are generally characterized by an abundance of systemic iron. The basic metabolic fault in vitamin $B_{12}$ and folic acid deficiency lies in a lessened capability of erythroid cells to synthesize nucleic acids and to divide.
Although the role of these substances in nucleic acid metabolism is not well understood, it is known that they are both involved in metabolic reactions involving one-carbon moieties (Wintrobe, 1961, p. 133). Folic acid is involved in transmethylation reactions, and vitamin B₁₂ in the de novo synthesis of labile methyl groups from the various one-carbon precursors. The one-carbon moieties are building blocks in the synthesis of purines, pyrimidines, and certain amino acids.

7. Erythropoietin and Humoral Factors

Towards the end of the last century, the hypothesis was proposed by Miescher (Miescher, 1893) that hypoxia stimulates red-cell production by acting directly on the red-cell precursors without intermediary agents. Carnot and Défandre (1906) reported experiments (later criticized on technical grounds) indicating that circulating blood carries an erythropoietic stimulating factor, "hemopoïétique." Their observation was that serum obtained from anemic rabbits and infused into normal rabbits caused doubling of the red-cell count within 3 days. Because of the technical shortcomings of their work and the inability of other investigators to obtain their result, however, the view of Miescher continued to be accepted.

In 1950 there was a report that normoblastic hyperplasia of the bone marrow developed in both partners of parabiotic pairs of rats, only one of which was subjected to anoxia (Reissman, 1950). This report renewed interest in the possibility that the hypoxic stimulus is mediated through a hormone-like factor in the blood. Many reports during the fifties provided convincing evidence for the presence of one or more such erythropoietic stimulating factors (ESF, erythropoietin) (Gordon, 1959). It has generally been found that plasma—obtained from animals made anemic by bleeding or other means—when injected repeatedly into test animals causes increased reticulocyte count, hemoglobin level, hematocrit, red-cell count, and uptake of radioiron by erythroid cells (Gray and Erslev, 1957). In addition, a patient having reversed blood flow due to patent ductus arteriosus has been studied (Stohlman et al., 1954). In this condition the upper part of the body is
normally oxygenated but the lower part is hypoxic. Erythroid hyperplasia developed not only in the hypoxic marrow, but systematically. Such studies have made untenable the hypothesis that oxygen tension alone controls red-cell production.

The kidney has recently been implicated as the primary, but not necessarily sole, source of erythropoietin production. The role of the kidney has been established by studies of erythropoietic responses inducible in nephrectomized animals, erythropoietin formation by perfused isolated organs, and experiments with parabiotic animals (Jacobson and Doyle, 1962, Sec. III).

The chemical nature of erythropoietin is not known. Opinion differs as to whether it is a single substance with a single mode of action, a single substance with more than one mode of action, or more than one substance (Gordon, 1959, and Fischer, 1962). Common sources of erythropoietin are the plasma and urine of animals and patients subjected to endogenous or exogenous erythropoietic stimulation (Gordon, 1959; Jacobson and Doyle, 1962, Sec. II). One, and perhaps the only, erythropoietic stimulating factor has been shown to be an appreciably heat-resistant, nondialyzable glycoprotein with sialic acid as a constituent (Wintrobe, 1961, p. 51). A number of bioassay schemes for erythropoietin have been worked out, although none are yet completely successful; some are based on the circumstance that hypophysectomy, transfusion-induced polycythemia, or acute starvation are effective for bringing about a suppression of erythropoiesis (Keighley et al., 1960). Induction of erythropoiesis, evidenced by factors such as radioiron uptake and reticulocytosis in an animal in which erythropoiesis has been suppressed, provides an indication and measure of the erythropoietic activity of an administered substance. In vitro assay methods have recently shown promise as a means of erythropoietin assay (Jacobson and Doyle, 1962, Sec. VIII).

A number of other factors, both humoral and chemical, are known to elicit an erythropoietic response. Cobalt has this property, and some workers have chosen to express the potency of experimental erythropoietin preparations in "cobalt units" (Goldwasser and White, 1959).
Corticosteroids and androgens have also been shown effective in promoting erythropoiesis (see Gardner, 1962), but such substances probably exercise their erythropoietic action by promoting a condition of relative hypoxia (Bothwell and Finch, 1962, p. 243).

8. Some Standard Clinical Procedures

Here considered are the routine clinical procedures of value in assessing erythropoietic function. Over the years these tests have constituted the basis for clinical diagnoses of anemia and related disorders. Newer methods of both clinical and experimental value are considered in the next section. The general reference for this section is Wintrobe (1961) though the information is available in most hematology textbooks.

a. **Reticulocyte count.** Reticulocytes in peripheral blood are demonstrated by vital staining with brilliant cresyl blue or methylene blue. Normally in adults about 1% of the circulating red cells are reticulocytes. Greater degrees of reticulocytosis are found in newborn infants. In adults an elevated reticulocyte count signifies hyperactivity of the erythropoietic system.

b. **Red-cell count.** The number of red cells in a given volume of blood is a primary parameter in the diagnosis of anemia. Conditions that may change the volume of circulating plasma affect this count.

c. **Marrow E/M (erythroid/myeloid) ratio.** Under conditions in which either the erythropoietic or the leukopoietic aspect of hematopoiesis is specifically taxed, a shift may occur in the ratio between the red-cell and the white-cell precursors. Determination of this ratio depends on the skill of the counting technician in discerning between the various stained marrow elements. An increase in the number of the earlier erythroid cells relative to the number of more mature erythroid cells is known as a "shift to the left."

d. **Hematocrit.** Mild centrifugation of whole blood (to which an anticoagulant has been added) can separate the red cells from the other blood elements. The volume of packed red cells compared to the total volume of the blood sample is expressed as the hematocrit. Most
textbooks discuss the optimum conditions of centrifugation; these conditions of course affect the degree of packing and thus the measurement.

e. **The size-number distribution.** The plot of cell number versus cell diameter is known as a Price-Jones curve. Such curves may approximate a Gaussian distribution. Younger reticulocytes tend to be larger than more mature ones; thus an unusually large number of large cells may have the same meaning as an increased reticulocyte count.

f. **Hemoglobin.** Hemoglobin in blood is conventionally determined in this country by cytolysing a blood sample, quantitatively converting hemoglobin to cyanmethemoglobin, and assaying this photometrically at an absorption peak. The result is expressed as mass of hemoglobin per volume of blood.

g. **Physiologic pigments.** Colorimetric or photometric assay of bile pigments in feces provides an indication of the rate at which hemoglobin is destroyed. Similar assay of heme precursors in urine can indicate blocks and irregularities in heme synthesis. Some instances of poisoning and of hereditary deficiencies in heme synthesis are indicated by spectral absorption analysis of abnormal pigments in the blood.

h. **Serum iron, and iron-binding capacity.** These parameters provide a measure of the dynamics of systemic iron allocation (see Sec. III. A. 5).

i. **Morphological irregularities.** To a skilled investigator, morphological irregularities of marrow erythroid cells and of circulating red cells are specifically indicative of hematologic disorder. Macro- and microcytoses, so-called megaloblastic conversions or shifts to the left, gross erythrocytic irregularities like those of sickle-cell trait, remnants of nucleic acid structures in erythrocytes, accumulations of ferritin or pigments in the cytoplasm, and nucleated red cells in the blood all can have meaning in terms of the underlying derangement.

j. **Derived quantities.**

\[
\text{Mean corpuscular volume (MCV, in cubic microns)} = \frac{\text{volume of packed red cells (ml per liter)}}{\text{red-cell count (millions per mm}^3)}
\]
Mean corpuscular hemoglobin (MCH, in µg)

\[
= \frac{\text{hemoglobin (g per liter)}}{\text{red-cell count (millions per mm}^3)}
\]

Mean corpuscular hemoglobin concentration (MCHC, expressed as wt/vol %)

\[
= \frac{\text{hemoglobin (g per 100 ml} \times 100)}{\text{volume of packed red cells (ml per 100 ml)}}
\]

Normally MCHC is very close to 34%; increases above this amount are uncommon, except in hereditary spherocytosis. In many anemias the MCHC decreases.

B. Erythropoiesis and Erythropoietin, Recent Findings

As discussed in the previous section, a new era in experimental hematology began in the 1950's, ushered in by renewed interest in erythropoietin. The predominant belief now is that erythropoiesis is regulated by erythropoietin, and is not directly responsive to hypoxia. The mechanism of marrow response to erythropoietin is discussed in this section. Results specifically based on techniques unknown to classical hematology have recently led some workers to suggest the possibility of a dual role for erythropoietin action. These various techniques, in context with the respective results, are discussed first (Sec. B.1). Then a broader picture of erythropoiesis according to the newer results is discussed (Sec. B.2).

1. Experimental Techniques and Findings

a. Tracer studies of glycine metabolism. Glycine is a precursor of heme as well as of globin. Heme is metabolically degraded after the destruction of red cells (or red-cell precursors). In 1950, it was observed that the amount of labeled fecal bile pigments excreted following the administration of \( ^{15}N \)-labeled glycine could provide a sensitive index of the catabolism of heme; labeled bile pigments appeared days after administration of labeled glycine rather than after the several month normal life span of erythrocytes (London et al., 1950; Gray et al., 1950). This finding is indicative of the premature death of
immature erythroid cells, which has been called "ineffective erythropoiesis" (Finch et al., 1956). Ineffective erythropoiesis is characteristic of conditions involving acute erythropoietic stress such as thalassemia and pernicious anemia (Schmid, 1962).

b. Radioiron uptake and re-emergence. As mentioned previously (Sec. III. A. 5) serum-bound radioiron administered intravenously is cleared from the plasma rapidly and approximately exponentially. Huff et al. (1950) initially reported simple exponential clearance. Pollycove and Mortimer (1961), however, elucidated additional iron-equilibration compartments, in particular, the "labile pool" probably associated with erythroid cell membranes. After an interval wherein erythrocyte radioiron levels are negligible but marrow radioiron is demonstrably high, red cells with radioiron incorporated into hemoglobin begin to appear. The plot of blood-cell radioactivity versus time can be interpreted as a measure of the time required for blood-cell maturation (Harris, 1963, p. 42). It is clear that the existence of iron pools and the possibility of ineffective erythropoiesis with reutilization of iron must be considered in the interpretation of radioiron reappearance or re-emergence data.

In several instances, stimulation of erythropoiesis has been found not only to enhance initial clearance of iron from plasma, but also to cause a decrease in radioiron re-emergence time (Stohlman, 1959; Van Dyke and Parker, 1964). This evidence suggests that stimulation leads to either a decrease in intermitotic generation time or to an acceleration of maturation.

c. Fe$^{59}$ radioautography. Radioautographic grain counts on marrow cells from rabbits to which Fe$^{59}$ was administered were determined by Lajtha and Suit (1955) and Suit et al. (1957). They found that the earliest erythroblasts took up iron most actively. Six hours after the administration of radioiron, the grain counts over blood reticulocytes were low, but by 24 hours blood reticulocytes appeared with grain counts similar to those of the early erythroblasts. As after six hours there was insufficient Fe$^{59}$ in the plasma to give a radioautograph, they argued that these heavily labeled reticulocytes could have originated
only from early erythroblasts that had matured without an intervening cell division (since every division would have halved the grain count) (see also Lajtha and Oliver, 1960).

Primary evidence for the conclusion that erythropoietic stimulation leads to differentiation of stem cells into the erythroid compartment was furnished by the radioautographic studies of Alpen and Cranmore (1959). At various intervals after the administration of $\text{Fe}^{59}$ to dogs, marrow-smear radioautographs were made and cell counts over pro-erythroblasts performed. After erythropoietic stimulation by severe bleeding, labeled cells were observed to disappear from the proerythroblast compartment at a rate that could be accounted for only by feed of new unlabeled cells from the stem-cell compartment. Furthermore, they demonstrated that erythropoietic stimulation did not decrease the grain-count halving time of the labeled cells; this finding led them to conclude that erythropoietic stimulation of this nature did not decrease the intermitotic interval.

d. Red-cell life span. There are a number of methods for the determination of the life span of red cells (Wintrobe, 1961). Most involve radioactive tracers, but a well-known one, the Ashby differential-agglutination method, does not. Of the radioisotope methods, the most widely used is based on the ability of red cells that are incubated in vitro with $\text{Cr}^{51}$-labeled sodium chromate to firmly bind the radioactive compound. Sequential counting of aliquots, or whole-body counting, after reinjection of the labeled cells determines a survival curve which may be analyzed to determine the life expectancy of an individual red cell. The $\text{Cr}^{51}$ method yields for human erythrocytes a life span of 109 to 127 days, whereas the Ashby method yields 108 to 120 days. The slightly shorter lifetime measured by the Ashby method may be due to the immunologically foreign nature of the injected cells. All the tracer methods are subject to label losses by processes other than red-cell death; the $\text{Cr}^{51}$ method is least offensive in this respect, and losses not due to red-cell death can be accurately estimated.

Numerous studies of anemias (such as pernicious anemia) have demonstrated that the life span of the erythrocytes of untreated patients
is significantly shortened (Harris, 1961, p. 176). In a preliminary report on erythropoiesis in rats treated with erythropoietin, Van Dyke (1959) reported shortened erythrocyte life span. However, such erythrocytes were later reported by Van Dyke and Berlin (1960) to have normal survival times.

e. **Tritiated-thymidine radioautography.** The work done by the group at Brookhaven National Laboratory (see Bond et al., 1962), has defined the kinetics of dog erythroblast maturation at later stages. The technique involves *in vivo* labeling of dividing cells by means of the specific DNA precursor, thymidine. Marrow-smear radioautographs made shortly after the administration of tritiated thymidine show both labeled and unlabeled erythroblast nuclei. However, if an interval corresponding to the erythroblast intermitotic generation time intervenes between tracer administration and taking of the marrow sample, erythroblasts of the initially nonlabeling type become labeled. The authors argue that the initially nonlabeling erythroblasts must therefore be those that will synthesize no DNA and can obtain label only through uptake in the prior generation. Among the results of experiments based on this technique are (a) an estimate of 9 to 10 hours, for the generation time of the last labeling erythroblast generation; (b) an estimate of 9 hours for the time during which the initially nonlabeling erythroblasts remain nucleated; and (c) establishment of extrusion as the mode of nuclear loss. Progression of the erythroid cells through the various maturation compartments was shown to be generally orderly, though a certain amount of "straggling" was demonstrated (Odartchenko et al., 1962).

f. **The electronic cell counter.** The Coulter Blood Cell Counter (Coulter Electronics, Hialeah, Florida) has been shown useful in clinical hematology (Brecher et al., 1956), and its application to experimental hematology has led to results of considerable interest. A flowing saline solution carries blood cells through an aperture, measured in tens of microns. The magnitude of an electrical current also passing through the aperture is affected by the passage of a cell, and the electrical disturbance is said to be proportional to the volume of the cell.
Brecher and Stohlman have used the Coulter Counter to elucidate an anomaly of red-cell-size distribution following erythropoietic stimulation (Stohlman, 1961; Brecher and Stohlman, 1961). In their studies, high doses of exogenous erythropoietin given to normal or hypertransfused rats caused short-lived macrocytes to appear in the blood. Phenylhydrazine, bleeding, and anoxia elicited a similar response (Brecher and Stohlman, 1962). In a very recent paper, Stohlman and coworkers demonstrated that erythropoetin stimulation of iron-deficient rats can produce microcytosis, normocytosis, or macrocytosis, according to the extent that iron balance is experimentally restored (Stohlman, Howard, and Beland, 1963). The results are in accord with the postulate that divisions may be skipped in the erythroid developmental sequence (Stohlman, Brecher, and Moores, 1962). The authors (Stohlman, Howard, and Beland, 1963) offer an explanation for their findings similar to one originally proposed by Lajtha and Oliver (1960); namely, that cellular hemoglobin may build up to a critical concentration which prevents cell division, and that such a concentration may be reached relatively early in the erythroid development sequence as the result of erythropoietic stimulation.

g. **Perfusion experiments.** In addition to demonstrating the role of the kidney in erythropoietin formation (Reissman and Nomura, 1962; Fischer, 1962), the technique of isolated-organ perfusion has recently led to a striking result. Gordon et al. (1962) perfused, on a once-through basis, isolated rat legs and femurs with blood depleted in reticulocytes and granulocytes. Administration of erythropoietin with the in-going blood led to the release of waves of reticulocytes, either immediately or within 2 hours. The release of reticulocytes was demonstrated both with a Coulter Counter by means of their larger-than-erythrocyte size, or by means of their content of Fe$^{59}$ following labeling of the marrow. The authors suggest that the erythropoietin may reduce the stickiness of marrow reticulocytes.

h. **A possible dual nature for erythropoietin.** Linman and associates have reported two separable components of serum ESF (erythropoietic stimulation factor) from rabbits made anemic with phenylhydrazine
A relatively thermolabile component, which is insoluble in ether, inactive orally, and probably a glycoprotein, enhances hemoglobin synthesis, probably by diverting multipotential stem cells into hemoglobin synthesis. The second component appears to govern mitotic activity and is more thermostable, ether soluble, and effective orally. When the second substance alone is given to rats, it induces microcytic plethora accompanied by reticulocytosis and erythropoietic hyperplasia, but does not increase hemoglobin or hematocrit levels. This result has not been confirmed by other workers (Wintrobe, 1961, p. 51).

2. Summary and Discussion of Current Ideas in Erythropoiesis
   a. Concise description of erythropoiesis. The following description of erythropoiesis is now generally accepted:
      (1) It normally occurs, in the mature animal, principally in the marrow (Sec. III. A. 1.2).
      (2) Erythroid cells are derived from stem cells, which are believed to be present in the marrow, but which may also conceivably arise from circulating lymphocytes (Sec. III. A. 1).
      (3) Erythropoiesis is under the control of a humoral factor (s), namely erythropoietin or ESF (Sec. III. A. 7).
      (4) Erythropoietin is produced primarily, if not exclusively, in the kidney (Sec. III. A. 7).
      (5) The stimulus to erythropoietin production is anoxia (Sec. III. A. 7).
      (6) A primary action of erythropoietin at the marrow level is the stimulation of stem cells to differentiate into the erythroid developmental compartment (Sec. III. B. 1. b).

   b. Other possible modes of erythropoietin action. The other possible modes of erythropoietin action are more controversial and have been reviewed in detail (Lajtha and Oliver, 1960; Lajtha, 1962; Fischer, 1962). Briefly erythropoietin, in addition to stimulating the differentiation of stem cells, may also lead to:
      (1) An acceleration of intermitotic growth (Sec. III. B. 1. b),
      (2) A decrease in the interval between the last division and the release of the cells into the blood stream (Sec. III. B. 1. g) (also see Stohlman, 1962),
(3) Stimulation of hemoglobin synthesis and/or stimulation of an enzyme system responsible for hemoglobin synthesis (Sec. III. B. 1. f),

(4) A stimulatory effect on cellular proliferation, which is separate from the above-mentioned stimulation of hemoglobinization (Sec. III. B. 1. h),

(5) The skipping of one or more divisions normally involved in the maturation sequence (Sec. III. B. 1. f),

(6) An enhancement in the efficiency of erythropoiesis, that is, a decrease in ineffective erythropoiesis (Sec. III. B. 1. a) (Fischer, 1962).

c. Other circumstances possibly modifying the response to erythropoietin.

It is possible that some of the modes of erythropoietin action listed in Sec. III. B. 2. b may be manifestations of erythropoietin's primary action plus one or both of the circumstances listed below:

(1) A limitation in the amount of space, available at a given time, for the maturation of erythroid cells (Lamerton et al., 1959),

(2) Impairment of nuclear division when hemoglobin exceeds a certain critical concentration (Sec. III. B. 1. f).

d. Some pertinent experimental and clinical evidence. Before proceeding with the discussion of the above items, some experimental and clinical evidence should be reviewed.

Experimental and pathologic anemias frequently lead to the appearance of normochromic, short-lived macrocytes in the peripheral circulation (Harris, 1963, p. 150; Wintrobe, 1961, p. 477; Stohlman, 1962). (See also Sec. III. B. 1. f.) Amb (1957) by microspectrophotometry demonstrated that young erythrocytes from bled or phenylhydrazine-treated rats may have amounts of cellular hemoglobin as much as 2.5 times the normal amount.

Erythroid stimulation may lead to the release from the marrow of relatively immature reticulocytes (Finch, 1962; Fischer, 1962; Bothwell and Finch, 1962, p. 239; Harris, 1963, p. 126). The megaloblastic anemias may be associated with (a) a "shift to the left" involving ineffective erythropoiesis and (b) skipping of one or more divisions in the erythroid development sequence (Lajtha and Oliver, 1960).

Borsook et al. (1962) observed that about half of the marrow reticulocytes produced by rabbits as the result of severe bleeding fail
to incorporate hemoglobin precursors, but that the synthetically active half incorporate considerably more label (tritiated leucine) than do reticulocytes from normal rabbit marrow. In the normal marrow, most of the reticulocytes are synthetically active. Though it is not necessarily in accordance with the view of Borsook et al., a possible interpretation of their data is that the abnormal reticulocytes that result from intense erythropoietic stimulation are highly active synthetically and have a high rate of mortality (evidenced here by the cessation of synthetic ability).

e. "Critical hemoglobin hypothesis," discussion. The above observations are in accord with the original suggestion of Lajtha and Oliver (1960), which has recently been substantiated by Stohlman et al. (1963). As previously mentioned, this suggestion was that erythropoietic stimulation may lead to the attainment, relatively early in the maturation sequence, of a critical concentration of hemoglobin above which further cell division is precluded. This would lead to skipping of subsequent divisions. Though these authors favor the idea that it is the terminal division that is skipped, it appears possible that early divisions may also be skipped, as originally proposed by Alpen and Cranmore (1959).

Reticulocytes produced by such a "shunted" process would be immature insofar as they had not completed their maturation normally, and very likely would have impaired viability. Also, because the quantity of cellular constituents is halved at division, such reticulocytes would have more hemoglobin and more RNA than is normal for cells of their age. If such a reticulocyte were able to continue to synthesize hemoglobin in spite of its immaturity, it might eventually accumulate considerably more than the normal amount of hemoglobin because of the increased quantity to start with, and because of the synthetic potential represented by the larger-than-normal RNA complement.

Thus, in accordance with the "critical hemoglobin hypothesis," retardation of the nuclear-synthesis-and-division cycle relative to the rate of hemoglobin synthesis should lead to large labile reticulocytes and to erythrocytes with abnormally large amounts of hemoglobin or impaired viability. Deficiency of vitamin B₁₂ or folic acid constitutes a
means by which such a retardation can occur. The complementary situation, in which hemoglobinization is retarded with respect to nuclear synthesis and division, is achieved by the deficiency of hemoglobin precursors, most commonly dietary iron. Iron deficiency was shown by Stohlman et al. to lead to microcytosis even under erythropoietic stimulation (Sec. III. B. 1.f). These authors suggested that supernumerary divisions may occur in the absence of the limiting effect of a critical hemoglobin concentration.

f. Ineffective erythropoiesis, discussion. A matter that has not been entirely clear in the literature relates to the possible role of ineffective erythropoiesis in the fine regulation of erythropoiesis. According to the mechanism postulated, erythropoietic output could be slightly increased by the channeling of erythroid cells, otherwise destined for death in the marrow, into viable pathways (see Fischer, 1962). But what is in fact seen under intense erythropoietic stimulation is the death of immature erythroid cells (Sec. III. B. 1.a). It seems reasonable to conclude that what causes impaired viability of immature cells (reticulocytes) in the peripheral blood will also cause poor viability of marrow cells (reticulocytes and erythroblasts). Thus lability of blood reticulocytes is probably due to the same cause as is the marrow-cell death evidenced by the early labeled-bile-pigment peak (Sec. III. B. 1.a). Ineffective erythropoiesis in anemia must aggravate the anemia. It does not seem reasonable that the same mechanism can also improve anemia.

g. Early release of erythroid cells from the marrow. By the above analysis, it may be concluded that ineffective erythropoiesis, macrocytosis, and impaired viability of reticulocytes are all phenomena that are consistent with the mechanism suggested to underlie the skipping of divisions—namely, the enforcement of an abnormally early cessation of erythroblast divisions. Though the "critical hemoglobin hypothesis" seems very attractive, another possibility must also be considered.

Under erythropoietic stimulation, erythroid cells may be obliged to leave the marrow earlier than normally. There is evidence that the immature cells (reticulocytes) may continue, outside of the marrow,
to incorporate iron and manufacture hemoglobin (Sec. III. A. 5), but it is unlikely that cell division of erythroid cells occurs in the blood. It is also possible that a prematurely released erythroblast could not metabolically support its nucleus. Thus such a nucleated cell, finding itself in the blood dissociated from its normal physiologic environment, might be obliged to give up its nucleus, and to do its best with its cytoplasmic equipment to prepare itself for a career as an erythrocyte. Because of the higher cytoplasmic quantity of RNA in the immature cells, and since the hemoglobin level may already be high (not having been reduced by the divisions that normally occur while the rate of hemoglobin production is decreasing), such a cell may be able eventually to accumulate considerably more than the normal amount of hemoglobin. But because its maturation ceased early, it may lack some of the subtle metabolic and physiologic mechanisms required for survival. Thus either early attainment of a critical hemoglobin concentration or enforced early release of erythroid cells from the marrow can lead to the same effect.

The possibility of early release of reticulocytes has been dramatically demonstrated in the perfusion experiments of Gordon (Sec. III. B. 1. g). (In that case, the release seemed to involve a reduction in reticulocyte stickiness mediated by erythropoietin.)

h. Growth pressure. Yet another mechanism can explain how erythropoietin might cause the release of reticulocytes prior to the time that reticulocytes, derived in the normal way from the directly stimulated stem cells, would be available. Such a mechanism has been described and is known as "growth pressure" (Lamerton et al., 1959). The difference between the capacities of large and small animals to expand erythropoietic function to meet crisis has already been discussed; both in situ expansion and peripheral expansion may occur in man, though in small experimental animals the erythroid marrow can probably expand at best only slightly under erythropoietic stimulation (Sec. III. A. 2).
3. **A Hypothetical Formulation to Explain Various Topical Aspects of Erythropoiesis**

I propose the following formulation: Stimulation of erythropoietic function, mediated by erythropoietin, creates a "pressure" in the marrow by increasing the number of erythroid cells. This "pressure" leads to an extrusion effect and, depending on the existent utilization of marrow space and on the capability of the erythropoietic marrow to expand, to an expansion effect. The extrusion effect may be visualized by invoking the erythroblastic islands of Bessis (Sec. III. A. 5). When proerythroblasts appear at the center of the islands, as the result of differentiation of stem cells, the more mature cells at the periphery are pushed off and find their way to the peripheral blood. Expansion of the erythropoietic marrow may occur either in situ or peripherally, by displacement of nonerythropoietic marrow elements (i.e., yellow marrow or granulopoietic elements).

Considering the extrusion and expansion effects, what does one expect to observe under various conditions of erythropoietic stimulation? 

(a) A very mild stimulation might have no perceptible effect because of the ability of normal marrow to accommodate a slight increase in the number of erythrocyte precursors. (b) Slightly more intense stimulation, however, leads to sufficient "pressure" to induce marrow expansion. This expansion, if it continues over a long enough interval, will be the demonstrable result of the expansion effect. After the marrow has expanded sufficiently to accommodate the increased demand, the stimulus (anoxia) subsides and thus the "pressure" subsides. Thereupon, both the extrusion effect and the expansion effect also subside. (c) Intense transient stimulation would lead not only to early release of reticulocytes, but probably also to the release of (nucleated) erythroblasts. If such nucleated cells had not completed their sequence of divisions, and cannot complete it outside the marrow (or at the periphery of the erythroblastic islands), such a process would lead to probably labile macrocytes of high hemoglobin-synthesizing potential.

If the stimulus continues long enough that the capacity for marrow expansion is saturated, or if the capacity for marrow expansion does
not exist, then the extrusion effect continues as long as erythropoietin levels remain high. As presented, this formulation allows the possibility of a "runaway" phenomenon; the prematurely released cells, especially the nucleated cells, have been described as having impaired viability; thus they do not serve well in their oxygen-carrying role. Anoxia induces erythropoietin formation, and thus induces stimulation of the process already impaired by excessive stimulation.

This formulation is consistent with the "panic" hypothesis attributed by Borsook et al. (1962) to Stohlman and Brecher, but it also suggests that enforced early departure of immature erythroid cells from the marrow causes division skippage. This formulation also takes into consideration the effects of growth pressure as a means for interpreting differences, in the nature of the response, which are dependent on the intensity and duration of stimulation and on the marrow expansion capability of the animal studies.

Chronic megaloblastic anemias, especially pernicious anemia (which can be related to an impairment of cell division) are more easily explained by the "critical hemoglobin hypothesis." However, even for pernicious anemia the above formulation may be applicable, for after the anemia is developed there may be a strong and continued stimulus to the erythropoietic system due to the shortened life span of the abnormal erythrocytes formed, and to the inability of the marrow to completely accommodate the increased erythropoietic demand by expansion.

The "critical hemoglobin hypothesis" is also consistent with the above formulation as follows: Assume that erythropoietin evokes the differentiation and proliferation of erythroblasts either by activation of an enzyme system responsible for the production of hemoglobin (Lajtha and Oliver, 1960; Stohlman et al., 1963) or by independent action of two components (Linman and Pierre, 1962). If the proliferation aspect of erythropoiesis is more sensitive to growth pressure than is hemoglobin synthesis, asynchrony would result, and the critical hemoglobin concentration would be reached relatively earlier. For instance, if a shortage of DNA precursors develops before the shortage of hemoglobin precursors becomes limiting, or if erythroblasts are
inhibited from dividing into an already crowded space, then hemoglobin synthesis may proceed at a near-normal rate while the cell division cycle is lengthened. Thus early attainment of a critical hemoglobin concentration might be secondary to an increase of erythropoietic function, but might also be a partial cause of division skippage.

In summary, the proposed formulation is as follows: (a) Erythropoietin stimulates erythropoiesis by increasing the feed of stem cells into the proerythroblast compartment. (b) Pursuant to the increase in physical size of the various proerythroblast compartments, a "pressure" is created. (c) This "pressure" may be relieved by the earlier-than-normal extrusion from the marrow of reticulocytes and other more-mature erythroid elements. (d) The "pressure" may also be relieved by marrow expansion. (e) If the "pressure" is so great that erythroblasts that have not completed all maturation divisions are obligated to leave the marrow (or are otherwise separated from the physiologic environment conducive to their normal maturation), large labile reticulocytes will result and some of these will accumulate an abnormally large amount of hemoglobin.
IV. CYTOPHOTOMETRIC HEMOGRAM: A STUDY OF THE NORMAL
AND THE BLED RAT

In the foregoing sections a cytophotometric technique for determining relative cytologic quantities of DNA and hemoglobin is described. This method allows determinations of cellular quantities -- that is, amounts per cell of absorbing substances.* Such determinations may be made in the presence of nonuniformity of distribution of absorbing constituents within the cells. Such nonuniformity is the case in erythroid marrow cells, and probably for this reason few microspectrophotometric studies on marrow cells have been made (Thorell, 1947; Lagerlöf et al., 1956; Sondhaus and Thorell, 1960; Korson, 1951; Reisner and Korson, 1951; also Carvalho -- see Lajtha and Oliver, 1960). Peripheral red blood cells have been a more popular microspectrophotometric subject (see, for instance Ambs, 1957; Sondhaus, 1958; Niemi, 1958; Latimer et al., 1963; Hale and Cooper, 1963). The discussion of the previous section has suggested that there are some immediate problems in experimental hematology that are amenable to the cytophotometric approach, and that the ability to make cytophotometric measurements on populations of marrow cells should be of value.

This section describes the design of a cytophotometric approach to marrow and blood-cell analysis through measurements of single-cell quantities of DNA and hemoglobin, and an experimental study that I carried out by means of this approach. In this study a striking result is obtained. This result is consistent with the hypothetical formulation given in the preceding section and is discussed in that context. Further, the adequacy of the method, the equipment, and the approach are discussed in terms of the data and conclusions obtained.

A. Cytophotometric Hemogram

How may the two parameters, total-cellular-hemoglobin content and total-DNA content, be used to clarify the developmental sequence

*Cellular quantities are expressed here in units of absorbance \( \times \) area (see Sec. IV. C. 8).
of erythroid elements in the marrow? Obviously one parameter can be plotted against the other. But it will be necessary to develop a rationale for doing this.

Call a clonal quantity the total quantity of a substance at a certain time in the group of nth stage decendents of a single stem cell. Assume, in accordance with Thorell (Sec. III.A.3), that RNA synthesis is complete early in development, and also assume that RNA is not lost (except for halving at division) until after the nucleus is extruded. (I suggest that a nucleus is required to sustain the functional integrity of cytoplasmic RNA.) The assumed variation of clonal RNA content with time is represented by the upper plot of Fig. 14(a). Assume that the clonal rate of synthesis of hemoglobin depends on the clonal amount of cytoplasmic RNA present at any time, as represented in the figure. (Actually, the RNA produces globin (Sec. III.A.4), and Thorell's evidence indicates that cytoplasmic globin is accumulated before its transformation into hemoglobin (Sec. III.A.3). However, it has also been reported that the formation of heme and globin are simultaneous (Nizet, 1957). The model outlined here oversimplifies this situation.) Thus the clonal hemoglobin plot is proportional to the integral of the clonal RNA plot. The cellular hemoglobin plotted in the figure is obtained from the clonal hemoglobin plot by halving the clonal amount at each division.

An assumed form of the DNA replication cycle is also shown on the figure, with allowance made for replication and for halving at division. By eliminating time between DNA content, or mode, and cellular hemoglobin, I obtained Fig. 14(b). There is no intention that these plots have any numerical significance. It would not be possible to rank erythroid cells according to their intermitotic stage by means of their cellular hemoglobin; an ambiguity would be generated in that a late-stage n-1 cell might have as much or more hemoglobin as an early-stage n cell. But, as indicated in the DNA vs cellular hemoglobin plot, this ambiguity should be removed by knowledge of the DNA content.

If the erythroid developmental sequence is entirely orderly, that is, if measurable quantities for a given cell are completely determined
Fig. 14. Derivation of the cytophotometric hemogram (schematic representation). (a) The amount of RNA in all of the cells derived from a single stem cell is estimated as a function of the time since differentiation, and shown (top) as clonal RNA. If it is assumed that the clonal rate of hemoglobin production is proportional to the clonal amount of RNA (see text for qualification of this assumption), the clonal hemoglobin content can be represented (middle plot) as the integral of the clonal RNA curve. The assumption of halving of cellular quantities at cell divisions allows estimation of the cellular hemoglobin content as a function of time, as shown. Nuclear DNA content is estimated as shown (bottom plot) on the basis of the replication-division cycle, and on the basis that erythroblasts mature to reticulocytes by nuclear loss. (b) Curve obtained by eliminating time between the cellular hemoglobin and the nuclear DNA plots. The resulting "pathways" are named in accordance with the nomenclature of the maturation sequence, adopted for this study. These plots are not intended to have quantitative significance, but only to serve as a conceptual reference for the interpretation of data.
by the time elapsed since its stem cell differentiated, and if measurements could be made with complete accuracy, all of the cells from an asynchronous marrow population should be representable as points lying on lines such as are shown on Fig. 14(b). These lines are called pathways; thus there will be poly I and poly II pathways corresponding to the early and late polychromatic erythroblasts, and a baso pathway corresponding to basophilic erythroblasts.

Given a reasonable amount of orderliness and accuracy of measurement, the various pathways should be represented by separate groups of points. Inasmuch as there should be twice as many stage n cells as stage n-1 cells if the intermitotic intervals were equal, the relative number of points on the two corresponding pathways should allow determination of the duration of stages n and n-1 relative to one another.

If there occur skipped divisions, say terminal divisions, the poly I pathway should disappear for those cells affected, and an abnormally high ratio should exist between the number of cells apparently on the poly II pathway and on the poly I pathway. If skipped divisions result in nucleated cells with abnormally high cellular hemoglobin, this condition should be reflected by points to the right of the nuclear-loss point. If such cells are tetraploid (Lajtha, 1962), this would be indicated by points to the right of the poly I pathway and above the poly II pathway.

If erythropoietic stimulation leads to the early release of reticulocytes, this should be readily apparent in the hemogram. Early release of nucleated erythroblasts should likewise be indicated.

This development is admittedly speculative, and it is uncertain whether sufficient orderliness of maturation exists, or whether sufficient accuracy of measurement can be achieved. But there seems to be enough promise of meaningful results to proceed with an experiment.

B. Purpose of Experiment

The primary purpose of the experiment is to establish the cyto-photometric hemogram for the normal rat, and to determine what changes, if any, can be detected in the hemogram of a severely bled
rat. An important secondary purpose is to test the cytophotometric method previously described (Sec. II). Because the previous discussion of the cytophotometric method has omitted the details of its practical application, such details are given in this section.

If the hemogram of the bled rat is seen to differ from that of the normal, it will be of interest to interpret the differences in terms of the foregoing discussion (Secs. III. B. 2, III. B. 3, and IV. A).

Severe bleeding was chosen as the means of erythropoietic stimulation in order to produce a maximum effect. Dr. Van Dyke (personal communication) found that moderate erythropoietic stimulation of the rat produced no detectable effect on the differential marrow-cell count and therefore made the suggestion that intense stimulation would probably be necessary to achieve an observable result.

The procedure was to bleed a rat severely over a period of days, to sacrifice both it and an unbled control rat (by a method designed to clear the marrow of recirculating erythrocytes), and to prepare marrow smears for microscopical study and for cell counts. Marrow smears were put through the cytophotometric procedure described previously, and cytophotometric hemograms were prepared for both rats. For convenience in describing and discussing the experiment, ungrammatical adjectival use will be made of the terms "bled" and "normal," e.g., the "bled" marrow will be used to signify the marrow obtained from the bled animal. Some data will be given for an animal designated "trial"; this animal was unbled -- I used its marrow in an initial trial of the cytophotometric procedure.

C. Experimental Method

1. Treatment of Rats

Male Buffalo rats that had been maintained on a diet of Purina Laboratory Chow were used. The trial and the normal rat weighed approximately 300 g when sacrificed. The bled rat weighed 316 g before bleeding and was one of six that had been subjected to daily blood-letting by heart puncture (five did not survive the bleedings). Prior to bleeding, the bled rat received subcutaneously 4 mg of iron
as Imferon (Lakeside Laboratories, Inc., Milwaukee, Wis.), a commercial preparation of complexed iron, to supplement body-iron stores.

The bleeding schedule was as follows:

<table>
<thead>
<tr>
<th>Day</th>
<th>Bled (cc)</th>
<th>Hematocrit</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.2</td>
<td>-</td>
<td>4 mg Imferon iron, sub. q.</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>22.3</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>16.8</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>13.5</td>
<td>Sacrificed</td>
</tr>
</tbody>
</table>

Five cc is roughly one-third of the blood volume of a 300-g rat. Normal hematocrit for a rat is approximately 45; the low hematocrit at the end of bleeding signifies that the rat was severely anemic at the time of sacrifice.

2. Means of Sacrifice

The trial rat was sacrificed by decapitation under ether anesthesia -- it was held by the tail until bleeding at the neck had ceased. The normal and the bled rat were sacrificed as follows: Following ether anesthesia, the abdomen was opened and the neck cut to expose a jugular vein. A #20 bevel-tipped hypodermic needle was inserted in the pelvic bifurcation of the abdominal aorta and 5 cc of blood withdrawn into a heparinized syringe. Part of this was centrifuged to separate the plasma, and several drops of the blood were treated with brilliant cresyl blue and/or smeared on clean microscope slides for reticulocyte counts. Then, by means of a "T" valve between the needle and the syringe, isotonic saline was delivered to the aorta through the needle. As the valve was turned, the jugular vein was cut. Perfusion was continued until the effluent from the jugular vein ran clear, the red coloration of the liver and spleen disappeared, and the heart ceased to beat. This procedure has been shown to clear the marrow of circulating blood cells (Van Dyke, Anger, and Pollycove, 1964).
3. Preparation of Marrow Samples

Following sacrifice, the femurs were excised, cleaned of flesh, and broken open to expose the marrow. A portion of the marrow from the upper end of the femur was placed on a cleaned microscope slide with a drop of plasma obtained from the withdrawn blood. The marrow was suspended in the plasma by repeatedly spreading the mixture to a thin film with another slide -- spicules present in the marrow prevented the slides from approaching contact close enough to injure the cells. The mixture was then smeared by drawing it across the slide with the edge of the other slide. The marrow smear so prepared was allowed to air dry. Several such slides were made -- for cytophotometry, differential counts, and spares.

A marrow specimen from each animal (trial, normal, and bled) was selected on the basis that the marrow cells should be numerous but reasonably well separated on the slide as viewed by microscope. The slides were fixed for 5 minutes in absolute ethanol.

4. Making the Phase Microscope "Maps"

A drop of Cargille (R. P. Cargille Laboratories, Inc., Cedar Grove, N. J.) immersion oil (viscosity A, refractive index 1.515) was placed on a selected portion of each smear and covered with a clean cover glass. The bottom of each slide was carefully cleaned and a slide placed on the stage of a Tiyoda phase microscope. A Miranda 35-mm camera body was prepared for photomicrography by replacing the Fresnel lens with a gasket, and cementing with Canada balsam a small cover glass over a pencilled "x" on the ground glass. The camera was mounted to the straight tube of the microscope trinocular with a Miranda microscope adapter.

Photomicrography was done on Eastman Kodak High Contrast Copy film with the Tiyoda 20X phase objective and a Leitz Periplan 10X ocular; exposure times (about 2 sec) had been predetermined by trial and error. Fine focusing was done by bringing the cell images into the plane of the "x" on the camera ground glass. Exposures were controlled with a shutter mounted close to the illuminator (a Bausch and Lomb research lamp fitted with a ribbon-filament bulb and a green filter) to avoid vibration.
The photomicrographic maps were prepared by serially photographing a single strip of each slide, advancing the stage micrometer between exposures an amount predetermined to move the slide just less than one field width. The stage coordinates for each exposure were recorded. It was found desirable to reduce the normal high contrast of the film somewhat by developing it for 10 min at 20°C in Agfa Rodinal diluted 1:100. The film strip was printed on 8-1/2 by 11-in. sheets of high-contrast photographic printing paper, and each print was labeled with the stage coordinates of the field.

The photomicrographic maps so prepared show hemoglobinized cytoplasm as quite dark, and show nonhemoglobinized cytoplasm as grey. Nuclei are shown light on the darker cytoplasmic background.

The well-focused central portion of each map depicts a section of the slide corresponding to a 3X3 array of nine fields of the photomicrographic set-up to be used for subsequent photography. The fields to be subsequently photomicrographed were indicated by pencilled lines on the maps. The pencilling was applied by means of a stencil overlay. Plate 3 shows a typical map. Note that the fields are lettered consecutively so that each field can be found by moving one field width or length from the preceding one.

5. Hemoglobin Photomicrography

Five smear preparations were photomicrographed at 412 μμ - trial, normal; and bled marrow, and normal and bled blood. The blood specimens, like the marrow, had previously been fixed for 5 min in absolute ethanol.

The equipment for photomicrography and sensitometry has been described (Sec. II.D). The stray-light filter, C.S. 7-59, was used for reasons given in Sec. II. E. 2. Prior to photomicrography, some tests were necessary. First, the proper exposure time was determined by photographing the same field of a marrow smear at various exposure times, developing by the standard procedure (as described in Sec. II. C. 1.b), measuring film transmittance in the background portion of the field images, making a plot of transmittance against time of exposure, and finding on the plot by extrapolation that exposure that
yielded 25 to 30% transmittance. Second, some of the more heavily hemoglobinized cell images on the most nearly correctly exposed frame were scanned. I did this using the smallest obtainable slit settings on the Jarrell-Ash microphotometer, to insure that all portions of these cells were represented within the film transmittance range for which t-A linearity is expected. Third, the monochromator-condenser-diaphragm settings for sensitometric exposures were determined by trial; the diaphragm was viewed through the microscope, its image superimposed on the micrometer reticle in a micrometer eyepiece; its diameter (on the reticle) recorded, the microscope swung away, and a sensitometric exposure made. The duration of this exposure was necessarily the same as that indicated for photomicrography (see Sec. II. D. 1.a). I repeated this procedure several times, changing the diaphragm setting each time and noting its diameter on the reticle. After standard development, the film strip was read with the Jarrell-Ash microphotometer, and the aperture setting which best bracketed the linear t-A range noted. Also determined were a diaphragm setting to give about 50% t with the sector wheel swung out, and settings to demonstrate the portions of the characteristic curve above 75% t and below 25% t. Thus four diaphragm settings for densitometric exposure were noted, though it became apparent that the one for 50% t was also suitable for demonstrating the high t end of the t-A curve. The high- and low-end exposures allow the shape of the curve to be accurately known at the bend-over points, thus aiding in assessment of the limits of linearity; they also provide a factor of safety against improper sensitometric exposures. The diaphragm settings were carefully chosen to yield overlapping sensitometric curves, which could later be superimposed if this became necessary because of improper exposure or uncertainty about the bend-over points. A sensitometer redesigned in the light of experience would cover a longer absorbance range.

A drop of immersion oil, refractive index 1.545, was placed on the previously photographed portion of a slide and covered with a cover slip. The slide was oiled to the condenser with type B Cargille oil (viscous to prevent running) and oiled to the objective with type A Cargille oil. The first field on the first map was located by prior
knowledge of the correspondence between the stage coordinate systems of the Tiyoda and of the Bausch and Lomb microscopes. Proceeding serially as indicated on the maps, 30 frames of film were exposed. When all of the fields on one map had been photographed, it was found that the first field on the adjacent map could be very easily located with the cell images used as guides, by virtue of the overlap of the maps. At the end of each roll (after 30 frames), the camera was mounted to the sensitometer and the sensitometric exposures made, with the diaphragm settings obtained as described above. It was necessary to set the diaphragm separately for each of three exposures by means of the micrometer ocular. The fourth exposure -- the one for 50\% t -- was obtained by swinging out the sector wheel after the exposure for the high-t end of the curve was made.

After the five slides were photographed, the films were developed and examined (a) for proper focus, (b) for uniformity of slit illumination (by examination of the densitometric tracing of the 50\% t frame), (c) for linearity of the t-A curve, and (d) for insurance that the absorbances of most of the cells fell upon the linear part of the curve. Several repeats were necessary to obtain proper exposure because of unexplained changes that occurred in the system. Development was in accordance with the procedure described in Sec. II. C. 1. b. The film strips were cut to six-frame lengths, indexed, and put aside in glassine envelopes.

6. Feulgen Staining

The slides designated trial, normal, and bled were refixed for 10 or 15 min in chloroform-Carnoy's solution to remove lipids, (see Appendix E), immersed in 1 N HCl at 50° C for 4.5 min (per Sec. II. F), and put through the staining procedure given in Appendix E.

7. Feulgen Photomicrography

After Feulgen staining, the three slides were photomicrographed at 555 m\(\mu\) with the apparatus described in Sec. II. D. It was necessary to go through the same procedure for exposure determination as for hemoglobin photomicrography described in Sec. IV. C. 5. The photomicrographic procedure was also the same as for hemoglobin, and the
same cell immersant was used. (The blue stray-light filter was of course removed.) The films were developed, checked, indexed, cut, and put in envelopes.

8. Photometry

Photometry was done on the enlarger-photometer described in Sec. II. D. 2. First the factor $b$ was determined for each roll of film by reading the sector-wheel images; for these readings a photocell aperture (aperture plate) was chosen that was smaller in diameter than the width of the projected sector-step images. The slope $b$ of the $t$-A curve was determined for each roll of film by plotting the transmittance values for the steps of the sector wheel against the absorbance values for the sector steps. For those rolls exposed at the same wavelength and developed together, the $b$'s were found to be identical.

To read the cell images, the apparatus was first carefully adjusted to the predetermined magnification for which conversion factors had been calculated. In reading cell images of various sizes, it was frequently necessary to change the size of the photocell aperture by changing the aperture plate. When this was done it was of course necessary to adjust the illumination to make the photometer galvanometer read full scale on the unexposed portion of the film between the frames. A coarse adjustment was made by varying the aperture setting of the enlarger objective, and a fine adjustment was made with the sensitivity control of the photometer unit. Also, the zero- and full-scale settings were routinely checked each time readings on a new frame were commenced.

The cell images were identified from the maps, and a symbol pencilled on the map adjacent to the image of each measured cell. The galvanometer readings for each cell image and for two diametric areas in the background adjacent to each cell were entered in the log with the appropriate identifying symbol, e.g., 3a16 for map 3, frame a, cell 16. The size of the photocell aperture used for each cell measurement was also entered.
The \( A \times F \) (absorbance \( \times \) area) values for each measured cell image are computed by means of the relationship

\[
AF = kM = \frac{F \Delta T}{b}.
\]

Recall that \( A \times F \) is the integrated absorbance over the area \( F \) (Sec. II. C). The area \( F \) is the area of the photocell aperture divided by the squared magnification of the system (enlarger plus microscope). The slope \( b \) was discussed directly above, and \( T \), of course, is the difference between the galvanometer reading with the cell in the aperture and the average of the readings for the background adjacent to the cell image.

The total absorbing mass for each cell could be computed at this point if \( k \), the absorption coefficient, were known. However, \( k \) is not known for the Feulgen-DNA complex (Sec. II. F) and the \( k \) calculated for hemoglobin is based on a number of assumptions (see Sec. II. E. 2). Therefore the \( A \times F \) values are used in the following presentation.

\section*{D. Graphical Presentation of Data}

The remaining step is to plot the \( A \times F \) values as hemograms. The marrow hemograms for the normal and bled rats are shown in Figs. 15 and 16. In these figures enucleate cells are represented as a histogram to be referred to the right ordinate, whereas nucleated cells are represented as a scatter diagram to be referred to the left ordinate.

The hemoglobin content of peripheral blood cells for the normal and bled rats is described by histograms [Fig. 17(a) and (b)]. For reference the corresponding marrow histograms are replotted from Figs. 15 and 16 to Fig. 17(a) and (b). Note that the blood histogram for the bled rat has been normalized to 0.33, as explained in the legend of Fig. 17, in order to take into account the depletion of red blood cells as reflected by the hematocrit.

Compare also the cellular-hemoglobin histograms for the enucleate marrow cells of the animals designated trial and normal (Fig. 18).
Fig. 15. Cytophotometric hemogram, marrow of normal rat. The points are plotted in accordance with the discussion of Sec. IV. A (see also Fig. 14) and should be referred to the left ordinate. The histograms represent the frequency distribution (with respect to cellular hemoglobin) of enucleate cells and should be referred to the right ordinate. The histogram has been normalized by dividing the number of enucleate cells in each interval of five abscissa units by the total number of erythroid cells measured for each animal. Thus, the area enclosed by the histogram is proportional to the fractional incidence of enucleate cells among erythroid marrow cells. (One abscissa unit equals $10^{-9}$ absorbance unit $\times$ cm$^2$.) There are 164 nucleated and 315 enucleate cells, making a total of 479 cells represented by the figure.
Fig. 16. Cytophotometric hemogram, marrow of bled rat. Same explanation as for Fig. 15, except for number of cells (266 nucleated, 115 enucleate, 381 total).
Fig. 17. Frequency distribution of enucleate blood cells with respect to cellular hemoglobin. Fig. 17(a) represents the normal animal and Fig. 17(b) represents the bled animal. The heavy-line histograms represent the peripheral blood-cell populations. For the normal rat, 166 red blood cells were measured, and for the bled rat 219 red blood cells were measured. For the normal blood, normalization has been carried out so that the sum of the distribution is 1.00, whereas for the bled blood the distribution has been normalized so that its sum is 0.33, corresponding to the reduction in the bled red-cell count estimated from the hematocrit. The light-line histograms represent the population of marrow enucleate cells and are reproduced from Figs. 15 and 16. These histograms are normalized, as previously, so that the area under the histogram is proportional to the fractional incidence of enucleate cells in the marrow erythroid cell population.
Fig. 18. Frequency distribution of enucleate marrow cells, with respect to cellular hemoglobin for the perfused (normal) rat (315 cells measured) and the nonperfused (trial) rat (111 cells measured). Normalization has been carried out so that the total number of enucleate cells measured in each animal corresponds to 1.00 on the ordinates of the plots. The histogram for the normal marrow has been renormalized from Fig. 17.
Before proceeding to a discussion of these data, a possible complication should be investigated. The overall low cellular hemoglobin of the bled marrow cells suggests that the bled animal was in iron starvation at the time of sacrifice.

Some differential-cell-count data for the normal and bled animals should also be presented.

Furthermore, the data shown on Figs. 15, 16 and 17 suggest the desirability of investigating some additional parameters, as described directly below.

E. The Effect of Severe Bleeding on Various Hematologic Parameters, Including Serum Iron

A 310-g male Buffalo rat designated as "iron-control bled" was given Imferon and bled by heart puncture according to the following schedule:

<table>
<thead>
<tr>
<th>Day</th>
<th>Bled (cc)</th>
<th>Weight (g)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>310</td>
<td>4 mg Imferon iron, sub. q.</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>294</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4.5</td>
<td>288</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>274</td>
<td>sacrificed (perfusion)</td>
</tr>
</tbody>
</table>

The control in this experiment was a 254-g male Buffalo rat designated "iron-control normal." Both normal and control received 0.25 μCi of serum-bound Fe⁵⁹ intravenously 3-1/2 h prior to sacrifice, and both were sacrificed by the technique described in Sec. IV. C. 2. The various determinations described below were made.
1. **Differential count data**

Differential count data are summarized in Table II.

**Table II. Differential counts**a (including normal and bled).

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Normal</th>
<th>Bled</th>
<th>Iron-control normal</th>
<th>Iron control bled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total leukocytes</td>
<td>100</td>
<td>90</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Normoblasts</td>
<td>30</td>
<td>117</td>
<td>87</td>
<td>145</td>
</tr>
<tr>
<td>Late erythroblasts</td>
<td>9</td>
<td>45</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Early erythroblasts</td>
<td>3</td>
<td>38</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Megaloblasts</td>
<td>0</td>
<td>10</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Ratio: normoblasts</td>
<td>0.71</td>
<td>0.53</td>
<td>0.95</td>
<td>0.92</td>
</tr>
<tr>
<td>to total erythroid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio: erythroid cells</td>
<td>0.30</td>
<td>0.70</td>
<td>0.48</td>
<td>0.61</td>
</tr>
<tr>
<td>to all cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. The differential-count data were kindly supplied by Mrs. Carol Bohlen, Medical Technologist with the Donner Laboratory, who stated that damage to some of the white cells made the counts somewhat uncertain.

2. **Fe$^{59}$ uptake at 3-1/2 h** (expressed as percent of injected dose)

<table>
<thead>
<tr>
<th>Uptake site</th>
<th>Iron-control normal</th>
<th>Iron-control bled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood cells</td>
<td>7.2</td>
<td>62.4</td>
</tr>
<tr>
<td>Marrow</td>
<td>35.2</td>
<td>22.0</td>
</tr>
</tbody>
</table>

The marrow uptake of Fe$^{59}$ was determined in each case by counting a cleaned femur in a well-type scintillation counter, comparing this count with that obtained on the dose before administration, and assuming that a femur contains 1/11 of the body marrow. Blood uptake of Fe$^{59}$ was calculated from the difference between scintillation counts of whole blood and of plasma.

*The iron-uptake data were kindly supplied by Miss Mary Lou Nohr, Medical Technologist with the Donner Laboratory.*
3. **Hematologic Values**

<table>
<thead>
<tr>
<th></th>
<th>Iron-control normal</th>
<th>Iron-control bled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit</td>
<td>33.2%</td>
<td>13.8%</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>13.1 g/100 ml</td>
<td>4.5 g/100 ml</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>1.8%</td>
<td>45.6%</td>
</tr>
<tr>
<td>No. of marrow cells</td>
<td>$1.87 \times 10^5$</td>
<td>$1.81 \times 10^5$</td>
</tr>
<tr>
<td>per femur</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The marrow cellularity, i.e., number of marrow cells per femur, was determined by suspending the marrow from one femur in a known volume of saline and counting cells in a hemocytometer. Approximately 500 cells were counted for each animal. Other values were determined by standard procedures (see Sec. III. A.8).

4. **Serum Iron at Time of Sacrifice**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron-control normal</td>
<td>147 µg/100 ml</td>
</tr>
<tr>
<td>Iron-control bled</td>
<td>23 µg/100 ml</td>
</tr>
</tbody>
</table>

The serum-iron values were obtained by the method of Peters (Peters et al., 1956). The amount of iron given with the tracer dose was sufficient to instantaneously raise the animal's serum iron by about one-tenth of the amount of iron normally present in the serum. However, in view of the extremely rapid iron turnover to be expected in the bled animal, it is unlikely that the tracer dose had an effect on the amount of serum iron present at the time that the samples were taken (at sacrifice).

5. **Absence of Nucleated Cells in Blood**

Nucleated blood cells would be demonstrated by brilliant cresyl blue staining and by photomicrographs taken at 412 µm. Examination of the stained slides and the photomicrographs indicated that nucleated erythroid cells comprised less than 1% of the blood-cell population of both bled and normal animals.

**Hematologic values were provided by Miss Nohr.**

**Serum-iron values were kindly provided by Mrs. Toscha Kusabov, Medical Technologist with the Donner Laboratory.**
6. **Difference in Spleen Size and Spleen Uptake of Fe$^{59}$**

At the time of sacrifice, the spleen of the iron-control bled animal weighed 1.62 g, representing 0.52% of the body weight before the initiation of bleeding, whereas the iron-control normal spleen weighed 0.61 g, representing 0.24% of the body weight. The iron-control bled spleen took up about 11.8% of the administered Fe$^{59}$ activity compared with 6.7% for the iron-control normal spleen.

**F. Discussion of Results and Experimental Conclusions**

Reference is made here to the data presented in Secs. IV. D. and E., and particularly to Figs. 15, 16, 17, and 18. Figures 15 and 16 should be compared with Fig. 14.

a. Examination of the hemograms (Figs. 15 and 16) suggests that a primary effect of severe bleeding is the disappearance of reticulocytes (here defined as enucleate cells with less than the mature amount of hemoglobin) and stage II polychromatic erythroblasts (cells on the poly II pathway) from the marrow.

b. The reticulocytes absent from the bled marrow are evident in the peripheral circulation [Fig. 17(b)]. They are distinguishable from erythrocytes by their considerably lower hemoglobin content.

c. There appears, in the bled animal, to be division skippage in the erythroid developmental sequence. The evidence for this is as follows:

1. Some red cells in the peripheral circulation of the severely bled rat have up to 1-1/2 times the average cellular hemoglobin normal for rat erythrocytes (see Fig. 17). No such cells, and indeed very few cells with even as much hemoglobin as is normal for reticulocytes, are seen in the bled marrow. This indicates that such cells matured outside the marrow from very immature reticulocytes or conceivably from cells released from the marrow as nucleated cells. It is difficult to find a reasonable explanation for the genesis of such cells other than that they arose from early erythroblasts that had not had their hemoglobin-synthesizing potential diminished by the normally ensuing mitoses (see discussion, Sec. IV. A).
(2) In the bled marrow, nucleated cells are seen with DNA content close to the 4C mode but with significantly higher cellular hemoglobin than is normal for dividing erythroblasts (compare Figs. 15 and 16). There is no evidence of a pathway leading to their coordinates except the suggestion of a horizontal pathway from the upper end of the dividing-cell pathways. This indicates that such cells are having trouble dividing and/or extruding their nuclei. Upon losing their nuclei, these such cells would be conspicuous in the bled hemogram as enucleate cells in a poorly populated portion of the enucleate-cell histogram. It is reasonable, therefore, to assume that these cells are released from the marrow either nucleated or directly after enucleation. It should be kept in mind, however, that if these cells did divide, their daughters would appear in a well-populated locus of the hemogram.

d. Though division skippage, implied in 3.b above, is consistent with the "critical hemoglobin concentration hypothesis", the most obvious and likely cause of division skippages is the conspicuous early release of immature erythroid cells from the stimulated marrow. The causative factor that leads to the observed early release of reticulocytes and stage II polychromatic erythroblasts probably also causes the premature release of some earlier erythroblasts.

e. With the exception of the few heavily hemoglobinized higher-than-2C erythroblasts discussed in 3.b above, there is no conspicuous difference between bled and normal in the distributions, with respect to cellular hemoglobin of the points representing 4C and DNA-synthesizing erythroblasts (Figs. 15 and 16). (In the case of the bled animal there does seem to be a slightly higher proportion of the erythroblasts at the 4C level, which again may suggest that there is some impairment of division process.) Iron starvation, or an acceleration of the cell-division process relative to hemoglobin synthesis, if they occurred, would be expected to lead to an overall lessening of cellular hemoglobin in the DNA-synthesizing and 4C erythroblasts.

f. In the iron-control bled experiment (Sec. IV.E) severe bleeding was found to lower serum iron in a rat, which, like the bled rat, had received 4 mg of Imferon iron to fortify its body stores (Sec. IV.E. 4).
Though the observed low serum iron may indicate no more than a high rate of iron utilization, it raises the possibility that the bled animal was in a state of iron starvation at the time of sacrifice. However, as stated in e, above, the possibility of iron starvation is not supported by the cytophotometric data.

g. Rat-marrow cellularity (nucleated cell count per femur) did not increase in the crisis of severe bleeding (Sec. IV. E. 3). Severe bleeding led to a considerable decrease in (3-1/2 h) of Fe$^{59}$ uptake in the marrow and a corresponding increase of incorporation by the peripheral blood cells. Though these observations are consistent with the assumption of a radically decreased marrow through-put time involving acceleration of maturation, the hemogram demonstrates the adequacy of another explanation; namely that severe bleeding causes the release of very immature cells that may continue hemoglobin synthesis, and thus iron uptake, in the peripheral blood.

h. In the normal rat, nuclear loss is not "orderly" (Fig. 15); it appears that it may occur at any stage of hemoglobinization of those cells that have been operationally designated as stage II polychromatic erythroblasts. This would indicate that the orthochromatic erythroblast stage (or normoblast stage) is not invariably involved in the erythroid developmental sequence.

i. There is no well-evident grouping of the DNA-synthesizing erythroblasts on the hemograms. Either the synchrony between DNA synthesis and hemoglobinization is poor, or there is scatter due to uncertainties in measurement, or both.

j. The abnormally low cellular hemoglobin for enucleate cells from the bled marrow is consistent with the low maximum cellular hemoglobin for the bled nucleated cells (Fig. 16). It has not been established that all or any of such enucleate cells are viable. This group of cells may be dying or dead and/or may represent a brief phase of residence in the marrow, between enucleation and exit from the marrow. The assumption that all of these cells are nonviable would imply that all viable cells pass nucleated into the blood. This possibility would be difficult to check experimentally because such cells may be sequestered in the
spleen for an interval during which maturation and nuclear loss may occur. Nucleated cells were not apparent in the peripheral blood. The weight of the spleen appeared to increase considerably as the result of severe bleeding (Sec. IV. E. 6).

k. The differential-count data are given as a matter of general interest though no specific attempt has been made to correlate them with the cytophotometric data. Severe bleeding tended to increase the proportion of erythroid cells to total cells, and, in the case of one of the experimental pairs, appeared to decrease the fractional incidence of erythroid cells morphologically identifiable as normoblasts, as would be expected from the cytophotometric data (Sec. IV. E. 1). However, in view of the cytophotometric data, it was surprising that any cells at all were identifiable as normoblasts in the bled marrow. (In the nomenclature of the Donner Laboratory Clinic, where the counts were made, a normoblast is a heavily hemoglobinized cell that has a nonbasophilic cytoplasm and is on the verge of losing its nucleus.)

l. The cellular hemoglobin histogram (representing enucleated cells) for normal marrow did not differ appreciably from that for trial marrow (Fig. 18). The perfusion step prior to sacrifice was omitted for the trial animal. If a significant number of mature erythrocytes had been present in the marrow of either animal, these would have been apparent by their higher hemoglobin content (cf Fig. 17). It is therefore concluded that no advantage is gained by perfusion.

G. Discussion of Experimental Conclusions

Insofar as the rat is atypical in not responding to erythropoietic stimulation by expansion of the marrow (Sec. IV. F. 7, Sec. III. A. 2; Van Dyke and Parker, 1964), it is difficult to generalize the results given above to other animals and humans. However, in acute-erythropoietic-stress duration that is short with respect to the time required for expansion of the erythropoietic marrow, larger organisms should respond in the same manner as the rat (Sec. III. B. 2).

Almost any erythropoietic stress should be acute stress to the rat. (The finding of Van Dyke and Berlin (1960) that normal-lived
erythrocytes can be produced by rats stimulated with moderate doses of erythropoietin is not inconsistent with this generalization. According to the mechanism proposed here, impairment of longevity of erythrocytes should occur only if prematurely released erythroid cells are very immature. A moderate increase in the number of earlier erythrocyte precursors in the marrow could well be accommodated by extrusion from the marrow of the more mature marrow reticulocytes.) Severe blood loss and intense phenylhydrazine anemia probably represent acute stress in any organism. Erythropoietin stimulation or moderate blood loss may or may not represent acute stress depending on the size of the dose or severity of blood loss, and on the capacity of the organism for marrow expansion.

It seems quite clear that severe erythropoietic stress in rats causes a shortening of the time between stem-cell differentiation and the release of erythroid cells to the blood, by evoking the release of very immature erythroid cells from the marrow. Apparently there is a finite space available for the maturation of erythrocyte precursors, and an increase in the number of precursors creates a "pressure" that drives the more mature precursors from the marrow. If such precursors have not, by the time of their release, completed the divisions that are normal in the erythroid developmental sequence, it is probable (1) that they will undergo no further divisions, will emerge enucleate from the marrow (or lose their nucleus very soon after release), and mature into cells with abnormally high hemoglobin content and/or (2) that they will die early, as indicated by other studies (see Secs. III. B. 1. a and f and Sec. III. B. 2).

The conspicuous early release of immature erythroid cells, which according to other studies (see Sec. III. A. 5) have the ability to take up radioiron from the plasma, is of considerable interest in the interpretation of data on the "re-emergence time" of radioiron. According to the conventional interpretation, the Fe$^{59}$ data of this experiment would have indicated, in the bled animal, considerable shortening of the residence time of erythrocyte precursors in marrow, and this indication is consistent with the observed early release of immature erythroid cells (Sec. III. B. 1. b). But considerable radioiron may
have gone from the demonstrably iron-deficient plasma directly to the immature, circulating, underhemoglobinized, hemoglobin-synthesizing red cells. To the extent that this occurs, "re-emergence time" is a very misleading misnomer.

There is no evidence in this study that the other mechanisms postulated to explain observations made under erythropoietic stress are not operative. Thus the "critical hemoglobin concentration" hypothesis is consistent with the observed results; also the possibility of a decreased intermitotic time is not inconsistent with them. Although I did not study ineffective erythropoiesis as a mechanism for increasing erythropoietic output under stress, it seems unreasonable (see Sec. III.B.2.f). Also, there is no evidence to suggest that stimulation has not directly caused the release of reticulocytes, as has been observed in another study (see Sec. III. B. 1.g).

The premature release of erythrocyte precursors has been definitely demonstrated in this study, however. A strong argument has been made that such premature release is due primarily to growth pressure, in turn due to expansion of the precursor pool probably resulting from increased stem-cell feed. This observation may call for a re-evaluation of the relative importance of the various mechanisms postulated to be involved in erythropoiesis.

H. Evaluation of the Cytophotometric Method in Terms of the Results

An estimate of the mean corpuscular hemoglobin content (MCH) in the normal rat can be made as follows: The estimated effective absorption coefficient is $7.25 \times 10^3$ (absorbance units)$(cm^2/g)$ (see Appendix D). The average value for the cellular hemoglobin of circulating red blood cells of the normal rat was $86 \times 10^{-9}$ (absorbance units) $(cm^2)$ (Fig. 17). Therefore the calculated value for MCH of the normal rat is $11.8 \, \mu g$. Correction for 1% flare would raise this to $12.0 \, \mu g$ (Sec. II.D. 1.c). A value for rat MCH of $17(15-19) \, \mu g$ has been reported in the literature (Spector, 1956).

On the basis of microspectrophotometric measurements, Thorell (1947) has reported a hemoglobin content of 28 $\mu g$ for human erythrocytes,
but he did not indicate the number of measurements on which this value is based. Ambs (1957) has simply referred his cytophotometric data to the clinically determined normal MCH value. Wintrobe (1961) gives the clinical value for human MCH as 30 µg. The cytophotometric data reported by Sondhaus (1958) for several normal humans is consistent with Thorell's value of 28 µg. Niemi (1958), who has given careful attention to the bandpass error as well as to other errors of cytophotometry, has reported an MCH of 21.94 ± 3.72 µg on the basis of measurements on a large number of human erythrocytes.

The MCH calculated above for the normal rat is just as far below the literature value for rat erythrocytes (17 µg) as Niemi's figure for human erythrocytes (22 µg) is below the clinical value (30 µg). The value I report in this study may be low as the result of atmospheric degradation of oxyhemoglobin to methemoglobin (see Heilmeyer, 1943, p. 105).

The sensitivity and accuracy of the method reported here suffer considerably from the uncertainties due to the nonuniformity of photomicrographic illumination, and the low hemoglobin content of rat erythrocytes makes them a less favorable subject than human erythrocytes. The mean abscissa value of 86 × 10⁻⁹ (absorbance units)(cm²) for the normal animal corresponds to a ΔT of about 0.20 under the conditions of measurement. But the irregularities of illumination due to pits and scratches on the optical surfaces of the monochromator registered as apparent ΔT's of 0.01 or 0.02. Also, the ΔT between the background on one side of the cell and that on the other side was generally of the order of 0.01, and frequently 0.02 and above. Thus the uncertainty in the measured value for a normal erythrocyte could be easily 10%. Cells containing as little as 5% of the mature amount of hemoglobin (i.e., 5% × 12.0 µg) could be identified as erythroblasts on the photomicrographs, and measurements were made on such cells though the uncertainty of measurement is of the order of the quantity measured.

The situation is somewhat better for Feulgen-DNA measurements. Under the conditions of measurement, the ΔT corresponding to a 4C nucleus was about 0.30, and for a 2C nucleus, 0.20 (a smaller aperture
was used in the photometry of the smaller 2C nuclei). Thus the $\Delta T$
uncertainty of as much as 0.02 might lead to uncertainties of as much
as 7% and 10% respectively. Essentially no cells had less than the 2C
amount, so the measurements allowed accurate grouping of the cells
according to their DNA content.

In view of the illumination irregularity, it seems unlikely that
measurements with the available monochromatic light source could
allow the assignment of erythroblasts to pathways as discussed in
Sec. IV. A.

With a higher quality source of monochromatic light, the error
of measurement could be reduced considerably, perhaps to 1 or 2% of
the total absorbance of a Feulgen-stained nucleus or a mature erythro-
cyte. Under this condition, it would be interesting to repeat the experi-
ments described here to determine whether orderliness of the erythroid
developmental sequence exists in various organisms, and to evaluate,
in terms of the cytophotometric hemogram, changes in the developmental
sequence that occur in experimental and pathological abnormalities of
erthropoiesis. If orderliness of erythroid-cell development exists,
and good measurement accuracy can be achieved, the cytophotometric
hemogram should allow the determination of the relative duration of
each developmental stage and the relative length of the DNA synthesis
phase of each stage, as discussed in Sec. IV. A. Also, if one works
backwards according to the scheme represented in Fig. 14, wherein
the basis of the cytophotometric hemogram is illustrated, it should be
possible to study the kinetics of RNA and hemoglobin synthesis during
erthroblast maturation.

A combination of microinterferometry with the methods described
could allow the "critical hemoglobin concentration" hypothesis to be
experimentally checked. (Recall from Sec. III. B. 1. f that Stohlman and
Lajtha have suggested that cell division, or nuclear DNA synthesis,
cannot take place in the presence of a certain limiting hemoglobin con-
centration.) This hypothesis cannot be directly evaluated with the
cytophotometric method described here because total cellular quantities
rather than concentrations are measured. But the application of micro-
interferometry allows the measurement of the cellular mass
(Barer, 1959), and the quotient \( \frac{\text{cellular hemoglobin}}{\text{cellular mass}} \) is an expression of concentration. A cytophotometric hemogram plotted on the basis of cellular hemoglobin concentration rather than cellular hemoglobin mass would allow much better separation of the pathways and would indicate, in the cases of various abnormalities of erythropoietic development, whether there is a certain maximum hemoglobin concentration above which either DNA synthesis or cell division cannot take place.

There is no doubt that the cytophotometric method described here is capable of allowing assignment of cells to their relative position in the DNA synthesis cycle. The ease of making these measurements, after the fairly complicated technique is mastered, and the fact that the data are recorded permanently on photographic film (whereas the dye in the nuclei may fade) should be of considerable interest in the study of DNA synthesis.
ACKNOWLEDGMENTS

In a research project of this sort, it is inevitable that the writer comes to appreciate the extent to which existing knowledge forms the necessary framework for development, and to fully understand the importance of knowledge and attitudes imparted to him by the individuals involved in his formal education. I wish to thank, in particular, the members of my Thesis Committee--Professors Max Alfert, Jonas Gullberg, and Howard C. Mel--for not only their personal counsel, but also their thoughts and knowledge as made available through academic instruction and written presentations. Furthermore, I would like to thank Dr. Charles A. Sondhaus and Professor Bo Thorell for their time spent in introducing me to the techniques and literature of microspectrophotometry and outlining various problems in the field. I am especially indebted to Dr. John Lawrence and Professor Cornelius A. Tobias for making the facilities for conducting this research available to me, to the Donner Laboratory Library Staff, and to Dr. Donald C. Van Dyke and his staff for their considerable assistance in formalizing and investigating the hematological problem.

This work was performed under the auspices of the U. S. Atomic Energy Commission.
APPENDICES

A. Testing Photographic Films

Figures 19 and 20 show the characteristic (t-A) curves obtained by the sensitometric analysis of four slow 35-mm films: Kodak Panatomic X, Adox KB-14, Adox Dokupan, and Kodak High Contrast Copy film. Film strips were exposed by "contact printing" a calibrated 21-step Kodak neutral density wedge onto them by means of a commercial xenon-flash unit designed for this purpose. All of the films were developed in Agfa Rodinal in Nikor tanks in a 20°C water bath. The other parameters of development are shown in the figures.

B. Correction of the t-A Curve for Flare

It is assumed that the fraction $f$ of flare light incident on the focal plane of the camera arrives from the ocular as if the ocular were a luminous source. When the sector wheel is mounted near the film plane, both flare light and image-forming light are sensitometrically identical. But if the sector wheel were mounted in the microscope specimen plane, as it should be if specimen absorbance were to be compared with sector-wheel absorbance without a correction for flare, then the following situation would exist (Fig. 21): A point $A_2 = -\log I_2$ on the abscissa of the t-A plot, as determined by film-plane sensitometry, would correspond to the same sector step as $A'_2 = -\log (I_2 + f)$ determined by specimen-plane sensitometry. But the transmittance value $t'_2$ would be found by reading the t-A curve (taken with the sector wheel in the film plane) at $A'_2 = -\log (I_2 + f)$. To calculate the specimen-plane t-A curve, first replot the point $t'_2$ at $A'_2$. This could be done for a series of points $(t'_1, A'_1)$, but experience has indicated that the new curve so defined is not perceptibly less linear than the measured curve. Therefore, one other point is sufficient to determine the new curve. It is convenient to take this point near the low $t$ limit of linearity, and arbitrarily set $A_1$ equal to zero. This operation may be represented analytically as follows:
Fig. 19. Characteristic (t-A) curves for slow films (Adox KB-14 and High-Contrast copy). Exposures were made by "contact printing" a neutral-density step wedge onto strips; illumination was from a xenon flash tube. Development was carried out at 20°C in Agfa Rodinal at the various dilutions and for the various times indicated on the respective curves.
Fig. 20. Characteristic (t-A) curves for slow films (Panatomic X and Adox Docupan). Explanation same as that of Fig. 19.
Calculated specimen plane t-A curve with slope = $b'$

Measured focal plane t-A curve with slope = $b$

$(A_2', t_2')$

$\Delta A = \frac{\Delta I}{b}$

$I_1' = 1 + f$

$A_1' = \log \left( \frac{1}{1+f} \right)$

$A_2 A_2'$

$A_1 = 0$

$(I_2)(I_2')$

$(I_1 = 1)$

$\Leftarrow A = -\log I$

Fig. 21. Derivation of flare correction. This figure is intended as a graphical illustration for the text and should be referred to the discussion under Appendix B.
The slope $b'$ of the calculated specimen-plane $t$-$A$ curve is

$$b' = \frac{t_2' - t_1'}{A_2 - A_1}$$

but $t = c + bA$ (Sec. II. C.1),

therefore $b' = b\left(\log \frac{1}{1 + f} \right) - b\left(\log \frac{1}{1 + f} \right)$

and $\frac{b'}{b} = b \frac{\log \left[\frac{1 + f}{1 + f} \right]}{\Delta t} = \frac{b \log \left[\frac{1 + f}{\log \left(\frac{1 + f}{1 + f}\right)} \right]}{\Delta t}$.\[149\]

C. Use of a Recording Microphotometer for Photometric Integration

It appeared initially that the microphotometer (Recording Console Microphotometer, Model 2310, Jarrell-Ash Co., 26 Farwell St. Newtonville, Mass.) might be used as an integrating photometer for the photometric interpretation of photographic cell images. The reasoning is straightforward. It is evident from the analysis of Sec. II. C.2 that the number $n$ of subareas into which the photometric area $F$ is divided does not enter into the computation of $M_k$ or $M$. Thus the entire cell image may be photometered at once, or the cell image may be broken down into subsections of arbitrary geometry for photometry. It would make no difference if the photometric area were rectangular. A rectangular area equal to $F$ could be further divided into narrow rectangles, or slits, of height equal to one dimension of the rectangle, and these slits photometered separately, the transmittances $T$ of the slits being averaged to provide the overall $T$ for the rectangular aperture $F$. It is also possible to perform such an operation continuously by means of a slit, which is longer than the height of the cell image being measured, to scan across the cell image. In this case, the light through the slit is measured by a photocell whose output is linear with transmittance, and the linearly amplified output of the photocell is fed to a chart recorder.
Assume that either the slit or the film-carriage drive is coupled to the horizontal axis of the recorder. The height of the recorder trace corresponds to $T_{c+bg}$ for a particular slice of the cell image. The background transmittance $T_{bg}$ is estimated by means of a straight line drawn to background traces on either side of the cell trace; thus $T_c$ is represented for any slice of the cell image by a vertical line between the background line and the cell trace. The average $T_c$ for all the slices is expressed as follows:

$$T = \frac{(\text{area})R}{F}$$

where

$(\text{area})$ is the area enclosed between the cell trace and the inserted baseline. It is convenient to express the dimensions of this area as vertical inches times horizontal inches,

$R$ is a factor expressing the ratio of transmittance units to vertical inches, and

$F$ is the same factor that appeared in the derivation of Sec. II. C. 2.

It describes the total aperture size (the photometric area). Its units in this case must be horizontal inches for correct dimensionality.

It is evident from dimensional considerations that horizontal inches on the chart correspond to an area on the film. This is readily understood when one considers that as the chart paper moves the slit of the microphotometer sweeps out an area in the plane of the photographic negative. It is necessary to convert chart and film dimensions to dimensions in the specimen plane of the microscope as follows:

$$FT = \frac{\text{area}(R)(h)}{(M_{fc})(M_{sf})^2}$$

where

$R$ is as defined above,

$h$ is the slit height in the film plane,

$M_{fc}$ is the magnification from film to chart, and

$M_{sf}$ is the magnification from microscope specimen plane to the photomicrographic film plane.
It is necessary only that the slit be long enough to accommodate the entire cell image. The only measurement that needs to be taken on the chart after the constants are evaluated is the measurement of the area between the cell trace and the base line. This measurement is readily accomplished by planimetry.

The Jarrell-Ash microphotometer allows selection of any of a number of slit heights between 0.1 mm and 2.0 mm; the slit width is continuously variable from 0 to 25 μ. That portion of the light that is intended for the photomultiplier tube is diverted by a mirrored surface (about as wide as a pencil line) in an optical rhomb. Undiverted light forms an image on a viewing screen of the optically superimposed mirrored surface and photographic film. Thus a cell image can be accurately located and scanned. The magnification from the film to the viewing screen of 15X facilitates measurements of small images on 35-mm film.

A carrier for a six-frame strip of 35-mm film was constructed to adapt to the carriage of the microphotometer (the carriage is intended for 4-in. -wide spectrographic plates). The carriage moves the film at a constant selected speed. To use the scanning method, a cell image is located next to the slit indicator (the viewing-screen image of the mirrored surface in the rhomb) and a slit height chosen that will accommodate the cell diameter. Then the drive is actuated so that the cell image is scanned and a tracing made by the chart recorder. A straight base line on the chart is inserted between the two regions of background trace on either side of the cell trace, and the chart area is determined by planimetry. The quantity \( F_T \), which corresponds to \( F_{AT} \) in the previous derivation, is found from the above expression, and the desired quantity \( M \), or \( M_k \), is found from the expression given in Sec. II. C. 2, namely,

\[
M_k = \frac{F_T}{b}
\]

Unfortunately, this method was found to be impractical with the Jarrell-Ash microphotometer because the image of the deflection mirror that appeared at the slit was not of uniform illumination. An attempt was made to correct this situation by installation of a new
rhomb and realignment of the instrument according to the procedure given by the manufacturer. But no improvement was noted.

The nature of the illumination irregularity is as follows: Illumination decreases by 30% or more from the center of the slit to the ends. This was determined by scanning a pinhole with different portions of the fully lengthened (2 mm) slit. Another test compared the amount of light transmitted at the various slit heights with that expected. This sort of illumination fall-off is consistent with the nature of lenses -- axial illumination is bound to be better than peripheral illumination. Thus it seems reasonable to conclude that this property of the microphotometer is inherent in the optical elements that focus the light from the mirrored surface onto the slit. The Jarrell-Ash microphotometer was designed for the evaluation of spectrographic plates and not for the purpose described here. Thus this property can not be construed as a deficiency.

However, there is the possibility that a unit that would serve the purpose described above could be constructed or obtained commercially. Thus it was deemed worthwhile to include the above discussion.

D. Estimation of the Effective Absorption Coefficient, and the Demonstration of Exponential Absorption

As discussed in Sec. II. E. 2, it was necessary to choose a wide opening of the monochromator slits in order to achieve sufficiently intense illumination and to illuminate the entire aperture of the substage condenser. In this section, an effective bandpass is estimated with regard to all of the components of the illumination system, and the interaction of light of this quality with the absorbing substance of the specimen as described by its absorption characteristic is considered. Exponential absorption is demonstrated over a range of at least one absorbance unit, and the composite absorption coefficient for hemoglobin is estimated to be \((7.25 \times 10^3)\) cm\(^2\)/g. The method of calculation is as follows (refer also to Fig. 22 and Table III):

1. Because the exit slit is focused on the plane of the diaphragm of the substage condenser, this diaphragm acts as an exit slit for the monochromator. The effective bandpass of this diaphragm is indicated
Fig. 22. Comparison of effective bandpass with Hb absorption spectrum. The effective bandpass (e) is obtained from (a) through (d) according to the method described in the text (Appendix D). The molar absorption coefficient for hemoglobin is represented by the dashed line in (e) as a function of wavelength. The effective absorption coefficient for hemoglobin is calculated from the two curves of (e) as described in the text.
by the semicircle on Fig. 22(a) to scale with the actual relative sizes of the slit image and the substage diaphragm.

2. Similarly, the bandpass of the monochromator is indicated as a triangle (see Hardy and Young, 1949) with a half width of 13 mμ [Fig. 22(b)] corresponding to a slit opening of 2 mm (for both entrance and exit slit) and a dispersion of 6.5 mμ/mm. (The dispersion of the Bausch and Lomb 250-mm monochromator as I aligned it was actually 6.38 mμ/mm by my measurement.)

3. The transmittance characteristic of the Dow-Corning C.S. 7-59 (used as a stray-light filter for hemoglobin photomicrography) is plotted in Fig. 22(c) from the data given by the manufacturer. The C.S. 1-69 filter, used as a heat-limiting filter, is spectrally flat in the region of interest.

4. The blackbody radiation characteristic for an assumed color temperature of 3000° K, which is typical for tungsten lamps (Eastman Kodak, 1962) is plotted in Fig. 22(d) for the spectral region of interest. Data for the blackbody characteristic was taken from Smithsonian Physical Tables (1934).

5. The above plots were read for 11 arbitrarily chosen equally spaced wavelengths, and the products of the four relative-intensity values were tabulated (π in Table III). These values were then normalized so that their sum was 1.0, and tabulated in the column denoted 'norm'. And from this column the effective bandpass plotted in Fig. 22(e).

6. In addition the hemoglobin absorption characteristic for this spectral region is plotted in Fig. 22(e), as extrapolated from the data of Jope given by Niemi (1958). The molar absorption coefficient for each of the 11 wavelengths chosen above was read from the plot, and tabulated in Table III along with the corresponding calculated absorption coefficient in units of cm²/gm.

7. The exponential absorption curves plotted from the values in the last and π norm columns of Table III (by means of the relationship log t = log π norm - k(m/a)) were added graphically to determine the composite absorption curve (see Fig. 23).
Fig. 23. Graphical determination of effective absorption coefficient.
The composite absorption curve (upper curve) has been determined
by graphically adding the eleven lower curves, which are the ab-
sorption curves for eleven selected wavelengths. See text (Appendix
D) for discussion; $k = (7.25 \times 10^3) \text{ cm}^2/\text{g} = 0.725 \mu^2/\mu g$. 
Table III. Part of calculation of bandpass and the effective absorption coefficient for hemoglobin.

<table>
<thead>
<tr>
<th>λ (μ)</th>
<th>Diaph.</th>
<th>Slits</th>
<th>Filter</th>
<th>Col. T</th>
<th>π</th>
<th>π norm</th>
<th>k mol</th>
<th>k (cm²/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>402</td>
<td>0.33</td>
<td>0.23</td>
<td>0.78</td>
<td>0.55</td>
<td>0.033</td>
<td>0.020</td>
<td>0.97</td>
<td>5.71</td>
</tr>
<tr>
<td>404</td>
<td>0.45</td>
<td>0.39</td>
<td>0.77</td>
<td>0.57</td>
<td>0.077</td>
<td>0.047</td>
<td>1.06</td>
<td>6.24</td>
</tr>
<tr>
<td>406</td>
<td>0.52</td>
<td>0.54</td>
<td>0.76</td>
<td>0.59</td>
<td>0.126</td>
<td>0.077</td>
<td>1.13</td>
<td>6.65</td>
</tr>
<tr>
<td>408</td>
<td>0.57</td>
<td>0.70</td>
<td>0.75</td>
<td>0.61</td>
<td>0.183</td>
<td>0.112</td>
<td>1.20</td>
<td>7.06</td>
</tr>
<tr>
<td>410</td>
<td>0.59</td>
<td>0.85</td>
<td>0.74</td>
<td>0.64</td>
<td>0.238</td>
<td>0.146</td>
<td>1.24</td>
<td>7.30</td>
</tr>
<tr>
<td>412</td>
<td>0.60</td>
<td>1.0</td>
<td>0.72</td>
<td>0.66</td>
<td>0.285</td>
<td>0.175</td>
<td>1.30</td>
<td>7.65</td>
</tr>
<tr>
<td>414</td>
<td>0.59</td>
<td>0.85</td>
<td>0.70</td>
<td>0.68</td>
<td>0.239</td>
<td>0.147</td>
<td>1.32</td>
<td>7.76</td>
</tr>
<tr>
<td>416</td>
<td>0.57</td>
<td>0.70</td>
<td>0.68</td>
<td>0.70</td>
<td>0.190</td>
<td>0.117</td>
<td>1.32</td>
<td>7.76</td>
</tr>
<tr>
<td>418</td>
<td>0.52</td>
<td>0.54</td>
<td>0.66</td>
<td>0.73</td>
<td>0.135</td>
<td>0.083</td>
<td>1.30</td>
<td>7.65</td>
</tr>
<tr>
<td>420</td>
<td>0.45</td>
<td>0.39</td>
<td>0.65</td>
<td>0.75</td>
<td>0.086</td>
<td>0.053</td>
<td>1.24</td>
<td>7.30</td>
</tr>
<tr>
<td>422</td>
<td>0.33</td>
<td>0.23</td>
<td>0.63</td>
<td>0.78</td>
<td>0.037</td>
<td>0.023</td>
<td>1.13</td>
<td>6.65</td>
</tr>
<tr>
<td>424</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Since the above calculation is based on several assumptions, it can provide no more than an estimate of the effective absorption coefficient. The primary purpose of this analysis is to demonstrate that absorption can be expected to obey an exponential law through the range where measurements are made. A similar analysis has not been made for the Feulgen-DNA complex, first because of the difficulty of estimating the color temperature at the attenuated lamp power used for the Feulgen measurements, but primarily because the Feulgen absorption characteristic is flatter in the bandpass used than is the hemoglobin characteristic. Due to the latter property, and in view of the demonstrated exponential absorption for hemoglobin, Feulgen-DNA absorption must also be exponential, conditions of measurement being the same.

E. Cytological Reagents and Procedures

The Feulgen staining procedure used was adapted from Darlington and La Cour (1962) and from the Sandoz Atlas of Hematology (1952).

1. Feulgen staining procedure
   Fix marrow smears in chloroform-Carnoy's, 15 min
   Air dry
   Immerse 5 min in distilled water
   Dip in 1N HCl at room temperature
   Hydrolyze for 4.5 min in 1N HCl at 50° C
   Dip in cold 1N HCl
   Dip in distilled water
   Immerse for 1 h in Feulgen reagent at room temperature
   Dip in distilled water
   Rinse three times, 2 min each time, in SO₂ water (see below)
   Rinse 5 min in running tap water
   Air dry.

2. Preparation of Feulgen reagent
   Dissolve 1 g of basic fuchsin (National Aniline) by pouring over it 200 cc of boiling water
   Shake well and cool to 50° C
Filter, and add 30 cc of 1N HCl to the filtrate
Add 3 g of K\textsubscript{2}S\textsubscript{2}O\textsubscript{5}
Allow solution to bleach for 24 h in a tightly stoppered bottle in the dark
Add 0.75 g of decolorizing carbon (Norit A), shake well for about 1 min, and filter through coarse filter paper (Whatman #42)
Store as long as 3 weeks in tightly stoppered bottle in refrigerator.

3. Preparation of SO\textsubscript{2} water

| 5 cc of 1 N HCl |
| 5 cc of 10% K\textsubscript{2}S\textsubscript{2}O\textsubscript{5} solution |
| 100 cc of distilled water |

4. Preparation of chloroform Carnoy's

| 6 parts absolute ethanol |
| 3 parts chloroform |
| 1 part glacial acetic acid |
GLOSSARY

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Term and Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><strong>Absorbance.</strong> ( \log_{10} \left( \frac{I}{I_0} \right) ) where ( I_0 ) is the intensity of the unattenuated light beam, and ( I ) is the intensity of the light beam attenuated by the specimen.</td>
</tr>
<tr>
<td>M</td>
<td><strong>Absorbing mass.</strong> The amount of absorbing material in a single biological cell. The absorbing mass is obtained by summation or integration of the mass per unit area ( (m/a) ) over the area of the cell.</td>
</tr>
<tr>
<td>k</td>
<td><strong>Absorption coefficient.</strong> See exponential absorption.</td>
</tr>
<tr>
<td>l</td>
<td><strong>Aperture plate.</strong> A square piece of metal with an accurately cut circular aperture in its center. This aperture is placed in the image plane during photometry, and defines the photometric area ( F ).</td>
</tr>
<tr>
<td>s</td>
<td><strong>Aperture stop or aperture diaphragm.</strong> In microscopy, the aperture stop is the diaphragm that determines the angular breadth of the cone of light that directly enters the objective. Conventionally, this diaphragm is located under the substage condenser.</td>
</tr>
<tr>
<td>t</td>
<td><strong>Apochromatic.</strong> This term describes highly corrected lens systems. An apochromatic lens is said to be spherically corrected for two wavelengths, and chromatically corrected for three.</td>
</tr>
<tr>
<td>b</td>
<td>The slope of the ( t-A ) curve (see ( t-A ) curve).</td>
</tr>
<tr>
<td>b</td>
<td><strong>Biconical error.</strong> Microspectrophotometric error that arises because the microscopical specimen generally presents a longer optical path length to oblique rays than to axial rays.</td>
</tr>
<tr>
<td>C</td>
<td>A complement of DNA, presumably the amount of DNA that represents a single set of genetic information. Thus gametes have one complement ( (1C) ), diploid cells have two complements ( (2C) ), and diploid cells that have completed DNA synthesis but have not yet divided have four complements ( (4C) ).</td>
</tr>
</tbody>
</table>
Cellular quantity (e.g., cellular hemoglobin). The quantity of a given substance in a single cell.

Cellularity of marrow. The number of nucleated cells in a given volume of marrow.

Chromophore. As used here, the molecular unit responsible for light absorption.

Clonal quantity. As opposed to cellular quantity, clonal quantity is the amount of a given material at a given time in the aggregate of cells that have developed from a single stem cell.

Critical hemoglobin concentration hypothesis. The hypothesis that in erythrocyte precursors there is a certain cellular hemoglobin concentration which is incompatible with further cell division, and which, under certain conditions, may be reached relatively early in the erythroid development sequence and lead to the skipping of divisions.

Cytophotometry. Microabsorption measurements made with visible light. Cytological stains are frequently used to develop optical density in the cytophotometric specimen.

Photographic density. The negative logarithm of the transmittance of developed photographic film.

D log E curve. The conventional way of describing the relationship between photographic exposure and the density D of developed photographic film.

Dispersion, optical. The variation of refractive index with wavelength of light.

Gamma. The slope, δD/δlog E, of the D log E curve.

Glare. As used here, glare refers to all registered non-image-forming light exclusive of that arising from lenticular flare.
Erythrocytes. Circulating red blood cells.

microcytes
normocytes (normal sized)
macrocyes or megalocytes
hypochromic
normochromic (containing a normal amount of hemoglobin)
hyperchromic

Erythroid cells. Red cells at varying degrees of maturity.

proerythroblasts
basophilic erythroblasts
polychromatic erythroblasts, stage I
polychromatic erythroblasts, stage II
(orthochromatic erythroblasts)
reticulocytes

Erythropoiesis. The genesis of red blood cells. Hematopoiesis refers generally to the formation of blood cells, and leuko- or granulopoiesis refers specifically to the formation of white blood cells.

Erythropoietin, or ESF. A humoral substance, crude preparations of which have been observed experimentally to increase erythropoietic activity. This substance is believed to be produced by the mammalian kidney, and to be involved in the endogenous regulation of erythropoiesis.

Expansion effect. As stipulated in this thesis, the expansion effect is one of the two effects of marrow "pressure" due to an increase in the number of erythrocyte precursors. This effect consists of the expansion of the erythropoietic marrow into marrow space previously occupied otherwise.

Exponential absorption, law of. As used here, this law is stated

\[ A = \log_{10}(I_t/I_0) = k(m/a) \]
where

A is absorbance,

$I_0$ and $I$ are, respectively, the intensities of the unattenuated measuring beam, and of the measuring beam as attenuated by the specimen,

$k$ is the absorption coefficient,

$m/a$ is the concentration, in terms of mass per unit area, of absorbing material in the path of the measuring beam.

**E** Exposure, photographic. A measure of the quantity of light falling onto a photographic emulsion; the integral of intensity times time, where intensity might be expressed as the number of photons per unit time falling on a unit area of emulsion. If exposure time is constant and intensity is not a function of time, exposure may be stated in terms of intensity $I$ or of relative intensity $I/I_0$, where $I_0$ is a reference intensity.

**Extrusion effect.** As stipulated in this thesis, the extrusion effect is one of the two effects of marrow "pressure" due to an increase in the number of erythrocyte precursors. This effect consists of the early release of relatively mature erythrocyte precursors from the marrow.

**F** Photometric area. The area defined by the aperture in the aperture plate placed directly above the photocell lens of the enlarger-photometer unit. The value of F is determined by division of the actual area of the opening in the aperture plate by the square of the linear magnification of the system (microscope and enlarger).

**Ferritin.** A complex of iron with a physiologic protein. This complex constitutes one form of stored systemic iron and may be involved in intracellular transfer of iron.

**Ferrokinetic.** Having to do with the kinetics of systemic iron allocation. By common usage, the term ferrokinetics implies measurements made through the use of radioiron tracers.
Feulgen technique. A cytological or histochemical staining technique based on the ability of colorless SO₂-bleached basic fuchsin dye to form a magenta-colored complex with the aldehyde groups of DNA made available by means of acid hydrolysis.

Field stop, or field diaphragm. In microscopy, and under the condition of Köhler illumination, the field stop is the diaphragm that limits the aperture of the lamp condenser.

Growth pressure. A hypothetical pressure created within the bone marrow by increase in the numbers of erythrocyte precursors.

Hematocrit. The volume of packed red cells compared with the total volume of blood sample, following centrifugation under specified conditions.

Hemogram. A graphical presentation of measurements made on elements of the blood or blood-forming system. In this study, the data for marrow erythrocyte precursors are presented as scatter diagrams, indicating, for each cell, the amount of cellular hemoglobin and the amount of cellular DNA. These diagrams are designated as cytophotometric hemograms.

Hemosiderin. A complex of iron with a physiologic protein and possibly other substances; a form in which iron is systemically stored.

Immersant. As used here, the fluid in which the microscopical specimen is immersed. To be contrasted with immersion fluids, which are used to form an optical seal between the slide or cover slip and the microscope optics.

Ineffective erythropoiesis. Premature death of erythrocyte precursors as measured by the early excretion of labeled bile pigments following the administration of a radioisotope-labeled heme precursor.
Köhler illumination. A condition of microscopical illumination whereunder an image of the light source appears at the sub-state condenser diaphragm of the microscope, and whereunder the image of the lamp condenser diaphragm is parfocal with the specimen.

Lenticular flare. Non-image-forming light arriving in the plane of registration and arising in reflections within air-spaced lens systems. As used here the term \( f \) specifies the ratio between flare light and the total light at the plane of registration.

Microspectrophotometry. Microabsorption analysis, generally of constituents of single biological cells, performed with a compound microscope in conjunction with a source of monochromatic light and a means of measuring light attenuation by the microscopical specimen.

N.A. Numerical aperture. A measure of the light-collecting ability and the resolving power of a microscope objective. Specifically, \( N.A. = n \sin \alpha \), where \( n \) is the refractive index of the immersing medium and \( \alpha \) is the angle subtended at the specimen plane between an axial ray and the most oblique ray that can enter the objective.

Orderliness of the erythroid development sequence. If the erythroid development sequence were perfectly orderly, all of the \( n \)th stage dependents of a given stem cell would be identical, at any time, with respect to all measurable parameters and observable features.

Pathway. A locus of points on a scatter diagram where cellular hemoglobin is plotted against nuclear DNA content, namely, on the cytophotometric hemogram. It is contended here that each such group of points represents a given stage in the erythroid development sequence.
Phenylhydrazine. A reagent used for the experimental induction of anemia. Administered intravenously, it destroys circulating erythrocytes.

Photographic characteristic, or photographic characteristic curve. The relationship between the blackening of a developed photographic emulsion and photographic exposure. This relationship is conventionally shown as the D log E curve, but in this thesis it is shown as the t-A curve.

Photometer. A device for measuring light intensity.

Ploidy. The ploidy of a biological cell is the number of complete sets of genetic information, thus the number of complements C of DNA contained within its nucleus.

Radioiron clearance. The disappearance of intravenously injected protein-complexed inorganic radioiron from the plasma. On the basis that this process occurs in an approximately exponential fashion with respect to time, a radioiron clearance time is given by the interval required for serum radioactivity to fall from the initial level to a stipulated fraction of the initial level.

Reciprocity law failure. It might be expected that in order to produce a given photographic density, the intensity of the exposing light should vary as the reciprocal of the duration of exposure. To the extent that this relationship is invalid, the reciprocity law fails.

Re-emergence time. A measure of the time required for intravenously-injected protein-complexed inorganic iron to be cleared from the plasma and to reappear as labeled red cells.

Sector wheel. A mechanical light attenuator.

Sensitometry. The determination of the photographic characteristic.

Soret band. A band of strong absorption in the violet end of the hemoglobin-absorption spectrum.
t-A curve, or transmittance absorbance curve. As used here, a manner of describing the photographic characteristic. The transmittance \( t \) of the developed film is plotted as a function of the absorbance \( A \) of a sector wheel used for sensitometric exposure.

**Transferrin.** A complex between a specific blood protein and inorganic iron. This complex functions as an iron carrier in the blood.

**Transmittance.** The ratio \( \frac{I}{I_0} \), where \( I_0 \) is the intensity of an unattenuated light beam, and \( I \) the intensity of the same light beam after attenuation by a specimen for which the transmittance is sought.
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