Standardization of Methyl Green for Specific Staining of Egg-shell Material in a Trematode

By J. D. SMYTH

(From the Department of Zoology, Trinity College, Dublin)

Summary

1. The specificity of a number of samples of methyl green for egg-shell material in *Fasciola hepatica* was investigated.

2. A method for distinguishing between specific and non-specific stains by paper chromatography is described.

3. Specific stains gave almost identical chromatograms with an Rf value of 0·83 for both green and violet constituents. Non-specific samples gave chromatograms with an Rf of 0·65 for the green constituent and an Rf of 0·84 for the violet constituent. The liquid phase used was a mixture of organic solvents in the following proportions: ethanol, 100; water, 80; *n*-butanol, 100; cellosolve, 100.

4. The application of this technique has led to the production of a methyl green, named Methyl Green M.2, specific for shell material in *Fasciola*.

Introduction

AQUEOUS methyl green has a marked affinity for the untanned egg-shell material in trematodes and pseudo-phyllidean cestodes, and under carefully controlled conditions acts as a specific stain for this material (Smyth, 1951*a*, 1951*b*).

Several workers have since reported (private communication) that the method has failed to work in their hands, in that nuclei and other organelles also stained green or blue-green by this technique. Since methyl green is notoriously inconsistent in its composition, it was suspected that the fault most likely lay in the sample of stain used. Samples of methyl green were obtained from a number of different manufacturers and their specificity for egg-shell material tested. The various samples were then investigated by paper chromatography; by this means it was possible to develop a method of standardizing a sample of methyl green with the required degree of specificity for egg-shell material.

Composition of Methyl Green

Data concerning the composition of methyl green, given by Conn (1946), may be briefly summarized here. Methyl green is one of the tri-amino-triphenyl-methane group, which includes a number of other well-known dyes. As the seventh methyl group is very loosely attached, there is always some methyl or crystal violet impurity present. Owing to its instability,

[Quarterly Journal of Microscopical Science, Vol. 94, part 3, pp. 243–6, Sept. 1953.]

standardization of this stain is difficult. It is soluble in water (4·8 gm. per 100 ml.) and alcohol (0·75 gm. per 100 ml.).

Testing Specificity of Methyl Green for Egg-shell Material

Fresh specimens of *Fasciola hepatica* were fixed in 5 per cent. formol-saline and embedded in wax; 7·5 μ sections were cut. These sections were stained in 0·5 per cent. *aqueous* solutions of the different samples of methyl green, according to the procedure in the original technique (Smyth, 1951*b*). It is particularly stressed that only neutral aqueous solutions were used. The acid solutions, in which methyl green is widely used as a nuclear stain, are unsuitable.

Results of Staining

The results of staining are summarized in Table 1. Four of the samples, obtained from two different manufacturers (A and E), stained the egg-shell material specifically. The eggs in the lower uterus (i.e. before tanning and hardening) and the shell globules in the vitelline cells and in the vitelline ducts, stained brilliant green or blue-green, the green being retained by no other tissues. The methyl violet impurity stained the nuclei violet. The remaining six samples of the stain, obtained from four manufacturers (A, B, C, D), were all non-specific in their staining reactions, acting mainly as nuclear stains but staining other organelles also.

Chromatographic Analysis

About 10 c.c. of saturated aqueous solutions of the various samples of methyl green were prepared and stored in a dark cupboard.

Battery jars were used as vessels and standard paper chromatography procedure (ascending method) was adopted. Small circular spots (2 μl.) of the dyes were placed on the starting-line of the paper with the aid of a fine pipette—one spot to each 3 inches of paper-width. Whatman No. 1 paper was used throughout. The chromatograms were allowed to run for a convenient time (about 24 hours); they were then removed and dried, and the frontier of each spot measured. A number of solvents and mixtures of solvents were tried as the liquid phase; the following mixture was found to give clear spots and was used throughout: ethanol 60 c.c.; *n*-butanol, 100 c.c.; cellosolve, 100 c.c.; water, 80 c.c.

Results of Chromatographic Analysis

The results are shown in Table 1. As is usual in chromatographic work, provided that the temperature was fairly constant and fresh solvents were used, the Rf values obtained were remarkably consistent. The figures quoted in table 1 are averages of at least three results, the experimental error being not greater than $\pm$0·02.

These figures revealed that the samples of methyl green investigated fell sharply into two groups giving very typical chromatograms; these are shown

diagramatically in fig. 1. Type I, containing all the samples that were specific for egg-shell material, showed a green and a violet spot occupying almost identical positions in the chromatogram. This overlap of the green and violet spots produced a bluish spot with a violet front edge and a green back edge, indicating that the Rf of the methyl violet is slightly higher than that of the methyl green.

TABLE 1. *Rf values for the green and violet ions separated from different samples of methyl green*

Temperature 18°–20° C. (For composition of liquid phase, see text)

Stain No.	*Specific for egg-shell material*	*Rf values*	
		Green spot	*Violet spot*
A 1	yes	0·84	none
A 2	yes	0·82	0·84
A 3	yes	0·83	0·83
E	yes	0·84	none
A 4	no	0·68	0·85
B	no	0·62	0·85
C 1	no	0·69	0·87
C 2	no	0·65	0·85
D 1	no	0·63	0·82
D 2	no	0·64	0·82

Type II, containing samples which stain non-specifically for egg-shell material, showed a characteristic methyl violet spot at about 0·84 (mean), as in the previous type, and a very elongated green spot with a mean Rf of 0·65 (range 0·62–0·69). The exact borders of this latter spot were often difficult to define, and in some cases it almost extended up to and bordered on the methyl violet spot.

DISCUSSION

Uncertainty regarding the composition of a stain is often a source of error in cytology, and most workers are familiar with stains that bear the same name but give widely different results. Typical examples are the basic fuchsin in the Feulgen reaction and the various sudans in the fat demonstration and associated techniques. The results obtained by paper chromatography suggest that this technique has many advantages in certain cases for testing such stains, and in the case of methyl green has proved particularly effective.

It has been shown that in the chromatogram of the specific stain, the green appears as a small spot. This result suggests that a fairly pure green substance is present. Moreover, since its Rf value is almost identical with that of methyl violet, it is likely that this stain is made up of almost pure methyl green, as the chemical composition of these two substances is very similar.

On the other hand, in the chromatograms of the non-specific stains, the green constituent appears as a long green streak—a result which indicates the presence of a number of green compounds, which may include some pure

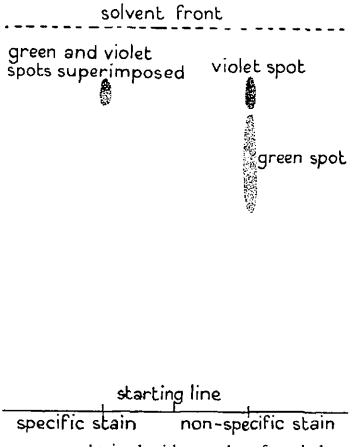

FIG. 1. Typical chromatograms obtained with samples of methyl green, specific and non-specific for egg-shell material in *Fasciola hepatica*.

methyl green. It is possible that this streak represents a number of breakdown products of methyl green.

From these results it was possible to conclude that in order to stain egg-shell material specifically in *Fasciola* (and possibly in trematodes in general) a methyl green with an Rf value of 0·83 in the stated solvent must be used.

Thanks to the co-operation of Messrs. G. T. Gurr, a methyl green with this characteristic has been produced: it has been named Methyl Green M.2.

I am indebted to the Medical Research Council of Ireland for financial aid in support of this work.

REFERENCES

CONN, C. J., 1946. *Biological stains*. N.Y. (Biotech Publications).
SMYTH, J. D., 1951*a*. Nature, **168**, 322.
—— 1951*b*. Stain Tech., **26**, 255.