Differential Collagen Stain by an Acid Fuchsin, Iron, Flavianic Acid Mixture. A Note on Ferrous Sulfate Hematoxylin [1]

R. D. LILLIE, A. GUTIERREZ AND R. W. PALMER
Department of Pathology, Louisiana State University School of Medicine, 1542 Tulane Avenue, New Orleans, Louisiana

ABSTRACT Substitution of flavianic acid (50 *mM*) or of a stoichiometric mixture of naphthol yellow S and hydrochloric acid, in place of picric acid in Van Gieson type mixtures gives deeper yellow colors to cytoplasm, muscle and erythrocytes. Higher concentrations of acid fuchsin can be used with consequent greater density of collagen fiber staining and improved contrast.

The familiar weakening of hematoxylin nuclear stains by exposure to Van Gieson mixtures can be largely avoided by inclusion of 0.1 *M* ferric chloride in the Van Gieson mixture. Alum hematoxylin can then be used in place of the unstable iron hematoxylin solutions, and the iron hematoxylin effect is attained by the iron postmordanting in the Van Gieson bath. A ten minute prestain in an alum hematoxylin containing 0.5% hematoxylin is adequate but density can be enhanced by longer staining or by staining at higher temperature; 5–10 min at 60°C is suggested. The iron containing flavianic or picric acid Van Gieson staining baths should be restricted to three minutes; longer exposures gradually weaken nuclear staining.

Substitution of 0.1 *M* copper sulfate for the iron in the Van Gieson bath also yields dark gray to black nuclei. Aluminum chloride (0.1 *M*) has an effect similar to the control hydrochloric acid, while the chromium ion seems quite inferior, even to the control HCl mixture.

A ferrous sulfate hematoxylin ripened overnight with a small amount of ferric chloride gives excellent progressive nuclear staining, adequate in 2–5 minutes, and not excessive in 30 minutes. The solution gradually deteriorates in 6–8 weeks.

The problem of satisfactory nuclear staining in combination with connective tissue stains of the Van Gieson and Mallory types has long excited the interest of morphologists. Alum hematoxylin prestains are regularly converted to reddish brown which contrast poorly. Iron hematoxylin prestains with such procedures as Regaud's, Weigert's or Lillie and Earle's ('39) redox buffered iron hematoxylin yield brown to gray tones, seldom black. Kattine's ('62) posttreatment of alum hematoxylin stains with a mixture of phosphotungstic and phosphomolybdic acids before Van Gieson staining did yield a dark gray to black nuclear stain which was more resistant to the Lillie-Van Gieson variant applied. But several extra steps were introduced into the staining procedure to achieve these results.

Lillie ('54, p. 81) had noted the conversion of blue alum hematoxylin stains of nuclei to black by postmordanting in a 4% dilution of the official ferric chloride solution. In his 1945 report he had used iron chloride + naphthol green B (C.I. no. 10020) in mixed solution as a connective tissue stain as well as substituting an iron chloride mordant bath for phosphomolybdic or picric acid in sequence procedures of the Masson trichrome type. These experiments suggested that an iron salt might be incorporated into the Van Gieson mixture and thus blacken alum hematoxylin prestained nuclei during the collagen fiber staining step of a simple Van Gieson type procedure.

It was further believed from Deitch's ('55) favorable results with its disodium salt naphthol yellow S, that flavianic acid might be substituted for picric acid with deeper yellow coloration of muscle and cytoplasms.

MATERIAL AND GENERAL TECHNIC

Dog stomach was selected as the histologic test material. The mucosa contains an abundance of inter and periglan-

[1] Supported by National Cancer Institute grant C-4816, National Institutes of Health.

dular, finely fibrillar stroma. The submucosa is thick and is composed of more coarsely fibrous collagen. There is very fine stroma between muscle fibers in the tunica muscularis and muscularis mucosae.

Dyes with lot numbers employed are as follows: Acid fuchsin LR-7, Hartman-Leddon Co., Philadelphia; Hematoxylin EH-4, Fisher Scientific co., Fair Lawn, N.J.; picric acid (2,4,6-trinitro-1-phenol) and flavianic acid (2,4-dinitro-1-naphthol-7-sulfonic acid) from Eastman (Distillation Products Industries, Rochester, N. Y.) and Naphthol yellow S, lot NX185 from Matheson, Coleman and Bell, Norwood, Ohio. Acids and ferric chloride were of reagent grade.

The general technic followed the usual pattern: deparaffinization and hydration through xylene and alcohols, prestaining with a hematoxylin solution, washing, staining in one of the Van Gieson mixtures, direct dehydration and differentation in 95% alcohol and final dehydration, clearing and mounting in a synthetic resin as usual.

EXPERIMENTAL STAINS

Picric acid mixtures. Lillie's ('65) table on page 529 suggests that increases in acid fuchsin content and in acidity intensify the acid fuchsin staining of collagen and sharpen the differential staining effect between collagen and other oxyphil tissue elements. Mixtures I and II in table 1 were compared previously (Lillie, '54; Kattine, '62; Lillie, '65) and it was found that sharper differentiation of red and yellow stained elements was attained with the more acid mixture. First in the present series mixtures III and V were compared as five minute counterstains after graded iron hematoxylin staining and with uncounter stained preparations.

The iron hematoxylin used was a new variant of the Lillie Earle ('39) and Weigert Lillie (Lillie, '65 p. 169) formulae, devised in the hope of attaining greater stability. The two solutions are 100 ml fresh 1% alcoholic hematoxylin and 100 ml of an iron solution containing 6.5 gm ferrous sulfate ($FeSO_4 \cdot 7\ H_2O$), 0.5 gm ferric chloride ($FeCl_3 \cdot 6\ H_2O$), 2 ml concentrated hydrochloric acid (12 *N*) and 98 ml distilled water. While the mixture of these two solutions may be used after an hour or two, superior staining is attained after 24 hours. The mixture deteriorates in a few weeks, but gives highly selective staining when fresh.

Without Van Gieson staining nuclei presented excellent blue black coloration at all staining intervals: 2, 5, 12 and 30 minutes, somewhat denser at five than two minutes on Carnoy fixed material, and somewhat denser on formalin fixed tissue than on Carnoy. Cytoplasms and erythrocytes remained only faintly gray up to 30 minute staining.

After five minute Van Gieson staining with either mixture (III or V) nuclear coloration was distinctly paler with shorter hematoxylin staining intervals, reaching deep gray at 12–30 minute times.

Submucosal collagen, mucosal reticular fibrils and basement membranes colored deep red with mixture III, deep purple red with mixture V. On formalin fixed material smooth muscle was yellow with III, slightly pink with V. Erythrocytes and epithelial cytoplasm were yellow with both mixtures.

In view of the weakened nuclear staining the HCl content of the iron solution was reduced to 1 ml. This second iron hematoxylin mixture still gave excellent nuclear detail with staining intervals of 2, 5, 12, or 30 minutes, and very little cytoplasmic or erythrocyte staining in uncounterstained preparations. For the Van Gieson stains mixture IV, with 125 mg acid fuchsin per 100 ml was used. Vivid red yellow contrasts were attained, with no pink coloration in smooth muscle.

Later trials with this same combination, using the second iron hematoxylin solution at 24 hours ripening interval and staining 30 minutes, followed by Van Gieson formula IV for five minutes gave even better black nuclear staining and the same good red yellow contrasts.

Addition of ferric chloride to the Van Gieson stain mixtures. In these procedures a level of 0.1 *M* ferric chloride was arbitrarily selected, both for picric and for flavianic acid and acid naphthol yellow S mixtures. A ten minute stain in the Lillie Mayer (Lillie, '65) or the Palmer Boehmer (Palmer and Lillie, '65) alum

TABLE 1

Composition of Van Gieson type acid fuchsin; picric and flavianic acid mixtures, with and without $FeCl_3$

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
Acid fuchsin	100 mg	100 mg	100 mg	125 mg	150 mg	200 mg	250 mg	150 mg	200 mg	200 mg	200 mg	200 mg	250 mg	200 mg
Picric acid	1.3 gm	1.25 gm	1.3 gm	1.25 gm	1.3 gm	1.25 gm	1.25 gm							
Naphthol yellow S								1.5 gm	1.5 gm	1.5 gm				
Flavianic acid											1.75 gm	1.75 gm	1.75 gm	1.75 gm
$FeCl_3 \cdot 6H_2O$						2.7 gm	2.7 gm	2.7 gm	2.7 gm	2.7 gm		2.7 gm	2.7 gm	550 mg
12 *N* HCl		0.25 ml	0.3 ml	0.3 ml	0.3 ml			0.6 ml	0.6 ml	1 ml				
Distilled water ad	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml
pH		1.59				1.70	1.70				1.61	1.22	1.14	1.31

Picric acid was saturated in all mixtures and may be reckoned at 47–50 *mM*. Flavianic acid (MW 350.25) concentration is also 50 *mM*. Naphthol yellow S (MW 412.25) at 1.5% is 36 *mM*. Ferric chloride is 100 *mM*, except in No. XIV, where 20 *mM* is used. The pH of saturated aqueous picric acid is 1.85, that of 0.1 M $FeCl_3$ is 1.24 (Lillie, '65). The pH readings in the tables were recorded electrometrically on a Model G Beckman pH meter at about 25°C.

TABLE 2

Composition of flavianic and picric acid, acid fuchsin mixtures with other metals

	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	XXIII	XXIV	Conc.
											mM
Acid fuchsin	200 mg	200 mg	200 mg	200 mg	200 mg	125 mg	125 mg	125 mg	125 mg	125 mg	
Flavianic acid	1.75 gm	1.75 gm	1.75 gm	1.75 gm	1.75 gm						50
Picric acid						1.25 gm	1.25 gm	1.25 gm	1.25 gm	1.25 gm	47.4
$FeCl_3 \cdot 6H_2O$	2.7 gm					2.7 gm					100
$CuSO_4 \cdot 5H_2O$		2.5 gm					2.5 gm				100
$CrF_3 \cdot 9H_2O$			2.7 gm					2.7 gm			100
$AlCl_3 \cdot 6H_2O$				2.4 gm					2.4 mg		100
12 *N* HCl					0.3 ml					0.3 ml	36
Distilled water ad	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	100 ml	
pH readings	1.20 1.30	1.25 1.30	1.50 1.54	1.00 1.05	1.58 1.60	1.00 1.02	1.35 1.40	1.58 1.60	1.15 1.20	1.28 1.30	

hematoxylin replaced the iron hematoxylin staining of the previous experiments, because of the greater convenience of stock alum hematoxylin solutions.

The first trials of the iron containing Van Gieson type mixtures were made with 1.5% naphthol yellow S solutions VIII, IX and X (table 1) using a five minute staining time. Gray to black nuclear staining was seen in all three tests, better with the less acid solutions VIII and IX. Mixtures IX and X with 200 mg acid fuchsin per 100 ml gave better red yellow contrast than mixture VIII with 150 mg acid fuchsin. A second series of stains with mixture IX were made, with Van Gieson staining intervals of one, two, three and five minutes. Nuclear staining was best at one minute, but still quite adequate at three minutes. The red yellow contrasts of collagen and muscle improved as the staining interval was increased. But there was good contrast at two minutes, and little difference was seen between three and five minutes staining intervals. Hence a three minute staining interval for the Van Gieson stain was adopted for further tests. Prolongation of the alum hematoxylin stain to 60 minutes at 25° and at 60°C increased the density of nuclear staining, but also produced brown tones in erythrocytes, muscle and cytoplasms at 60°. Limitation of alum hematoxylin staining to 5–10 minutes at 60° gives black nuclei and leaves muscle, cytoplasm and erythrocytes fairly clear deep yellow.

It was thought that it would be more convenient to use flavianic acid directly as such, rather than to make an acid solution of naphthol yellow S. Hence a supply of this reagent, 2,4-dinitro-1-naphthol-7-sulfonic acid, was obtained and tested at about 50 *mM* concentration in mixtures XI, XII, XIII, and XVI in comparison with picric acid mixtures II, VI and VII.

The four mixtures containing 0.1 *M* $FeCl_3$ (VI, VII, XII, XIII) yielded dark gray to black nuclei. With mixture XIV, iron content 0.02 *M*, only lymphocyte nuclei were described as black, others as moderate to dark gray. The iron free mixture XI left nuclei reddish brown, as in the usual alum hematoxylin Van Gieson stain.

All tests gave red to deep purplish red collagen, reticulum, mucosal basement membranes and even partial demonstration of smooth muscle stroma (fig. 3). All of the flavianic acid mixtures yielded deep yellow muscle. With picric acid the 200 mg acid fuchsin level (VI) produced pink to gray pink cytoplasm and erythrocytes, with 250 mg acid fuchsin (VII) pink tinging was more pronounced. Also with flavianic acid the 250 mg acid fuchsin level (XIII) produced orange yellow cytopalsm and erythrocytes (figs. 1, 2).

Since dilute hematoxylin staining yields blue green, dark blue and blue lakes respectively on tissues premordanted with Cu^{++}, Cr^{+++} and Al^{+++} (Lillie, '65; Pizzolato and Lillie, '67), it was thought that inclusion of these metals in the Van Gieson mixtures might also prove valuable. Accordingly, a series of ten mixtures was prepared (table 2, XV-XXIV). The usual technic was employed, except that a ten second rinse in fresh distilled water was interpolated after the Van Gieson bath, and fresh 95% alcohol was used for differentiation after each of the ten stains, to avoid any metal carryover. The hematoxylin staining was for ten minutes with the Lillie Mayer formula, one series at 25°C and one at 60°C.

In general the five picric acid mixtures XX to XXIV gave somewhat denser nuclear staining than the flavianic acid mixtures XV to XIX. The iron mixtures XV and XX and the copper mixtures XVI and XXI yielded good deep gray nuclear stains with the 25°C hematoxylin staining, deepening to almost black with the 60°C stain. The chromium mixtures XVII and XXII yielded gray to light gray rather poorly stained nuclei, both after 25°C and 60°C hematoxylin staining. Results with aluminum postmordanting (XVIII and XXIII) were quite closely similar to those with the hydrochloric acid controls XIX and XXIV; poor nuclear staining after the 25°C, quite good after the 60°C staining.

DISCUSSION

From the experiments reported herewith it is shown that by adding 0.1 *M* ferric chloride to a Van Gieson type mixture nuclei may be shown in black or dark gray after an ordinary alum hematoxylin

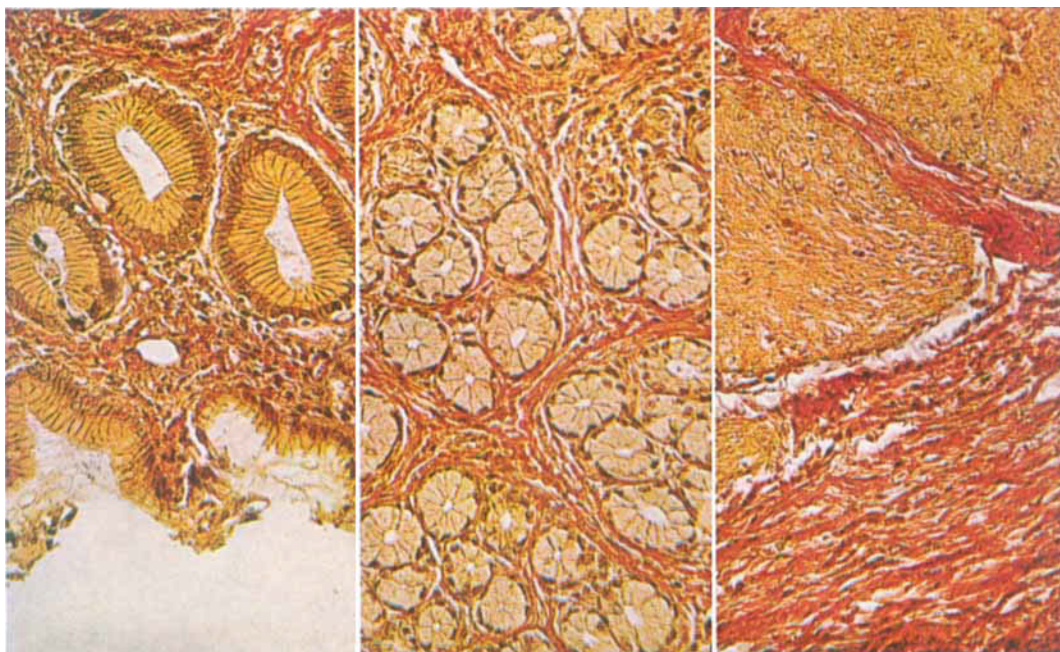

Figure 1 Figure 2 Figure 3

All figures are from a dog's stomach, buffered formalin fixation, paraffin sections, flavianic acid iron acid fuchsin stain × 210.

Fig. 1 Pyloric mucosa at surface with foveolae and supporting stroma.
Fig. 2 Pyloric glands with interglandular stroma.
Fig. 3 Submucosa and adjacent muscularis.

prestain. Substitution of flavianic acid or its disodium salt naphthol yellow S for picric acid in such mixtures materially deepens the yellow staining of muscle, cytoplasm and erythrocytes. Addition of 0.1 *M* ferric chloride to the mixtures brings their pH to about 1.2 and permits the use of 200 mg acid fuchsin per 100 ml solution, with enhanced brilliancy of collagen staining and increased contrast.

An iron hematoxylin containing chiefly ferrous iron gives excellent nuclear staining, but only moderate stability. Alum hematoxylin staining for longer periods or at higher temperatures gives improved nuclear staining in Van Gieson technics when iron containing Van Gieson mixtures are employed.

Recommended technics are as follows.

Flavianic acid, acid fuchsin, iron chloride method.

1. Deparaffinize and hydrate sections as usual.

2. Stain ten minutes at 25°C in an alum hematoxylin of 0.5% hematoxylin content; for increased density of nuclear staining, stain 5–10 minutes at 60°C.

3. Wash briefly in water.

4. Stain three minutes in flavianic acid 1.75 gm, acid fuchsin 200 mg, ferric chloride ($FeCl_3 \cdot 6\ H_2O$) 2.7 gm and distilled water to make 100 cm^3. The solution keeps.

5. Dehydrate and differentiate in two changes of 95% alcohol.

6. Complete dehydration with absolute alcohol, clear with xylene and mount in synthetic resin (Permount, Harleco, cellulose caprate, etc.) Naphthol yellow S 2 gm may be substituted for the flavianic acid, but a stoichiometric equivalent ($Na_2C_{10}H_4O_8\ N_2S$, M. W. 412.25) of HCl (0.8 cm^3 conc) should be added to bring the pH to about 1.2.

It is a familiar fact that alum hematoxylin is a progressive stain, becoming denser with longer exposures. That it should also

become denser on staining at elevated temperatures is also to be expected. The extraction in Van Gieson solutions ranging in acidity from pH 1.0 to 1.6 is in essential agreement with the well known acid alcohol differentiation effect and with the complete decolorization observed in an hour's exposure to a pH 4 McIlvaine buffer (Lillie, '54). It is also familiar that iron hematoxylin stains are relatively more resistant to the acid differentiation occurring in iron alum solutions.

It appears that increased resistance to decolorization as well as deeper color is conferred on the hematoxylin by partial or complete substitution of ferric or cupric for aluminum ions in the tissue dye lake complex. With aluminum ions in the Van Gieson solution no ion substitution occurs and decolorization proceeds just as in the similarly acid metal free solutions.

In regard to the chromium, it would appear that the pH 1.0–1.6 range is too low for Cr^{+++} tissue binding and that consequently the chromic ion acts as an extracting agent for the hematoxylin, taking it out of the previous Al tissue binding more rapidly than metal free or aluminum chloride solutions of about the same pH. It is noted in the Colour Index that chromium acetate, which gives relatively neutral solutions, is the usual textile chromium mordant.

LITERATURE CITED

Deitch, Arline D. 1955 Microspectrophotometric study of the binding of the anionic dye, naphthol yellow S, by tissue sections and by purified proteins. Lab. Investig., *4:* 324–351.

Kattine, Vicki C. 1962 Retention of nuclear staining by phosphomolybdic-phosphotungstic mordanting. Stain Techn., *37:* 193–195.

Lillie, R. D., and W. R. Earle 1939 Iron hematoxylins containing ferric and ferrous iron. Amer. J. Path., *15:* 765–770.

Lillie, R. D. 1945 Studies on selective staining of collagen with acid anilin dyes. J. Tech. Meth. Bull. Int. Assoc. Med Mus., *25:* 1–47.

——— 1954 Histopathologic Technic and Practical Histochemistry. 2nd ed. The Blakiston Co., New York.

——— 1965 Histopathologic Technic and Practical Histochemistry. 3rd ed. McGraw-Hill Book Co., New York.

Palmer, R. W., and R. D. Lillie 1965 Spectroscopic and staining studies of the ripening and overripening of aluminum hematoxylin. Histochemie, *5:* 44–54.

Pizzolato, P., and R. D. Lillie 1967 Metal salts-hematoxylin staining of skin keratohyalin granules. J. Histochem. Cytochem., *15:* 104–110.

Soc. Dyers and Colourists and Amer. Assoc. Textile Chemists and Colourists 1956 Colour Index. 2nd edition. Bradford, Yorksh. and Lowell, Mass.