Fluorescence microscopic demonstration of rat mast cells stained with acid dyes

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The amphoteric acid dyes acid fuchsin, aniline blue WS, azocarmine G, fast green FCF, light green SF and the wholly acid dye thiazine red R were used for fluorochromy of mast cells in sections of formalin or alcohol-formalin fixed rat tongues. By ordinary light the mast cell granules were barely visible. By UV or blue light exitation, however, a selective, secondary fluorescence of varying intensity and contrast against the tissue background manifested itself. By thin layer chromatography each dye revealed 3–7 components. Except for aniline blue WS, the main component(s) of each dye conferred to the granules the same fluorescence as the corresponding whole dye. These results of fluorochromy, together with the findings of methylation and deamination experiments, allowed the assumption that the anionic groups of the dyes were bound to unoccupied basic groups of the mast cell granules.

Key-words: Histochemistry; chromatography

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INTRODUCTION

Basic or neutral dyes have been mandatory for selective staining of mast cells. Metachromatic basis dyes have played a dominant role by examination of these cells with ordinary light (22). The literature concerning the secondary fluorescence of mast cells was reviewed by Hals (10) who also described some new methods for fluorochromy of rat mast cells with basic dyes. However, as indicated previously (9) some wholly acid or amphoteric acid dyes, when used as fluorochromes, were well suited for selective demonstration of mast cells in the rat. Further studies on this subject will be reported here.

MATERIAL AND METHODS

Preparation of tissues

The 100–250 g Sprague-Dawley rats stemmed from two experimental series. In one of these (12) dental changes following ligation of the bile duct, in the other (13) dental changes following intraperiotoneal injection of carbon tetrachloride were produced. Several control animals from both series were also used. No difference was observed between these categories with regards to fluorochromatic features of mast cells.

The material consisted mainly of tongues, which in the rat is known to be rich in mast cells, salivary and lymph glands, and jaws. Immediately following sacrifice of the ani-

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mals, small pieces of tissue were fixed for 24 hours: 1) either in 10 % aqueous neutral (pH 6.9) solution of formaldehyde (1 part of a 35 % w/v reagent grade formaldehyde solution with three parts of water saturated with calcium carbonate and stored in brown bottles over a layer of solid calcium carbonate), or 2) in absolute alcohol- 10 % formaldehyde 4 : 1 (22).

Five μm thick paraffin sections were used. The jaws were demineralized in 5 % nitric acid, embedded in paraffin, and sectioned. Most of the sections were stained in the period 1968–1970. Some years later it was found necessary, for supplemental and control purposes, to stain further sections from the same material. The staining technique was the same, and these sections showed identical fluorochromatic features.

Table 1. *Data on dyes used*

Dye	Type of dye: D, diachrome F, fluorochrome ( ) rarely used
Amphoteric acid:	
Acid fuchsin (M)	D (F)
Aniline blue WS (G)	D (F)
Azocarmine G (M)	D (F)
Fast green FCF (G)	D (F)
Light green SF (M)	D
Wholly acid:	
Thiazine red R (G)	F D
Basic (control):	
Acridine orange (G)	F (D)
Toluidine blue (M)	D

G = G.T. Gurr
M = Merck

Dyes used

The dyes used in the present investigation are listed in Table 1. The sections were examined both by ordinary and UV/blue light. The concentration of the dye solutions ranged from 0.01 to 0.5 %. For simplicity the terms "dye" and "staining" are used below also to indicate "fluorochrome" and "fluorochromy". This is justifiable because the "Results" are subdivided into observations by UV/blue light and by ordinary light. In most current hand books aniline blue WS, light green SF yellowish, fast green FCF, acid fuchsin and azocarmine G are listed as acid dyes. In the classification of Gurr (7) they are defined amphoteric anionic dyes and should be distinguished from the wholly acid anionic dyes, like thiazine red R. Puchtler & Isler (20) claimed that the former dyes, having strong basic groups, are amphoteric in nature against collagen, reticulin fibers and basal membranes which have been pretreated with phosphomolybdic acid. It will be shown that to the untreated mast cells in the present study they acted as acid (anionic) dyes. Below they are reffered to as amphoteric acid dyes.

The dyes were submitted to purity determinations with thin layer chromatography using silica plates (MN Polygram Sil N–HR). The solvent system was n-butanol, acetic acid and water in proportions 40 : 10 : 50, the upper phase of this solution being used.

Staining procedures

When solutions of the dyes in distilled water, usually 0.1 %, were used, the staining procedure, 1–2 minutes, was followed by differentiation in acid alcohol for 1/2–1 minute, whereafter the sections were embedded in Eukit® or Fluormount®. In a few cases the FALG method (double staining with acid fuchsin and light green SF) (7) was used.

When buffered solutions of dyes (usually 0.01 % at pH range 0.5–6.0) were used, the sections were kept in the pertinent buffer solution (Theorell Stenhagen buffer solutions, Sweden), or solutions made from Coleman buffer tablets (*citricacid-sodium* diphosphate, Citricacid-sodium citrate) (Coleman Instruments Corp., Maywood, Ill., USA), for 1–2 minutes, then in the buffered dye solution for 1–2 minutes. After rinse and mounting in buffer solution, the section was examined. With respect to the staining results reported below, no difference between the buffers used was observed.

The dyes were also used as a 0.03 % solution in 5 % aluminium sulphate following the method of Heath (15). In these cases the sections were stained for 5–10 minutes, rinsed in water, differentiated for 20–30 seconds in 70 % alcohol, and finally mounted. The identification of mast cells was secured by the use, in control sections, of toluidine blue and acridine orange, the latter in ultraviolet light. These dyes, at low pH, are known to stain selectively the mast cells in the rat (10, 22, 24).

Histochemical procedures

Methylation was done with methanol containing 0.1 N HC1 (18). The sections were immersed for increasing intervals, starting with 15 minutes at 60 °C and 90 minutes at 23 °C.

Deamination was done according to Lillie (18) by immersing in van Slyke's nitrous acid reagent [10 cm^3 concentrated (6 g in 10 cm^3) sodium nitrite, 5 cm^3 glacial acetic acid and 25 cm^3 distilled water] 1–12 hours at 20–25 °C.

Fluorescence microscopy took place in a Leitz Ortholux microscope equipped with an Osram HBO 200 W lamp, a Ploem-type vertical illuminator and Leitz immersion objectives. UV light was obtained with the exiter filter UG1, and blue light with the filter Bg 12. As barrier filters were used K460 and K490, respectively. Photographs were taken with Kodak Ektachrome High speed color reversal film for daylight.

RESULTS

Amphoteric acid dyes

The results were basically the same with all the amphoteric acid dyes used in this study.

Ordinary light

The mast cell cytoplasm was only faintly stained, in some instances barely visible (Fig. 1, bottom, 4). Differentiation between granules and intergranular substance could hardly be made. One or two round bodies, distinctly stained like nuclei, were often seen in the cell bodies. The tissue background was heavily stained in the pH range 2–4, at pH 5 markedly reduced, and at pH 6 barely visible, except with fast green FCF, which caused a slight staining.

UV/blue light

At pH 2–3, the mast cell cytoplasm revealed a selective, secondary fluorescence which contrasted more or less distinctly with that of the tissue background (Table 2, Fig. 1). In some instances the cytoplasm revealed a mottled appearance, apparently caused by the presence of fluorescent granules, separated by non-fluorescent areas (Fig. 2). In other instances such granules were observed outside the mast cell (Fig. 1, upper left and right). The nucleus and the mentioned round bodies showed the same fluorescence color as the tissue background.

With acid fuchsin, the selective fluorescence of the cytoplasm could be demonstrated even at pH 0.5. The other dyes were not used at this low pH.

Common to the secondary fluorescence caused by these dyes was the rapid fading on UV/blue light radiation. This was especially the case with the yellow colors caused by aniline blue WS and acid fuchsin, which changed respectively to a greenish blue and a greyish blue (UV light). However, even the

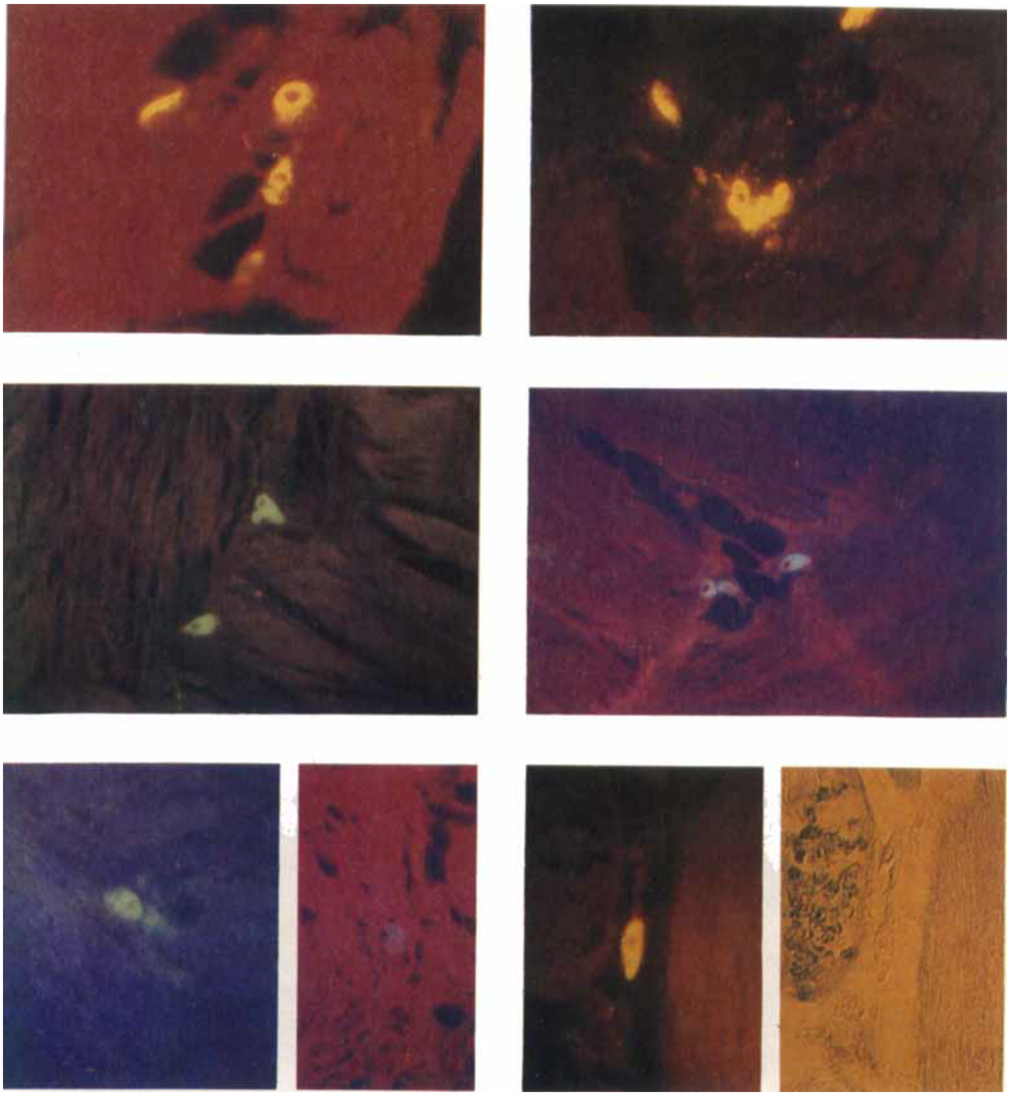

Fig. 1. Color photomicrographs of mast cells from rat tongue. Paraffin sections. Original magnifications stated.
Upper left: Acid fuchsin, pH 2, blue light (B). X 400
Upper right: Fast green FCF, pH 2 (B). X 400
Center left: Light green SF, pH 2 (UV). X 400
Center right: Azocarmine G, pH 3 (UV). X 250
Bottom from left to right:
1: Aniline blue WS, pH 2 (UV). X 400
2. *Thiazine red R, pH* 3 (UV). X 250
3. Fast green FCF, pH 2 (B). X 400
4. Same area as in (3) by ordinary light. Mast cell cytoplasm practically unstained. X 400.

Fig. 2. Mast cells from rat tongue. Mottled appearance of cytoplasm, probably due to fluorescent granules and nonfluorescent intergranular substance. Paraffin section. Light green SF. Blue light. X 500.

latter color contrasted very well with the other tissue.

The results obtained by light green SF yellowish and fast green FCF which are chemically almost identical, were very similar.

Results similar to those shown in Table 2 were obtained by staining with 0.5 % solutions of all dyes of this group (azocarmine G: 0.1 %), followed by differentiation for 1/2–1 minute in acid alcohol and mounting in Fluormount.

The results from the use of amphoteric acid dyes in 5 per cent aluminium sulphate were negative.

By staining with the FALG method (7), the mast cell cytoplasm, when irradiated with UV/blue light, proved to emit the yellow fluorescence ordinarily produced by acid fuchsin alone.

Wholly acid dye

Ordinary light

The staining with thiazine red R followed the pattern characteristic of the amphoteric acid dyes.

UV/blue light

At pH 0.5–3.0 thiazine red R conferred to the mast cell cytoplasm a blue (UV light) or green (blue light) fluorescence whereas the nuclei stood out as red dots. The mast cells formed a weak contrast only against the reddish fluorescence of the tissue background. (Fig. 1, Table 2). This contrast was further reduced at pH 5 and entirely lost at pH 6.

A selective fluorescence, corresponding to that seen at pH 3, was achieved by staining sections with a 0.1 % watery solution of thiazine red R, differentiating in acid alcohol and mounting. With thiazine red R, all amphoteric acid dyes and the FALG method, mast cells showed selective fluorescence even in demineralized material.

Thin layer chromatography

By thin layer chromatography the dyes revealed 3–7 components, the width and color intensity of which varied within the single dye (Fig. 3). With the exception of thiazine red R all dyes contained at least one component with a shade differing both by ordinary and blue light examination from

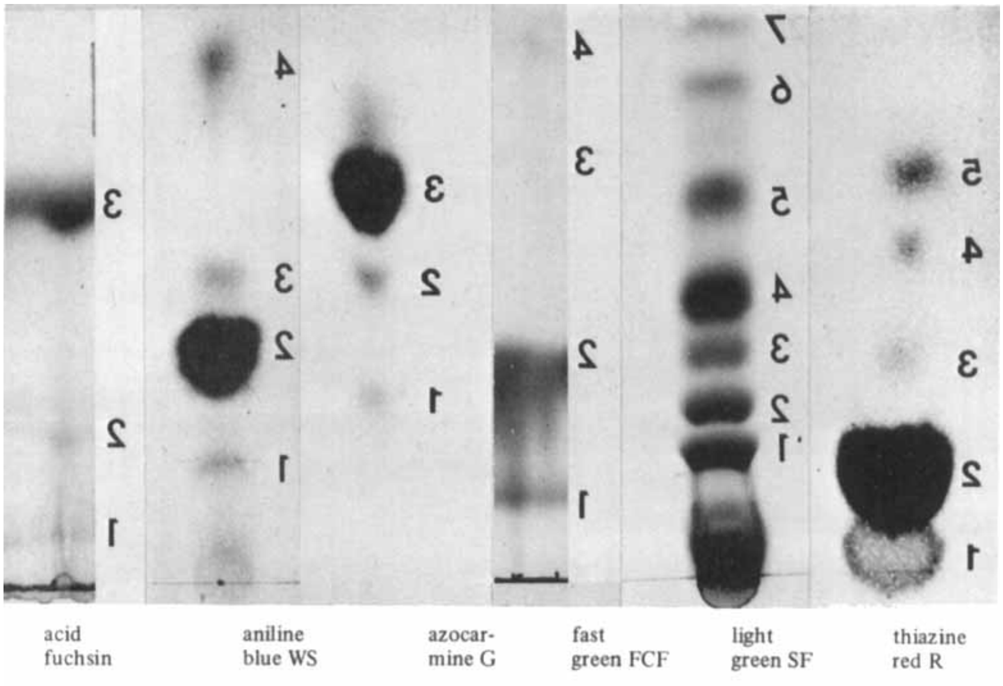

Fig. 3. Thin layer chromatographs of the dyes used. For description of the colors of the various dye components as revealed by ordinary, UV or blue light, see Table 3.

Table 2. *Secondary fluorescence of rat mast cells stained with amphoteric acid or wholly acid dyes at pH 2–3*

Dyes 1. *Amphoteric acid*	Preferred radiation type	Cytoplasm-fluorescence color and intensity	Nucleus	Tissue background	Contrast mast cells/ background
Acid fuchsin	blue light	yellow +++*)	red	dark red	+++*)
Fast green FCF	»	yellow +++	dark reddish	brownish red	+++
Light green SF	UV	light greenish +++	dark reddish	dark reddish	+++
Azocarmine G	»	grey blue ++	bluish red	bluish red	++
Aniline blue WS	»	dull yellow +	blue	blue	+
2. *Wholly acid* Thiazine red R	»	blue +	red	reddish	+

*) + = weak intensity/contrast
++ = moderate intensity/contrast
+++ = high intensity/contrast

Table 3. *Components of the dyes used in this study as occurring by thin layer chromatography. Fluorescence induced in mast cell cytoplasm by these components*

	Ordinary light	UV (Blue light)	Fluorescence induced in mast cell cytoplasm
Acid fuchsin (Merck)	1, 2, 3 and 5 red 4 light yellow red	1, 2, 3 and 5 red 4 reddish yellow (B)	1, 2, 3 and 5 yellow 4 greenish yellow (B)
Fast green FCF (G.T. Gurr)	1 green 2 green 3 bluish green	1 dull greenish 2 brick red (B) 3 dull greenish	1 yellow 2 greenish (B) 3 yellow
Light green FCF (Merck)	1 weak green 2 heavy green 3 barely visible green 4 bluish green	1 reddish 2 reddish 3 dull reddish (UV) 4 barely visible	1 not isolated 2 greenish 3 not isolated (UV) 4 not isolated
Azocarmine G (Merck)	1 pink 2 pink 3 yellow reddish 4 yellow reddish	1 yellow red 2 yellow red 3 yellow (UV) 4 yellow	1 grey blue 2 grey blue 3 not isolated (UV) 4 no effect
Aniline blue W.S. (G.T. Gurr)	7 blue components, number 3 of which with a reddish shade	1–7 blue (UV)	2, 4 and 5 greenish 1, 3, 6 and 7 no effect (UV)
Thiazine red R	1 weak red 2 weak red 3 heavy red	1 red 2 red (UV) 3 red	1 no effect 2 no effect (UV) 3 blue

that characteristic of the main components (Table 3).

Secondary fluorescence of mast cell cytoplasm following staining with isolated components of the various dyes (Fig. 3) are recorded in Table 3. In acid fuchsin, fast green FCF, azocarmine G and thiazine red R 1–4 isolated single components conferred to the mast cell cytoplasm a selective fluorescence of the same color as that caused by the respective whole dyes. The components acid fuchsin no. 4, fast green FCF no. 2, light green SF no. 2 and aniline blue WS nos. 2, 4 and 5 caused fluorescence colors slightly differing from those caused by the whole dyes. A few components caused no effect and still a few others were found too weak for isolation.

Histochemical reactions

In methylated sections, stained at pH 2–3 with acid fuchsin, the mast cell cytoplasm displayed a red fluorescence not discernible from that of the nucleus or the tissue background. Thus, methylation blocked the selective fluorescence which could be induced with this dye in non-methylated sections. Also in the control sections stained with the basic acridine orange, methylation blocked the selective fluorescence which could be induced in non-methylated sections (Table 4). Deamination by exposure for about 4 hours to van Slyke's reagent abolished the secondary fluorescence that normally would be caused by acid fuchsin. The mast cells were not discernible.

DISCUSSION

The fact that the amphoteric acid dyes at low pH caused heavy staining of the acidophil connective tissue, while the basophil mast cell cytoplasm was practically unstained, shows that they acted as acid dyes. The initial report of mast cell fluorescence in *unstained* sections (4) clearly linked this

Table 4. *Effect of methylation on stainability and secondary fluorescence of the cytoplasm of rat mast cells*

	Acid dye (acid fuchsin)		Basic dye (acridine orange)	
	Color by ordinary light	Fluorescence color by blue light	Color by ordinary light	Fluorescence color by blue light
No methylation	Practically unstained	Yellow, selective	Ochre	Metachromatic, red, selective
Methylation	Red, no differentiation from tissue background	Red, no differentiation from tissue background	Practically unstained	Ortochromatic, green, no differentiation from tissue background

reaction to the use of formalin as a fixative. Benditt et al. (2) have proved low concentrations of serotonin (5–HT) in rat mast cells. Others have shown that treatment of rat mast cells with formaldehyde vapour induces a yellow fluorescence of the granules that is explained by the content of serotonin (1, 5, 19). The yellow fluorescence of mast cells which was observed in some unstained sections in the present formalin-fixed material is, therefore, best explained by the serotonin content.

The difference between this type of fluorescence and that which occurred following staining with acid dyes was marked. In the latter instances the fluorescence color varied with the dyes used, and its intensity was much higher than that caused by serotonin. It is concluded that the acid dyes caused a secondary fluorescence due to an effect on structures within the cytoplasm of rat mast cells.

The chemical composition of rat mast cell granules is very complex. The main structural elements are protein and heparin, the two components each amounting to about one third of the dry weight of the granules. The heparin-protein complex is believed to form the matrix of the histamine storing granule. The strong acidic groups of heparin may be expected to be attracted to the basic protein groups and thus be unavailable for the binding of histamine, which is thought to have an ionic binding to carboxyl groups in the granule heparin-protein complex (3). The protein is very alkaline with the isoelectric point around pH 9 (3, 17). The number of basic groups of the histamine plus serotonin exceeds the number of acid groups of the heparin (6). Staining of tissue elements with acid dyes at low pH values means a maximum of adsorption to all basic groups in an amphoteric substrate (14). Acid groups of the dyes used in this study are probably bound to occasionally occurring unoccupied amino groups in the granules. Presumably, due to the scarcity of free amino groups the concentration of dye will be so low as to make the staining barely visible.

The image seen in Fig. 1 (upper right), was interpreted as degranulation of a mast cell. However, whether the round particles represent the granules proper, or the granules with a coating of intergranular substance, could not be stated.

The selective secondary fluorescence induced in the mast cell cytoplasm seems to be conditioned by a low concentration of the dye. This is evident from the color change which took place by staining of methylated sections with acid fuchsin. By this method the acid sites of the tissue were occupied by methyl groups, which resulted in loss of tissue basophilia. Concomitantly, however, due to the increased amounts of basic groups, the acidophilia of the tissue increased. This explains why the mast cell cytoplasm by ordinary light showed a distinct red color and by blue light exitation a red fluorescence, in neither of the cases to be distinguished from the color of the tissue background.

In non-methylated sections the mast cell cytoplasm stained heavily with the basic acridine orange. Generally, small amounts of adsorbed particles of this dye to tissue elements will exhibit a green fluorescence, higher amounts, however, a metachromatic red fluorescence [concentration effect (23)]. This explains the selective red fluorescence of mast cell cytoplasm stained with acridine orange at low pH (Table 4) which has been shown by Schümmelfelder (21), Zachrisson (24) and Hals (10). The loss of tissue basophilia by methylation explains why the acridine orange, during these circumstances only caused a barely visible staining of the mast cell cytoplasm, which, therefore, by blue light exitation displayed a non-selective green fluorescence color. It is seen that with acid dyes, the selective secondary fluorescence of mast cell cytoplasm occured by low dye concentration while with basic dyes a high concentration was necessary.

The possibility that selective fluorescence could be due to dye impurities have been suggested (16). In the present study, however, a fluorescence color of mast cell cytoplasm similar to that displayed by staining with the whole dyes also occurred by staining with the isolated main components of these dyes. In all instances this fluorescence color was different from that shown by the components when still in situ at the chromatographs. Probably this may be explained by the low concentration of the components which, judging from the examination by ordinary light, had adsorbed to the cytoplasm. It is of interest that a selective fluorescence of rat elastic fibers stained with acid fuchsin or fast green FCF at neutral or higher pH values (11) also seems to have connection with low concentration of dyes in the (dense) fibers.

With the isolated main components of aniline blue WS the mast cell cytoplasm did not reveal the same fluorescence color as conferred by the whole dye. Possibly, therefore, the latter color is the result of interaction of all the components of this dye.

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