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Review

Cellulose bearing covalently linked copper phthalocyanine trisulphonate as an adsorbent selective for polycyclic compounds and its use in studies of environmental mutagens and carcinogens

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ABSTRACT

A method useful as a preconcentration technique for isolating mutagens and carcinogens is described. Cotton bearing covalently linked copper phthalocyanine trisulphonate as ligand (blue cotton) can adsorb selectively compounds having three or more fused rings. The adsorption takes place in aqueous media, involving 1:1 complex formation between the ligand and the polycyclic compound. The desorption can be done by elution with organic solvents, most effectively with methanol containing ammonia. As many important environmental mutagens and carcinogens are polycyclics, this adsorption is useful as a means of extracting this class of materials from crude samples such as food, urine and river waters. The use of copper phthalocyanine as a ligand for chromatographic supports has recently been initiated, yielding promising results for the effective separation of polycyclic aromatic compounds from each other.

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1. INTRODUCTION

1.1. Need for developing methods for detection and identification of environmental mutagens and carcinogens

The scope of studies on mutagens widened greatly when the notion that carcinogens overlap with mutagens was introduced in 1973 by Ames *et al.* [1]. The detection of mutagens has become increasingly important since the discovery of highly potent mutagens in cooked food and the subsequent demonstration of their carcinogenicity in rodents [2]. It seems now that the environment contains a great number of mutagens and humans live surrounded by mutagens/carcinogens [3–5]. As a result, the importance of assessing environmental mutagens both qualitatively and quantitatively has become realized by researchers worldwide, and great efforts have been made to accomplish this task. A difficulty one encounters in attempting this assessment is that mutagens, either those of known structures or of unknown nature, are present only in minute amounts in environmental materials. Consequently, the separation and identification of these substances require the extensive use of chromatographic techniques. The original materials are often sampled from the environment in very large amounts, *e.g.*, river waters and foods, and in such instances preconcentration of the desired components is essential before the materials are subjected to chromatographic procedures.

Adsorption by cellulose-supported copper phthalocyanine trisulphonate is one such means for preconcentration [6]. This technique is relatively new, but has already been used extensively for isolating mutagens and for detecting mutagenicity in the environment. Further, the use of copper phthalocyanine as an affinity ligand in chromatographic procedures has recently been developed. This review summarizes the present status of these methods.

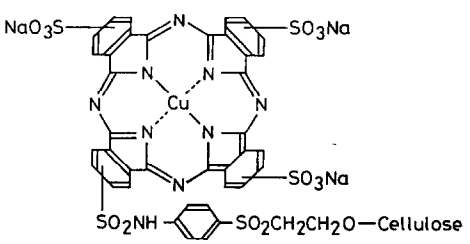
1.2. Chemical structure of cellulose–copper phthalocyanine trisulphonate (blue cotton)

Cotton bearing covalently linked copper phthalocyanine trisulphonate is named "blue cotton" after its colour [6]. The linkage connecting the blue pigment to cellulose is illustrated in Fig. 1. The synthesis of blue cotton can be done by using a simple one-step reaction as shown in Fig. 2.

The usefulness of blue cotton lies in its unique property to adsorb aromatic compounds having three or more numbers of fused rings. The adsorption capacity is strong and the selectivity for adsorbing this class of compound is high. The fact that many such polycyclic aromatic compounds in the environment are mutagenic and often carcinogenic makes blue cotton very useful as a means of efficiently purifying these mutagens from crude samples.

1.3. Historical background

The innovation of blue cotton occurred by luck. We had been studying modulators of mutagenesis by using the assay on *Salmonella* [7]. Our first finding in this study was the ability of hemin to inhibit the mutagenicity of polycyclic aromatic compounds [8,9]. For example, the mutagenicity of Trp-P-1 in *S. typhimurium* TA98 in the presence of microsomal activating enzymes is 50% inhibited by addition of only 2 equiv. of hemin [8]. On the other hand, hemin does not inhibit the mutagenicity of MNU, 4-



Blue Cotton

Fig. 1. Structure of blue cotton.

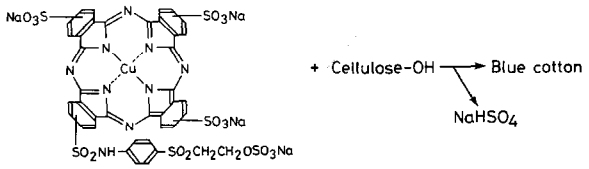


Fig. 2. Synthesis of blue cotton.

nitroquinoline 1-oxide or several other compounds that do not possess structures with three rings or more [9]. It was found that an equimolar mixture of hemin and Trp-P-1 gives an absorption spectrum significantly different from the sum of the spectra of the individual compounds. We interpreted this as indicating the formation of a complex between these two planar molecules [8].

Interest had arisen in whether chalks as used in teaching were mutagenic. Chalk powder collected in a lecture room was found to be mutagenic in the *Salmonella* test. It turned out that blue chalks were mutagenic but white chalks were not. Further studies showed that the blue pigment used to manufacture the chalks contained a mutagenic contaminant. After some discussions with the manufacturers of the pigment the contaminant was removed and, as a result, most of the blue chalks collected in the city of Okayama a few years later were no longer mutagenic [10].

During this investigation, the chemical nature of the blue pigment was a focus of discussion. The pigment was copper phthalocyanine (Fig. 3). The structural similarity to hemin was obvious. At that time we already knew that hemin can form complexes with polycyclic compounds; therefore, it was a straightforward guess that copper phthalocyanine might behave like hemin towards polycyclic aromatic compounds. An interesting question was whether these molecules can serve as a ligand to adsorb polycyclic aromatic compounds. To explore this possibility, we wanted to have these molecules connected to some carrier. Our attempt at that time to link hemin covalently to a solid support had failed. Therefore, we decided to investigate the use of copper phthalocyanine or its derivatives as a ligand on a solid support for adsorbing polycyclic mutagens. As the support, absorbent cotton appeared to be an attractive candidate because it swells in water to allow efficient contact with molecules in solution, and because the manipulation is expected to be easy. Fortunately, there was a copper phthalocyanine derivative perfectly suited for our purpose. C. I. Reactive Blue 21 can react with hydroxyl groups to form a stable ether linkage. It bears three sulphonate substituents on the ring, so that it has a strong hydrophilic character, a property that seemed important for a ligand intended to be used in aqueous media. As described below, blue cotton prepared from absorbent cotton and C. I. Reactive Blue 21 has proved to be an excellent adsorbent for polycyclic aromatic compounds.

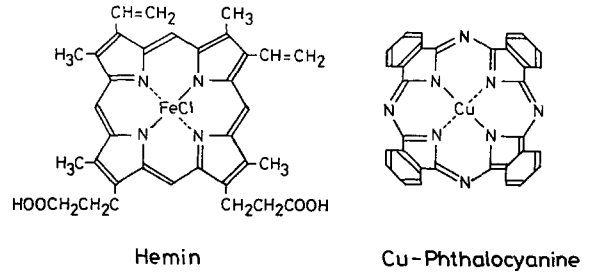


Fig. 3. Structures of hemin and copper phthalocyanine.

2. PREPARATION AND PROPERTIES OF BLUE COTTON AND BLUE RAYON

Phthalocyanines are representatives of blue and green pigments that have commercial value [11]. There are a large number of phthalocyanine derivatives potentially useful for the purpose of our present interest, namely as an adsorbent of polycyclic compounds. In addition, the support to carry the phthalocyanine ligand can be anything from cellulose to glass beads. Therefore, the combination of C.I. Reactive Blue 21 plus cotton is only one of many possibilities.

2.1. Preparation

As the scheme in Fig. 2 shows, the reaction between C.I. Reactive Blue 21 and cellulose is of a Schotten–Baumann type. The condensation proceeds in an aqueous medium under alkaline conditions (sodium carbonate) at 70°C [6]. The whole process requires about 2 h, during which period the absorbent cotton is gently swirled in the reaction flask. The stained cotton is collected on a filter and washed with water and then with dimethyl sulphox-

ide. The dimethyl sulphoxide washing is a necessary step to remove unbound pigment efficiently from the cotton. Generally, several repeated soakings of the cotton in dimethyl sulphoxide are required to obtain a very faint blue washing. To ensure removal of any polycyclic aromatic compounds that may have been adsorbed on the blue cotton during these processes, the cotton is then washed with a mixture of methanol and concentrated ammonia (50:1, v/v) and finally with methanol.

The dried sample may be analysed by determining the copper content by atomic absorption spectrometry. A simpler method is currently used in our laboratory (unpublished work). It consists of decomposing the cellulose with 60% perchloric acid by heating at 90°C for 30 s, making the solution alkaline by diluting with an excess of NaOH and finally subjecting the solution to spectrophotometric determination of the blue pigment. For this spectroscopic determination, a calibration graph is obtained by submitting C.I. Reactive Blue to the same treatment. The values thus obtained by this method are consistent with those found by the copper determination.

The blue cotton samples prepared under the conditions specified in the literature [6] showed reproducible copper phthalocyanine contents of *ca.* 10 μmol per gram of dried material. Although the phthalocyanine content in the cotton can be increased by submitting the stained cotton to further treatment with C. I. Reactive Blue 21, we considered that the once-stained material was satisfactory for use in mutagen studies.

An obvious extension of this synthesis is to explore the variation of the kind of cellulose employed as the carrier of the ligand. For example, cellulose powder can be stained similarly, and the copper phthalocyanine content of the blue cellulose powder prepared is approximately the same as that of blue cotton. An interesting observation was recently made when we used a special kind of rayon. This rayon was obtained from Daiwabo (Kakogawa, Hyogo, Japan), a material called by the manufacturer "amorphous rayon staple fibres". This fibre lacks the rigid filamentous structure that can be found in regular rayon fibers, and consequently provides better access for reagent molecules to its surface. On staining this rayon fiber with C. I. Reactive Blue 21 using the conditions described above, about three times the amount of pigment was bound to the cellulose compared with the pigment content of blue cotton.

2.2. Properties

Phthalocyanine show an outstanding stability to light and heat [11]. The pigment content of blue cotton, as determined by perchloric acid solubilization, did not change after allowing the cotton to stand on a laboratory bench in an open container for 6 months without particular precautions to protect it from light. A gradual reduction in the colour occurs when blue cotton is exposed to a continuous flow of tap water. This decoloration is apparently due to chlorination of the pigment; treatment of blue cotton with water containing 10 ppm of chlorine results in a rapidly deminishing blue colour.

Because of its strong capacity to adsorb polycyclic aromatics, it is advisable that old samples of blue cotton be refreshed before use by washing with methanol–ammonia and methanol.

Blue cotton contains a small amount of non-bonded pigment that has remained in the material even after exhaustive washing. The blue pigment eluted with methanol–ammonia from a blue cotton preparation corresponded to 6 ppm of the cotton.

Blue rayon prepared from amorphous rayon fibres contains 30 μmol of copper phthalocyanine per gram. Because of its particular physical properties, this fibre can be efficiently washed with solvents. The free blue pigment content that has been held in the rayon is less than 1 ppm. Amorphous rayon fiber is slightly weaker than regular rayon fibre against tensile forces: the amorphous rayon has a tensile strength of 1.97 g/denier, whereas regular rayons have a strength of 2.5–3.1 g/denier and cotton 3.0–4.9 g/denier [11a]. Therefore, if a suspension of blue rayon in water is shaken vigorously, a portion of the rayon may be split to form short pieces of fibres. In our hands, however, these fines are generally less than 0.5% (w/w) of the rayon placed in the solvent. When necessary, the fines may be collected by use of a gauze.

3. SPECIFICITY AS A MUTAGEN ADSORBENT

Techniques widely used for the preconcentration of organic compounds from crude materials include solvent partitioning and adsorption to activated

carbon, silicates and organic polymers. In these techniques, the specificity regarding the structures of target compounds is generally low. In contrast, blue cotton adsorption offers a high selectivity toward polycyclic aromatic compounds.

3.1. Classification of adsorbable and non-adsorbable compounds

Various mutagens dissolved in saline can be adsorbed on blue cotton and can be recovered from the cotton by elution with an organic solvent [6]. The adsorption is achieved simply by a batch process, shaking the mutagen solution with the blue cotton added. Elution with a mixture of methanol and a small amount of concentrated ammonia (usually used in a ratio of 50:1) generally gives excellent recoveries of adsorbed mutagens. Table 1 gives results for several typical compounds and Fig. 4 summarizes experiments for 62 compounds. Compounds with three or more fused rings in their structures are adsorbed efficiently on blue cotton and can be recovered satisfactorily, whereas compounds with two, one or no rings are adsorbed only to a small extent. There are several exceptional cases. 1,N^6-Ethenoadenosine (No. 17) is not adsorbed on blue cotton although it has a three-ring system. 1,N^6-Ethenoadenine, the base portion of No. 17, is also not adsorbed (data not shown). The reason for this phenomenon is not clear; this three-ring system may have some structural anomaly [12]. PhIP (No. 50) and quercetin (No. 51) appear also to be exceptional; however, they have two rings and one ring directly linked and conjugated, and therefore would possess planar sizes comparable to a three-ring system. Fecapetaene (No. 62) seems to have a weak affinity to the blue dye. The linear but planar structure of this aliphatic compound may contribute to the affinity found. Control experiments using plain absorbent cotton (Table 1) clearly shows that the adsorption on blue cotton is dependent on the pigment bound to cotton. A cotton sample that had undergone the sodium carbonate treatment in the absence of the dye was also tested as a control. No significant difference was found between the carbonate-treated and the plain cotton samples in their adsorption of compounds. The effect of pH of the mutagen solution on the adsorption was studied for Trp-P-2, and the adsorption was equally efficient in the pH range 4–10. It was also demonstrated that [^{3}H]Trp-P-2 and [^{14}C]acetylaminofluorene can be similarly recovered by blue cotton from solutions of these compounds in human urine or bovine serum.

Most of the mutagens that have been found to adsorb on blue cotton are those which give positive

TABLE 1

ADSORPTION AND RECOVERY OF COMPOUNDS BY BLUE COTTON

A 5-ml volume of solution of a compound in 0.15 *M* NaCl (concentrations 0.1–100 m*M*) was treated with blue cotton (50 mg) by shaking at room temperature for 30 min. The cotton was taken up and squeezed to combine the cotton-held portion of the solution with the mother liquor. A fresh batch of blue cotton (50 mg) was added, and the treatment was repeated. The extent of adsorption was determined by measuring the compound that remained in the solution (by either spectrophotometry, fluorimetry or radioactivity measurement). The two 50-mg cotton samples were combined, wiped with a paper towel, washed twice with 0.15 *M* NaCl, freed from moisture by use of a paper towel and then eluted with 5 ml of methanol–concentrated ammonia (50:1) with gentle shaking for 30 min at room temperature. This elution was repeated once more, and the combined eluate was evaporated under reduced pressure. The residue was analysed for the compound. For the histidine adsorption, 1 m*M* [^{3}H]histidine at 230 000 dpm was used, and the radioactivity recovered was 146 dpm, corresponding to a 0.06% overall recovery.

Compound	Adsorption (%)		Overall recovery by blue cotton (%)
	Blue cotton	Plain cotton	
MeIQx	94	12	94
Daunorubicin	99	22	65
Aflatoxin B_1	84	26	63
4-Nitroquinoline 1-oxide	11	0	Not done
p-Nitrophenol-Na	5	0	Not done
[^{3}H]Histidine	–	Not done	0.06
[^{14}C]Nitrosodimethylamine	0	Not done	Not done

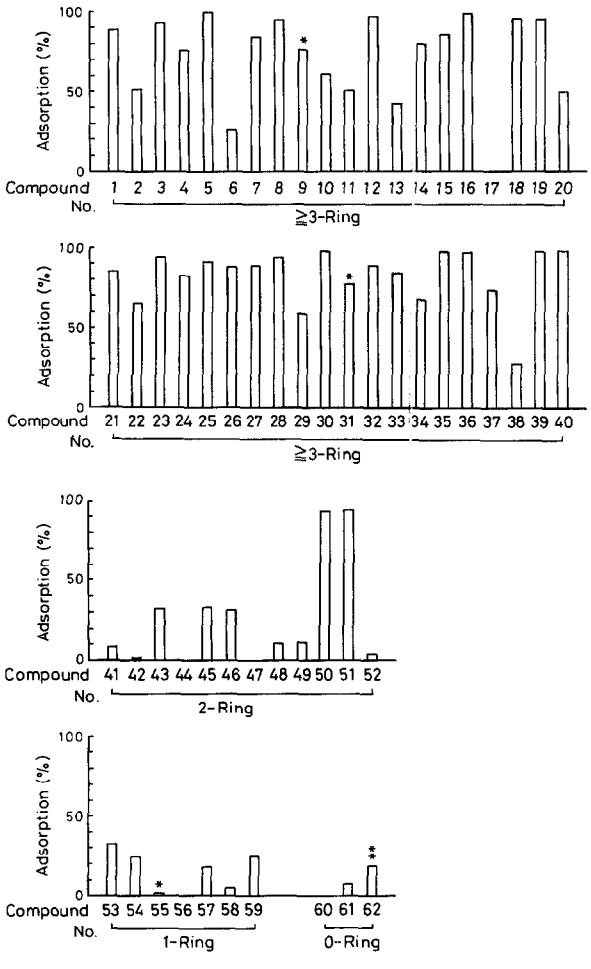


Fig. 4. Adsorption of 62 compounds on blue cotton. The experimental procedure is described in Table 1. The single asterisks indicate overall recovery, rather than the extent of adsorption. The double asterisks indicate that the experiment for fecapentaene-12 was done with blue rayon: 20 mg of rayon for 43 nmol of fecapentaene-12 in 5 ml of saline under an argon atmosphere, and the fecapentaene-12 was determined spectrophotometrically. The fecapentaene-12 used was a generous gift from Dr. K. Wakabayashi of the National Cancer Center Research Institute (Tokyo), who prepared the sample as reported [13]. Compound numbers and names are as follows: 1 = AαC; 2 = 2-acetylaminofluorene; 3 = N-acetyl-Trp-P-1; 4 = N-acetyl-Trp-P-2; 5 = acriflavine hydrochloride; 6 = actinomycin D; 7 = aflatoxin B_1; 8 = 9-aminoacridine; 9 = 2-aminoanthracene; 10 = bellidifolin (courtesy of Dr. H. Kanamori, Hiroshima Institute of Hygiene); 11 = benz[*a*]anthracene 5-methylenesulphate (courtesy of Professor T. Watabe of Tokyo College of Pharmacy); 12 = benzo[*a*]pyrene; 13 = carminic acid; 14 = chlorpromazine hydrochloride; 15 = copper phthalocyanine tetrasulphonic acid; 16 = daunorubicin; 17 = 1,N^6-ethenoadenosine; 18 = ethidium bromide; 19 = ethyl eosin; 20 = fluorescein; 21 = Glu-P-1; 22 = Glu-P-2; 23 = harman; 24 = hemin; 25 = IQ; 26 = MeAαC; 27 = MeIQ; 28 = MeIQx; 29 = 8-methoxypsoralen; 30 = methylene blue; 31 = 1-nitropyrene; 32 = norharman; 33 = phenazine A (courtesy of Professor T. Okuda of Okayama University) [14]; 34 = phenazine C [14]; 35 = quinacrine hydrochloride; 36 = rhodamine 6G; 37 = riboflavin; 38 = swertianolin (courtesy of Dr. H. Kanamori); 39 = Trp-P-1; 40 = Trp-P-2; 41 = adenine; 42 = adenosine; 43 = amaranth; 44 = ATP; 45 = carbadox; 46 = 6-dimethylallylaminopurine; 47 = NADH; 48 = naphthalene 1-methylenesulphate; 49 = 4-nitroquinoline 1-oxide; 50 = PhIP; 51 = quercetin; 52 = tryptophan; 53 = 4-aminobiphenyl; 54 = furylfuramide; 55 = histidine; 56 = mitomycin C; 57 = 4-nitro-*o*-phenylenediamine; 58 = *p*-nitrophenol-Na; 59 = phenolphthalein; 60 = nitrosodimethylamine; 61 = oleic acid-Na; 62 = fecapentaene-12.

mutagenicity in the *Salmonella*/microsome test using the strain TA98 in the presence of S9 mix. The compounds that can interfere with the *Salmonella* test, *i.e.*, histidine and oleic acid [15] are practically non-adsorbable on blue cotton.

As the ligand has sulphonate groups, blue cotton is expected to have a cation-exchange capacity. However, the adsorptions in the experiments shown in Fig. 4 took place in 0.15 *M* NaCl solution, and therefore such ionic affinities would have contributed little to the observed adsorptions. In fact, many of the adsorbable compounds do not have dissociable groups, *e.g.*, acetylaminofluorene and benzo[*a*]pyrene. It should be borne in mind, however, that blue cotton has this ionic property and that such a property may become manifest in solutions of low salt concentrations.

Our experimental results, together with those reported from other laboratories (see Section 4), indicate that blue cotton has a selective affinity to compounds having three or more fused rings. These compounds are commonly planar in their molecular forms. Consequently, it is conceivable that they can form face-to-face hydrophobic complexes with copper phthalocyanine moiety, which has a large planar surface in the molecule.

As expected, blue rayon shows similar adsorption characteristics. Blue rayon has a higher capacity and a lower free-pigment content than blue cotton, as already discussed in Section 2. Although its mechanistic strength is lower than that of blue cotton, it does not seem to create a serious problem; for example, blue rayon did not show any weight loss when allowed to hang in river water for 2–3 days [16].

Table 2 shows the results of a series of experiments in which (1) the efficiency of adsorption was compared between blue rayon and blue cotton and (2) the efficiency of elution was compared between methanol and methanol–ammonia for various compounds on blue rayon and blue cotton. (1) With equal masses of the rayon and cotton, the extents of adsorption are generally greater with the rayon (note that the molar amounts of blue pigment used in these experiments are different between the rayon and the cotton; the molar amounts of pigment in the cotton are three times lower than those in the rayon, and in some instances are less than the stoichiometric amount from the compound used). (2) Many of the compounds can be efficiently eluted with methanol alone, but Trp-P-1 and quinacrine were not elutable, but could be quantitatively eluted from the cotton with methanol–ammonia. For benzo[*a*]pyrene, and probably also for other polycyclic aromatic hydrocarbons, elution with dioxane appears to be the most appropriate.

3.2. Mechanism of adsorption

At the time when we made these observations in 1983, there appeared to be no report on the affinity of aromatic compounds to copper phthalocyanines, except for the literature on the adsorption of gaseous benzene derivatives to the surface of crystalline copper phthalocyanine [17,18]. This lack of information is possibly due to the fact that copper phthalocyanine sulphonates (hereafter referred to as Cu-pc-SO_3^-) form dimers and higher aggregates in aqueous solutions in a reversible manner [19,20]. Any attempt to study quantitatively complex formation between Cu-pc-SO_3^- and another substance would have been hampered by this complicated situation.

In blue cotton, Cu-pc-SO_3^- is fixed on a solid sup-

TABLE 2

COMPARISON OF BLUE COTTON AND BLUE RAYON IN THEIR ADSORPTION CAPACITIES, AND THE EFFICIENCY OF ELUTING SOLVENTS

A 5-ml volume of saline solution of a compound was treated once with 20 mg of blue cotton or blue rayon for 5 min with mechanical shaking. The extent of adsorption was measured spectrophotometrically. The cotton or rayon was taken out, washed with 1 ml of water and dried by use of a paper towel. The cotton or rayon was then eluted with 5 ml of methanol by shaking for 5 min. The eluted compound was determined spectrophotometrically, and then to the methanol–cotton (or –rayon) mixture was added 0.1 ml of concentrated ammonia solution. The mixture was shaken for a further 5 min and the eluted compound was determined.

Compound	Amount (μmol)	Adsorption (%)		Overall recovery (%)			
		Cotton	Rayon	Elution with methanol		Elution with methanol–NH_3	
				Cotton	Rayon	Cotton	Rayon
Trp-P-1	0.1	89	95	15	10	90	94
Glu-P-1	0.25	30	61	18	44	22	52
Glu-P-2	0.25	20	45	15	34	15	36
MeIQx	0.1	59	83	43	51	57	78
IQ	0.04	58	79	60	73	72	81
Quinacrine	0.5	33	83	4	0	34	73
Chlorpromazine	0.2	25	61	24	39	26	66
PhIP	0.01	–	–	67	69	72	75
4-Nitroquinoline 1-oxide	0.3	2	6	–	–	–	–
p-Nitrophenol-Na	0.2	0	2	–	–	–	–
Benzo[*a*]pyrene	0.0004	88	96	76	63 (Dioxane	87 90	68 87)[a]

[a] For adsorption of benzo[*a*]pyrene, a solution in dioxane–saline (2:8) was used because of the insoluble nature of this compound in saline.

TABLE 3

ANALYSIS OF COMPLEX FORMATION BETWEEN THREE-RING COMPOUNDS AND COPPER PHTHALOCYANINE TRISULPHONATE COVALENTLY LINKED TO CELLULOSE (BLUE COTTON)[21]

The adsorption is in 0.05 *M* sodium phosphate buffer (pH 7.4) containing 0.2 *M* sodium chloride at 25°C. k_d = [C] [Cu-pc-SO_3^-]/[C Cu-pc-SO_3^-], where [C] is the concentration of the compound that remained in the solution after equilibration, [Cu-pc-SO_3^-] the concentration of Cu-pc-SO_3^- at equilibrium (assuming that these residues are in solution) and [C Cu-pc-SO_3^-] the concentration of the complex, which is identical with the concentration of the compound adsorbed.

Compound	Dissociation constant for complex formation, $k_d \times 10^6$ (*M*)	Molar ratio at saturation (compound/Cu-pc-SO_3^-)
Trp-P-2	2.90	1.25
Quinacrine	4.83	1.03
9-Aminoacridine	5.90	1.10
2-Acetylaminofluorene	18.5	0.96
2-Aminofluorene	73.1	1.00

port; hence aggregations of these Cu-pc-SO_3^- residues would be difficult. It should be noted that the procedure for preparing blue cotton involves washing the material with dimethyl sulphoxide and methanol [6], solvents in which such aggregates would be dissociated [19,20]. Further, in blue cotton a complex already formed between a Cu-pc-SO_3^- moiety and a given compound is expected to show little interference with other Cu-pc-SO_3^- moieties to make complexes.

Based on these considerations, we analysed quantitatively the adsorption of five different three-ring compounds on blue cotton [21]. The medium used for the adsorption was 0.05 *M* sodium phosphate–0.2 *M* NaCl (pH 7.4), and two types of titration were done. First, a fixed amount of blue cotton was titrated with a compound, and second, a fixed amount of a compound was titrated by adding increasing amounts of blue cotton. Both of the titrations indicated 1:1 complexing, and the results obtained by the second type of titrations are given in Table 3. All five compounds examined show qualitatively the same behaviour, forming 1:1 complexes with Cu-pc-SO_3^-, with dissociation constants in the range 10^{-6}–10^{-5} *M*.

Further evidence for the presence of molecular interactions between these adsorbable compounds and Cu-pc-SO_3^- was obtained in spectroscopic studies. Fig. 5 shows visible absorption spectra of copper phthalocyanine disulphonate (spectrum 1), Trp-P-1 (spectrum 2) and their mixture (spectrum 3), each at 20 μM concentration in 50 m*M* sodium phosphate buffer (pH 7.0). A large shift of the peak at 606 nm to a longer wavelength is seen. Similar measurements were made with other compounds, and the results are summarized in Table 4. Compounds that can adsorb on blue cotton induce spectral shifts, but those which do not adsorb can induce no shift in the copper phthalocyanine spectrum. It is noteworthy that there is a tendency for strongly adsorbable compounds to induce large spectral changes.

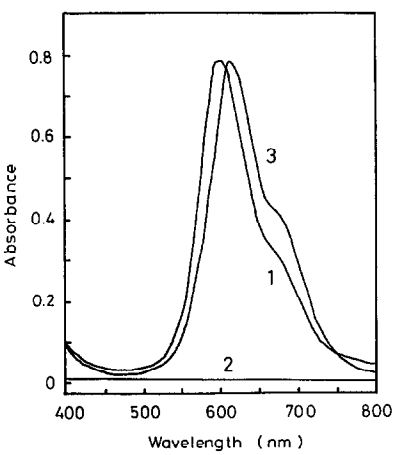


Fig. 5. Visible absorption spectra of (1) 20 μM copper phthalocyanine disulphonate, (2) 20 μM Trp-P-1 and (3) 20 μM copper phthalocyanine disulphonate plus 20 μM Trp-P-1. The solutions were in 50 m*M* sodium phosphate buffer (pH 7.0).

TABLE 4

PARALLELISM BETWEEN ADSORPTION OF COMPOUNDS ON BLUE COTTON AND THEIR ABILITY TO CHANGE THE ABSORPTION SPECTRUM OF COPPER PHTHALOCYANINE DISULPHONATE

The solutions were 20 μM copper phthalocyanine disulphonate Na_2 (Cyanine Blue S-1, Sumitomo Chemical Industries) and 20 μM test compound, dissolved in 50 mM sodium phosphate buffer (pH 7.0). The extents of adsorption were determined by the procedure described in Table 1, but with a single blue cotton (50 mg) treatment instead of a double treatment as in the experiments of Table 1. The data in this table are from H. Kobayashi and H. Hayatsu (unpublished results).

Compound	Adsorption (%)	Shift of λ_{max} at 606 nm (nm)
Trp-P-1	96	+12
Glu-P-1	49	+4
AαC	49	+2
9-Aminoacridine	78	+10
Daunorubicin	82	+14
2-Acetylaminofluorene	20	+4
Ethidium bromide	92	+14
Quinacrine	75	+18
8-Methoxypsoralen	33	+2
Carbadox	8	0
4-Nitroquinoline 1-oxide	1	0
p-Nitrophenol-Na	0	0
Tryptophan	5	0
Histidine	0	0

In the experiments described in Table 1 and Fig. 4, no spectral changes were detected for the compounds before and after the blue cotton treatment, an indication that the pigment did not catalyse chemical transformations such as aerobic oxidation. During these treatments, no particular care was taken to protect the materials from light.

The fact that the compounds adsorbed on blue cotton can be recovered by elution with hydrophilic organic solvents indicates that the complex formation does not occur in these solvents. As already discussed, methanol–ammonia is the most effective among the eluents examined. It appears possible that ammonia helps to dissociate the complex by coordinating itself to the central metal of the ligand.

4. USE OF BLUE COTTON IN STUDIES OF MUTAGENS/CARCINOGENS

A large number of environmental mutagens and carcinogens have polycyclic aromatic structures: polycyclic aromatic hydrocarbons and food pyrolysis products belong to this class of compound. Mycotoxins, such as aflatoxin B_1, can adsorb on blue cotton (Table 1), although its structure is not perfectly planar. It can therefore be expected that blue cotton is useful in extracting these types of mutagenic compounds from complex mixtures.

4.1. Mutagens in food

Mutagenic heterocyclic amines that can be formed by pyrolysis of foods, proteins and amino acids are shown in Fig. 6. Most of them are proven carcinogens in rodents [2].

A typical example of the use of blue cotton to demonstrate the mutagenicity of cooked food is the following [6]: a hot-water extract of pan-fried ground beef is prepared and treated with blue cotton at room temperature. The cotton is taken up, washed with distilled water to remove the beef soup, dried with a paper towel and then eluted with methanol–ammonia. After removal of the solvent by evaporation, the residue is submitted to the Ames test on *S. typhimurium* TA98 with metabolic activation. With this simple work-up, mutagenicity of cooked beef can be demonstrated in a clear-manner: a linear dose response with respect to the amount of beef, and an increase in mutagenicity with the increase in cooking time.

For the extraction of mutagenic compounds from Difco Bacto beef extract, the sample was first diluted by dissolution in water, and the solution was then treated with blue cotton [24]. In this study of beef extract mutagens, we carried out several cycles of blue cotton adsorption, *i.e.*, a blue cotton extract was again dissolved in water, and the solution was treated with a new batch of blue cotton. With this repetition of the cycle, an efficient concentration of mutagenic component was achieved with only a small loss of total activity (Table 5). By further purification using high-performance liquid chromatography (HPLC), the presence of MeIQx and IQ in the beef extract was demonstrated, a result consistent with the literature reporting the presence of these mutagens in cooked beef [25,26].

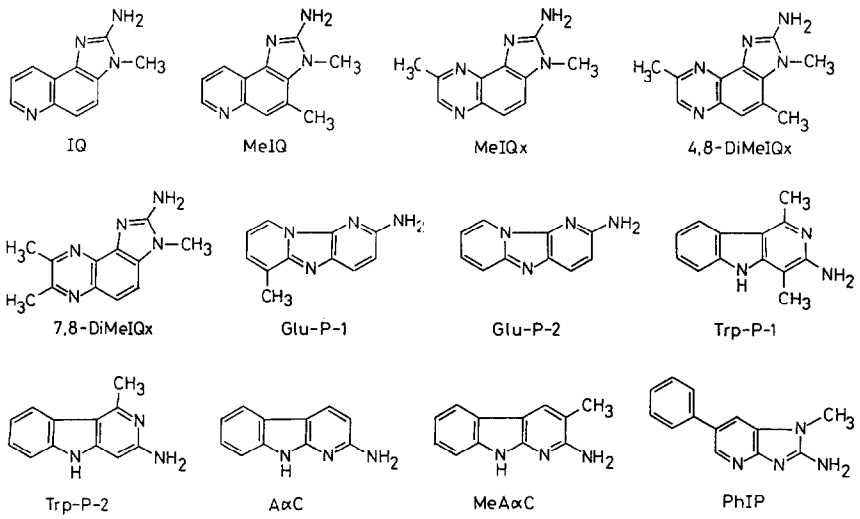


Fig. 6. List of food pyrolysate mutagens. The drawing of compounds except PhIP is taken from Sugimura [22] and that of PhIP from Felton *et al.* [23].

More recently, Sugimura and co-workers applied blue cotton adsorption in the first step of quantifying IQ and MeIQx in beef extract [27]. They were able to achieve 10^4-fold concentration of the MeIQx-containing fraction by the use of two cycles of blue cotton extraction, with an overall recovery of 71% with respect to MeIQx. By a similar procedure, 4,8-Me_2IQx in beef extract was quantified [28]. These workers also succeeded in detecting Trp-P-2 in blue cotton extracts of both bacteriological-grade beef extract and broiled beef [29].

When one intends to detect mutagenicity in a crude sample, a necessary step is to reduce the amount of the sample to make it suitable for subjecting to an assay. For such a purpose of concentrating mutagenic fractions, blue cotton offers a

TABLE 5

EFFICIENCY OF REPETITIVE BLUE COTTON PROCESSING IN PURIFICATION OF MUTAGENS IN BEEF EXTRACT [24]

Cycle No.	Sample	Blue cotton used	Material obtained	
			Weight (mg)	Mutagenicity[a]
1	10 g of Difco Bacto beef extract in 100 ml of water	0.8 g × 3[b]	15.6	150 400 (100%)
2	15.6 mg in 100 ml of water	0.4 g × 2	0.3	118 200 (78%)
3	0.3 mg in 50 ml of water	0.2 g × 2	0.1	101 800 (68%)

[a] The mutagenicity was assayed for small fractions of the samples using *S. typhimurium* TA98 with metabolic activation. The numbers represent those of revertants corresponding to the total material.

[b] This expression means that the blue cotton adsorption was done three times, with 0.8 g of fresh cotton in each adsorption.

simple means, as exemplified in the efficient concentration of mutagens from beef extracts. Crude materials such as food often contain inhibitors for mutagens. Free unsaturated fatty acids, such as oleic acid and linoleic acid, are ubiquitously present in fats and oils, and they can mask the mutagenicity of compounds [30]. We have shown that oleic acid interferes with the detection of mutagenicity of various compounds in the Ames *Salmonella* test, and have presented evidence to indicate that this inhibition occurs by entrapping the mutagens in micelles of the fatty acid [31]. As these fatty acids are only very poorly adsorbed on blue cotton, samples prepared by the blue cotton method are usually free from this problem.

With these advantages of blue cotton in mind, we started a survey of mutagenicity in processed food commercially available in the market. A food sample was extracted with boiling water, and the aqueous solution was subjected to the blue cotton procedure to prepare a material for assays in *S. typhimurium* TA98 and TA100, with and without metabolic activation. Soon we found that smoked, dried bonito (*katsuobushi*, in Japanese) is mutagenic [32]. This item is a traditional, popular food in Japan. The blue cotton extract was further fractionated by chromatographic techniques, and MeIQx was identified as a major mutagenic component [33]. A minor mutagenic component was also isolated, and was suggested to be 4,8-Me_2IQx. Later, MeIQx and 4,8-Me_2IQx were also found in other Japanese smoked, dried fish products [34]. As raw bonito does not contain these mutagens, an investigation was made to find which stage of processing is responsible for the mutagen formation. It turned out that the process called *baikan*, in which the fish is dried at 80–120°C for several days, is responsible [35]. Consistent with this finding, heating various fish at 100°C for 48 h, which does not result in charring of the fish, can give rise to the formation of MeIQx and 4,8-Me_2IQx [36]. Throughout these studies, the blue cotton method was used extensively.

Blue cotton has since been utilized by many researchers as a regular step for preparing partially purified samples of food pyrolysate heterocyclic amines to be submitted to HPLC analysis. Thus, Edmonds *et al.* [37] quantified IQ and MeIQ in broiled salmon, Aeschbacher and co-workers

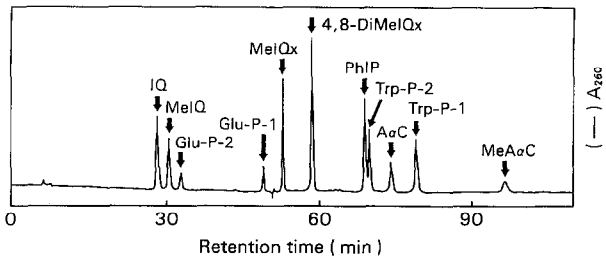


Fig. 7. Elution profile of heterocyclic amines by HPLC on an ODS column [41].

[38,39] determined IQ and MeIQx in cooked beef products and Manabe *et al.* [40] detected Glu-P-1 and Glu-P-2 in Worcestershire sauce. Zhang *et al.* [41] analysed Chinese cooked fish and meat, and detected IQ, MeIQ, MeIQx, 4,8-Me_2IQx and PhIP.

A very efficient HPLC separation of these heterocyclic amines is illustrated in Fig. 7. With the use of blue cotton preconcentration followed by the HPLC, a comprehensive list of heterocyclic amine contents in cooked meats has been produced [42].

The survey of mutagenicity in food by the blue cotton method has continued to offer information about new sources of mutagens. While boiled rice is free from mutagenicity, a broiled rice ball is positive in the Ames test, Muraoka *et al.*'s observation of this [43] is important because broiled rice ball is a popular item of Japanese food. Treatment of boiled rice with nitrous acid followed by extraction with blue rayon resulted in the detection of several mutagenic products [44]. Although the mutagenic products are suggested to be polycyclics, their structures are unknown. Coffee contains blue cotton-adsorbable mutagens: MeIQ-like mutagens are present in roasted coffee beans as a tightly bound form, extractable only with methanol–ammonia [45,46]. The same group found that IQ and Glu-P-1 occur in oil of charred egg yolk, a health food in Japan [47].

An important question in food mutagen problems is how these heterocyclic amines are formed. This question was answered by a series of work performed in Sugimura's laboratory. Thus, on heating a mixture of creatinine, D-glucose and glycine, MeIQx [48] and 7,8-Me_2IQx [49] are formed. Another mutagen, 4,8-Me_2IQx, can be formed by replacing glycine in the mixture with threonine [50,51]. These model reactions indicated the pos-

sible routes of formation of these mutagens in the cooking of food. In these studies, the blue cotton procedure was used in the isolation of the products.

4.2. Cigarette smoke and opium pyrolysate

Cigarette smoke intake is associated with human cancer development [52]. An association is known between the ingestion of opium pyrolysates and the incidence of oesophageal cancers in Iran (references cited in ref. 53). Mutagenic components in these plant pyrolysis products have been studied by use of blue cotton.

Yamashita and co-workers [54,55] subjected a basic fraction of cigarette smoke condensates to blue cotton extraction, and from the extract they isolated IQ by liquid chromatography. The mutagenicity of the IQ accounted for 1% of the activity of the basic fraction, which in turn corresponded to 42% of the activity of total cigarette condensates. The recovery of IQ during the whole process, including the blue cotton extraction and the chromatography, was estimated to be 70% by an experiment in which an authentic IQ was added to the smoke condensate. In addition to IQ, Trp-P-1, Trp-P-2, AαC and MeAαC were detected in cigarette smoke condensates, again by use of blue cotton [55].

A useful application of blue cotton was in the isolation and identification of new mutagens from opium pyrolysates [53]. In this study at the International Agency for Research on Cancer (Lyon, France), advantage was taken of the selective adsorbability of blue cotton for planar compounds. The prepared morphine pyrolysates were subjected to blue cotton extraction. As Fig. 8 shows, gas chromatographic analysis of morphine pyrolysate and its blue cotton extract indicated that a selective concentration of compounds I–IX took place. These compounds were further purified and identified as substituted hydroxyphenanthrenes having strong mutagenicities in bacteria. The structure of compound IX, which has the strongest activity among these hydroxyphenanthrenes, is also illustrated. It is remarkable that whereas morphine families, all retaining the morphine D-ring perpendicular to the phenanthrene ring system, are recovered only poorly by this extraction procedure, almost 100% of compounds I–IX, which are planar, are extractable.

4.3. Urines and faeces

Analysis of these excretions gives important information about the metabolisms of mutagens. The results of analysis may also provide a glimpse of the internal exposure of the human body to mutagens; there is a review on this subject [56]. Human excretions, especially faeces, are materials for the analysis of which the availability of a method with easy manipulation, such as blue cotton extraction, is obviously valuable.

In an early report [6], we showed that blue cotton extracts of smoker's urine are mutagenic in the *Salmonella* test on strain TA98 with metabolic activation, a result consistent with the known mutagenicity of smoker's urine [57]. The ease of manipulation has allowed time-course studies on urinary mutagenicity during smoking and no smoking sessions over a period of several days [58]. It was found that mutagenicity in the urine appears and disappears rapidly in response to the starting and stopping of cigarette smoking. In this study, a comparison was made of the efficiency of blue cotton, XAD-2 resin [57] and Sep-Pak C_{18} column [59] extraction methods. Among six individual after-smoking samples, five gave the highest responses with blue cotton and one gave the highest response with XAD-2 (Table 6). With blue cotton, mainly polycyclic mutagens are monitored, whereas with XAD-2, organic substances in general would have been included in the assay samples [57]. While the blue cotton method is biased in this sense, its high sensitivity is undoubtedly useful in a general survey of urinary mutagenicity.

Mohtashamipur *et al.* [60] reported that with either the blue cotton method or the proposed chloroform extraction procedure, a clear correlation can be found between urine mutagenicity and the number of cigarettes smoked. In contrast, with the use of XAD-2 chromatography, they were unable to find a clear dose response between the number of cigarettes smoked and the urinary mutagenicity.

Mutagenicity of smokers' urine can be demonstrated with high sensitivity by the use of blue rayon adsorption followed by assays on recently developed new *Salmonella* strains that are exceptionally sensitive towards aromatic amines and nitroaromatics [61].

Ingestion of foods containing mutagens may be expected to result in excretion of those mutagens.

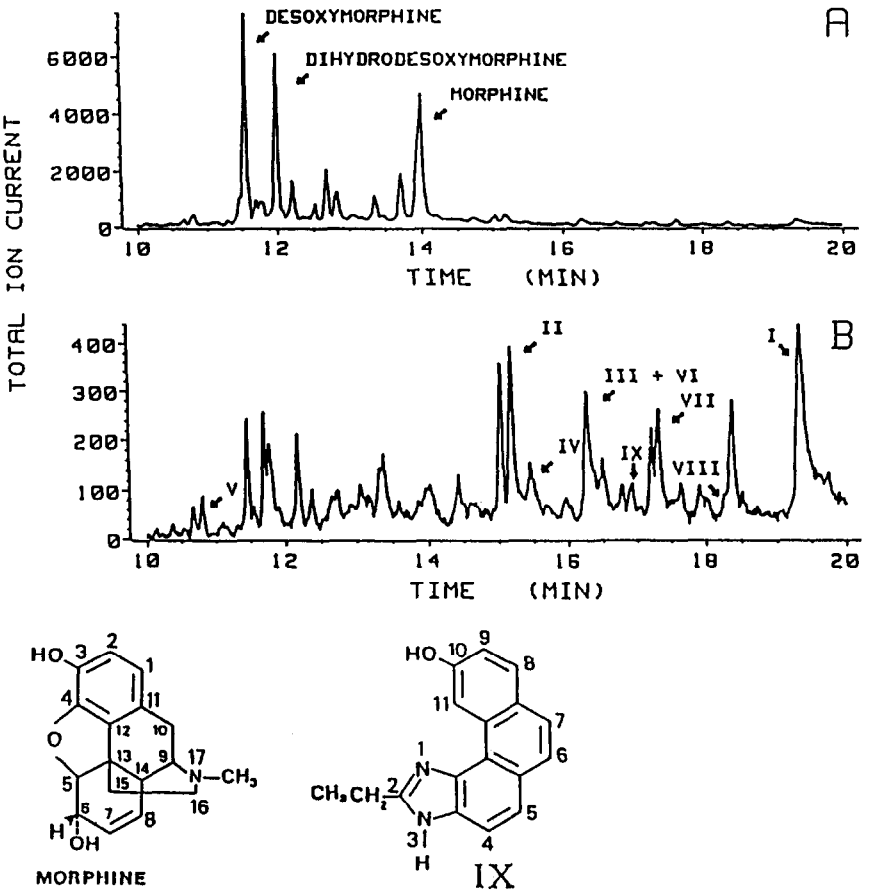


Fig. 8. Total ion current mass chromatograms of (A) morphine pyrolysate and (B) morphine pyrolysate blue cotton extract [53].

TABLE 6

COMPARISON OF EFFICIENCY OF METHODS FOR MONITORING URINARY MUTAGENICITY OF CIGARETTE SMOKERS [58][a]

Method	His^+ revertants per plate					
	HK-1[a]	HK-2[a]	HK-3[a]	SH[a]	YF[a]	TK[a]
Blue cotton	190	216	274	480	392	674
XAD-2 resin	30	175	491	386	158	497
Sep-Pak C_{18}	31	ND[b]	64	69	37	60

[a] Each urine sample (300 ml), which was collected in 1 day, was divided into three 100-ml portions, and the 100 ml were processed by one of the three methods. Assay was done on *S. typhimurium* TA98 with S9 mix. Solvent controls (dimethyl sulphoxide only) gave 32 ± 5 revertants per plate. HK was male, aged 22, smoking 10–20 cigarettes per day (the three samples were taken on different days); SH, aged 58, 20–30 cigarettes per day; YF, aged 56, 20–30 cigarettes per day; TK, aged 57, 20–30 cigarettes per day;

[b] ND = Not done.

We examined the mutagenicity of urines and faeces of humans to determine the effect of ingesting fried ground beef. Both urine and faeces became mutagenic in this feeding experiment [62–64] (Fig. 9). Analysis of the mutagenic fractions by HPLC indicated that the mutagenic compounds in urine and faeces may be mostly metabolites of MeIQx, although more recently MeIQx itself was found in human urine [65,66]. When rats were fed MeIQx and the urine and faeces of these rats were extracted with blue cotton and the mutagens were fractionated by HPLC, three mutagenic metabolites were isolated: 8-hydroxymethyl-IQx, N-acetylated MeIQx and N^3-demethyl MeIQx [67]. Bashir *et al.* [68] have shown that when IQ is treated with human faecal flora and the product is extracted by blue cotton, an oxidized IQ is obtainable, *i.e.*, 2-amino-3,6-dihydro-3-methyl-7*H*-imidazo[4,5-*f*]quinolin-7-one.

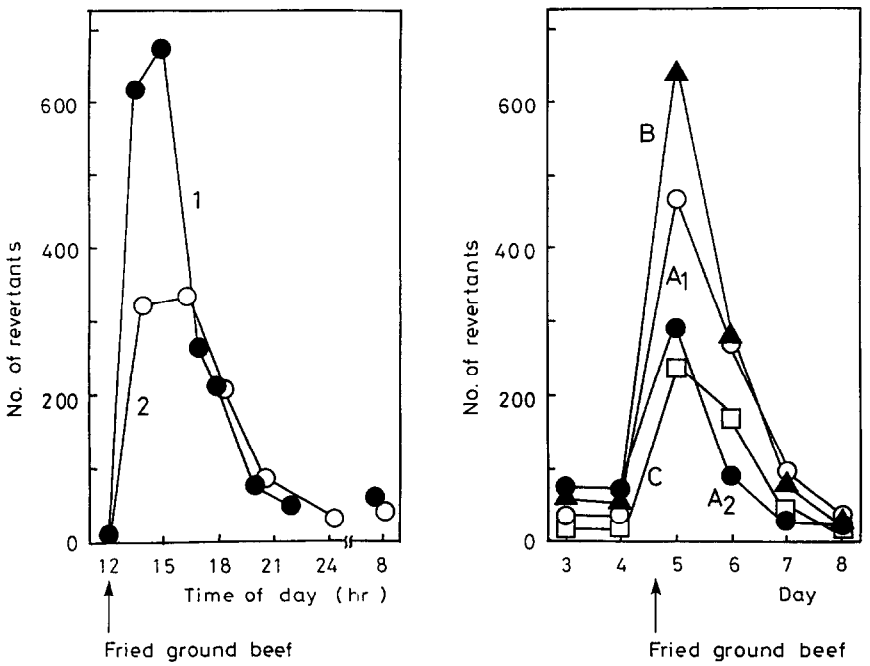


Fig. 9. Mutagenicity of human urine (left) and faeces (right) arising from ingestion of fried ground beef. Fried ground beef corresponding to 130–150 g of raw meat was eaten, and the blue cotton extracts of urine and faeces were assayed on *S. typhimurium* TA98 with metabolic activation ([62] urine; [63] faeces). The volunteers who participated in these studies were three adult male Japanese (1, 2 and A; A_1 and A_2 show results of two independent experiments with A), an adult female Japanese (B) and an adult male white American (C).

Blue cotton has also been used in studies on the metabolism of IQ in the rat [69].

More recently, it was shown, by use of blue rayon extraction, that the mutagenicity of urine and faeces in eleven human volunteers detectable after the intake of cooked beef could be diminished by administration of *Lactococcus acidophilus* fermented milk together with the meat [69a].

4.4. Body fluids and tissues

Trp-P-1 and Trp-P-2 were found in the dialysis fluid of patients with uraemia [70]. In this study, a large volume of the dialysis fluid (40 l) was subjected to blue cotton extraction and the mutagens in the extract were determined by HPLC. High recoveries (60–70%) were noted for both Trp-P-1 and Trp-P-2. Likewise, MeIQx has been detected in the blue cotton extract of dialysis fluid of uraemic patients [71]. Glu-P-1 and Glu-P-2 were found in the blue cotton extracts of human plasma [72]. Furthermore, Trp-P-1 and Trp-P-2 can be detected in normal human plasma [73]. In the urine of healthy humans, Trp-P-1, Trp-P-2, PhIP and MeIQx were found and determined by use of blue cotton extraction [66]. It is noteworthy that the level of MeIQx in the urine is much higher than that of other heterocyclic amines [66]. Glu-P-1 and Glu-P-2 are present in human cataractous lens [74].

4.5. Water and air

Monitoring of the mutagenicity in ambient water and air may be facilitated by the use of the blue cotton method.

Blue cotton is hung in river water and the mutagenicity is assayed in the methanol–ammonia eluate of the cotton collected after 1 day; in this way, water from the river Asahi of Okayama was examined, and the mutagenicity, as assayed on *S. typhimurium* TA98 with metabolic activation, was about 2000 revertants per gram of blue cotton near the factory area of the city and 40 revertants per gram at an upstream location [6].

Since this measurement was made, we have continued this type of work using several different sites

on the river to establish a standard procedure to monitor the mutagenicity of ambient waters. The following is the one we currently use [75]: (1) six or more batches of 0.5 g of blue rayon are placed in meshed nylon bags, together with some weights (usually stones); (2) the bags are hung in the water for 24 h; (3) the blue rayon is washed with distilled water and eluted with methanol–ammonia (50:1), and then, after drying, is weighed to confirm that there has been no loss of rayon during the hanging in water; (4) the mutagenicity is assayed with *S. typhimurium* TA98 and TA100, with and without metabolic activation. In the mutagenicity assay, a dose response (with respect to blue rayon equivalence) is usually measured.

By this method, we measured the mutagenicity of waters from the rivers Yodo, Katsura, Kitsu and Uji of Osaka, and found that at several locations the activities were very high (up to 4000 revertants in TA98, +S9, per 0.1 g equivalent of blue rayon). The study has shown that the source of the mutagenicity is the discharge from a sewage plant, at least four strongly mutagenic polycyclic substances are present in the discharged water and these mutagens appear to have continuously polluted the river for years [75].

In a follow-up study, a comparison was made of the efficiency of mutagen adsorption between the blue rayon technique and the conventional XAD-2 column method. It was demonstrated that blue rayon was much more efficient than XAD resin in recovering these river mutagens, in terms of both the total mutagenic activities and the activities per unit organic material recovered [76].

Recently, a survey of sea-water mutagenicity by use of the blue rayon technique was carried out in Galveston Bay, USA, and several locations near the industrial area were found to be mutagenic [77].

This simple monitoring would provide information as to where and when mutagenicity is present in a given water. With the currently practised analysis of river water, in which a large volume of water is taken out of the river and processed, the time and labour required to obtain data are enormous.

As already discussed in Section 2.2, blue cotton is decomposed by the action of chlorine, and therefore is not suitable for exposure to large amounts of tap water which contains chlorine. A trial of the use of blue cotton to examine the mutagenicity of tap water in Finland resulted in the detection of no positive activity [78].

Blue cotton is also useful in detecting the mutagenicity of ambient air. For measuring the mutagenicity of airborne particulates, a large volume of air is filtered through a glass-fibre filter and the particulates on the filter are extracted by sonication in organic solvents. Working on such samples collected in the city of Okayama, we have encountered many cases where linear dose responses were not obtained in the Ames assay; at higher doses extensive cell killings took place. By submitting the samples, suspended in water, to blue cotton extraction, we were able to remove the cytotoxic substances, and linear dose responses were then obtained [79]. Further, the substances that had masked the mutagenicity of the airborne particulates were identified as long-chain fatty acids, such as stearic and oleic acids [80].

4.6. Others

As described in the Section 4.5, a lipophilic sample, such as an air particulate extract, may be subjected to the blue cotton treatment by first preparing an aqueous suspension of the oily material. By using such a manipulation, the mutagenicity of used machine oils has been monitored, and several samples showed positive responses [81]. Alternatively, such oily materials may be extracted first with aqueous media, and then the extracts can be submitted to the blue cotton treatment.

5. OTHER APPLICATIONS

Ethidium bromide is widely used in biochemical laboratories as an agent for visualizing nucleic acids. As ethidium bromide is a powerful mutagen [82], its removal from wastes before disposal is desirable. The use of blue cotton for this purpose has been tested. Although blue cotton did adsorb ethidium from its aqueous solutions, the swelling of cotton with the solution precluded the practical use of the cotton for this particular purpose [83].

An interesting application of the blue cotton method was proposed by Povey and co-workers [84,85]. They demonstrated binding of benzo[*a*]pyrene metabolites in the rat intestinal rumen to magnetic polyethyleneimine microcapsules, and are investigating the use of copper phthalocyanine tetra-

sulphonate as an ingredient of the capsules, aiming at making the capsules act as a selective, planar-mutagen trap [4,86].

6. CHROMATOGRAPHY WITH COPPER PHTHALOCYANINE AS LIGAND

Now that the molecule of copper phthalocyanine has been revealed to possess a particular affinity to polycyclic aromatics, an obvious question is whether this molecule has any use in chromatography. So far, the use of copper phthalocyanine as a ligand in chromatography has been tested with cellulose powder, silica gel and aromatic resin as supports. Although these studies are still immature, this area of blue pigment application seems to have a promising future.

6.1. Chromatography on blue cellulose powder

An example of separating mutagens on a column of blue cellulose powder is shown in Fig. 10. In this model experiment, three mutagens having a low affinity (Carbadox), a moderate affinity (2-aminofluorene) and a high affinity (Trp-P-1) to the ligand were separated by stepwise elution. Twelve different compounds were examined for their affinity to the column and also to a control column of underivatized cellulose. As Table 7 indicates, several compounds having no affinity to cellulose were shown to be retained on the blue cellulose. Trp-P-1, ethidium bromide and 9-aminoacridine have some affinity to cellulose and they were strongly retained on the blue column. Blue cellulose column chromatography may thus be expected to be useful in separating polycyclic mutagenic compounds, although this aspect needs further studies.

6.2. High-performance liquid chromatography

For the preparation of silica gel bearing covalently bound copper phthalocyanine as a ligand, two

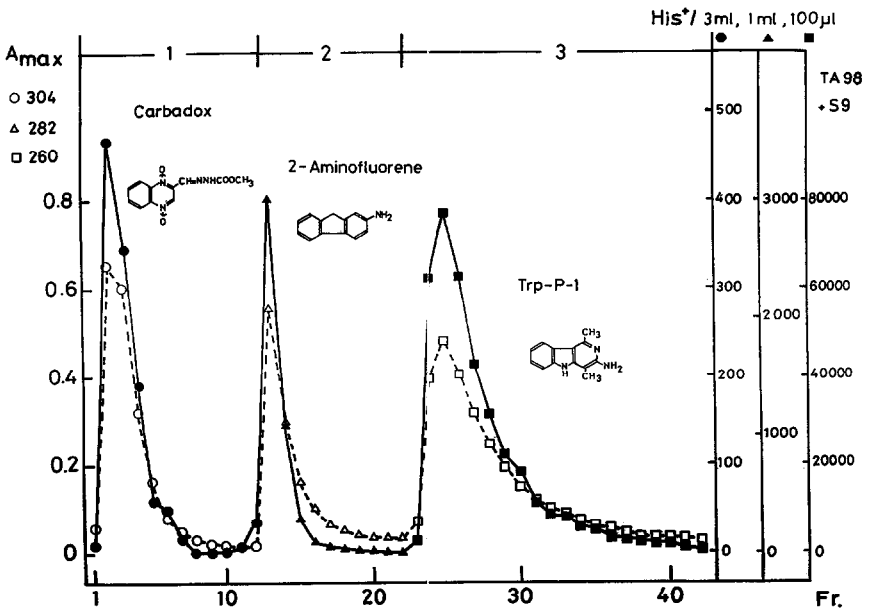


Fig. 10. Separation of mutagens on a blue cellulose column. Cellulose powder (Whatman CF11) was treated with Reactive Blue 21 to prepare blue cellulose bearing 9 µmol of pigment per gram, and the powder was packed in a tube to form a column (2.0 × 0.7 cm I.D.). The elution was done at a rate of 1 ml/min with 3 ml per fraction. The elution solvents were (1) water, (2) 50% aqueous methanol and (3) 50% methanol–concentrated ammonia (20:1). The elution sequence with these solvens is shown on the top of the figure. Compounds eluted from the column were determined by use of absorption spectra. Also, the mutagenic activity on *Salmonella* (TA98 with S9) was recorded. The solid symbols represent mutagenicity. The scales for the individual symbols are given on the right-hand side of the figure. The open symbols represent absorbance (the left-hand side scale) at the absorption-maximum wavelengths of the compound (304 nm for carbadox, 282 nm for 2-aminofluorene and 260 nm for Trp-P-1). The recoveries of the compounds were 94% for carbadox, 85% for 2-aminofluorene and 97% for Trp-P-1. The data presented here are from H. Kobayashi and H. Hayatsu (unpublished results).

TABLE 7

ELUTION OF COMPOUNDS FROM A BLUE CELLULOSE COLUMN

The column and the elution were as described in Fig. 10. The data are from H. Kobayashi and H. Hayatsu (unpublished results).

Elution solvent	Compound eluted	
	From blue cellulose	From cellulose
Water	Tryptophan, Carbadox, AF-2	Triptophan, Carbadox, AF-2, 2-aminofluorene, 2-acetylaminofluorene, Glu-P-1, IQ, MeIQ, MeIQx
50% Methanol	2-Aminofluorene, 2-acetylaminofluorene, Glu-P-1, IQ, MeIQ	9-Aminoacridine, Trp-P-1, ethidium bromide
50% Methanol–concentrated ammonia (20:2)	9-Aminoacridine, MeIQx, Trp-P-1, Ethidium bromide[a]	

[a] Eluted with methanol–concentrated HCl (100:1).

different procedures have been used, both employing aminopropylsilica gel as the starting material. In one procedure, Reactive Blue 21 and the aminoalkylsilica gel were reacted as in the preparation of blue cotton (unpublished work) and in the other procedure, copper phthalocyanine tetrasulphonyl chloride was reacted with the aminoalkylsilica gel [87]. Both procedures proved to be satisfactory.

As shown in Fig. 11A, benzene, napthalene, anthracene and phenanthrene were eluted in this order from the HPLC column of blue silica gel, whereas no retention took place on the HPLC column of underivatized aminopropylsilica gel [87]. Furthermore, *o*-terphenyl, in whose molecule the three benzene rings are not coplanar, was not retained, whereas triphenylene was retained by the column (Fig. 11B). As Fig. 12 shows, separation between compounds having similar structures can be achieved with the HPLC (unpublished work). The order of elution for each pair of compounds is in agreement with the affinity of the compounds towards blue cotton, *i.e.*, the stronger the affinity, the more slowly the compound was eluted.

These results show clearly the potential usefulness of blue silica gel in HPLC.

An anion-exchange silica gel with positively charged amino functions can be loaded with copper phthalocyanine tetrasulphonate to give a material on which the blue pigment is fixed with strong ionic

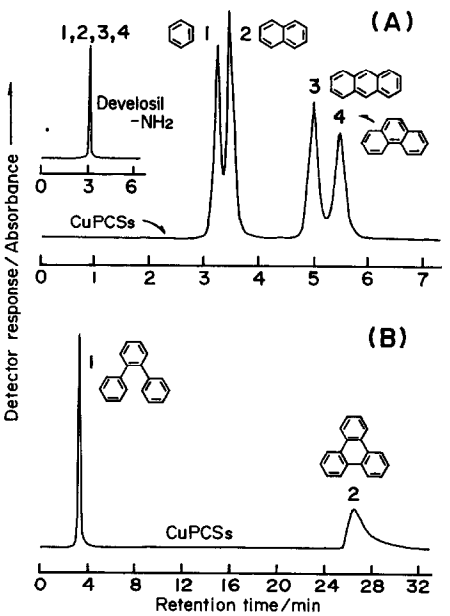


Fig. 11. HPLC on blue silica gel [87]. (A) 1, benzene; 2, naphthalene; 3, anthracene; 4, phenanthrene. (B) 1, *o*-terphenyl; 2, triphenylene. Results with an underivatized resin column are given on the upper left of the figure. The columns were prepared by packing into a stainless-steel tube (15 × 0.4 cm I.D.) Develosil-NH_2 (Nomura Kagaku, particle size 5 μm) that had been treated (or not treated, for the control experiment) with copper phthalocyanine tetrasulphonyl chloride (6 μmol/g). Elution was with methanol–water (8:2) at a flow-rate of 0.5 ml/min.

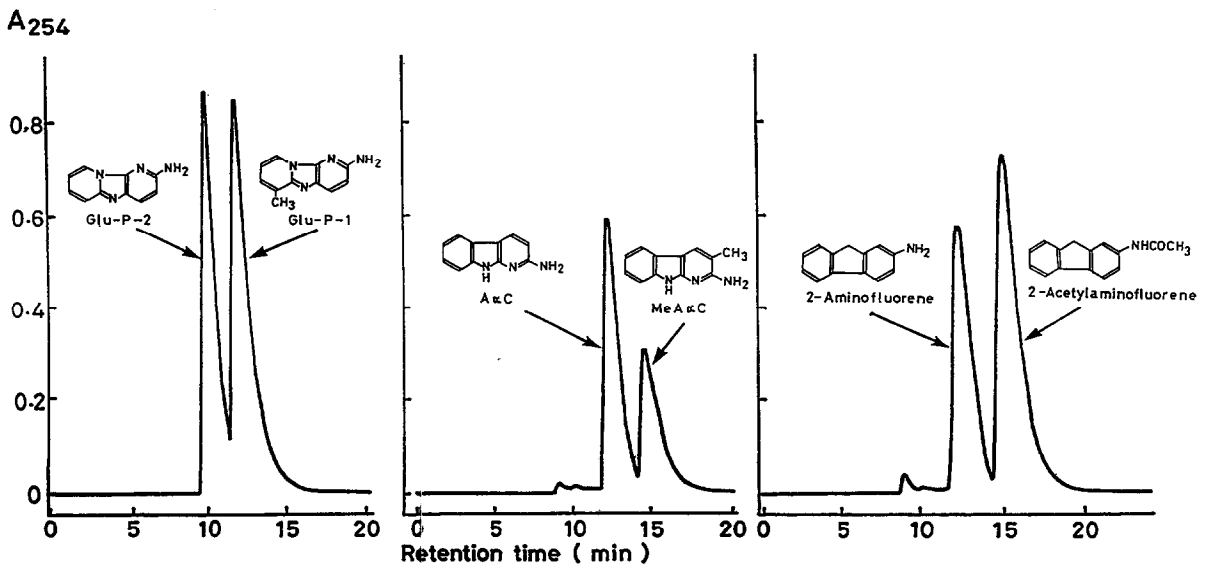


Fig. 12. HPLC of mutagens on blue silica gel. The gels were prepared from LiChrosorb-NH_2 by treatment with Reactive Blue 21. The blue gel having the ligand at 22 μmol/g was packed into a stainless-steel tube to form a column of 25 × 0.4 cm I.D. The elution of compounds was done with a linear gradient from 50% aqueous methanol to 100% acetonitrile for the first 10 min and then with 100% acetonitrile. The flow-rate was 1 ml/min. The data were taken from H. Kobayashi and H. Hayatsu (unpublished results).

interactions. This material was used in an HPLC column to explore the affinity of polycyclic aromatics to the column [88]. As Fig. 13 shows, retention took place and separation between four anthracene derivatives was observed, whereas with the control column no retention of these compounds was detected. These preliminary observations again show that this system appears to have promising potential for future development.

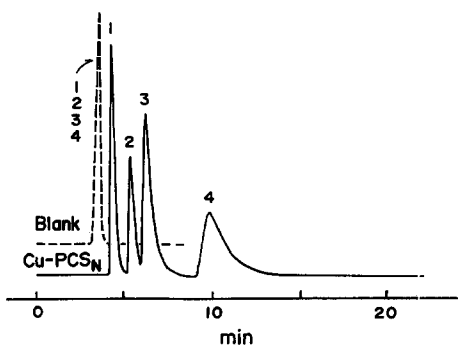


Fig. 13. Chromatography of anthracenes on blue silica gel [88]. Nucleosil 5SB bearing 25 μmol/g of copper phthalocyanine tetrasulphonate was used in a column of 15 × 0.4 cm I.D. for HPLC. Elution was with methanol at a flow-rate of 0.5 ml/min. For a blank, a column of Nucleosil 5SB alone was used. Peaks: 1 = 9-phenylanthracene; 2 = anthracene; 3 = 2-ethylanthracene; 4 = 9-methylanthracene.

7. CONCLUSIONS

The power of blue cotton resides in the ligand molecule copper phthalocyanine sulphonate, which is now revealed to have a strong affinity to polycyclic aromatics. However, the chemistry of this affinity has not yet been adequately studied. A systematic search for more powerful ligands seems important in view of the abundance in the environment of mutagens/carcinogens with polycyclic structures.

The development of this affinity principle in the field of chromatography is very important, but requires further work, as discussed in the preceding section.

8. ABBREVIATIONS

Trp-P-1 = 3-Amino-1,4-dimethyl-5*H*-pyrido-[4,3-*b*]indole; Trp-P-2 = 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole; Glu-P-1 = 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole; Glu-P-2 = 2-amino-dipyrido[1,2-*a*:3′,2′-*d*]imidazole; AαC = 2-ami-

no-9*H*-pyrido[2,3-*b*]indole; MeAαC = 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole; IQ = 2-amino-3-methylimidazo[4,5-*f*]quinoline; MeIQ = 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline; MeIQx = 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; 4,8-Me_2IQx = 2-amino-3,4,8-trimethylimidazo-[4,5-*f*]quinoxaline; 7,8-Me_2IQx = 2-amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline; PhIP = 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; MNU = N-methyl-N-nitrosourea.

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