expressed as the activation coefficient $\alpha = a_+/a_o$ has proved useful as an index of the riboflavin status in the case of EGR and has been tried also for whole blood in animals[4].

For the blood samples from 45 schoolchildren the activation coefficients for the stimulation of the glutathione reductase in erythrocytes ($0.96 \leq \alpha_{EGR} \leq 1.46$) and whole blood ($0.92 \leq \alpha_{BGR} \leq 1.28$) showed a good correlation with a coefficient $r = 0.96$ (Pearson-Bravais).

The Figure presents the stimulation of EGR and BGR for the blood drawn from 203 pregnant women. For the two collectives, very similar equations between the activation coefficients were calculated:

$$\text{Schoolchildren} \quad \alpha_{BGR} = 0.67\,\alpha_{EGR} + 0.29$$

$$\text{Pregnant women} \quad \alpha_{BGR} = 0.67\,\alpha_{EGR} + 0.33$$

Because of the increased stability in our test system of glutathione reductase from whole blood as compared to erythrocytes, it appears reasonable to calculate activities for the BGR. One may argue about the proper basis for the activities to rely on, especially when there are anaemic persons in the group investigated, as was the case with the pregnant women (DECKER, GLATZLE, HINSELMANN, to be published).

Whether the activities were based on hemoglobin[4], hematocrit, blood volume or erythrocytes, the activities a_o (without FAD addition) were negatively correlated with α_{BGR} (Tables II and III). This means that, in the case of high activation coefficients, the enzyme activities a_o of the flavoenzyme glutathione reductase were low, indicating a biochemical riboflavin deficiency. On the hemoglobin basis, considering the correlation coefficients r (Pearson-Bravais) in Table III, there was no statistically significant correlation between the activities a_+ (after FAD addition) and the activation coefficients α_{BGR}. On the other hand, a negative correlation was found between α and a_+ when the activities were calculated for 1 l of blood, 1 ml of erythrocytes or 10^{11} erythrocytes, respectively. Depending on the basis for the activities, this might point towards either no or some loss of glutathione reductase protein (apoenzyme plus holoenzyme) in riboflavin deficiency[4]. More work has to be done to elucidate this problem which is complicated by various circumstances affecting glutathione reductase activity[8].

Table III. Correlation coefficients r (Pearson-Bravais) for blood and erythrocyte glutathione reductase and blood flavin ($191 \leq n \leq 203$)

	α_{BGR}	f_B
a_o (BGR, per g Hb)	−0.67	+0.33
a_o (BGR, per ml erythrocytes)	−0.71	+0.34
a_o (BGR, per l blood)	−0.78	+0.52
a_o (BGR, per 10^{11} erythrocytes)	−0.79	+0.36
a_+ (BGR per g Hb)	−0.11 n.s.	+0.13 n.s.
a_+ (BGR per ml erythrocytes)	−0.19	+0.16
a_+ (BGR per l blood)	−0.39	+0.47
a_+ (BGR per 10^{11} erythrocytes)	−0.41	+0.23
α_{BGR}		−0.39
α_{EGR}	+0.96	−0.36

—, Significant; n.s., not significant.

In cross-sectional studies – contrary to controlled ones – SHARADA and BAMJI[9] reported that with their test system a correlation between glutathione reductase activity and red cell flavin levels is often not seen. In our study with pregnant women, we found statistically significant, albeit not high, correlations between flavin levels in whole blood and the enzyme parameters α_{EGR}, α_{BGR} and a_o which are presented in Table III and were also established by the Kendall rank correlation test: Low enzyme activities a_o – or high activation coefficients – corresponded to low flavin levels in blood and high enzyme activities a_o – or low activation coefficients – to high flavin levels.

Zusammenfassung. Eine vereinfachte Variante des Glutathionreduktasetests zur Erfassung des Riboflavinstatus wird beschrieben, für die nur 0,05 ml Gesamtblut anstelle von gewaschenen Erythrozyten benötigt werden. Beim Vergleich der Stimulierbarkeit der Glutathionreduktase durch das Coenzym FAD ergab sich eine sehr gute Korrelation zwischen den Aktivierungskoeffizienten bei Verwendung von Gesamtblut und Erythrozyten. Diese zeigten statistisch signifikante Korrelationen zum Flavingehalt im Blut.

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20 December 1973.

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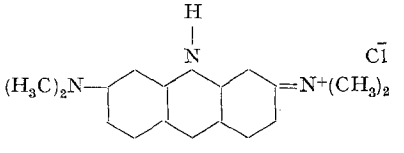
Aqueous Solution of Acridine Orange in the Staining of DNA-Aldehyde

The attempt to stain DNA-aldehyde in tissue sections with the fluorescent dye, acridine orange (AO) following Feulgen procedure led to unsuccessful results. The present communication embodies results of a study with acridine orange and its use in the staining of acid hydrolyzed DNA.

Two samples of AO were used in this investigation. One was manufactured by the National Aniline Division, New York, USA, batch No. 11538 and the other by G. T. GURR, London, batch No. 55744. It is a basic dye (C.I. No. 46005) of the acridine group with the following structural formula:



For cytochemical detection of DNA this dye was used as 0.1% aqueous solution. When freshly made the pH of the American dye was found to be 6.5. The pH of the English dye solution was 4.0. Staining involved prior acid hydrolysis in 6 *N* HCl at room temperature for 10 min followed by staining with AO for 10 min. The sections were differentiated in amyl alcohol or dehydrated through a graduated series of ethyl alcohol, cleared in xylol and mounted in DPX. Paraffin sections (12 μm) of several different tissues from a Holtzman strain of rat, fixed in 10% buffered neutral formalin and Zenker's fixative, were used throughout.

A Beckman DB spectrophotometer was used to record the absorption data of this dye. Observations were made with an American Optical Corporation Fluorester microscope in conjunction with a Fluorolume illuminator fitted with a mercury super pressure lamp, HBO 200 W. Fluorescence of the dye-DNA complex was obtained with an exciter filter Schott BG-12 and barrier filter, either Schott GG-9 or Schott OG-1.

Staining of mammalian tissues fixed in formalin and Zenker's fixative was perfect with AO after acid hydrolysis of the tissues. The stained slides showed a deep yellow coloured nuclei with a clear cytoplasm when viewed through a light microscope. When viewed through the fluorescence microscope with exciter filter, Schott BG-12 and barrier filter, Schott GG-9, the nuclei exhibited a deep reddish-orange colour without any colour in the cytoplasm. When barrier filter, Schott OG-1 was used with exciter filter, Schott BG-12, the nuclei exhibited an yellow-orange colour against green cytoplasm. Staining intensity with an aqueous solution of the American product of AO at pHs 3.0, 5.0 and 7.0 was more or less similar. In the case of the English product, staining intensity was best at pH 7.0. At lower pH, staining was not uniform. Acid hydrolyzed tissues, when stained with AO that was shaken with activated charcoal, revealed perfectly stained nuclei. Tissue sections, when stained with AO containing 250 mg of sodium hydrosulphite per 50 ml of AO solution, exhibited perfect yellow coloured nuclei only. Sections in which the nucleic acids were extracted with boiling 5% trichloracetic acid (TCA), then acid hydrolyzed and stained, revealed no staining of the nuclei and the cytoplasm. Unhydrolyzed sections stained with AO revealed perfect staining of the nuclei and the cytoplasm. When viewed through the fluorescent microscope employing exciter filter, Schott BG-12 and barrier filter, Schott GG-9, the nuclei and the cytoplasm exhibited a deep orange colour. With barrier filter, Schott OG-1, the nuclei were of orange colour and the cytoplasm of an orange-green colour. Acid hydrolyzed sections after treatment with aldehyde blocking reagent, phenylhydrazine at 60 °C for 1 h and then stained, revealed no staining of the nuclei. Sections after treatment with hot distilled water at 70 °C for 30 min, then hydrolyzed in hydrochloric acid and stained, revealed perfect staining of the nuclei indicating that staining with AO is possible even with depolymerized DNA.

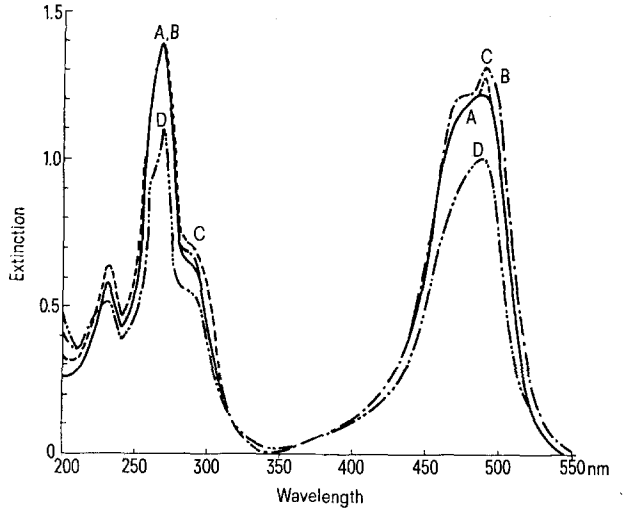


Fig. 1. Absorption characteristics of purified and unpurified acridine orange. A) Only dye; B) after chloroform extraction; C) after amyl alcohol extraction; D) after butanol extraction.

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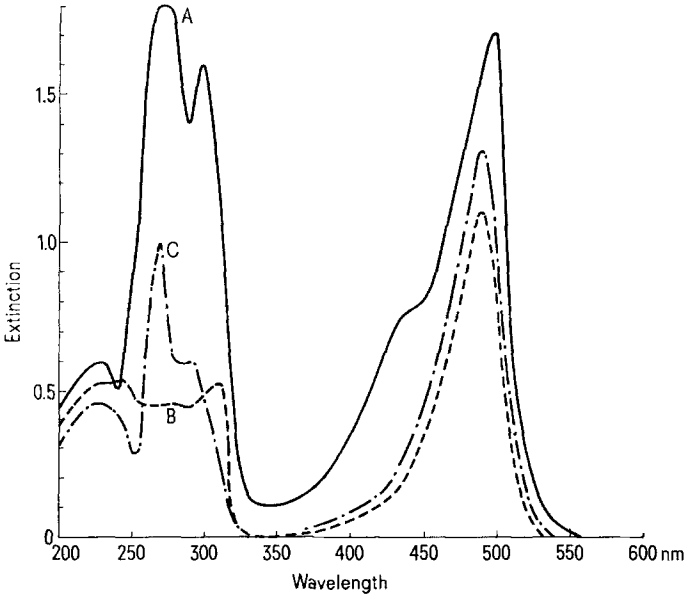

Fig. 2. Absorption characteristics of the fraction of acridine orange in solution in different purification agents. A) Chloroform soluble fraction; B) amyl alcohol soluble fraction; C) butanol soluble fraction.

Acridine orange as a pseudo-Schiff reagent[1] was first tried by KASTEN[2] and he obtained negative results. He therefore concluded that dyes without any primary amino group cannot be suitable for Feulgen type of staining. A more recent study with AO by KASTEN[3] indicated that different batches of this dye, manufactured by different firms, contain contaminants with primary amino group as judged by thin layer chromatography. Following this technique, the present author noted 4 distinct fluorescent zones in the chromatograms of the American product for which the solvent used consisted of *n*-butyl alcohol, ammonium hydroxide, ethyl alcohol and water in the proportion of 16:0.15:5:5. To obtain a dye that would show a single spot in the chromatogram, the dye solution was thoroughly washed with chloroform or amyl alcohol or *n*-butyl alcohol. The spectrophotometric analyses of the dye thus purified, as well as the unpurified dye solution (Figure 1), revealed 2 peaks in the UV-regions, one at 230 nm and the other at 270 nm and a 3rd peak in the visible region, viz. at 490 nm. It is important to mention here that in the visible region the curves for the unpurified dye and the butanol extracted dye differ from those of chloroform and amyl alcohol extracted dyes in not showing a secondary shoulder. Similar analyses of the fraction of the dye solution that went into solution in chloroform or amyl alcohol or *n*-butyl alcohol also revealed some difference (Figure 2). Results of staining with the unpurified dye, as well as the purified dye, indicated that perfect staining of DNA is possible with both. These results, therefore, do not corroborate KASTEN's[4] contention that dyes without any primary NH_2 group are not suitable for staining DNA aldehyde. Carbylamine reaction, to test the presence of primary NH_2 group as impurities in AO, also indicated that the samples of AO used in this investigation did not contain any contaminant with primary amino group, as judged by the absence of an offensive odour of carbylamine. It is pertinent to mention here that the present author has also obtained successful staining of acid hydrolyzed DNA with basic dyes, thionine blue and methylene blue[5] and rhodamine B[6], all of which are without any primary NH_2 group in their molecules.

Résumé. Après hydrolyse par l'acide hydrochlorique et teinture subséquente par une solution aqueuse d'acridine orange suivie de déshydratation régulière par l'alcool éthylique, des coupes de tissu, examinées au microscope fluorescent montrent des noyaux brillants fluorescents rouge-orange. On considère que cette teinte produite par la solution aqueuse d'un colorant qui ne contient aucun groupe aminé primaire dans sa molécule, est due à la réaction Feulgen modifiée.

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Standardized Method for the Production of Experimental Atrial Fibrillation

Several methods have been proposed for the experimental study of auricular fibrillation. Most of them are based on the original observations of BURN et al.[1]. These authors demonstrated in the dog's heart-lung preparation that during the infusion of acetylcholine, electrical stimulation of the right auricle caused the onset of atrial fibrillation, which continued as long as acetylcholine was infused. This method was applied with some modification by others. SCHERF and CHICK[2], NADEAU et al.[3], and NAHUM and HOFF[4] placed acetylcholine or some other cholinergic drug directly on the anterior surface of the right atria. HASHIMOTO et al.[5] and NAKAYAMA et al.[6] induced fibrillation by means of the administration of acetylcholine into the sinus node artery under constant pressure perfusion. Other cholinergic drugs have been used by CHIBA and HASHIMOTO[7] for the same purpose. Production of an hipokalemic state has been utilized by LEVEQUE[8] for the maintainance of the acetylcholine-induced fibrillation.

When the effects of antiarrhythmic drugs on auricular fibrillation are considered, the duration of the experimental arrhythmia is a critical parameter to be measured. In this communication, we attempt to present an experimental model based on the facts summarized above, in which the time of persistance of the arrhythmia is a highly predictable value and an appropriate criteria for the assessment of the antiarrhythmic activity of a drug.

Method. Experiments were carried out on 30 dogs of either sex, weighing 6 to 15 kg, anesthetized with morphine-chloralose-urethane (morphine 1 mg/kg s.c. followed 30 min later by chloralose 3%-urethane 30%, 2 ml/kg i.v.). The trachea was cannulated and artificial ventilation was initiated with a positive pressure pump, in order to allow a chest opening through the 4th right intercostal space. The pericardium was incised parallel and just anterior to the right phrenic nerve, exposing the right atria, and the heart was suspended loosely in a pericardial cradle. Two electrodes were implanted in the anterior surface of the right atria, near to the base of the appendage and about 5 mm apart. They were connected to a Grass S4 stimulator through nickel-steel alloy electrodes. A 3rd similar electrode was inserted 5 mm apart and connected to an ECG preamplifier related to a Sanborn two-channel recorder. Electrodes consisted of coil-springs conductors covered with silicone rubber. 2 mm of the tip were denudated from the cover and the coil-spring was stretched in order to form a pointed terminal. After insertion, a gently clockwise rotation allowed penetration of the tip into the atrial wall with a minimal trauma.

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