

151. *Vital Stains. Part I.*

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Vital stains of the trypan-blue type are selectively deposited in tumour tissue when administered parenterally to tumour-bearing animals. A series of such stains has been synthesised bearing iodine or arsenic as a substituent part of the molecule in order to utilise the dye as a carrier of iodine or arsenic into the site of the tumour, where these elements may exert some therapeutic action.

THE intravital staining of malignant neoplasms as a therapeutic measure has been studied with a number of dyes in both animals and man, although in general the results have been negative as far as retardation of tumour growth is concerned (Weil, *J. Cancer Research*, 1916, **1**, 95; Marsh and Simpson, *ibid.*, 1928, **11**, 417; Engel, *Z. Krebsforsch.*, 1925, **22**, 365; Munck, *ibid.*, 1928, **26**, 377; Glasunow, *Z. Zellforsch.*, 1928, **6**, 773). This property of selective intravital deposition is possessed by trypan-blue to a high degree: after injection of the dye into a tumour-bearing animal the tumour stroma becomes heavily stained and there is a general deposition in the reticulo-endothelial cells (Ludford, *Proc. Roy. Soc.*, 1929, **B**, **104**, 493). Evans-blue—an analogue of trypan-blue—when administered intravenously to mice and rabbits bearing spontaneous or implanted tumours, produces general staining with very marked selective deposition of the dye in and about the neoplasms and their metastases; although the dye does not penetrate the cancer cells themselves, the necrotic areas become uniformly stained and there is an abundant segregation of the dye in the fibroblasts and macrophages of the stroma (Duran-Reynals, *Amer. J. Cancer*, 1939, **35**, 98). Similar observations on the intravital activity of isamine-blue and Evans-blue in tumour-bearing humans have been made respectively by Bernhardt (*Z. Krebsforsch.*, 1928, **27**, 221) and Brunschwig, Clark, and Schmitz (*Arch. Path.*, 1940, **30**, 902).

It was therefore of interest to examine the intravital activity of a series of dyes of the trypan-blue type bearing iodine or arsenic as a constituent part of the molecule; provided there was no impairment in the selective substantivity of the dye molecule, the latter would be used as a carrier of iodine or arsenic into the site of the tumour, where these elements may exert some therapeutic action.

The dyes synthesised were of the extended trypan-blue type containing iodine in the terminal and/or the central components; in the case of the arsenical dyes the terminal components alone were arsenated. In general an iodinated arylamine (terminal component) was diazotised and coupled with a heteronuclear aminonaphtholdisulphonic acid in mineral acid solution in the *o*-position to the amino-group; two molecules of this monoazo-dye were then coupled in alkaline solution with a tetrazotised diaryldiamine (central component) in the *o*-position to the hydroxyl groups.

5-Iodo-*o*-toluidine was a suitable terminal component; arylamines containing two iodine substituents, such as the di-iodotoluidines, were too sparingly soluble for the purpose, since even when dissolved in cold sulphuric acid and diazotised with nitrosylsulphonic acid it was not possible to attain the physical conditions necessary for coupling. Introduction of solubilising groups, however, overcame this difficulty, since 2:6-di-iodosulphanilic, 2:6-di-iodoarsanilic, and 4:6-di-iodoanthranilic acids diazotised and coupled smoothly in mineral acid solution with the aminonaphtholdisulphonic acid.

For the central component benzidine, *o*-toluidine, and dianisidine were employed, giving a series of vital stains of the extended trypan-blue type bearing iodine in the terminal components alone. In order to obtain dyes carrying iodine in the central component it was necessary to obtain iodinated derivatives of benzidine. The iodination of benzidine with iodine or iodine monochloride gave products which could not be obtained pure and were too sparingly soluble for the purpose in mind. Attempts to prepare a heteronuclear di-iodobenzidine of known orientation by reduction of *m*-iodonitrobenzene to the corresponding hydrazo-compound, followed by a benzidine transformation, were not successful. Benzidine-2:2'-disulphonic acid, however, was iodinated smoothly with iodine monochloride to yield a soluble di-iodobenzidinedisulphonic acid; this resisted further iodination and in consequence it seems probable that the iodine occupies the 5:5'-positions and that entrance of iodine into the 3:3'-positions is sterically prevented.

Iodination of 4:4'-diaminodiphenylmethane yielded a *di-iodo*- and a tetraiodo-derivative, presumably the 3:3'- and the 3:3':5:5'-compound, respectively. The former is sufficiently soluble for tetrazotisation and coupling; dyes prepared from this central component, however, are not strictly of the trypan-blue type.

Physiological work carried out by Drs. Pick, Scholl, and Wiesner (to be published elsewhere) with some of these vital stains shows that the presence of iodine does not impair in any way the property of selective deposition in tumour tissue which appears to be characteristic of all dyes of the trypan-blue structure. Doses of 0.75 g./kg. per day of the iodinated vital stains A and B (pp. 715 and 716) may be administered subcutaneously to mice on 3 consecutive days without toxic symptoms arising. In addition to general staining (blue with A and red-purple with B) there is a rapid segregation of stain in the cells of the reticulo-endothelial system; the dye accumulates in the kidney cortex, liver, spleen and testes of normal mice, and with mice bearing implanted tumours of the Crocker and Ehrlich type there is marked localisation of the dye in those parts of the tumour where the tumour cells penetrate into the muscular and connective tissue of the host.

EXPERIMENTAL.

5-Iodo-*o*-toluidine Hydrochloride (compare Wheeler and Liddle, *Amer. Chem. J.*, 1909, **42**, 498).—Iodine (60 g.) and precipitated calcium carbonate (60 g.) were ground together and added to a mixture of *o*-toluidine (30 g.), ether (75 c.c.), and water (45 c.c.), which was then refluxed, with shaking at intervals, for 4 hrs. Next day water was added, the unchanged toluidine distilled in steam, and the residual calcium carbonate and 5-iodo-*o*-toluidine collected and extracted with boiling alcohol. On evaporation of the alcohol (charcoal) the iodotoluidine crystallised in purple needles (55 g.), which were dissolved in the minimal amount of dilute hydrochloric acid (1:6) and filtered to remove any di-iodotoluidine, the hydrochloride of which was almost insoluble. The filtrate (charcoal) was chilled, and hydrochloric acid (50 c.c.) added; the iodotoluidine hydrochloride then separated in almost white needles (42 g.), m. p. 214—216°.

Sodium 2-(5'-Iodo-*o*-tolueneazo)-1-amino-8-naphthol-3:6-disulphonate.—A fine suspension of 5-iodo-*o*-toluidine hydrochloride (26 g.) in water (380 c.c.) and 10*N*-hydrochloric acid (60 c.c.) was diazotised just below 0° with sodium nitrite (7.5 g. in the minimum amount of water); after 30 minutes' rapid stirring the diazonium chloride formed a thin brown suspension. An ice-cold solution of sodium hydrogen 1-amino-8-naphthol-3:6-disulphonate (36 g.) in water (150 c.c.)

and 5N-sodium hydroxide (20 c.c.) was added slowly, and stirring continued for 5 hrs. at 0—5°, any tendency to frothing being suppressed by the addition of a few drops of octyl alcohol. 5N-Sodium hydroxide (40 c.c.) was added, and the dark red liquid kept for 12 hrs. and then warmed at 40° for 1 hr. in order to complete the coupling. 5N-Sodium hydroxide (40 c.c.) was again added, the mixture chilled in ice-salt, and the dark precipitate removed, and dissolved in boiling water (210 c.c.) and the theoretical amount of sodium hydroxide, and reprecipitated in the cold with a small excess of dilute sulphuric acid as the sodium hydrogen salt; on drying at a low temperature the monoazo-dye was obtained as a maroon-black microcrystalline powder (25 g.). For analysis it was converted into the *disodium* salt and recrystallised four times (Found: I, 20.3. $C_{17}H_{12}O_7N_3I_2S_2Na_2$ requires I, 20.9%). The disodium salt is very soluble in water with production of a deep red solution.

Sodium 3:3'-Dimethyldiphenyl-4:4'-bis-[2''-azo-8''-amino-1''-hydroxy-3'':6''-disulphonaphthalene-7''-(5'''-iodo-*o*-azotoluene)] (A).—*o*-Tolidine (4.24 g.) was kept in water (120 c.c.) and 10N-hydrochloric acid (18 c.c.) for several hours to facilitate subsequent dissolution, then warmed with rapid stirring and quickly cooled below 0°; the fine suspension of the hydrochloride thus obtained was tetrazotised with a cold concentrated solution of sodium nitrite (2.4 g.). After 15 mins., the solution was added slowly to a rapidly stirred, ice-cold solution of the foregoing monoazo-dye (24 g.) in water (280 c.c.) and 5N-sodium hydroxide (100 c.c.). The mixture, which gradually became deep purple was stirred at a low temperature for 6 hrs., kept overnight, warmed at 50° for 2 hrs., and chilled; the tetrakisazo-dye, precipitated by cold hydrochloric acid (60 c.c.), was collected, dried at a low temperature, and obtained as a blue-black powder (17 g.). For analysis it was precipitated from a cold solution in water (200 c.c.) and 5N-sodium hydroxide (10 c.c.) with dilute sulphuric acid and converted into the *tetrasodium* salt, which was dialysed and recrystallised four times (Found: I, 18.0; Na, 6.7. $C_{48}H_{34}O_{14}N_{10}I_2S_4Na_4$ requires I, 17.5, Na, 6.35%). The tetrasodium salt is very soluble in water, giving a fine purple solution.

The benzidine and dianisidine analogues of the above tetrakisazo-dye were similarly prepared.

2:6-Di-iodoarsanilic Acid (compare Berthelm, *Ber.*, 1910, **43**, 535).—Potassium iodide (35 g.) in water (100 c.c.) was added during 30 mins. to a warm solution of arsanilic acid (11 g.), potassium iodate (10 g.), and sulphuric acid (35 c.c.) in water (500 c.c.); the white precipitate formed slowly turned dark brown. The mixture was heated, with frequent shaking, on the water-bath for 3 hours and cooled, the dark residue extracted with warm dilute sodium hydroxide solution, and the insoluble tri-iodoaniline (5 g.) removed. Sodium sulphite (*ca.* 1 g.) was added to the filtrate and then an excess of hydrochloric acid; the di-iodoarsanilic acid was precipitated as a white flocculent mass (12 g.) (Found in material recrystallised from dilute alcohol: I, 54.3. Calc.: I, 54.1%).

Sodium 2-(2':6'-Di-iodo-4'-*o*-arsonobenzeneazo)-1-amino-8-naphthol-3:6-disulphonate.—To a strongly cooled solution of di-iodoarsanilic acid (18.8 g.) in water (200 c.c.) and 5N-sodium hydroxide (10 c.c.), sodium nitrite (3.0 g., in a small amount of water) was added, followed dropwise, with stirring, by 10N-hydrochloric acid (25 c.c.). The heavy yellow precipitate formed redissolved with production of a thin yellow suspension of the diazonium salt. An ice-cold solution of sodium hydrogen 1-amino-8-naphthol-3:6-disulphonate (14.4 g.) in water (200 c.c.) and 5N-sodium hydroxide (25 c.c.) was added during 60 mins., the deep red liquid stirred at 0° for 10 hrs., kept overnight, heated at 40° for 1 hr., and chilled, and the bulk of the dye collected. The filtrate was neutralised with sodium carbonate, evaporated to smaller volume, and cooled, and a small excess of hydrochloric acid added; a further crop of the dye was then obtained. The combined precipitates were dissolved in boiling water (125 c.c.) and the smallest amount of sodium carbonate to effect solution and, after filtration, reprecipitated in the cold with a slight excess of dilute sulphuric acid; the dye, dried at a low temperature, was obtained as a dark maroon powder (15 g.). For analysis the monoazo-dye was converted into the *tetrasodium* salt, which was recrystallised several times (Found: I, 27.8. $C_{14}H_8O_{10}N_2S_2AsNa_4$ requires I, 28.6%).

Sodium 3:3'-Dimethyldiphenyl-4:4'-bis-[2''-azo-8''-amino-1''-hydroxy-3'':6''-disulphonaphthalene-7''-(azo-2'''-6'''-di-iodobenzene-4'''-*o*-arsionate)].—*o*-Tolidine (4.2 g.) in water (75 c.c.) and hydrochloric acid (15 c.c.) was tetrazotised (sodium nitrite, 2.1 g.), and the solution added during 30 mins. to a stirred ice-cold solution of the foregoing monoazo-dye (25 g.) in water (130 c.c.) and 5N-sodium hydroxide (80 c.c.). After the usual procedure the dye was precipitated in the cold by hydrochloric acid (75 c.c.) as the sodium hydrogen salt, which was collected by centrifuging, drained (pump), and dissolved in boiling water and the minimum amount of sodium carbonate to form the octasodium salt. The dye was reprecipitated in the cold with a small excess of dilute sulphuric acid, collected, and reconverted into the *octasodium* salt, which was dialysed, recrystallised twice from the smallest amount of water, and obtained in dark maroon leaves (8 g.), which formed a deep brown-red solution in water (Found: I, 24.4; S, 6.8. $C_{46}H_{26}O_{20}N_{10}I_4S_4As_2Na_8$ requires I, 25.3; S, 6.4%).

3:3'-Di-iodo-4:4'-diaminodiphenylmethane.—Diaminodiphenylmethane (20 g.), iodine (50 g.), calcium carbonate (30 g.), water (50 c.c.), and ether (30 c.c.) were refluxed for 7 hrs., the viscous mass extracted with boiling alcohol (600 c.c.), the calcium carbonate removed, and the filtrate evaporated to small volume under reduced pressure. On addition of water a thick black tar was precipitated which became friable on standing. This was ground and warmed with water (500 c.c.) and hydrochloric acid (75 c.c.); the di-iodo-derivative dissolved, leaving a residue (4 g.) of more highly iodinated compounds. The filtered solution (charcoal) was basified with cold sodium carbonate, the precipitated base removed and suspended in water (250 c.c.), and sulphuric acid added (5 c.c.); the di-iododiaminodiphenylmethane sulphate separated as a light brown powder (the sulphate of diaminodiphenylmethane is soluble under these conditions). The sulphate (18 g.) was collected, washed, and suspended in cold water, and sodium carbonate added; 3:3'-*di-iodo-4:4'-diaminodiphenylmethane* was then obtained as a purple-black micro-crystalline powder (16 g.), m. p. 80—85° after recrystallisation from alcohol (Found: I, 56.4. $C_{12}H_{12}N_2I_2$ requires I, 56.5%).

Potassium 1-(4'-*o*-Arsonobenzeneazo)-2-amino-8-naphthol-3:6-disulphonate.—Arsanilic acid (10.9 g.) in water (100 c.c.) was diazotised with sodium nitrite (3.5 g.) and 10N-hydrochloric acid (25 c.c.), and an ice-cold solution of potassium hydrogen 2-amino-8-naphthol-3:6-disulphonate (18 g.) in water (100 c.c.) and 5N-potassium hydroxide (10 c.c.) added. After 8 hrs.' stirring, the scarlet liquor was kept overnight, warmed at 50° for 1 hr., and exactly neutralised, and the dye salted out with potassium chloride (20 g.). After standing on ice for 12 hours, the precipitate was collected and recrystallised from the minimum amount of boiling water, the *monoazo-dye* forming red-black leaves (13 g.), very soluble in water, giving an intense scarlet solution (Found in recrystallised salt: S, 9.6. $C_{16}H_{10}O_{10}N_2S_2AsK_4$ required S, 9.2%).

Potassium 3:3'-Di-iododiphenylmethane-4:4'-bis-(2''-azo-7''-amino-1''-hydroxy-3'':6''-disulphonaphthalene-8''-*o*-azobenzene-4'''-*o*-arsionate).—Di-iododiaminodiphenylmethane (4.5 g.) in water (100 c.c.) and 10N-hydrochloric acid (20 c.c.) was tetrazotised at 0° (sodium nitrite, 1.4 g.) and after 15 mins. the clear solution was added slowly to an ice-cold solution of the foregoing dye (12 g.) in water (130 c.c.) and 5N-potassium hydroxide (80 c.c.), the colour becoming blood-red. After 16 hrs.' stirring, the solution was warmed at 50° for 1 hr., and the dye isolated as its *octapotassium* salt, a dark micro-crystalline powder (5 g.) very soluble in water (Found: I, 14.3; S, 7.3. $C_{45}H_{26}O_{20}N_{10}I_2S_4As_2K_8$ requires I, 13.6; S, 6.85%).

2:6-Di-iodosulphanilic Acid (compare D.R.-P. 129,808).—A solution of sodium sulphanilate (19.5 g.) in water (50 c.c.) at 60° was added during 30 minutes to a rapidly stirred solution of iodine monochloride (33 g.) and hydrochloric acid (37 c.c.) in water (250 c.c.) at 60°. After 3 hrs.' stirring at 70—80°, the liquid was kept overnight and filtered, the precipitate dissolved in boiling water (300 c.c.) and 5N-sodium hydroxide (25 c.c.), and a little sodium bisulphite added

(charcoal), followed by an excess of hydrochloric acid; the di-iodosulphanilic acid separated in heavy white needles (36 g.) (Found in recrystallised material: I, 59.5. Calc.: I, 59.7%).

5:5'-Di-iodobenzidine-2:2'-disulphonic Acid.—A solution of benzidine-2:2'-disulphonic acid (34.4 g.) in water (50 c.c.) and 5*N*-sodium hydroxide (30 c.c.) at 80° was added during 60 minutes to a solution of iodine monochloride (65 g.) in water (200 c.c.) and hydrochloric acid (100 c.c.) at 80°. After 4 hrs.' stirring, the dark liquid was kept overnight and chilled, and the precipitate collected; a further crop was obtained by evaporation of the filtrate to 100 c.c. and cooling. The combined precipitates were suspended in hot water (200 c.c.) and treated with sulphur dioxide to remove free iodine, and 5*N*-sodium hydroxide added until a clear solution at p_H 8.0 was obtained. This solution (charcoal) was evaporated until crystallisation on the water-bath began (120 c.c.), a saturated solution of sodium chloride (20 g.) added, and the orange crystalline precipitate (40 g.) collected after standing on ice for 16 hrs. Recrystallisation from the minimal amount of boiling water (120 c.c.) gave sodium 5:5'-di-iodobenzidine-2:2'-disulphonate in pale orange leaves (28 g.) (Found: I, 39.4. $C_{12}H_8O_6N_2I_2S_2Na_2$ requires I, 39.7%). From the mother-liquor a further quantity of almost pure substance (14 g.) was obtained. The salt was very soluble in water, giving a pale orange solution.

Sodium 2:(2':6'-Di-iodo-4'-sulphobenzeneazo)-1-amino-8-naphthol-3:6-disulphonate.—To an ice-cold solution of 2:6-di-iodosulphanilic acid (42.8 g.) in water (240 c.c.) and 5*N*-sodium hydroxide (20 c.c.) was added a concentrated solution of sodium nitrite (7.2 g.), followed by 5*N*-hydrochloric acid (80 c.c.); the diazonium chloride formed a bright yellow suspension. After 30 mins.' stirring, an iced solution of sodium hydrogen 1-amino-8-naphthol-3:6-disulphonate (36 g.) in water (120 c.c.) and 5*N*-sodium hydroxide (40 c.c.) was run in during 1 hr. Stirring was continued at 0° for 30 hrs. in all and then the red liquid was kept for 12 hrs., warmed at 50° for 1 hr., exactly neutralised with sodium carbonate, evaporated to small volume, and cooled, and 10*N*-sulphuric acid (20 c.c.) added. After standing on ice for several days the acid sodium salt was collected and converted into the trisodium salt, which was recrystallised several times from boiling water and obtained as a black powder very soluble in water (Found: I, 31.8. $C_{16}H_8O_6N_3I_2S_3Na_3$ requires I, 30.9%).

Sodium 5:5'-Di-iodo-2:2'-disulphodiphenyl-4:4'-bis-[2''-azo-8''-amino-1''-hydroxy-3''':6''-disulphonaphthalene-7''-(azo-2''':6''-di-iodobenzene-4''-sulphonate)] (B).—To a solution of sodium di-iodobenzidinedisulphonate (13 g.) and sodium nitrite (2.9 g.) in water (110 c.c.) in a freezing mixture, 5*N*-hydrochloric acid (40 c.c.) was slowly added; the tetrazonium salt formed a light orange suspension. After being stirred for 1 hr., this was added slowly to an ice-cold solution of the foregoing monoazo-dye (33 g.) in water (120 c.c.) and 5*N*-sodium hydroxide (100 c.c.). Stirring at a low temperature was continued for 6 hrs., a little octyl alcohol being used to prevent frothing. After standing overnight, the dark maroon-red liquid was warmed at 50° for 1 hr., and the acid sodium salt (22 g.) isolated as above described. A solution of this in the minimal amount of boiling water and sodium carbonate (2 g.) was evaporated to small volume; the octasodium salt of the tetrakisazo-dye was obtained as a black powder (8 g.), extremely soluble in water, yielding a deep brown-red solution. For analysis it was dialysed and recrystallised from water (Found: I, 32.3; Na, 8.6. $C_{44}H_{16}O_{26}N_{10}I_6S_8Na_8$ requires I, 33.1; Na, 8.0%).

4:6-Di-iodoanthranilic Acid (compare Wheeler and Johns, *Amer. Chem. J.*, 1910, **43**, 405).—A solution of sodium anthranilate (31.8 g.) in water (200 c.c.) at 60° was added during 1 hr. to a stirred solution of iodine chloride (80 g.) in water (350 c.c.) and hydrochloric acid (150 c.c.) also at 60°. The mixture, which rapidly threw down a dark precipitate, was stirred for 3 hrs. at 70—80°, kept overnight, and filtered. The brown residue was dissolved in dilute sodium hydroxide solution (charcoal) and precipitated with an excess of hydrochloric acid in the presence of a small quantity of sodium sulphite to remove free iodine. Recrystallisation from a large volume of alcohol gave di-iodoanthranilic acid (51 g.) in light brown, felted needles, m. p. 228—230° (Found: I, 65.5. Calc.: I, 65.3%).

Sodium 2:(2':4'-Di-iodo-6'-carboxybenzeneazo)-1-amino-8-naphthol-3:6-disulphonate.—Di-iodoanthranilic acid (19.6 g.) in warm water (360 c.c.) and 5*N*-sodium hydroxide (10 c.c.) was stirred in an ice-bath, and sodium nitrite (3.6 g. in aqueous solution) added, followed by 5*N*-hydrochloric acid (40 c.c.); the diazonium chloride formed a yellow suspension. After 2 hrs. an ice-cold solution of sodium hydrogen 1-amino-8-naphthol-3:6-disulphonate (18.2 g.), in water (100 c.c.) and 5*N*-sodium hydroxide (20 c.c.) was slowly added, and stirring continued at 0° for 30 hrs. and at 30—40° for 3 hrs. The dark maroon liquor was centrifuged, and the slurry drained (pump) and dissolved in boiling water (80 c.c.) and sodium carbonate (5 g.). The bright red solution was filtered, evaporated to small volume, and chilled; the monoazo-dye was obtained in micro-needles (14 g.); recrystallisation from water gave the pure dye (Found: I, 31.6. $C_{17}H_8O_9N_3I_2S_2Na_3$ requires I, 32.3%).

Sodium 5:5'-Di-iodo-2:2'-disulphodiphenyl-4:4'-bis-[2''-azo-8''-amino-1''-hydroxy-3''':6''-disulphonaphthalene-7''-(azo-2''':4''-di-iodobenzene-6''-carboxylate)].—The foregoing monoazo-dye (31 g.; 2 mols.) was coupled in alkaline solution with 1 mol. of tetrazotised sodium di-iodobenzidinedisulphonate (13 g.) in the manner described for the analogue above. The product, isolated in a similar manner, gave the tetrakisazo-dye as a maroon-black microcrystalline powder soluble in water with production of brown-red solutions (Found: I, 33.3; Na, 8.0. $C_{46}H_{16}O_{24}N_{10}I_6S_8Na_3$ requires I, 34.0; Na, 8.2%).

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