CERTAIN ASPECTS OF BROMOPHENOL BLUE STAINING DEDUCED FROM SPOT TESTS ON FILTER-PAPER

G. G. SELMAN
Institute of Animal Genetics, Edinburgh (Great Britain)

(Received September 1st, 1959)

INTRODUCTION

That bromophenol blue stains proteins is common knowledge; that bromophenol blue can stain proteins only is not true. BAKER[1] has recently questioned the reliability of bromophenol blue as a histochemical agent for the recognition of protein. The present paper aims at an increase in understanding of the chemistry of staining with bromophenol blue. It describes spot tests for organic compounds of moderately low molecular weight, but the results are believed to be relevant to the interpretation of the results obtained by various established methods of staining proteins on filter-paper or starch gel, and they also suggest ways in which bromophenol blue methods could usefully be developed for the histological location of concentrations of specific organic groups.

EXPERIMENTAL

A wide variety of methods exist for staining proteins on filter-paper with bromophenol blue. In its usual form the staining reagent is made up in ethyl alcohol saturated with mercuric chloride, and contains 1 % bromophenol blue as employed by CREMER AND TISELIUS[2], FLYNN AND DE MAYO[3], and KUNKEL AND TISELIUS[4], or 0.1 % bromophenol blue as used by DURRUM[5], GESHWIND AND LI[6] and HARDWICKE[7]. After this reagent the filter-papers are washed to a white background with tap-water after DURRUM[5] or with 1 % mercuric chloride in methyl alcohol after FLYNN AND DE MAYO[3] and CREMER AND TISELIUS[2], or with 0.5 % (or 2 %) acetic acid as in KUNKEL AND TISELIUS[4] and HARDWICKE[7]. In addition reagents have been used containing bromophenol blue, acetic acid and mercuric chloride in aqueous solution by KUNKEL AND TISELIUS[4] and DURRUM, PAUL AND SMITH[8], and also with the mercuric chloride replaced by zinc sulphate by JENCKS, JETTON AND DURRUM[9]. These were all followed by rinses in dilute acetic acid.

In the present work a series of drops, each of volume one twentieth of a millilitre approximately, of certain compounds in aqueous solution were placed separately on Whatman 3MM filter-paper strips to give a series of spots. The papers were then oven-dried, stained, rinsed and dried again according to whatever procedure was being investigated. After drying the papers were read, and when necessary the visibil-

ity of the bromophenol blue spots was enhanced by passing the paper through the fumes of concentrated ammonia immediately before observing. The papers were finally dried, sprayed with 0.2 % ninhydrin in *n*-butyl alcohol, dried again, steamed if necessary and observed. In this way the degree to which amino acids were leached out of the paper into the rising solutions could be followed. The compounds tested were commercial samples of the common amino acids and also included some purines, pyrimidines, vitamins, nucleosides, nucleotides and nucleic acids.

Tests were first made with the standard staining reagent of 1 % bromophenol blue in ethyl alcohol saturated with mercuric chloride used with a staining time of ten minutes. This was followed by washing in steadily running tap-water of pH about 6.5 until a white background was just obtained. This staining method showed a useful specificity, since of the amino acids only histidine and cysteine gave clear blue spots on a white background. The method detected down to 50 γ of either histidine or cysteine, and 200 γ of these amino acids was detected easily. (Using ninhydrin 12 γ of histidine was detected under similar conditions and 25 γ was detected easily.) GESHWIND AND LI[6] have already demonstrated this bromophenol blue test for histidine and its peptides, and for peptides of cysteine, although they did not actually test cysteine itself. They also showed that histamine behaves similarly to histidine. They demonstrated that the test depended upon bromophenol blue coupling to the organic compound through mercury to form a complex which is relatively insoluble in the rinsing water. Other amino acids are not retained on the paper. The present work showed in addition that the purines guanine, adenine, hypoxanthine and xanthine gave a positive test, while the pyrimidines uracil and thymine were negative. The purines tested all gave blue spots except guanine which gave the orange colour of the dye in its acid form. Benzimidazole gave a blue spot but uric and orotic acids, the ribose nucleosides xanthosine and adenosine, the nucleotides guanylic, cytidylic and adenylic acids, vitamins B_2 and B_6, choline and cholesterol, all gave negative tests. From inspection of the structural formulae of these compounds one may conclude that the staining method gives a positive test for imidazole compounds provided that their imidazole ring is not substituted at the position of the nitrogen atom in the nine position, to use the usual numbering for a purine compound. This nitrogen atom is the third atom of the imidazole ring according to FOX[10] and in the unsubstituted compounds there is a double bond between it and the carbon atom two. For imidazole compounds where such a substitution does occur so that the double bond is absent, as in uric acid, xanthosine, adenosine, guanylic and adenylic acids, the test is negative. Tests with DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) gave a very weak blue colour, such as was obtained by MAZIA, BREWER AND ALFERT[11]. The faint dye binding here may be due to Van der Waals forces. The absence of a strong blue colour with nucleic acids is to be expected because of the β-configuration of their nucleosides, see for example CLARK, TODD AND ZUSSMAN[12].

In addition to the above, the compound aneurine hydrochloride or thiamine, which contains a thiazole ring, gave a very strong blue spot. Indeed this was the only compound which gave a strong blue colour with all the various combi-

nations of reagent and rinses which were tried. It even showed blue while it was in a rinse of 2 % acetic acid. Other compounds with thiazole rings have not been tested.

When the same staining reagent was used followed by rinses in 0.5 % acetic acid, after KUNKEL AND TISELIUS[4], less amino acid was leached out so that arginine, lysine, aspartic acid and proline were also detectable with bromophenol blue in addition to histidine and cysteine. When rinses in 2 % acetic acid were used nearly all the amino acids were retained on the paper to some extent, but the use of the acetic acid made the blue colour difficult to develop even after ammonia fumes. Histidine and cysteine for instance did not give as strong a blue colouration after acetic rinses as when tap-water was used. When rinses of 1 % mercuric chloride in methanol were used, after FLYNN AND DE MAYO[3], more amino acid was retained on the paper, as detected by the final ninhydrin spray, and arginine and lysine again gave blue spots with bromophenol blue, but although histidine and cysteine also gave blue spots they were not as easily detectable as with the tap-water rinse.

The method of KUNKEL AND TISELIUS[4] also stained the more basic amino acids but the staining of histidine and cysteine was less intense in comparison.

The method of JENCKS, JETTON AND DURRUM[9], which uses no mercuric chloride, did not give a blue spot with either histidine or cysteine, although a subsequent spraying with ninhydrin showed that these were retained on the paper after the rinses. Arginine, lysine, tryptophan, phenylalanine and proline did give blue spots with this method.

When, however, the staining solution consisted of a saturated solution of bromophenol blue in acetic alcohol (consisting of 3 parts of 95 % alcohol to 1 part of glacial acetic acid) and the rinsing was done in 0.5 % acetic acid, blue spots were given by arginine, lysine, thiamine and histidine but not cysteine. Presumably the staining of histidine in this case was through its amino group.

DISCUSSION AND CONCLUSIONS

Now the above staining methods were all originally developed for heat-coagulated proteins on filter-paper after separation by paper electrophoresis. In the tests described above, whatever the method of rinsing, there was a greater loss of amino acid during the rinsing procedure than would be the case with proteins stained in the same manner. Nevertheless, since the amino acid composition of each protein is different, then it follows from the above observations that proteins will differ in their bromophenol blue staining intensity per gram of nitrogen. This has been shown directly by JENCKS, JETTON AND DURRUM[9] for the electrophoretically separable protein constituents of serum. Of course proteins may also differ in the availability of their staining groups. In addition the present observations indicate that each different staining procedure will be expected to give a different staining intensity for the same protein. Several authors have described their particular methods as quantitative, for example, FLYNN AND DE MAYO[3], KUNKEL AND TISELIUS[4] and JENCKS, JETTON AND DURRUM[9], but

unless correction factors are applied, firstly for the individual proteins or classes of proteins and secondly for the staining and rinsing procedures used, no quantitative comparison can be made between different sets of results.

The method of GRASSMANN AND HANNIG[13], used by FLYNN AND DE MAYO[3], employs Naphthalene Black as a stain for proteins on filter-paper. Using a sample of Naphthalene Black 12B 200 from Imperial Chemical Industries Ltd., in exactly the same manner as the latter authors, it was found that all the amino acids, purines, pyrimidines etc. gave darker blue spots on a pale blue background. It was not found possible to detect less than 250 γ of histidine with this method because the blue background limited sensitivity. However, since the method detects a wider range of compounds than does ninhydrin for example, it has obvious value for detecting compounds whose chemical natures are unknown. Its lack of specificity suggests also that Naphthalene Black may be superior to bromophenol blue for semi-quantitative work when comparisons are required between proteins of widely different amino acid composition. Amidoschwarz 10B is the same dyestuff as Naphthalene Black 12B or 10B.

Bromophenol blue has been used as a histological stain by MAZIA, BREWER AND ALFERT[11]. For histochemical purposes it is useful to remember that this dye can couple to the imidazole ring of histidine and to the sulphydryl group of cysteine, provided that mercuric chloride is present. For simple acid staining of the more basic amino acids and for the case of thiamine it does not matter whether mercuric compounds are present or not. It is easy to see how proteins are stained in these ways, but it also follows that other compounds which are not proteins but which contain sulphydryl groups, imidazole rings which have not been substituted in the manner described above, amino groups, or perhaps thiazole rings, may also stain. MAZIA and his colleagues suggested that coupling to free carboxyl groups via mercury may also occur but no case of this was noticed in this work. Of course, after hot trichloracetic acid extraction (see *e.g.* TAFT[14]) there may be little left to stain except protein, but interesting cytological results are usually obtained by less drastic methods. It is suggested that if the amino groups are first blocked or changed, then bromophenol blue with mercuric chloride could be used to stain for the sulphydryl groups and histidine, or for either separately by the use of further blocking reagents. Guidance in the choice of appropriate blocking reagents for achieving this, and for confirming the results, is obtainable from a study of DANIELLI[15], BOWYER[16] and MUNDAY AND GIERER[17].

SUMMARY

Spot tests are described using bromophenol blue for low molecular weight organic compounds of biological importance. One method for carrying out the spot test detects sulphydryl groups, imidazole rings provided that they are not substituted at a particular nitrogen atom, and thiamine. A comparison of the different ways of using bromophenol blue leads to conclusions relevant to the use of the dye for staining proteins etc. on filter-paper or starch gel and its use in histochemistry.

REFERENCES

1 J. R. BAKER, *Quart. J. Microscop. Sci.*, 99 (1958) 459.
2 H. D. CREMER AND A. TISELIUS, *Biochem. Z.*, 320 (1950) 273.
3 F. V. FLYNN AND P. DE MAYO, *Lancet*, 261 (1951) 235.
4 H. G. KUNKEL AND A. TISELIUS, *J. Gen. Physiol.*, 35 (1952) 89.
5 E. L. DURRUM, *J. Am. Chem. Soc.*, 72 (1950) 2943.
6 I. I. GESHWIND AND C. H. LI, *J. Am. Chem. Soc.*, 74 (1952) 834.
7 J. HARDWICKE, *Biochem. J.*, 57 (1954) 166.
8 E. L. DURRUM, M. H. PAUL AND E. R. B. SMITH, *Science*, 116 (1952) 428.
9 W. P. JENCKS, M. R. JETTON AND E. L. DURRUM, *Biochem. J.*, 60 (1955) 205.
10 S. W. FOX, *Chem. Revs.*, 32 (1943) 47.
11 D. MAZIA, P. A. BREWER AND M. ALFERT, *Biol. Bull.*, 104 (1953) 57.
12 V. M. CLARK, A. R. TODD AND J. ZUSSMAN, *J. Chem. Soc.*, (1951) 2952.
13 W. GRASSMANN AND K. HANNIG, *Z. physiol. Chem.*, 290 (1952) 1.
14 E. B. TAFT, *Exptl. Cell Research*, 2 (1957) 312.
15 J. F. DANIELLI, *Cold Spring Harbor Symposia Quant. Biol.*, 14 (1950) 32.
16 F. BOWYER, *Nature*, 174 (1954) 355.
17 K. W. MUNDAY AND A. GIERER, *Z. induktive Abstammungs- u. Vererbungslehre*, 89 (1958) 614.