A SELECTIVE STAINING METHOD FOR THE BASIC PROTEINS OF CELL NUCLEI

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Histochemical staining methods combined with specific enzymatic hydrolysis and chemical substitution procedures have permitted the cytologist to visualize, and in some instances to quantitate by microphotometric methods, many cellular constituents. Methods for histochemical detection of specific proteins or of characteristic groups in proteins are few, and some of those presently available are somewhat cumbersome for routine histological use, or result in formation of highly unstable color complexes whose quantitative relationship to the substances tested is often unknown. As far as the distinction between histones and non-histones, or, in general, between proteins of different isoelectric points, is concerned, the methods previously used have included u.v. absorption-spectroscopy,¹ a modification of the Millon reaction,² and staining with acid and basic dyes at a series of different pH's.³ The last-named method, which makes use of the amphoteric nature of proteins and their resulting ability to form salts with acid and basic dye ions, has been used extensively in the past. However, up to now it has not been possible to define any set of conditions under which a particular type of protein could be visualized selectively by a single staining operation involving only one dye. The procedure described herein represents an empirical method which grew out of a series of staining experiments at controlled pH. It is believed to afford a simple, stable, and specific staining procedure for histones and, when present, protamines in cell nuclei, since other proteins with high isoelectric points (such as cytochrome C), which were found to stain under the specified conditions, do not occur in cells in sufficient concentration to affect the staining picture. Moreover, this method permits relative quantitation of the stainable material by microphotometric procedures.

The method consists of the following steps:

1. Tissues are fixed for three to six hours in 10% neutral formalin, washed overnight in running water, dehydrated, and embedded in paraffin.
2. Sections on slides are rehydrated and immersed for 15 minutes in a 5% solution of trichloroacetic acid (TCA) in a boiling water-bath, for removal of nucleic acids. The TCA is subsequently washed out by three changes of 70% alcohol for ten minutes each, followed by distilled water.

3. Slides are stained at room temperature for 30 minutes in a 0.1% aqueous solution of the acid dye Fast green FCF (National Aniline Division; 96% dye content) adjusted to pH 8.0–8.1 with a minimum of NaOH; the sections are then washed for five minutes in distilled water, followed directly by 95% alcohol, and mounted after complete dehydration and clearing.

By this method a specific nuclear, i.e., chromosomal stain, has been obtained in a variety of vertebrate tissues and in onion root tips. The following comments to this technique are relevant:

1. A number of routine histological fixatives such as Zenker's, Susa's, and Carnoy's do not allow specific nuclear staining. The presence of mercuric chloride in Zenker's and Susa's fluids is apt to introduce a staining artifact since a divalent metal ion might serve as a link between carboxyl groups and acid dye ions. Carnoy's fluid, on the other hand, has been claimed to cause a shift in the isoelectric point favoring acid dye uptake.

2. No stain is retained by the tissues unless nucleic acids are extracted prior to staining. Desoxyribonuclease can replace TCA in this respect, but ribonuclease is ineffective. It is a well-known fact that nucleic acids can interfere competitively with the staining of proteins, and vice versa.

3. Variations of the staining conditions were attempted in several ways: Increase of the dye concentration to 0.25% and, independently, of the time of staining and differentiation to two hours, produced no noticeably different results. Only freshly prepared and unbuffered dye solutions (to avoid interference by buffer ions) were used. The pH of the solution did not change during the staining procedure. Although the dye solution at pH 8 is distinctly bluish in color and its absorption curve is shifted to lower wave-lengths, the dye bound by nuclei appears green and has an absorption peak close to 635 mμ, similar to that of tissue elements stained at pH 2.2.

Figures 1 to 4 illustrate the difference in the staining picture between routine Fast green staining at low pH (in N/100 HCl, pH 2.2) and at pH 8, both after extraction of nucleic acids by hot TCA. It will be noted that the basic hemoglobin, formerly classified as a histone, does not stain at high pH, that the stain is restricted to chromosomes in dividing cells, and that nucleoli fail to stain. This last fact is of special interest. Although nucleoli were said by Caspersson to contain histones on the basis of spectrophotometric analysis, this claim has not been substantiated by the histochemical studies of Pollister and Ris, nor by direct chemical analysis of isolated nucleoli by Vincent. That only chromatin stains in cells where
Photomicrographs of animal and plant cells stained with Fast green at pH 2.2 (Figs. 1 and 3) and at pH 8 (Figs. 2 and 4). × 550. Figures 1 and 2 are blood smears of the lizard *Sceloporus occidentalis* prefixed in absolute methanol to prevent hemolysis; figures 3 and 4 are sections of onion root tips.
mitotic figures are evident (which aside from the onion root tips was also the case in activated rat thyroid glands) is of considerable importance since chromosomes are mainly composed of nucleic acid, histone and "residual protein," or "chromosomine." Hamer has recently reviewed the available evidence for the acidic nature of nuclear and chromosomal non-histone proteins. On the basis of the model experiments reported below, proteins of this type would not be expected to stain.

In order to test this staining procedure model experiments on known proteins, protamine (salmine), desoxyribonucleic acid (DNA) and protamine-nucleate were undertaken. Commercial crystalline proteins and edestine were dissolved in suitable aqueous media in amounts calculated to provide solutions containing the same concentrations of total basic groups (6 μM basic groups/ml.). Six μl. drops of these were spotted on filter paper, fixed in 80% alcohol followed by formalin, washed and stained with Fast green at pH 2.2 or 8. Typical results are summarized in figure 5.

It appears evident that the ability to bind acid dye at high pH is correlated with a high isoelectric point of the protein itself, rather than with the presence of any particular basic group: Protamine, histone, lysozyme, and cytochrome C (not illustrated) are able to stain at pH 8 although, with the exception of protamine, considerably less intensely than at pH 2.2. Globulins and albumins do not retain dye at high pH but they become stainable following methylation of carboxyl groups, a procedure which results in a considerable shift of the isoelectric point into the alkaline range. The fact that globin and edestine (a seed protein of unusually high arginine content) fail to stain, or retain at most only traces of dye, at pH 8 indicates that the absolute number and type of basic groups present are of less importance than the over-all balance of acid to basic groups, i.e., the net positive charge of the protein.

At pH 8 the groups mainly responsible for staining by acid dye ions are the guanidine groups of arginine and the ε-amino groups of lysine. Only about one-third of the terminal α-amino groups and less than one-tenth of the imidazole groups of histidine can be expected to bind dye. The binding is further dependent upon the number and position of carboxylate ions which may exert a force opposing the approach of dye anions, and upon the degree of hydrogen bonding in the protein.

Competitive interference with acid dye binding can be demonstrated by alternately spotting a solution of protamine and a solution of DNA (2.3 mg./ml.), thereby allowing formation of a precipitate of protamine-nucleate on the filter paper. Such a precipitate retains considerably less dye at pH 8 than a spot containing an equivalent amount of protamine alone. Protamine is also able to combine with and precipitate less basic proteins such as insulin. A protamine-insulin complex formed on filter paper in the same manner as the protamine nucleate was found to stain at pH 8 with
almost undiminished intensity when compared to an equivalent amount of protamine alone. It appears thus that the dye at high pH is able to compete successfully with insulin but not as well with DNA for binding sites on the protamine. The inhibitory effect of DNA is not as pronounced in these model systems when protamine nucleate is stained at low pH. How-

**FIGURE 5**

A series of drops of model-proteins stained with Fast green at pH 2.2 and pH 8. Photographed in red light, reduced (actual size of filter paper squares 13 × 13 mm.).

ever, we have observed that two different samples of DNA tested on filter paper were themselves able to bind dye at pH 2.2. (Two samples of ribonucleic acid could not be tested since they diffused from the filter paper during the staining procedure.) It is not known whether nucleic acids in fixed tissues retain acid dye when staining is performed, as custom-
ary, at low pH. If this were the case, it would be exceedingly difficult to detect since this phenomenon can be expected to be overcompensated by a stronger opposite effect, namely, the partial inhibition of protein staining in presence of nucleic acid.\textsuperscript{5} Although the results of the model experiments reported here may not apply exactly to observations made on fixed and stained tissues, they still make it doubtful whether acid staining at low pH can be used for quantitative determination of basic protein groups in tissue sections unless nucleic acids were removed prior to staining.

It should be added that the staining of model proteins has proved to be quite reproducible. In all instances was it possible to demonstrate by re-staining at pH 2.2 that the failure to stain at pH 8 was not due to elution of the stainable material. Exposure of the paper strips to hot TCA, as is done in the histological application of the procedure, does not alter the results except that protamine is almost totally extracted. Since no protamine containing fish sperm was available to us, we do not know whether protamine would be lost from cell structures during this procedure. If this were found to be the case, it might be circumvented by enzymatic removal of DNA in such cases.

The assumption that histones or histone-like proteins are mainly responsible for the histological staining picture is thus supported by the model experiments and can be subjected to some further verification based on known analytical findings. Recent biochemical data\textsuperscript{11} indicate that histones occur in a constant quantitative ratio to DNA in cell nuclei; neither of these compounds varies in amount when cells undergo physiological changes that bring about considerable variations in nuclear non-histone protein content. We have consequently determined the "DNA-histone ratios" of several series of tissue nuclei by microphotometric estimations of Feulgen-dye, and of Fast green bound at pH 8. The methods used have been described previously\textsuperscript{12} and the results are summarized in table I.

It is evident that the Feulgen-Fast green ratios are quite similar in the somatic mammalian and two of the reptilian tissues tested. The polyploid series of liver nuclei as indicated by their DNA content is equally well detectable on the basis of Fast green measurements. The three series of thyroid nuclei represent cells in very different physiological states whose nuclei exhibit striking differences in size; the same is also true for the three types of mouse kidney nuclei which are reported here, but there is no detectable change of Feulgen dye or Fast green content within either series.\textsuperscript{13} Lizard erythrocyte nuclei exhibit a markedly lower Feulgen-Fast green ratio and onion root tip nuclei show an intermediate ratio.

Vendrely and Vendrely\textsuperscript{14} have recently reported an unusually high arginine-DNA ratio in certain fish sperm, as well as in beef sperm, when compared to other types of nuclei. By our method beef sperm has been
Optical conditions of microphotometric measurements: Beckman B spectrophotometer used as light source; animal nuclei were measured at 550 mμ for Feulgen-, and at 630 mμ for Fast green determinations, with 0.3 mm. slit width. To avoid stray light error in measuring the very dense root tip nuclei, measurements were conducted at 600 mμ, close to 50% of peak absorption for both dyes.

* Amounts of dye are given in arbitrary units calculated according to the formula: amount = (0.6 average nuclear diameter)^2 × optical density × 100. Numbers in parentheses are numbers of measurements.

† Since these ratios are based on arbitrary units of dye content, they do not indicate the ratios of actual amounts of DNA and histone and are only of value for relative comparison.

The Feulgen-Fast green ratio of sperm was calculated on the basis of average optical density (Em) of central 2 μ cores. Since these sperm cells are of very regular size and shape, unaffected by the treatments used, this method results in a ratio directly comparable to all the others given.

Blood smears, prefixed in methanol, were used. In these the nuclei are flattened to various extents. The computation of amounts of dye will in such a case result in values which are low compared to those obtained on spherical nuclei. Consequently the Feulgen values of erythrocytes cannot be compared to those of liver and kidney cells. Fast green values are affected to the same extent so that the Feulgen-Fast green ratio remains directly comparable to that of other nuclear types. Two different samples, stained at different occasions, were measured to test the reproducibility of the results.
found to possess the lowest Feulgen-Fast green ratio of all tissues tested, which agrees with these findings. Preliminary observations on mouse sperm tend to indicate a similar situation.

Assuming the Feulgen reaction to allow a correct quantitative estimate of nuclear DNA content even among different organisms, a change in the Feulgen-Fast green ratio may indicate a difference in the amount of nuclear basic protein, a different composition of the basic protein, or both. The exact significance of changes in this ratio will only be understood when more extensive analytical data on histones from different sources become available.

As has been pointed out earlier, this staining procedure cannot serve to titrate all basic groups that occur in the proteins which can be visualized by it. However, the results of the photometric measurements make it likely that a constant proportion of the available groups is stained when nuclei of related cell types are compared. This method should consequently prove to be of value for such relative quantitative comparisons, as well as provide a simple routine procedure for visualization of histones.

Summary.—Treatment of formalin-fixed tissue sections with Fastgreen at pH 8 after extraction of nucleic acids results in a selective chromatin stain. Model experiments were undertaken on a series of proteins to determine the specificity of the staining procedure, and microphotometric determinations of the Feulgen-Fast green ratios in nuclei of various cell types were made. All experiments indicate that histones or protamines are responsible for the histological staining picture.

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THE EVALUATION OF THE KINETIC CONSTANTS OF ENZYME CATALYZED REACTIONS

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For an enzyme-catalyzed reaction that can be described by equations (1a), (1b), (1c), etc., and where the experimental conditions are such that

\[
\begin{align*}
E_f + S_f & \rightleftharpoons k_1 \quad k_3 \quad ES \rightarrow E_f + P_1 + P_2 + \ldots \\
E_f + P_1 & \rightleftharpoons k_4 \quad k_5 \quad EP_1 \\
E_f + P_2 & \rightleftharpoons k_6 \quad k_7 \quad EP_2 
\end{align*}
\]  

the course of the reaction is given, within the limits of experimental error, by the integrated rate equation (2), the evaluation of the kinetic

\[
k_3[E] = K_S \left( 1 + [S]_0 \sum \frac{1}{K_P_j} \right) \ln \frac{[S]_0}{[S]_t} + \left( 1 - K_S \sum \frac{1}{K_P_j} \right) (S)_0 - (S)_t 
\]

constants \( K_S = (k_2 + k_3)/k_1 \) and \( k_3 \), and eventually of \( K_P_1 = k_5/k_4, K_P_2 = k_7/k_6 \) etc., is a problem of considerable practical importance which is complicated not only by the possible competitive interaction of the free enzyme with one or more of the reaction products but also by the fact that the integrated rate equation contains both a zero and a first order term.

The traditional solution of this problem has been to study the reaction in its initial stages so as to minimize the difficulties arising from the possible interaction of the free enzyme with one or more of the reaction products and to estimate the initial velocities, at the various initial specific substrate