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The Accentuators, pH and the Principles of the Effect in Histological Staining

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Abstract. A study was made of the influence of accentuators (phenol, aniline) and pH on the stainability of vegetable tissue (roots of *Allium cepa* L.) with light green and methylene blue with reference to the theory of the electrostatic basis of staining. — The results obtained on the whole confirm PISCHINGER's theory of the electrostatic basis of staining for light green only, but with methylene blue the situation was found to be much more complicated. Only the alkalization of a watery solution of methylene blue increases its staining ability, whereas the accentuators markedly disturb staining. Prischinge's theory is thus not of general validity, but applies only to one factor of this complicated process. Electrostatic influences acting on staining ability are not so important as other factors in this complicated process in some dyes.

In histological staining technique, auxiliary substances, which are known to improve the staining of the preparations, have been used for a number of years (from general studies e.g. HERTWIG 1929, SINGER 1952, DAVENPORT 1960). These substances may be divided into two groups; i.e. caustic dyes which in combination with a coloured substance form soluble substances only with difficulty. To this group belongs e.g. iron alum in Heidenhain's stain. These substances are supposed to be of chemical importance. The other group which is the subject of this paper is formed by the so-called accentuators (SINGER 1952). They are essentially two groups of substances (to the one belongs the usually applied aniline, to the other phenol, which have been frequently used in histological technique and have become an indispensable part of some staining processes.

The explanation of the action of these substances is, however, unsatisfactory. Older theories (e.g. PAPPENHAIM 1901) that they may effectively lower the surface tension on the staining solutions and thereby facilitate intense adsorption of the dyes on the preparations is difficult to maintain. If it were so, other substances with a high surface tension would have been discovered, e.g. tributyline or taurocholates, which would also act as accen-

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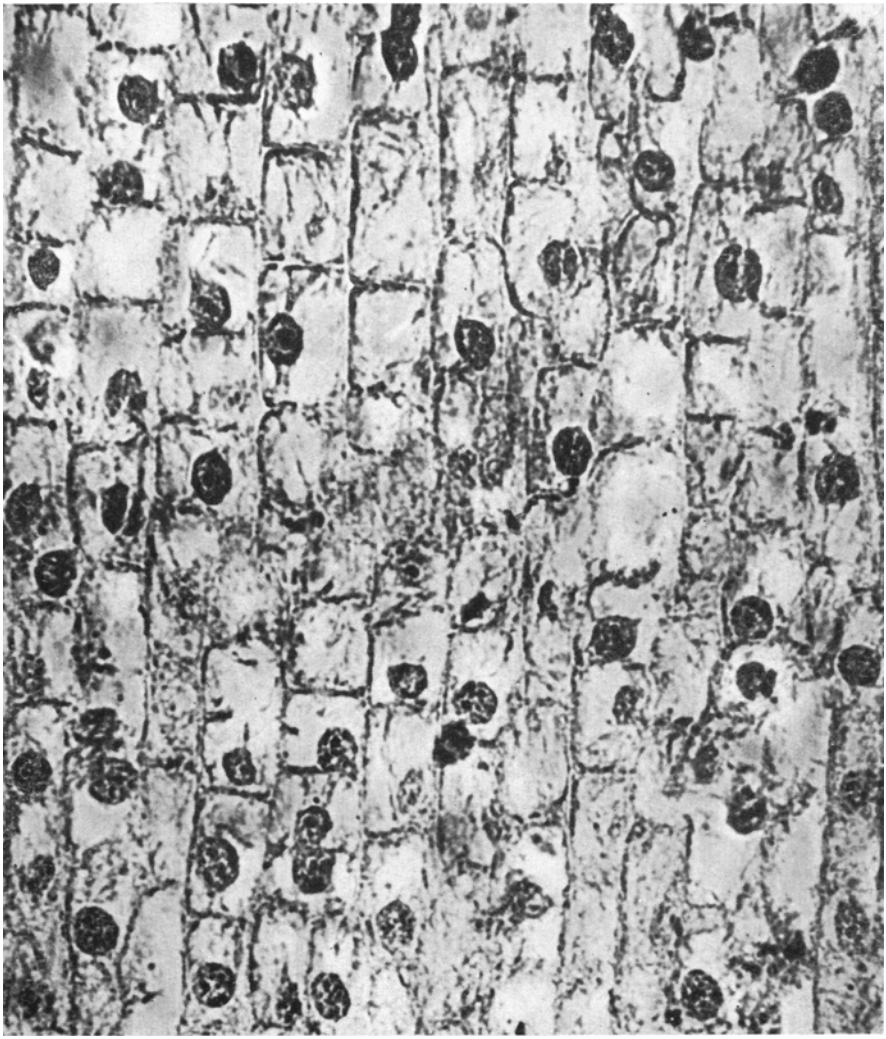

Fig. 1. Interphase intervals in the root of *Allium cepa* L. stained with light green (1% light green + 1% phenol + 1 gt HCl). The plasma is less stained, mitoses are not stained. Thickness of sections is 7 μ, time of staining 30 sec., time of differentiation in 96% alcohol 23 min. 20 sec. (obj. 20×, Hom. II., without filter, exp. 3 sec., photoplates Repro Ortho, negative developer PD:).

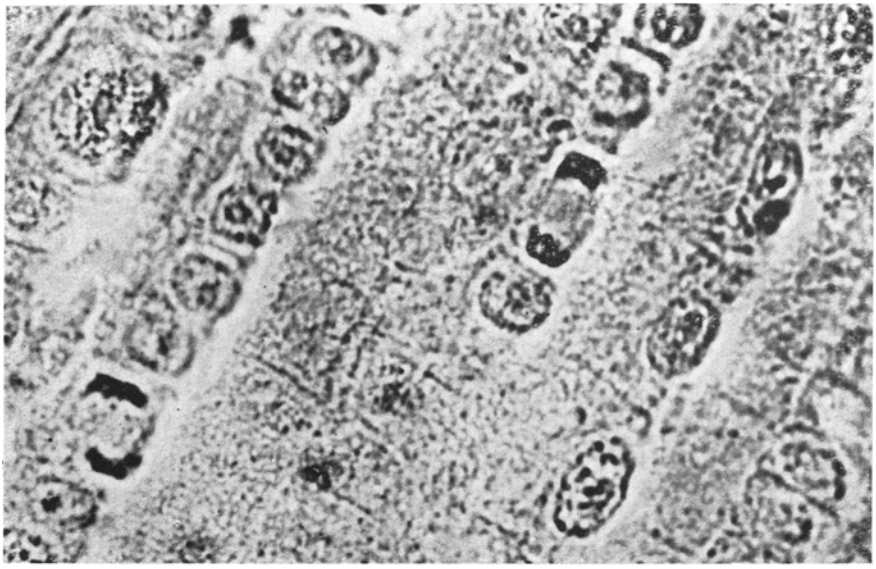

Fig. 2. Mitoses on root tip of *Allium cepa* L. stained with methylene blue (1% methylene blue + + 1% aniline water + 2 gtt KOH). The plasma is well stained, interphase nuclei are not stained Thickness of section is 7 μ, time of staining 30 sec., time of differentiation in 96% alcohol is 31 min. 30 sec. (obj. 45×, Hom. II., without filter, exp. 3 sec., photoplates Repro Ortho, negative developer PD_5).

tuators. This, however, is not the case. Experience has shown that only substances belonging to the above groups can be used for these purposes. There may be a further objection that both of these accentuators would have to act analogically, if only the surface tension were in question. Even in this case, however, it has been found that this is not so; in some mixtures only aniline, in others only phenol is effective.

When studying this problem we proceeded from the electrostatic theory of staining, worked out chiefly by Pischinger (PISCHINGER 1926). This author considers that the decisive factor for staining is the isoelectric point of proteins or other coloidal substances in the preparations. If a staining mixture is provided with a suitable pH by a certain buffer system, the preparation stains much more intensely than at other pH values. This theory has found ample support. Today staining processes are known in which regulation of pH for the improvement of the staining results is successfully used.

From this standpoint the action of the above accentuators presents quite a new aspect. Phenol in watery solutions is regarded as a weak acid, aniline as a weak base. It was our task to examine the influence of changes in acidity or basicity on the effect of these accentuators with regard to the theory of the electrostatic basis of staining.

Materials and Methods

The experiments were carried out on the roots of the onion (*Allium cepa* L.), fixed by Nawashin's fluid and embedded through methylbenzoate into paraffin. The 7 μ thick sections were stained for a period of 30 seconds which was chosen experimentally as the most suitable, and then differentiated for a certain time in 96% alcohol, until 1) the staining of the preparation was constant, or 2) differentiation was interrupted, as the preparation was in danger of being completely bleached.

For staining we used methylene blue which is known to be a base or light green which on the contrary is an acid dye. The dyes were applied in 1% solutions, the concentrations also being chosen experimentally as the most suitable. To the 1% solutions of the above dyes both accentuators were added, also in 1% solutions. Both series were gradually acidified or alkalized by 10 N hydrochloric acid (HCl) or by 10 N potassium hydroxide (KOH). To 50 ccm of dyes 1 to 4 drops of this acid or base were gradually added. The controls were stained in watery solutions of these dyes, i.e. without accentuators.

The results were evaluated according to the following principles:

1. The resistance of the dye or the staining of the preparation to differentiation with 96 % alcohol, which is expressed by the time of differentiation.
2. The evaluation of the staining of the cell components. The evaluation was carried out in 4 degrees (weakly stained, well stained, intensely stained, intensely overstained).

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Results and Discussion

The results obtained are shown in Tab. 1. The intensity of the staining is marked with crosses, the time of differentiation is given in minutes.

As shown by the experiments, light green is unusually accentuated by phenol, so that even after one hour's differentiation with 96 % alcohol a very

Tab. 1. Influence of accentuators and pH on staining and time of differentiation

(The intensity of staining is expressed by crosses, time of differentiation in 96% alcohol in minutes.) — Explanations: 0 — not stained, + + — well stained, + + + and + + + + — intensively stained, + + + + + — intensively overstained. — Numbers: time of differentiation in 96 % alcohol in minutes — Note: 0 — value was lower than 1 min. and moved within the limit of seconds

Accentuators / Reaction	Aniline		Phenol		Control (water)	
	light green	meth. blue	light green	meth. blue	light green	meth. blue
Neutral	0 0′	+ 27′	+ + + + + 60′	+ + 25′	+ + + 1′	+ + 20′
Acid	+ → + + 1—2 gtt 0′ 3—4 gtt 35′	+	+ + +	+ → 0	+ + + +	+ + +
		8′	38′	2′	16′	0′
Alcalic	0	+ + → 0 1—2 gtt 23′ 3—4 gtt	+ → 0	+ + → +	+ → 0	+ + + +
	0′		0′	8′	0′	23′

intense staining of the preparation is evident (Fig. 1). The control preparation stained with a watery solution of light green, however, tolerates differentiation much worse, and is practically bleached as early as after 1 minute. Aniline, on the contrary, prevents staining with light green. These results lead to the assumption that the influence of pH is really important in this case especially as the watery solutions or light green stain very intensely after merely acidifying with HCl and that the time of differentiation after this staining is quite long, too (16 minutes). Light green stains well in a neutral environment, the time necessary for differentiation is, however, very short (about 1 minute). Our assumption can also be confirmed by the fact that aniline lowers stainability with light green down to zero value and that the stainability is partly regenerated by adding HCl. After adding 1—2 drops the staining begins to appear, the preparation, however, is quickly differentiated, but after adding 3—4 drops, stainability increases and the time of differentiation is also considerably prolonged (35 minutes). Nevertheless, even the added HCl cannot counteract the depressive effect of aniline.

Experiments with methylene blue yielded much more varied results. In control experiments the watery solution in a neutral environment stains well and differentiation lasts about 20 min. On alkalizing the staining ability becomes much more intense and the time of differentiation is also prolonged. On the basis of these experiments aniline would be expected to increase the staining ability of methylene blue. In these experiments, however, just the contrary was proved, aniline essentially lowered this ability. Better stainability was achieved only after adding 1—2 drops of KOH (Fig. 2), whereas after further alkalization the staining ability again decreased.

On adding phenol in a neutral environment the stainability does not change when applying methylene blue, after acidifying it decreases and after alkalizing it is regenerated, but not to the same extent as in control experiments. It is interesting that the staining ability of watery solutions of methylene blue also increases with acidifying, but the time of differentiation in this environment is very short (2 min.). The degree of colloidity (sol — gel) does not seem to be a decisive factor in methylene blue. The experiments showed that after adding 3 drops of KOH to the solution of methylene blue with phenol the dye begins to precipitate reversibly. After 4 drops of KOH reversible precipitation occurs almost immediately. On the basis of the electrostatic theory methylene blue with phenol should stain best after alkalization since it is close to the isoelectric point. We proved, however, that its staining ability in these conditions is not good. On the contrary, it does not stain well and in alcohol it dissolves immediately. From the results it can be concluded that the accentuators lower the staining ability of methylene blue respective of the actual acidity of the dye, but that they improve the bond of the dye with the substrate.

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Studovali jsme vliv akcentuátorů (fenol, anilin) a pH na barvitelnost rostlinné tkáně (kořínky *Allium cepa* L.) světlou zelení a methylenovou modří vzhledem k Pischingerově teorii elektrostatické podstaty barvení. Získané výsledky potvrzují správnost uvedené teorie pouze u světlé zeleně, avšak u methylenové modři jsme nalezli poměry mnohem komplikovanější. Pouze alkalizace vodního roztoku methylenové modři stupňuje její barvicí schopnost, zatímco akcentuátory ji výrazně porušují. Není tedy Pischingerova teorie o elektrostatické podstatě barvení obecná, ale představuje pouze jednu složku tohoto složitého procesu. Elektrostatické vlivy, působící na barvicí schopnost, jsou u některých barviv dalekosáhle překonány jinými faktory tohoto složitého procesu.

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Изучалось влияние акцентуаторов (фенол, анилин) и pH на окрашиваемость растительной ткани (корни Allium cepa L.) светлой зеленью и метиленовой синью с точки зрения теории электростатической сущности окрашивания PISCHINGER'a.

Полученные результаты подтверждают правильность этой теории PISCHINGER'a только по отношению к светлой зелени, между тем как для метиленовой сини положение гораздо более сложно. Красящую способность водного раствора метиленовой сини повышает только алкализация, тогда как акцентуаторы ее резко нарушают. Итак, теория роли электростатических процессов в окрашивании, как ее предложил PISCHINGER, имеет только ограниченное значение. Это только один из компонентов всего сложного процесса. Электростатистические факторы, влияющие на окрашивающую способность, у некоторых красителей в значительной степени отступают на второй план перед другими компонентами этого процесса.