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ANALYSIS OF THE STAINING PROPERTIES OF ACRIDINE ORANGE IN EPITHELIAL CELLS

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Classical histology has been enriched with new histotopochemical features by the introduction of fluorescence microscopy. The use of the fluorescence microscope covers a wide range, and a special field consists of the utilization of fluorescent dyes for the investigation of tissue structures. While a great number of fluorochromes is known, only a few of these dyes are being employed. This must be attributed to the fact that the staining and physico-chemical properties of many fluorescent dyes have not been properly elucidated and we are still ignorant of the possibilities concerning the differentiation of fluorochromes; nor do we know those biochemical structures which — forming physical connections or chemical reactions with the dye molecules — emit specific rays of light if exposed to ultraviolet irradiation.

Acridine orange is the most extensively used fluorochrome. Literature contains very controversial reports on its staining properties. Considering that contradictory interpretations of the mechanism of staining lead to different evaluations of the results obtained, we have designed experiments with a view to arriving at definite conclusions regarding the histochemical reactions of acridine orange. It was hoped that they would enable us to facilitate an unequivocal interpretation of the microscopic picture of preparations stained.

It is claimed by British and North American authors (Armstrong, Bertalanffy, De Bruyn, Hendley, Morthland, Steiner-Beers) that

(i) on account of its adequately located amino groups, acridine orange may be bound to any tissue structure of free acid character;

(ii) in a medium with an adequate pH, DNA and RNA emit different colours;

(iii) the binding of acridine orange to structures of an acid character is not merely due to the interaction between acid and basic radicals but also, and in the first place, to complex formation;

(iv) the reactivity of acridine dyes to nucleic acids is considerably diminished by the enzymatic degradation of the latter;

(v) it is the nature of the reaction which determines the colour of the dissolved dye.

German authors (KRIEG, KREBS-GIERLACH, SCHULER, SCHÜMMELFEDER, STRUGGER, ZEIGER) on the other hand declare that

(i) apart from nucleic acids, it is only to mucopolysaccharides that acridine orange displays affinity;

(ii) of the nucleic acids, DNA and RNA stain differently in a medium with an adequate pH;

(iii) the binding of the dye is partly electrostatic, chemical, and partly adsorptive, physical;

(iv) depolymerized and poorly polymerized nucleic acids are staining similarly to RNA;

(v) the colour of the dye solution is determined by its concentration and the degree of its dissociation.

We instituted the following experiments with the object to reconcile these differing opinions.

Method

Using the vaginal secretion of women between 20 and 30 years of age who were in the same hormonal cycle (follicular phase), we performed our experiments on morphologically uniform epithelial cells with non-pycnotic nuclei, from the second superficial layer.

1. The dye was dissolved in distilled water resp. 96 per cent ethyl alcohol, at pH 7. The light emitted by various concentrations (1 : 100, 1 : 1000, 1 : 10,000, 1 : 100,000, 1 : 1,000,000) of the solution in ultraviolet resp. blue light was studied *in vitro*. Estimation was performed in 100 ml cuvettes with a Zeiss standard arc lamp as the source of light, 5 per cent acid copper-sulphate as the heat filtre and an 1.5 mm *UG*1 (Schott) filtre for ultraviolet rays resp. a 4 mm BG4 filtre for blue light. The colour of the emitted light was compared with the naked eye to a standard colour scale in incident light.

2. Unfixed cells of the vaginal epithelium as also cells fixed in ether-alcohol in a wet state were stained with each of the said solutions. Time of staining was 3 minutes in every case, followed by rinsing with distilled water or alcohol.

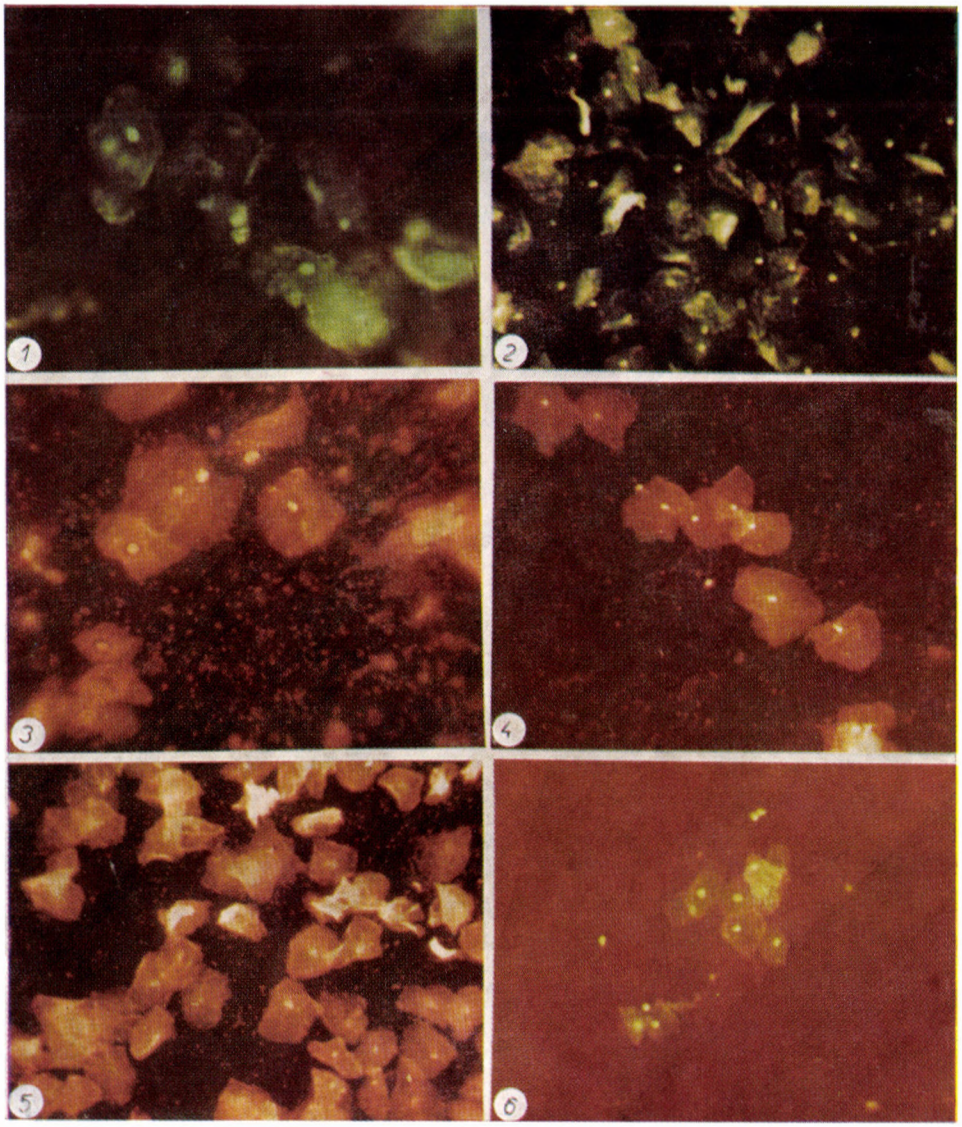
3. We studied the light emitted by the aqueous and alcoholic solution (concentration 1 : 10,000, pH from 2 to 9) of the dye under exposure to ultraviolet rays and blue light. Solutions of sodium tetraborate resp. oxalic acid and potassium hydrocarbonate, mixed in adequate proportions, served as buffer. The pH of the buffered dye solutions was determined by means of an electric apparatus adjusted to an accuracy of ± 0.05.

4. Unfixed vaginal epithelial cells and cells fixed in a wet state in ether-alcohol were stained simultaneously with each of the above solutions. Unfixed cells were examined in a wet chamber. A buffer solution, adjusted to the same pH as the dye solution, was used for covering the preparations. Fixed smears, after having been rinsed with a buffer solution of adequate pH, were left to dry at room temperature.

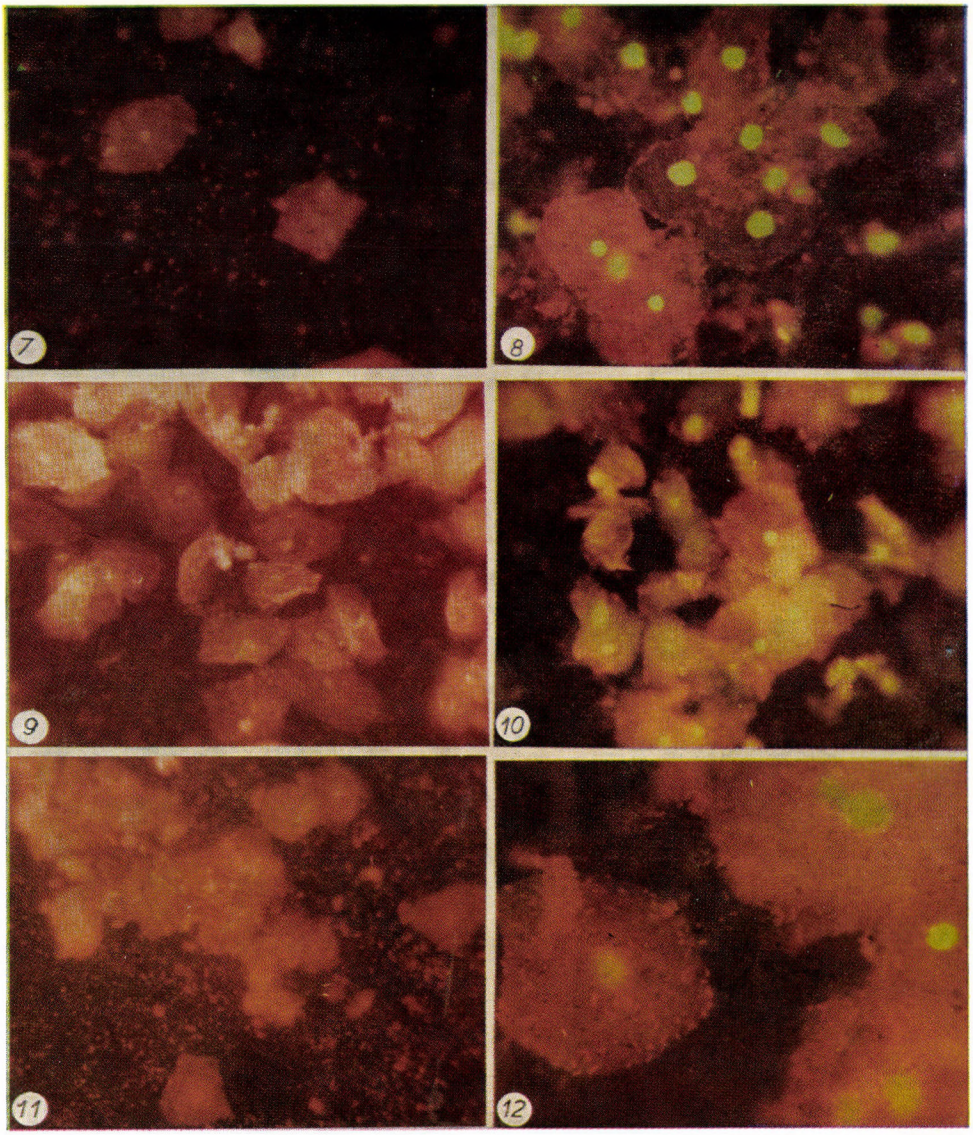
5. To ascertain the specificity of acridine orange for nucleic acids, we observed the staining of fixed and unfixed epithelial cells by an 1 : 10,000 aqueous dye solution

(a) after the digestion of DNA,
(b) ,, ,, ,, ,, RNA,
(c) ,, ,, ,, ,, both DNA and RNA,
(d) ,, resynthesis and repolymerization by means of active ATP, subsequent to enzymatic degradation.

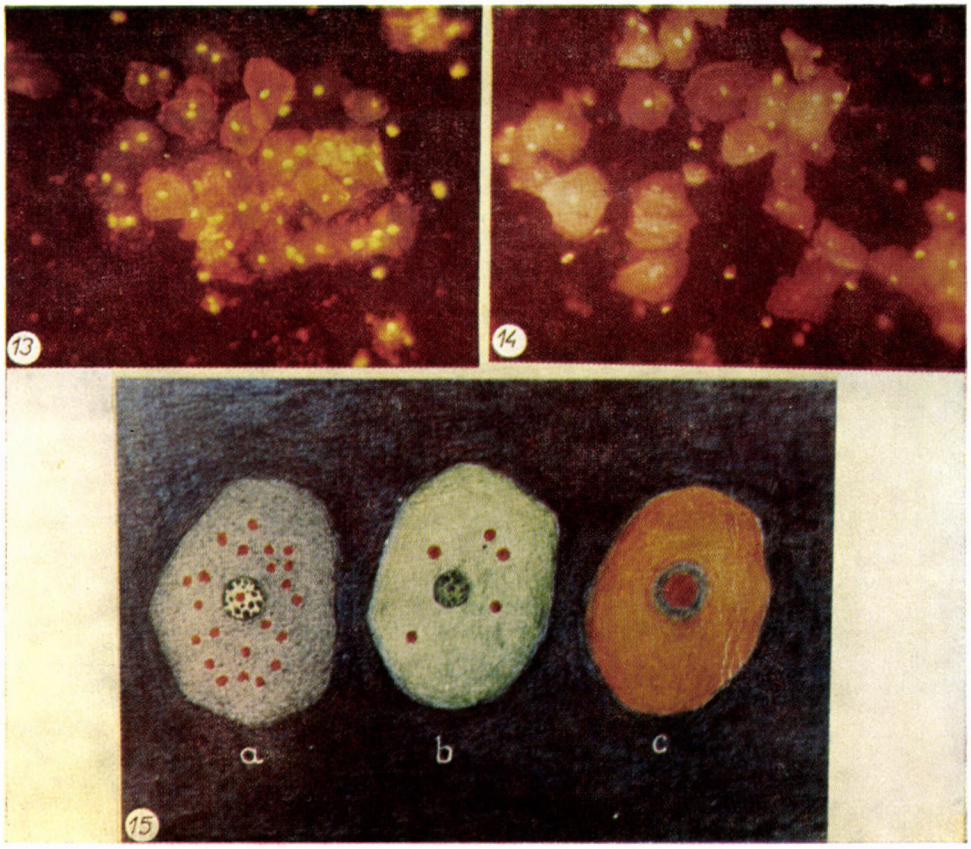
Enzymatic degradation of DNA was achieved by freshly dissolved crystalline desoxyribonuclease applied for an hour in a concentration of 0.005 per cent, at 37° C. The desoxyribonuclease was dissolved in a veronal-acetate buffer of pH 7.4, which contained 0.01 per cent gelatin and 0.003 molar magnesium sulphate. Following digestion, the preparations were stained with acridine orange 1 : 10,000, at pH 2, 6.7 and 9.



1—4. Fixed cells of vaginal epithelium stained with acridine orange solutions at pH 2, 5, 6 and 9
5. Fixed vaginal smear. Staining at pH 6
6. Fixed vaginal smear. Staining at pH 6 followed by alcoholic extraction



7. Fixed epithelial cells stained at pH 7
8. Unfixed wet-chamber preparation stained at pH 7
9. Preparation stained at pH 2 after digestion by desoxyribonuclease
10. Preparation stained at pH 9 after digestion by ribonuclease
11. Preparation stained at pH 6.7 after complete digestion by desoxyribonuclease
12. Preparation stained at pH 6.7 after partial digestion by desoxyribonuclease



13. Digestion with desoxyribonuclease and resynthesis with ATP. pH of dye solution, 6.7
14. Digestion with ribonuclease and treatment with ATP. pH of dye solution, 6.7
15. Staining of living, dying and dead epithelial cells
(a) Green fluorescence: chromatic substance; red fluorescence: nucleolus. No cytoplasmic fluorescence. The majority of red granules is dye stored by the living cell; some of the cytoplasmic red granules consist of RNA
(b) Green cytoplasmic fluorescence due to diffusely spread dye in low concentration. The few red granules in the cytoplasm represent RNA
(c) At high concentration, the dye is physically adsorbed to dead cells. Brilliant red cytoplasmic and nuclear fluorescence with a non-fluorescing plasmic zone around nucleus. RNA granules blotted by intensive plasmic fluorescence

A 0.02 per cent ribonuclease extract, prepared from homogenized small intestine applied in the above concentrations and under the said conditions, served for the enzymatic degradation of RNA.

Both enzyme solution — first the one and then the other — were applied for the combined splitting of DNA and RNA. The technique was as described above, and the preparations were placed into an enzyme-free stock solution (veronal acetate, pH 7.4) for 3 minutes between the two digestions.

Digestion of DNA or RNA or of both having been completed, the preparations were kept in an active ATP solution (20 mg of ATP per ml) for 3 hours at 37° C, and then stained.

Acridine orange NO Gurr (C. I. No. 788) was used in the experiments.

Results

Ad 1 and 3

In vitro, all aqueous solutions of acridine orange between the dilutions of 1:100 (limit of solubility) and 1:1000 give purple, or red and orange-red fluorescence in ultraviolet or blue light. At dilutions above 1:1000 fluorescence

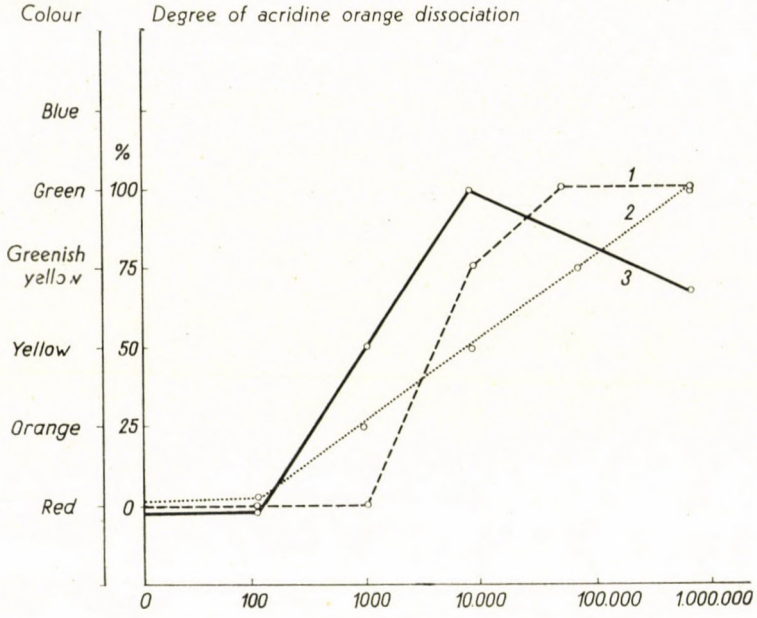


Diagramm. Curve 1 — — — —: fluorescence of dye in vitro
Curve 2 .....: dominant colours of the microscopic picture
Curve 3 ————: number of dissociated acridine orange molecules

turns into a yellowish green. Assuming various shades of green and greenish grey, the colour becomes paler and paler with increasing dilutions.

The dye dissolves considerably less readily in alcohol (limit, 1:10,000). Its fluorescence in ultraviolet and blue light is likewise yellowish green, green and greenish grey according to the degree of dilution.

The colour of the 1:10,000 aqueous and alcoholic solutions depends greatly on the pH of the solvent. It is pale green or yellowish green in the pH range of 2—3, greenish yellow in that of 4—7, and is shifted towards orange red in the range of 8—9.

Ad 2

Fixed and unfixed preparations give more or less the same fluorescence with different concentrations of the dye. Stained with a solution of 1:1000, the epithelial cells emit a homogeneous red colour. A differential pattern results on staining with acridine orange diluted at 1:10,000; nuclear fluorescence is yellowish green, cytoplasmic fluorescence is dark green with red granules (in unfixed preparations). At concentrations of 1:100,000 and 1:1,000,000 fluorescence is an uniform green.

Treatment with an alcoholic solution of 1:100 results in an uniform green fluorescence and only superficially situated Döderlein's bacilli and the nuclei of certain leucocytes give a red fluorescence. At 1:1000 even this red fluorescence disappears, while the hue of the green fluorescence becomes deeper. Only dark green and greenish grey fluorescence is observable beyond the dilution of 1:10,000.

Ad 4

It is up to pH 5 that 1:10 000 aqueous solutions of the dye yield differentiated and reliable pictures. The nucleus of the epithelial cells gives a moderate pale green fluorescence, the cytoplasm is dark green with red granulations, superficial Döderlein's bacilli show a brilliant red fluorescence. Red becomes dominant at pH 6: the nuclei emit a pale red, while the cytoplasm of the epithelial cells a greenish brown colour. In the pH range of 6.9 only red fluorescence, then a deepening of this colour and an increase in the intensity of fluorescence occur.

With alcoholic solutions, the sequence of the emitted colours is similar but shifted by two pH degrees towards alkalinity in favour of green. By using a 20 per cent alcoholic solution of pH 7 or an aqueous buffer of acid pH, it is possible to differentiate the excess dye from preparations stained at strongly alkaline pH. No such elimination is possible in the reverse case, i. e. in that of preparations stained at an acid pH.

Ad 5

On the digestion of desoxyribonuclease, the green fluorescence disappears almost completely even below pH 6: both nuclear and cytoplasmic fluorescence are pale red. Above pH 6 the intensity of red fluorescence increases. In case of

incomplete desoxyribonuclease digestion (*i. e.* one of 45 minutes) the cytoplasm is pale red at a time when the nucleus still gives green fluorescence. It does not become pale red until after complete digestion, *i. e.* in about 90 minutes. Treatment with ribonuclease gives uniformly green fluorescence at pH 6.7: the nucleus and Döderlein's bacilli stain light, the cytoplasm dark green, and it is only above the inversion zone, near the neutral point, that a red fluorescence appears. Combined digestion with desoxyribonuclease and ribonuclease reduces the intensity of fluorescence and the morphological pattern also becomes indistinct. A homogeneous red fluorescence comes to dominate the picture at values above pH 7.

Our attempts at resynthesis by means of ATP resulted in that both nuclear and cytoplasmic fluorescence became green again and brilliant red granules appeared in the cytoplasm after digestion with desoxyribonuclease, while after ribonuclease digestion a red fluorescence was observed. The reappearing colours were much more brilliant than before resynthesis, in other words, the intensity of fluorescence was increased.

Discussion

Ad 1

It follows from our first experiment that the spectrum emitted by acridine orange in ultraviolet or blue light varies according to the proportion of dissociated and non-dissociated dye molecules per units of the solvent. At low concentrations the number of dye molecules per unit is small and they are dissociated. Ionized, dissociated dye solutions give a green fluorescence.

Beyond a certain degree of concentration, dissociation is mutually inhibited by the molecules contained in the solution. In other words, there exists a limit concentration at which the number of dissociated molecules per unit reaches a peak.

Increasing the concentrations, the number of dissociated molecules becomes less and less not only in proportion to the number of non-dissociated molecules but absolutely as well. At the concentration of 1:1000 the dissolved acridine orange is in the normal molecular state. Molecular dye solutions show a red fluorescence.

Ad 3

We examined the connection between pH and fluorescence at a given dye concentration. One member of the buffered dye solution was the solvent, the other the dye. According to the law regarding the dissociation of buffer systems, acidly buffered solvents promote the dissociation of the acridine orange molecules, these being of an alkaline character and the fluorescence is

green in such cases. On the other hand, solvents treated with alkaline buffers inhibit dissociation of the likewise alkaline dye, and the fluorescence is red in such cases.

Thus, the colour of fluorescence of acridine orange *in vitro* depends on the dissociation of the molecules, since the degree of concentration and the changes in pH are both affecting dissociation.

Ad 2 and 4

Our further experiments had the object to examine the binding of acridine orange by living, necrotizing and necrosed cells, at various concentrations and different pH values. Considering that fixation stabilizes a given histochemical state, it is not unessential in which vital phase the cells have been fixed. Permeability being different in living, dying and dead cells, they take up different amounts of dye from solutions of identical concentration and pH.

Living cytoplasm does not stain and gives no fluorescence. Acridine orange, being alkaline, appears in the cytoplasm of the living cell in the form of masses and its red fluorescence is misleadingly similar to that of RNA granules.

Examined in a wet chamber, necrotizing epithelial cells show a green cytoplasmic fluorescence. We think that the dye which stains the cytoplasm of these cells a diffuse green is quantitatively equal to the aggregated dye observed in living cells.

The cytoplasm of necrosed cells displays a diffuse red fluorescence.

Its substance being DNA, the nucleus of living cells gives a green fluorescence in the pH range of 2—6, and a red one above pH 6. Since the selective permeability of the cell membrane disappears with the death of the cell, Strugger's concentration effect causes their nucleus to fluoresce red already at pH 6.

Ad 5

A mixture consisting principally of mononucleotides and, to a smaller extent, of oligonucleotides is formed from the ribonucleic acids under the prolonged action of ribonuclease which causes the decomposition of the 3′-phosphate bond of pyrimidine nucleotides. Prolonged action of desoxyribonuclease on the other hand induces the formation of oligonucleotides consisting of several members. Being of alkaline character, these products possess no affinity to acridine orange. The paucity in colour of the microscopic pictures observed after combined digestion with both enzymes may be due to this circumstance.

A partial disintegration of the polymeric structure of nucleic acids occurs if desoxyribonuclease is allowed to act for a short time only. A disruption of the sequence of linked long basic chains seems to affect the hydrogen bonds,

which stabilize the relative position of the chains, in such a manner as to promote their readiness for dissociation. This acidifies the entire system so that its affinity to acridine orange is increased, its staining properties become similar to those of RNA and its fluorescence becomes red. This would well explain why desoxyribonuclease was seen in our experiments to cause the disappearance of green and the intensification of red fluorescence. Apart from the existing RNA structures, depolymerized DNA molecules too, give a red fluorescence.

The first to lose its affinity to acridine orange is the RNA in the nucleoli while RNA in the cytoplasm is much more resistant to enzymatic action. This would confirm the correctness of observations that seem to point to a difference in the molecular structure of nucleolar and cytoplasmic RNA.

As regards our attempts at repolymerization, we refer to the statement of GRUNBERG and MANAGO who claim that polynucleotide phosphorylase performs the degradation and synthesis of nucleic acids in a reversible manner with the aid of the corresponding nucleotide diphosphate. Reaction tends towards the synthesis of nucleic acids when phosphate is split off and towards decomposition when phosphate is taken up. What occurs in the course of reaction is not merely a simple resynthesis, a reunion of split nucleic acids, but repolymerization as well. It is safe to suppose that — if the reaction is capable of producing from any nucleotide diphosphate the polymer of the corresponding nucleic acid — also high nucleic acid polymers or, possibly, even nucleic acids with a different basic chain sequence may be formed. This would explain that characteristic brightening of colours which occurred in all our resynthesis experiments.

When interpreting our results of resynthesis the possibility of a simple adsorption of ATP molecules to the split nucleic acids might arise. This was, however, disproved by the observation that the staining capacity of acridine orange was not intensified by raising the concentration of ATP.

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Our experimental results allow to distinguish three components in the staining effect of acridine orange.

(I) The dye, if applied in an alkaline medium or in a molecular state in concentrated solutions is adsorbed to any morphological element. The bond so formed is of a physical nature, and the fluorescence is red in colour.

(II) The dye, if applied in an acid medium or in an ionized state in solutions of low concentration, can be linked with any morphological element of acid character. Green fluorescence results from this non-specific salt linkage. The red fluorescence seen in the preparations is due to a complex having been formed between dye and RNA.

(III) After the disruption of the said physical and salt linkages, or in strongly acid media, a complex is formed between the dissociated dye molecules and the DNA and RNA molecules. This process is influenced by the extent of nucleic acid depolymerization. No other chemical structures are stained any longer in this case, and the fluorescence given by DNA is green, that by RNA red.

Summary

The staining effect of acridine orange both *in vitro* and in exfoliated fixed and unfixed vaginal epithelial cells has been investigated with special regard to the histochemical reactions of the dye. The demonstrability of nucleic acid by means of acridine orange is claimed on the evidence of experiments with enzymatic degradation. Differences in the reaction of living, necrotizing and necrosed cells to acridine orange at different concentrations and pH values are discussed. The practically complete bibliography on acridine orange is presented.

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АНАЛИЗ АКРИДИН-ОРАНЖЕВОГО ОКРАШИВАНИЯ НА ЭПИТЕЛИАЛЬНЫХ КЛЕТКАХ

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Авторы исследовали эффект окрашивания акридин-оранжевым в опытах in vitro и на отслоенных фиксированных и нефиксированных эпителиальных клетках влагалища, и высказывают свое мнение в связи с гистохимическими реакциями данного красителя.

На основе экспериментов энзиматическим перевариванием демонстрируется выявляемость нуклеиновых кислот при помощи акридин-оранжевой краски. Обсуждаются резко отклоняющиеся друг от друга реакции связывания краски живых, отмирающих и омертвелых эпителиальных клеток, при изменении химической реакции и концентрации. Приводится полная доступная литература относительно акридин-оранжевого красителя.

ANALYSE DER AKRIDINORANGEFÄRBUNG VON EPITHELZELLEN

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Es wurde an exfoliierten unfixierten und fixierten vaginalen Epithelzellen der Färbungseffekt von Akridinorange untersucht.

Auf Grund der Enzymverdauung wird die Nachweisbarkeit von Nukleinsäuren mit Hilfe von Akridinorange demonstriert. Die verschiedenen Farbstoffbindungs-Reaktionen von lebenden, absterbenden und abgestorbenen Epithelzellen bei verschiedenen Reaktionen und Konzentrationsänderung werden demonstriert und die gesamte zugängliche Literatur über Akridinorange angeführt.

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