STUDIES WITH RADIOACTIVE DI-AZO DYES. II. THE SYNTHESIS AND PROPERTIES OF RADIOACTIVE DI-BROM TRYPAN BLUE AND RADIOACTIVE DI-BROM EVANS BLUE [1]

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Investigations from these laboratories (1, 2) deal with the accumulation of radioactive di-brom dyes in abscesses and tumors. The radio-dyes are also useful for studies of capillary permeability in so far as their permeation from blood into lymph, too slight to be detected visually in the normal animal, may be quantitatively measured on samples of lymph (3). Dyes of this group make a bond with plasma protein (4), becoming preferentially associated with albumin, and they therefore constitute a radioactive "tracer" for protein under certain experimental conditions.

In view of the interest attaching to such matters, as well as the fact that a large group of chemically related but biologically highly varying compounds—the di-azo dyes—may be made radioactive by this technique, the present paper will describe the method used in rendering the molecule radioactive. Chemical and biological properties of the brominated dyes will also be discussed.

The molecule is made radioactive by the incorporation of two atoms of radioactive bromine in the di-phenyl portion of the molecule (Figure 1). Bromine atoms, so incorporated into an aromatic ring, do not ionize or dissociate. Thus in using the radio-dye for "tracer" experiments, measurements for radio-bromine constitute measurements for the dye molecule as a whole.

The steps involved in this synthesis are as follows:

1. The production of radioactive bromide.
2. Oxidation to radioactive bromine.
3. Bromination of ortho-tolidine.
4. Coupling of this radioactive di-brom tolidine with an amino-naphthol-sulfonic acid to make the finished dye.

DETAILS OF SYNTHESIS

1. Production of radioactive bromide

We have investigated the production of radio-bromide for this synthesis by three methods: the deuteron bombardment of selenium or sodium bromide, and the neutron bombardment of ethyl bromide. The latter method has been found to be the most satisfactory because it produces large total activities at a high specific activity, both factors being important for such a synthesis as this.

From 20 to 40 kgm. of ethyl bromide [3] are placed by the cyclotron in such a fashion as to secure maximum surface exposure to the neutron beam. The neutron beam, in our experience, has been the secondary result of deuteron bombardment of other elements, beryllium yielding the greatest neutron-intensity. An amount of activity adequate for these purposes lies in the range of 5 to 8 mc.,[4] and will produce a dye which is useful in animal

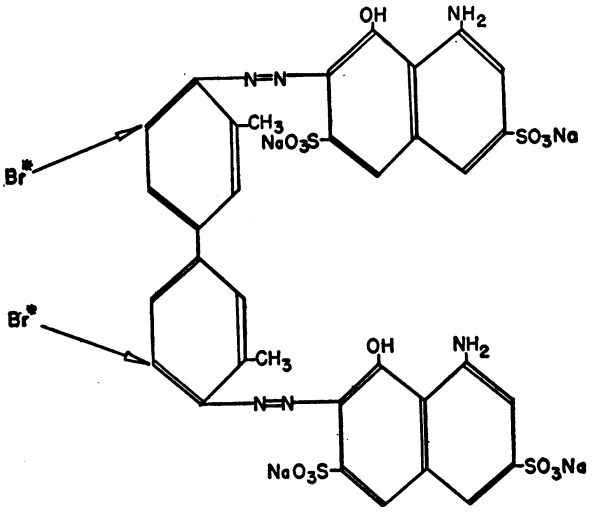

FIG. 1. THE STRUCTURAL FORMULA OF TRYPAN BLUE, SHOWING THE POINTS AT WHICH RADIO-BROMINE ATOMS ARE INCORPORATED INTO THE MOLECULE TO RENDER IT RADIOACTIVE

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[3] Other organic bromides such as bromoform or tetrabromethane, may be used instead of ethyl bromide.

[4] As used here, the term mc. (milli-curie) and μc. (micro-curie) represent an arbitrary correlation with the radiation emitted from a uranium standard. One μc.

experiments for about one week after synthesis. Such an amount of activity may be produced by the deuteron bombardment of beryllium on a probe target, at 250 to 300 micro-amperes, in about 5 hours. Using an external beryllium target as the neutron source, bombardment at 35 to 50 micro-amperes, for about 7 hours, is sufficient. The half-life of Br^{82} is 34 hours.

The organic bromide is then extracted in a large separatory funnel with half its volume of water, three extractions being sufficient to secure a large proportion of the extractable radioactivity. The water extract contains the active Br atoms (5, 6). The extract is made alkaline to litmus and then concentrated to a small volume (100 to 200 cc.) by boiling.

2. *Oxidation to elementary bromine*

The concentrated aqueous extract is then transferred to the pot of a small distilling apparatus, the two traps of which are immersed, respectively, in ice-water and in dry ice (in a carbon tetrachloride-chloroform bath). The bromide is oxidized by the dropwise addition of a suspension of MnO_2 in H_2SO_4, and the resultant bromine condensed and recovered in the two traps, the majority remaining in the first trap, layered beneath water. About 1.0 to 1.5 grams of Br_2 are distilled, this amount being the result of hydrolysis of the ethyl bromide, the active atoms alone naturally constituting only an extremely small weight of bromine.

3. *Bromination of ortho-tolidine*

One gram of ortho-tolidine (3,3′ di-methyl benzidine), recrystallized from toluene or xylene, and dissolved in 75 to 100 cc. of glacial acetic acid, is placed in a 250 cc. three-necked flask. The flask is fitted with a stirring motor, a dropping funnel for the addition of the bromine, and a lead-off to a gas trap.

The radio-bromine is removed from the traps of the distilling apparatus by washing with glacial acetic acid, and this solution is added slowly to the three-necked flask containing the *o*-tolidine solution. The latter immediately turns a dark greenish-purple as the bromine is added. If any bromine beyond that obtained from the distillation is needed to make up a 10 per cent excess, it is now added, and stirring is continued for an hour. The flask is heated to 85° C. in a water-bath to complete the reaction. The resultant suspension is cooled and transferred to a 500 cc. Erlenmeyer flask, and a small amount of $SnCl_2$ added slowly, with shaking. This reduces the excess bromine to bromide, and changes the green suspension to a white crystalline precipitate in a clear supernatant. The volume is made up to about 500 cc. with water and the flask is cooled for two hours.

The radioactive di-brom *o*-tolidine (5,5′ di-brom, 3,3′ di-methyl benzidine) is then collected by filtration, washed with 50 per cent acetic acid, and with water, and then dried. The product is a grayish powder with a melting point of 195 to 195.5° C., conforming to a previous description in the literature (7), and may be used without further crystallization. The yield is 85 to 90 per cent of the theoretical, on the basis of tolidine.

The precipitation of this product from a large volume of water, and the thorough washing with water, eliminate any radioactivity in the precipitate which might be due to water soluble forms of the isotope, *i.e.* bromide ion.

Successful runs of this bromination have been carried out with as little as 0.15 gram of *o*-tolidine.

4. *Coupling*

The coupling of the radioactive di-brom tolidine with an amino-naphthol-sulfonic acid involves three substituent steps (8).

a. Hydrochloride formation. The dried, ground, di-brom tolidine is dissolved in toluene. Dry HCl is bubbled through this solution until no further precipitate forms. This is collected by filtration, washed with toluene and ether, and dried. The yield of amine hydrochloride is 90 per cent of the theoretical.

b. Diazotization. All of the hydrochloride is suspended in 150 cc. of water in the presence of four equivalents of HCl, stirred, and cooled to 0 to 15° C. A solution of $NaNO_2$, slightly in excess of the calculated amount (two equivalents), in 10 cc. of water, is run in rapidly. A deep greenish-blue color appears as the diazonium salt is formed. Throughout diazotization, an excess of both HCl and $NaNO_2$ should be maintained. The solution is stirred for one-half hour and then about one gram of urea is added to decompose excess HNO_2.

c. Coupling. In a beaker, a paste is made of the acid to be used for the coupling, consisting of two equivalents in about 50 cc. of water. "H-Acid" (1-amino,8-naphthol, 3,6-di-sulfonic acid) is used for radioactive di-brom trypan blue; "Chicago acid" (1-amino,8-naphthol, 2,4-di-sulfonic acid) is used for radioactive di-brom Evans blue. "Chicago acid" is more water-soluble than "H-Acid" and solution of the latter is affected by adding one equivalent of NaOH in 10 cc. of water, testing with litmus to be sure that the solution is still acid. In computing the weight of acid to be used, account is taken of the fact that the technical grade of the acids is only 88 per cent pure.

The solution is cooled to below 15° C.; 4.0 grams of anhydrous sodium carbonate are added to make the solution alkaline. With vigorous stirring, the diazonium salt is run in rapidly and the solution turns a deep purple. After one-half hour of stirring, the solution is tested for alkalinity and more Na_2Co_3 is added if necessary.

At this point, the solution will approximate 200 cc. of a 1.5 per cent solution of radioactive brom-dye. It may be partially purified by dialysis against running tap water. However, it is more desirable to precipitate the dye and make up the solution for injection from pure, dry dye.

This precipitation may be carried out by adding 60 grams of sodium acetate for each 100 cc. of the dye solution, heating to 85° C. and centrifuging while hot. The

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of Br^{82} by this method gives in the vicinity of 1.5×10^5 counts per minute on our Geiger counter, under the geometric conditions we employ.

precipitate is dried overnight at 100° C. and freed of acetate by repeated washings with small amounts of hot 95 per cent alcohol until the washings are acetate-free by test with a drop of H_2SO_4.

A more satisfactory method consists in concentrating the dye-solution by boiling, to a volume of 50 to 100 cc. Absolute alcohol is then added to a final concentration of 90 per cent. The dye is only sparingly soluble in this strength of alcohol and may be centrifuged. This sediment is dried in the oven, the resultant cake is ground up in a mortar and a 1 per cent solution made.

COMMENTS

1. *Time consumed*

In the synthesis of a radioactive compound containing a 34 hour isotope, the time element becomes of prime importance. In our experience, the solution of pure dye, ready for animal injection, may be obtained in 24 to 36 hours from the time the radio-bromide is received from the cyclotron. The dye is then useful for about one week for animal experimentation involving determinations of radioactivity in tissues or body fluids.

2. *Radioactivity*

The strength of the dry dye at the time it is obtained is in the order of 0.5 μc. per mgm. This is such that 0.00025 mgm. may be measured on the counter, an amount well below the visible range. Higher activities are obtainable with longer bombardments, or by bombarding larger amounts of organic bromide.

Radioactivity measurements may be made on counter or electroscope, the latter instrument being better adapted for the stronger samples. The technic for measurement in tissues or in the intact animal is described in other publications (1, 2). Body fluids containing the dye may be measured directly by drying the sample in a small receptacle which can be placed under the detection instrument in a standard fashion. Care must be taken to use the same amount of fluid for each measurement in a series so as to avoid errors due to self-absorption. For tissue measurements, or where varying amounts of fluid are to be used, a calibration curve may be made to correct for self-absorption.

3. *Properties of the dye*

If an organic compound is rendered radioactive by the addition of an extraneous element for purposes of biological investigation, it is essential that the radioactive compound have the same biological properties as the non-radioactive compound one-wishes to study. In this case, it is important to contrast the chemical and biological behaviour of the brominated dyes with their non-brominated counterparts, trypan blue and Evans blue (T–1824).

Colloidal di-azo acid dyes of this group exhibit the following biological properties:

First, slow disappearance from the bloodstream; second, slow or negligible appearance in lymph, urine, and cerebrospinal fluid, under normal conditions; third, accumulation in areas of inflammation and neoplasia; fourth, uptake by the reticulo-endothelial system; and, fifth, staining of certain other cells, such as the tubular epithelium of the kidney.

These biological properties are, in turn, related in some manner to the following physico-chemical properties:

First, the dyes are of a colloidal character in aqueous solution. They are non-diffusable, and congo red, for example, has a particle-size (9) of around 13 Å, considerably larger than one would expect from the molecular weight of around 700. Second, the charge on the colloidal particle is negative (10). In contrast, positively charged dyes, such as Bismarck brown, act quite differently in the organism. Third, they combine with plasma and other body proteins, through acidic or basic attaching groups or adsorption (12). And, fourth, they possess a hydrophobic-hydrophilic molecular configuration of the type shown by Höber (11) to be associated with active transfer in living cells.

The brominated dyes herein discussed exhibit the following comparable properties:

1. The brominated dyes are colloidal in aqueous solution, both as evidenced by their relative non-diffusibility through cellophane membranes, and their conduct on attempts at filtration. The rate of diffusion of the dye through cellophane is in the order of 3 to 6 per cent in a 24-hour period. Under the experimental conditions employed, this is the same order of magnitude as trypan blue and Evans blue. When the dye is in concentrations of

1.0 per cent or higher, it is virtually unfiltrable, using ordinary suction filtration apparatus.

2. The charge on the colloidal particle was determined by placing a drop of the dye in an electric field in a simple apparatus such as that described in Burrows (10). The charge on the particle in the case of the two brominated dyes is negative.

3. The dye-protein bond is a matter which has until recently been the subject of little study. Chapman, Greenberg and Schmidt (12) titrated dyes against proteins and showed the union to be chemical (rather than adsorptive) in nature, and stoichochemical in proportion. Rawson's recent work (4) has cast more light on this field. She has kindly carried out tests with our dyes by the Tiselius technique, and by using ultracentrifugation and a cellophane-staining test which she has devised.[5] She finds that the bromo-dyes, like their non-brominated counterparts, migrate preferentially with albumin if the concentration in plasma is in the vicinity of 0.05 per cent or lower. The bromo-dyes are less soluble in water than the non-brominated dyes and stain cellophane to a greater extent.

4. Höber (11) has shown that mono-azo dyes, possessing a structure characterized by a water-soluble "head" and a water-insoluble "tail," have properties in regard to active transfer in living cells not shared by dyes in which both ends of the molecule are either water-soluble or lipoid-soluble. Trypan blue and Evans blue possess this structure, the di-amine portion of the molecule being hydrophobic, and the sulfonic-acid-salt, hydrophilic. The brominated dyes likewise share this property, the bromine on the amine rendering it, if anything, less soluble in water.

5. The addition of two bromine atoms produces a dye which is more red than the non-brominated dyes. This is associated with a shift of the peak absorption from 630 mμ. for Evans blue (13) to 545 mμ. for di-brom Evans blue, and from 600 mμ. for trypan blue (13) to 550 mμ. for di-brom trypan blue. Di-brom Evans blue also shows a color-change with pH at about 8.0. On the alkaline side of this point the dye is quite red, on the acid side, purple. Di-brom trypan blue shows color intensification with addition of small amounts of either acid or base, with a sharper absorption peak.[6]

6. Dialysis experiments demonstrate that a red component dialyzes through cellophane more rapidly than the purple color comprising the main portion of the dye. This red component is perhaps analagous to the "red impurity" found in trypan blue or unpurified Evans blue (13). This red substance, however, is both colloidal and radioactive and may result from the coupling of the di-brom tolidine with an oxidation-product of the sulfonic acid.

[5] The authors wish to express their thanks to Dr. Rawson for carrying out these studies.

[6] Absorption studies were generously carried out for us on the Hardy Self-Recording Spectrophotometer at the Color Measurement Laboratory of the Massachusetts Institute of Technology.

SUMMARY

(*1*) The synthesis of radioactive derivatives of trypan blue and Evans blue is described.

(*2*) The physico-chemical and biological properties of the brominated dyes are discussed.

(*3*) A means of studying the *in vivo* conduct of colloids of this group is offered, as well as a means of "tagging" plasma protein.

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