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# STUDIES IN FUNGITOXICITY I.—Fungitoxicity of Certain Carbocyanines

By M. PIANKA and J. C. HALL

Thirty-five carbocyanines were examined for fungitoxicity and some were found to be active. This newly found biological property was limited to carbocyanines of a fairly low molecular weight, and was dependent on the heterocyclic base or bases from which the carbocyanine was derived. Alkyl substituents enhanced the fungitoxicity of the dyes irrespective of their position. Tests with some dyes showed that these penetrated to the protoplasts of the spores, staining them irreversibly.

## Introduction

Paul Ehrlich's early work lay in the field of dyes, which he tested for their potential chemotherapeutic properties. Sulphonamide drugs originated from dyestuffs chemistry, Prontosil (2 : 4-diaminoazobenzene-4'-sulphonamide) having been found active against streptococci in 1935.[1] In a series of papers published between 1924 and 1934, Browning and his collaborators[2, 3, 4] described the antiseptic properties of the dyes anil- and styryl-quinolines and cyanines, all of which contain the conjugated system

$$-\overset{+}{N}R{:}\overset{|}{C}-(CH{:}CH)_n-N<$$

This also occurs in the well-known antiseptic, acriflavine (in acriflavine $n = 1$, in carbocyanines $n = 2$). Some cyanines, e.g. 1 : 1'-dimethyl-6 : 6'-dimethoxycarbocyanine iodide[2] and some thiacarbocyanines[5] were found to inhibit greatly the growth of staphylococci.

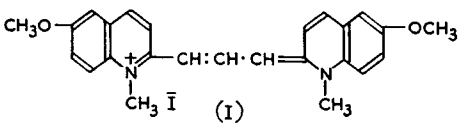
Ogata[6] found that neocyanines—trinuclear cyanines with two acidic radicals—had antileprotic properties. Other workers found that certain cyanine dyes possessed antifilarial activity.[7]

The above reported findings prompted us to submit certain carbocyanines to testing for fungitoxicity, with the hope of establishing some correlation between chemical structure and effect on fungal spores.

## Experimental

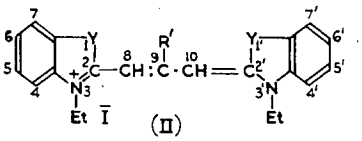
*Nomenclature of carbocyanine dyes*

Carbocyanines are cyanine dyes linked by a chain consisting of three methine groups (methine chain). Thus the previously mentioned 1 : 1'-dimethyl-6 : 6'-dimethoxycarbocyanine iodide has the formula **I.**




(I)

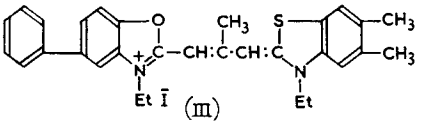
With other nuclei a suitable prefix is used, thus thia- denotes a benzthiazole, oxa- a benzoxazole, selena- a selenazole and thiazolo- a thiazole nucleus. The numbering used in this paper is based on the general scheme shown in **II**.



(II)

The symmetrical carbocyanine, in which Y = O, S or Se and R′ = Et is termed 3 : 9 : 3′-triethyloxa- or thia- or selena-carbocyanine iodide.

The unsymmetrical carbocyanine of the formula **III**



(III)

is termed 3 : 3′-diethyl-9 : 5′ : 6′-trimethyl-5-phenyloxathiacarbocyanine iodide.

*Preparation of the dyes*

The preparation of dye No. 2 (see Results) was by the method of Kendall & Fry ;[8] the preparation of the dyes Nos. 3–6, 11–16, 22–24, 28–29, 31–35 was described by Barany & Pianka.[9] Dye No. 7 was prepared by the method of Mills,[10] dye No. 25 by that of Clark ;[11] dyes Nos. 17–19 were synthesized by the methods of Brooker & White,[12] whose method[13] was also used for dye No. 16.

All the dyes were recrystallized twice from methanol.

3 : 3′-*Dimethyloxacarbocyanine iodide* (*Dye No.* 1)

2-Methylbenzoxazole (1·3 g.) and methyl toluene-*p*-sulphonate (1·9 g.) were kept at 140–150° for 4 h. Pyridine (6 c.c.) and ethyl orthoformate (6 c.c.) were added to the cooled reaction mixture, which was then kept for 6 h. at 120–140°. The hot solution was added, with vigorous stirring, to a solution of potassium iodide (6·5 g.) in water (32·5 c.c.) and alcohol (32·5 c.c.). The dye separated instantly. After 4 h. it was filtered and recrystallized. Purple-violet needles were obtained, melting at 274–275° (Found : N, 6·39. $C_{19}H_{17}O_2N_2I$ requires N, 6·48%).

3 : 3′-*Diethyl*-8-*methylthiacarbocyanine iodide* (*Dye No.* 8)

*Preparation of the intermediate 2-2′-acetanilidovinyl-3-ethylbenzthiazolium ethyl sulphate.*—2-Methylbenzthiazole (7·4 g.) and diethyl sulphate (8 g.) were heated at 140–150° for 30 min. The quaternary salt thus obtained was heated with *NN′*-diphenylformamidine (10 g.), acetic anhydride (5 c.c.) and glacial acetic acid (5 c.c.) for 3 h. in an oil-bath kept at 100°. The cooled reaction mixture was repeatedly triturated with methanol and ether (1 : 2). The yellow solid that separated was filtered, washed with ether and recrystallized from methanol. Yellow crystals, m.p. 200°, were obtained (Found : N, 6·20. $C_{21}H_{24}O_5N_2S_2$ requires N, 6·25%).

*Preparation of the dye.*—2-Ethylbenzthiazole (3·2 g.) and diethyl sulphate (2·3 c.c.) were heated at 150° for 20 min. To the cooled quaternary salt were added the above 2-2′-acetanilido-vinyl-3-ethylbenzthiazolium ethyl sulphate (2·8 g.) and pyridine (10 c.c.), and the mixture was heated at 120–140° for 1 h. The dye was then converted to its iodide, as with the previous dye. On recrystallization, dark green crystals, m.p. 254°, were obtained (Found : N, 5·71. $C_{22}H_{23}N_2S_2I$ requires N, 5·53%).

3 : 3′-*Diethyl*-8 : 10-*dimethylthiacarbocyanine iodide* (*Dye No.* 9)

2-Ethylbenzthiazole (3·26 g.) and diethyl sulphate (2·3 c.c.) were heated at 150° for 20 min. Pyridine (7 c.c.) was added and then ethyl orthoformate (2·7 c.c.), and the mixture was heated at 140–150° for 1 h. On pouring into excess of 10% aqueous potassium iodide, an oil separated.

This was well washed with water, dissolved in hot methanol and poured again into excess of 10% aqueous potassium iodide. After 24 h. at 0° a very small amount of solid separated, which was filtered and recrystallized. A black microcrystalline powder, m.p. 241–242°, was obtained (Found: N, 5·30. $C_{23}H_{25}N_2S_2I$ requires N, 5·38%).

3 : 3′-*Diethyl*-8 : 9 : 10-*trimethylthiacarbocyanine iodide* (*Dye No.* 10)

The conditions were as described above, except that ethyl orthoacetate (3·4 c.c.) was used instead of ethyl orthoformate. The condensation to the dye took 3 h. A minute amount of solid separated after 48 h. at 0°; it was filtered and recrystallized from methanol and a little ether. Dark purple crystals, m.p. 233–234°, were obtained (Found: N, 5·21. $C_{24}H_{27}N_2S_2I$ requires N, 5·24%).

When ethyl orthopropionate (3·2 c.c.) was used instead of ethyl orthoacetate, no 3 : 9 : 3′-triethyl-8 : 10-dimethylthiacarbocyanine iodide could be recovered.

3 : 3′-*Diethyl*-4 : 5 : 4′ : 5′-*dibenzthiacarbocyanine iodide* (*Dye No.* 20)

This dye was prepared by the method of Brooker & White,[12] but the dye was converted to the iodide instead of the bromide. On recrystallization, a dark blue powder, m.p. 238°, was obtained.

3 : 3′-*Diethyl*-9-*methyl*-4 : 5 : 4′ : 5′-*dibenzthiacarbocyanine bromide* (*Dye No.* 21)

2-Methyl-β-naphthathiazole (16·5 g.) and ethyl toluene-*p*-sulphonate (16·5 g.) were heated at 180° for 5 h. A solid was obtained. The 3-ethyl-2-methyl-β-naphthathiazolium toluene-*p*-sulphonate (6 g.), pyridine (25 c.c.) and ethyl orthoacetate (5·5 c.c.) were heated at 120–140° for 3 h. The dye was converted to the bromide.[12] On recrystallization a dark purple powder was obtained, m.p. 236–238°.

3 : 9 : 3′-*Triethylselenacarbocyanine iodide* (*Dye No.* 27)

2-Methylbenzselenazole (4·5 g.) and diethyl sulphate (3·1 c.c.) were heated at 160° for 15 min. Pyridine (16 c.c.) and ethyl orthopropionate (2·8 c.c.) were added and the mixture was heated at 120–140° for 2 h. The dye was converted to the iodide. On recrystallization an olive-green powder was obtained, m.p. 234–237° (softens at 140°) (Brooker & White[12] gave m.p. 146–148°; U.S.P. 2,378,783 reported m.p. 210–211°).

3 : 9 : 3′-*Triethyl*-5′ : 6′-*dimethyl*-5-*phenyloxathiacarbocyanine iodide* (*Dye No.* 30)

*Preparation of the intermediate* 2-(2-*anilinobut*-1-*enyl*)-3-*ethyl*-5-*phenylbenzoxazolium ethyl sulphate.*—2-Methyl-5-phenylbenzoxazole (6 g.) and diethyl sulphate (4·5 g.) were heated at 160° for 20 min. Ethyl *N*-phenylthiolpropionimidate (6·1 c.c.) was then added and the mixture was heated in an oil-bath at 170° for 30 min. The mixture was repeatedly triturated with methanol and ether (1 : 1). The solid that separated was filtered off, washed with ether until the washings were colourless, and recrystallized twice from methanol. Yellow crystals, m.p. 197°, were obtained. On keeping, the solid became gummy and turned to a mass of glass-like appearance.

*Preparation of the intermediate* 3-*ethyl*-2 : 6-*dimethylbenzthiazolium iodide*

2 : 6-Dimethylbenzthiazole (11·1 g.) and ethyl iodide (6·3 c.c.) were heated at 100° in a closed tube for 48 h. The contents of the tube were ground with excess of acetone, filtered, washed with acetone and then with ether and recrystallized from ethanol. Crystals, m.p. 157–158°, were obtained.

*Preparation of the dye.*—The above-mentioned freshly prepared anilinobutenyl compound (1·85 g.), 3-ethyl-2 : 6-dimethylbenzthiazolium iodide (1·7 g.) and pyridine (15 c.c.) were heated at 130° for 2 h. The dye was converted to the iodide and recrystallized. Plum-red crystals, m.p. 360°, were obtained (Found: N, 4·87. $C_{31}H_{33}ON_2SI$ requires N, 4·61%).

*Fungitoxicity tests*

All the tests were carried out by the well-established Montgomery–Moore[14] slide germination technique: 0·015 c.c. of a solution of a dye in methanol at the required concentration was spread

uniformly over circular areas, 15 mm. across, delimited on 3 × 1 in. microscope slides. The methanol was allowed to evaporate. To the dry slides 0·04 c.c. of a suspension of spores, diluted to contain 15–20 spores per low-power field of the microscope ($\frac{2}{3}$ in. objective, × 10 eye-piece), was applied to the treated circular areas. The slides were then placed in moist Petri dishes and kept in an incubator at 21° for 20 h. Counts for relative percentage germination of the spores were then carried out. The concentrations of the dyes required to kill 95% of the spores ($LD_{95}$) were obtained by plotting probits of percentage mortality against the appropriate log concentration values. Goodness of fit was checked by means of the $\chi^2$ test. Tests were repeated a sufficient number of times until reproducible results were obtained. The average $LD_{95}$ values are reported for the following fungi: *Venturia inaequalis* (Cooke) Wint.; *Botrytis cinerea* Pers.; and *Fusarium bulbigenum*, Cooke and Massee, var. *lycopersici* (Brushi) Wollenw. ($LD_{95}$ values were chosen as they give greater critical difference in activity levels than $LD_{50}$ when no slope figures are appended.)

## Results

Table I shows the results of fungitoxicity tests on 35 cyanine dyes prepared by methods described above or in the literature.

*Imbibition of the dyes by spores from aqueous solutions*

Two simple dyes, Nos. 1 and 7, were dissolved in distilled water and to the coloured solution a suspension of spores of *Botrytis* was added. After setting aside for 20 h., the spores were washed repeatedly by centrifuging with distilled water. Under the microscope, the protoplasts of the spores were found to be deeply stained, whereas spores devoid of cell contents were either colourless or only very faintly coloured. The protoplasts of the germ tubes of those spores that had germinated in sub-lethal concentrations of the dyes were also found to be stained.

## Conclusions

The following points on the relationship between fungitoxicity and chemical structure of carbocyanines emerge from a study of the results reported above:

(*a*) Only changes in the cationic part of the carbocyanine have an effect on the activity; as far as one can judge from the limited results available, the anion does not appear to influence the activity.

(*b*) Alkyl substituents appear to enhance the activity of the carbocyanines irrespective of their position in the methine chain or the nucleus. Thus 3 : 3′-diethylthiacarbocyanine iodide (No. 7) is inactive, whereas the 8-methyl-, 8 : 10-dimethyl-, 8 : 9 : 10-trimethyl-, 6 : 6′-dimethyl-, 6 : 9 : 6′-trimethyl- (Nos. 8–12), 5 : 6 : 5′ : 6′-tetramethyl- and 5 : 6 : 9 : 5′ : 6′-pentamethyl-derivatives (Nos. 14 and 15) are all active. Among the oxacarbocyanines the 5 : 6 : 5′ : 6′-tetramethyl- and 5 : 6 : 9 : 5′ : 6′-pentamethyl-derivatives (Nos. 5 and 6) are highly active. Also in the unsymmetrical oxathia- (Dyes Nos. 28 and 29), oxaselena- (Dyes Nos. 31 and 32) and selenathia-carbocyanines (Dyes Nos. 33–35), alkyl substitution enhances the activity of the parent compounds. Sen & Joshi[15] also observed that alkyl substitution generally increased toxicity.

(*c*) Oxacarbocyanines are more active than the corresponding thia- and selena-carbocyanines. Katz[16] also found that derivatives of 2-hydrazinobenzoxazole possessed marked, whereas the benzthiazole analogues had little, antifungal activity.

(*d*) Dibenzthiacarbocyanines and tetraphenylthiazolocarbocyanine (Dye No. 24) are inactive. In general, increase in molecular weight and in the bulk of the molecule causes a decrease in biological activity. Thus even alkylated 3 : 3′-diethyl-4 : 5-4′ : 5′-dibenz- and -6 : 7-6′ : 7′-dibenzthiacarbocyanines (Dyes Nos. 18, 19 and 21) are inactive, though alkylation rendered the inactive 3 : 3′-diethylthiacarbocyanine iodide very active. Also 3 : 3′-diethyl-5 : 5′-diphenyl-oxacarbocyanine iodide is much less active than its parent compound. Edgerton & Burckhalter,[17] who studied the amoebicidal activity of derivatives of 8-hydroxyquinoline, itself fungicidal, also observed a decrease in the activity with increasing molecular weight; and Sen & Joshi[15] observed that higher ether-esters of cresotinic acid were much less fungicidal than the lower ones.

(*e*) Selenacarbocyanines are fairly fungitoxic.

## Table I

*Results of fungitoxicity tests*

| No. of dye | Name of dye | M.p. | *Venturia* $LD_{95}$ values, p.p.m. | *Botrytis* $LD_{95}$ values, p.p.m. | *Fusarium* $LD_{95}$ values, p.p.m. |
|---|---|---|---|---|---|
| | *Oxacarbocyanine iodide* | | | | |
| 1 | 3 : 3′-Dimethyl- | 274–275° | 50 | 100 | 30 |
| 2 | 3 : 3′-Dimethyl-9-(*p*-toluidino)- | 251–253° | 50 | 55 | 35 |
| 3 | 3 : 3′-Diethyl-5 : 5′-diphenyl- | 240° | 260 | 325 | 90 |
| 4 | 3 : 3′-Diethyl-9-methyl-5 : 5′-diphenyl- | 273–274° | 150 | 160 | 100 |
| 5 | 3 : 3′-Diethyl-5 : 6 : 5′ : 6′-tetramethyl- | 280–281° | 25 | 30 | 25 |
| 6 | 3 : 3′-Diethyl-5 : 6 : 9 : 5′ : 6′-pentamethyl- | 253–256° | 20 | 25 | 20 |
| | *Thiacarbocyanine iodide* | | | | |
| 7 | 3 : 3′-Diethyl- | 269°(d.) | >1000 | >1000 | >1000 |
| 8 | 3 : 3′-Diethyl-8-methyl- | 254° | 60 | 75 | 65 |
| 9 | 3 : 3′-Diethyl-8 : 10-dimethyl- | 241–242° | 75 | 70 | 75 |
| 10 | 3 : 3′-Diethyl-8 : 9 : 10-trimethyl- | 233–234° | 80 | 80 | 70 |
| 11 | 3 : 3′-Diethyl-6 : 6′-dimethyl- | 275–276° | 35 | 85 | 45 |
| 12 | 3 : 3′-Diethyl-6 : 9 : 6′-trimethyl- | 274° | 185 | 110 | 90 |
| 13 | 3 : 9 : 3′-Triethyl-6 : 6′-dimethyl- | 245–247° | 25 | 30 | 40 |
| 14 | 3 : 3′-Diethyl-5 : 6 : 5′ : 6′-tetramethyl- | 265–267° | 70 | 65 | 45 |
| 15 | 3 : 3′-Diethyl-5 : 6 : 9 : 5′ : 6′-pentamethyl- | 275–276° | 65 | 75 | 65 |
| 16 | 3 : 3′ : 9-Triethyl-5 : 6: 5 ′ : 6′-tetramethyl- | 254–255° | 190 | 110 | 775 |
| | *Thiacarbocyanine bromide* | | | | |
| 17 | 3 : 3′-Diethyl-6 : 7-6′ : 7′-dibenz- | [278–280°(d.) | >1000 | >1000 | >1000 |
| 18 | 3 : 3′-Diethyl-9-methyl-6 : 7-6′ : 7′-dibenz- | 267–268°* | >1000 | >1000 | >1000 |
| 19 | 3 : 3′ : 9-Triethyl-6 : 7-6′ : 7′-dibenz- | 290–292°† | >1000 | >1000 | >1000 |
| 20 | 3 : 3′-Diethyl-4 : 5-4′ : 5′-dibenz-(iodide) | 238° | >1000 | >1000 | >1000 |
| 21 | 3 : 3′-Diethyl-9-methyl-4 : 5-4′ : 5′-dibenz- | 236–238° | >1000 | >1000 | >1000 |
| | *Thiacarbocyanide iodide* | | | | |
| 22 | 3 : 3′-Diethyl-6-methyl-4′ : 5′-benz- | 216° | 460 | 150 | 770 |
| 23 | 3 : 3′-Diethyl-6 : 9-dimethyl-4′ : 5′-benz- | 252–254° | 250 | 140 | 140 |
| 24 | 3 : 3′-Diethyl-4 : 5 : 4′ : 5′-tetraphenyl-thiazolocarbocyanine iodide | 247–250° | >1000 | >1000 | >1000 |
| | *Selenacarbocyanine iodide* | | | | |
| 25 | 3 : 3′-Diethyl- | 276–277°‡ | 115 | 105 | 100 |
| 26 | 3 : 3′-Diethyl-9-methyl- | 292–293° | 460 | >1000 | >1000 |
| 27 | 3 : 9 : 3′-Triethyl- | 234–237° | 130 | 145 | 150 |
| | *Oxathiacarbocyanine iodide* | | | | |
| 28 | 3 : 3′-Diethyl-5′ : 6′-dimethyl-5-phenyl- | 270° | 110 | 125 | 80 |
| 29 | 3 : 3′-Diethyl-9 : 5′ : 6′-trimethyl-5-phenyl-(perchlorate) | 250–253° | 90 | 95 | 70 |
| 30 | 3 : 9 : 3′-Triethyl-5′ : 6′-dimethyl-5-phenyl- | 360° | 105 | 105 | 160 |
| | *Oxaselenacarbocyanine iodide* | | | | |
| 31 | 3 : 3′-Diethyl-5′-methoxy-5-phenyl- | 262° | 425 | 170 | 500 |
| 32 | 3 : 3′-Diethyl-9-methyl-5′-methoxy-5-phenyl- | 247° | 235 | 150 | 180 |
| | *Selenathiacarbocyanine iodide* | | | | |
| 33 | 3 : 3′-Diethyl-5-methoxyselena-4′ : 5′-benzthiacarbocyanine | 237–238° | >1000 | >1000 | >1000 |
| 34 | 3 : 3′-Diethyl-9-methyl-5-methoxyselena-4′ : 5′-benzthiacarbocyanine | 227–228°(d.) | >1000 | 100 | 150 |
| 35 | 3 : 9- 3′ :Triethyl-5-methoxyselena-4′ : 5′-benzthiacarbocyanine | 210–213°(d.) | 135 | 90 | 125 |

* Brooker & White[12] reported m.p. 261°(d.)
† *idem, ibid.*, reported m.p. 296°(d.)
‡ Clark[11] reported m.p. 270–271°(d.)
(d.) = melting with decomposition

(*f*) In general, the activity of the unsymmetrical dyes lies midway between those of the symmetrical parent dyes. It follows, therefore, that the activity is a function of the properties of the terminal heterocycles from which the dye is derived rather than of the shape of the dye.

As has been seen, the dyes were irreversibly adsorbed by the spores, irrespective of their activity. In consequence, the differences in activity of the dyes cannot be ascribed to their powers of penetration. Thus the explanation for their mode of action has to be sought in terms of interference with the vital processes connected with the metabolism of the protoplast.

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# A RAPID METHOD FOR THE DETERMINATION OF LACTOSE IN MILK AND CHEESE

By A. JOHN G. BARNETT and G. ABDEL TAWAB

A colorimetric method is described for the determination of lactose in milk and cheese. It appears to be both speedy and accurate, as is evidenced by the facts that a lactose determination in milk takes about 15 min. to complete, while suitably designed experiments indicate a satisfactory degree of recovery of lactose. As illustrations of the potential uses of the method, figures are given for the lactose content of different types of cheese at different stages of maturity and for milk undergoing the process of souring.

## Introduction

During the course of a study of the ripening of some locally-made type cheeses, it became necessary to measure the lactose contents of the different samples. Official methods[1] are somewhat laborious and, as far as the authors are aware, there has been only one attempt in recent years to evolve a reliable method capable of giving results at both routine and research levels. This is the method suggested by Fagan, Sibbach & Hussong,[2] in which anthrone, a reagent specific for carbohydrates, is used in sulphuric acid solution. Use has been made of the anthrone method in these laboratories for the determination of soluble carbohydrates in grass and silage,[3] but as the reagent does not keep for any length of time and as the technique involves heating and cooling procedures, attention was turned to the method of Dubois *et al.*[4] which has been used satisfactorily here for similar purposes.[5] The phenol–sulphuric acid reagent appears to be quite specific for carbohydrates, and equivalent amounts of different carbohydrates, under the same experimental conditions, give very similar colorimetric readings. As the purpose of the present work was concerned with the carbohydrates in milk and cheese, the results described below were obtained with the use of lactose as a standard. It has been possible to show that