THE SYNTHESIS OF PROPIDIUM IODIDE

CH 80 THESIS

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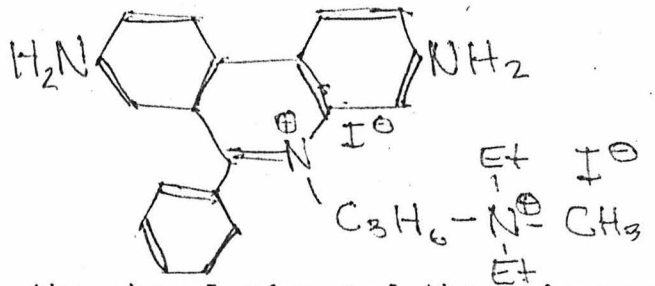
One of the problems approached in this research was to check the validity of a previously published synthesis of 3,8-Diamino-5,3'-diethylaminopropyl-6-phenylphenanthridinium Iodide Methiodide and to prepare the material for use in the laboratory. We have named this compound Propidium Iodide. Its structure is:



Excepting the phenyl ring and the aminopropyl side chain, Propidium is a flat molecule and is one of several which have been found to bind to DNA in a particular manner known as intercalation. The molecules are arranged perpendicularly to the axis of the DNA and lie between adjacent base-pairs of amino acids.

The first of these chemicals widely used in centrifugation was Ethidium Bromide. Identical to Propidium except that the sidechain is only a methyl group, it was first used as a Trypanocide, and then as a dye for DNA. It was especially useful for its fluorescent properties:

only weakly radiant in the free state, it fluoresced many times more strongly while bound to the DNA. It is now suspected that this is caused by a flattening of the phenyl ring relative to the plane of the molecule on intercalation. As the ring approaches the same plane, it becomes a more active part of the resonance in that plane, and the fluorescent properties of the molecule are enhanced.

Ethidium was put to a new far-reaching use in 1967 by Radloff, Bauer, and Vinograd.[1] They developed a method for the separation of linear and closed-circular DNA's which was dependent on the fact that as the Ethidium binds to the DNA, it causes the helix to unwind. In linear DNA this is not particularly significant, though it does result in some strain and a corresponding lowering in the free energy of binding. But in the case of circular DNA the total number of turns in the molecule must remain constant. In naturally occuring molecules, the sense of the superhelical twisting is opposite to that of the helix itself. Thus a small amount of dye will unwind the superhelices, producing a relaxed circle. The free energy contribution of this relaxation makes the circular form, for that first small amount of dye, more easily bound. But as more dye is added, superhelicas of of the opposite sense form and soon seriously restrict intercalation. So at commonly used dye concentrations,

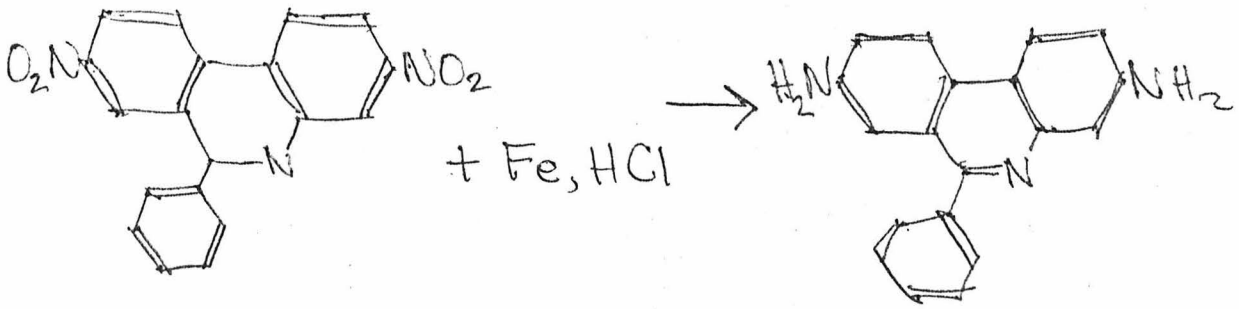
a greater amount, relative to the number of DNA base-pairs, is bound to linear DNA than circular DNA. Similar results to those in the linear case are produced in circular molecules that have been "nicked", or had one phosphate linkage broken. This provides a method of internal rotation, removing the restriciton to a constant number of turns.

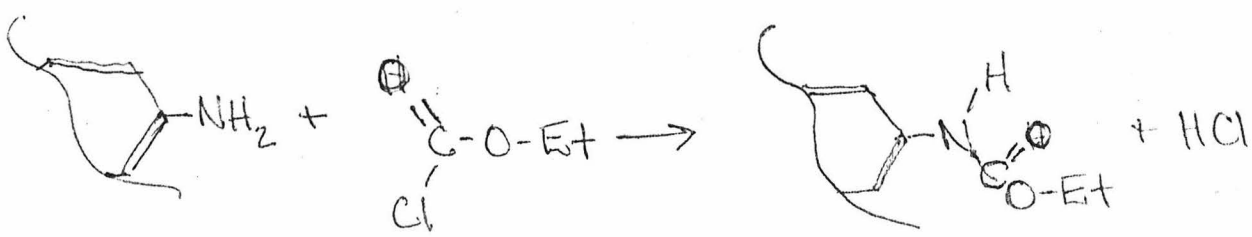
The effective buoyant density of the Ethidium molecule is less than that of DNA. Since the linear DNA-dye complex contains more dye, it is less dense than the circular complex. The Bauer-Radloff-Vinograd method is to put dye, DNA, and Cesium Chloride in a water solution and to spin it at high speeds in the preparative ultracentrifuge for twenty-four to forty-eight hours. The Cesium Chloride forms a density gradient, and the DNA-dye complexes form bands at the appropriate position. The tube is then empties slowly through a hole punched in the bottom and the two types of DNA appeared in separate fractions.

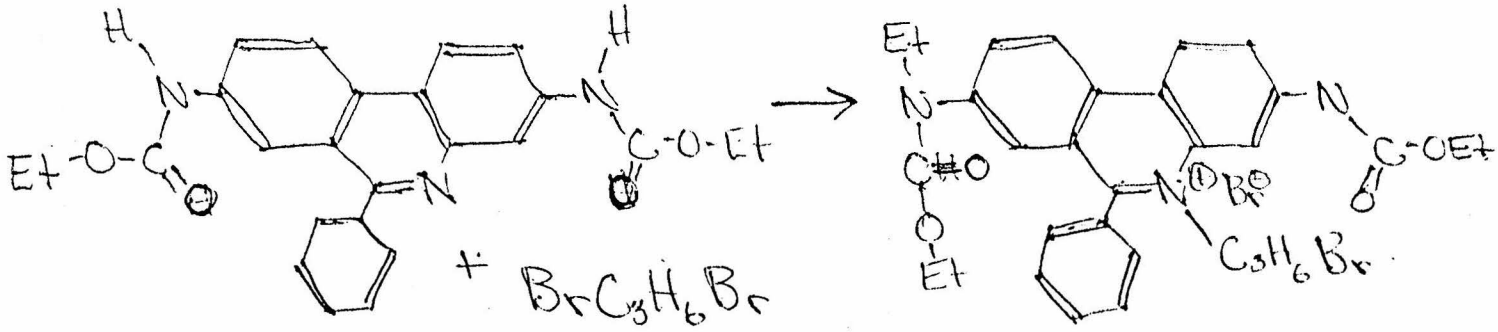
T. I. Watkins of Boots Drug and Chemical Company, who was interested in Ethidium because of its value as a Trypanocide in treating the diseases of cattle, reported in 1952 the synthesis of eight analogs, mainly differing in the sidechain? Bruce Hudson, a member of the Vinograd group, tested these for their DNA-binding properties.

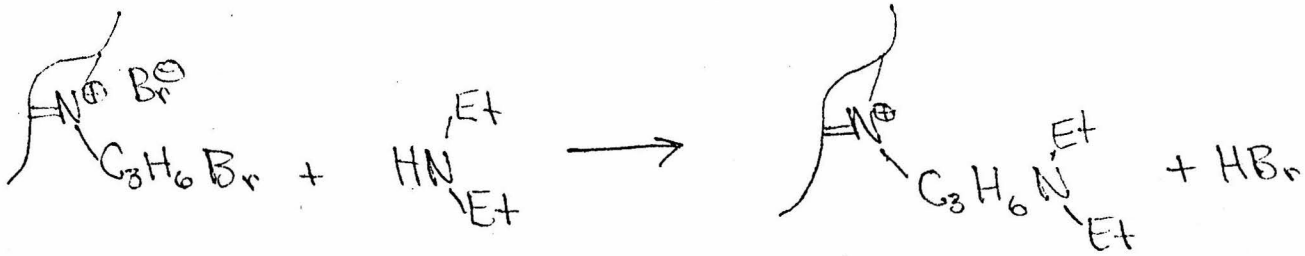
One of these was Propidium. The results showed that in the dye-buoyant-density separation it produced a difference 1.5 to 1.8 times greater than Ethidium. This greater separation would be of value in general use for two reasons: the DNA bands are not perfectly sharp--the balance between the buoyant forces and the process of diffusion produces a Gaussian distribution of molecules, and other separations--involving varying base-pair composition or catenanes--may produce much smaller differences and thus not be resolvable with Ethidium.

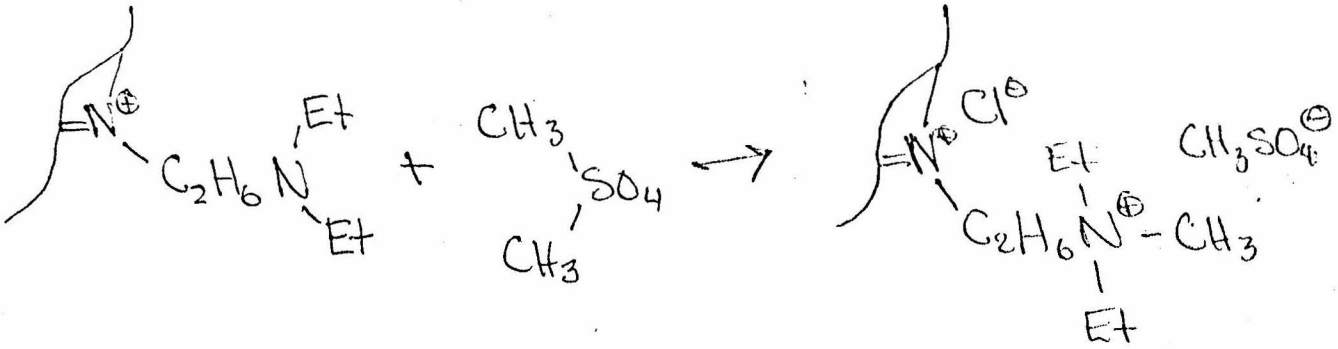
But for the Propidium process to be generally practical, it was necessary to know how difficult it was to produce the chemical itself. Duplication of Watkin's synthesis would tell. We began, as he did, with 3,8-dinitro-6-phenylphenanthridine. The nitrate groups were reduced to amines, then blocked to prevent unwanted side reactions, before the quaternization was attempted. The scheme is as follows:



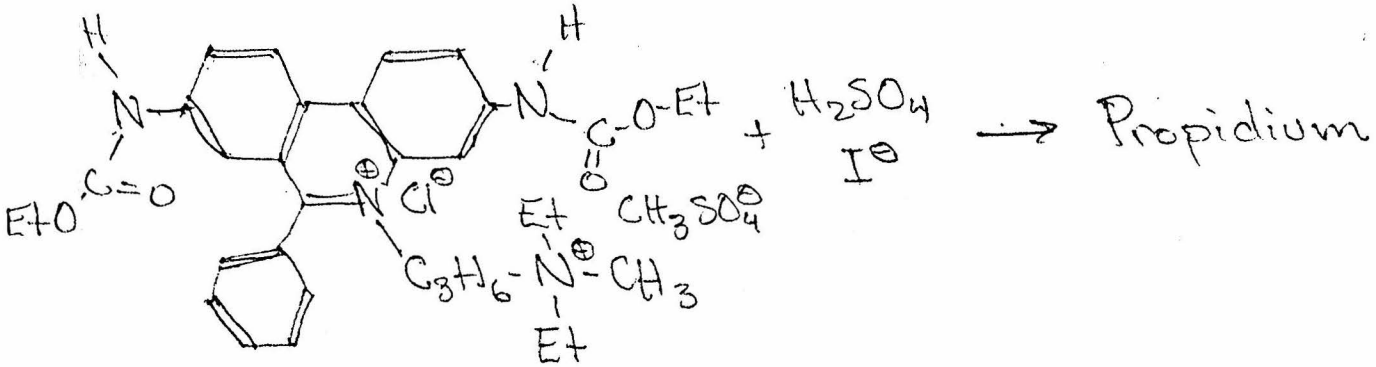








unblocking, and precipitating with iodide,



Watkins' descriptions of the procedures are below, with the difficulties I encountered. Most of the steps were done at least twice, and several were done three times.

2 : 7-*Biscarbethoxyamino-9-phenylphenanthridine.*—This compound is described by Walls (*J.*, 1947, 67). It was found that a purer product was more readily obtained by reduction of 2 : 7-dinitro-9-phenylphenanthridine and subsequent carbethoxylation. Powdered 2 : 7-dinitro-9-phenylphenanthridine (14·3 g.) (Walls, *J.*, 1915, 294) and iron powder (17 g.) were stirred together in water (300 c.c.), alcohol (50 c.c.), and 5*N*-hydrochloric acid (2 c.c.), and the mixture was heated under reflux for 5 hours. The whole was then cooled, made alkaline with ammonia solution, and filtered, and the residue washed with water. 2 : 7-Diamino-9-phenylphenanthridine was extracted from the dried solid residue with chloroform and, on concentration of the extract, separated in yellow prisms, m. p. 197—198° (10·75 g., 91%) (Walls, *loc. cit.*, records m. p. 198°). A stirred solution of the diamine (10·4 g.) in dry pyridine (75 c.c.) was treated with ethyl chloroformate (7·5 c.c.) at 25—30°. The dark red solution was slowly stirred at room temperature for 5 hours, then poured into water, and the yellow solid collected. It crystallised from aqueous methanol as white needles, m. p. 138° (not sharp) (Found : C, 70·3; H, 5·2; N, 10·0. Calc. for $C_{23}H_{23}O_4N_3$: C, 69·9; H, 5·4; N, 9·8%). This compound, which is not described by Walls, was also obtained by his procedure. Heating with water for 2 hours converted it into Walls's high-melting compound, m. p. 218—220° (decomp.) (12·5 g., 80%).

This was the most troublesome step. The result of the first reaction was a dirty black substance which yielded only small amounts of product even when extracted with large amounts of chloroform. Strong improvement was noted when the extraction was done with methanol, and the product precipitated with water. The solid residue was noted to contain yellow lumps, presumably of starting material, which were difficult to avoid because the nitrate is insoluble in water. Possibly some more violent method of stirring would increase the yield. My best effort produced about 9.1 grams, below Watkins' 10.75 grams.

In the second part of this step it should be noted that the ethyl chloroformate reacted violently when added to the pyridine solution, and so should be dropped in

gradually, with care. I also met with difficulty in the methanol recrystallization, and my first results were characterized by poor purity and low yield. Since I also found that the "heating with water" improved purity, I left the methanol recrystallization out completely the second time, and did the latter step twice with a change of supernatent. The results were fair, my melting point being 6° low, and my yield corresponding to 11.5 grams rather than 12.5 grams.

I noted throughout the synthesis a common difficulty with crystallization procedures. The substances crystallized poorly, or if forced suddenly out of solution, in very small particles. My conclusion was that the rate of crystallization was small, and that improvement would result from very gradual cooling or from reheating and slow cooling of small-particle colloids. This was confirmed by trials. Even so, however, in almost all cases I was unable to get the clearly defined crystal forms described by Watkins.

10-3'-Bromopropyl-2 : 7-biscarbethoxyamino-9-phenylphenanthridinium Bromide.—2 : 7-Biscarbethoxyamino-9-phenylphenanthridine (22 g.) was heated with stirring with 1 : 3-dibromopropane (60 c.c.) in an oil-bath at 170—175° for 60 minutes. Yellow plates were deposited after 20 minutes. The mixture was cooled, and dry ether (40 c.c.) was added, and the mixture filtered. The yellow solid was washed with dry ether and dried at 100° [yield 30 g.; m. p. 195—200° (decomp.)]. Recrystallisation of the *bromide* from a large volume of ethanol yielded yellow plates, m. p. 235—236° (decomp.) (27·5 g., 84%) (Found : C, 52·7; H, 5·0; N, 6·5; Br, 23·8. $C_{28}H_{29}O_4N_3Br_2$ requires C, 53·3; H, 4·6; N, 6·7; Br, 25·4%).

This step went well as described. "A large volume" of ethanol was about two liters, and evaporation of part of the supernatent was required to retrieve all of the

product. I got about 21 grams, rather than 27.5, but the purity was good.

2 : 7-*Biscarbethoxyamino*-10-3'-*diethylaminopropyl*-9-*phenylphenanthridinium Chloride Hydrochloride*.—The above bromopropyl quaternary compound (20 g.) was stirred under reflux with diethylamine (150 c.c.). The original compound dissolved and a white crystalline solid was deposited. The heating was continued for 1 hour and the mixture was filtered. The solid (8·1 g.) was diethylamine hydrobromide, plates (from ethanol–ether), m. p. 212° unchanged on admixture with the authentic material. The diethylamine filtrate was evaporated nearly to dryness under reduced pressure and the residue treated with water. The insoluble material (halogen-free) was purified by dissolution in acid and reprecipitation with alkali as the pseudo-base (II).

The only problem encountered here was the "dissolution in acid." I used 10 N HCl and was not able to obtain complete dissolution. However, reprecipitation with saturated NaOH seemed successful never the less.

2 : 7-*Biscarbethoxyamino*-10-3'-*diethylaminopropyl*-9-*phenylphenanthridinium Hydrogen Sulphate 3'-Methomethylsulphate*.—2 : 7-Biscarbethoxyamino-10-3'-diethylaminopropyl-9 : 10-dihydro-9-hydroxy-9-phenylphenanthridine [pseudo-base (II)] (6 g.) was dissolved in ethyl acetate (100 c.c.) and ethanol (60 c.c.), and methyl sulphate (2·8 c.c., 2·5 mols.) was added. The solution was refluxed for 30 minutes and cooled but no deposit formed. It was accordingly diluted with benzene–ether, to give a yellow solid *salt* which crystallised from ethanol–ethyl acetate as yellow acicular prisms (6·3 g.), m. p. indefinite, *ca.* 250° (decomp.) (Found : C, 53·7; H, 6·1; N, 7·4. $C_{31}H_{46}O_{12}N_4S_2$ requires C, 53·3; H, 6·0; N, 7·3%).

This step worked as described, except for a disappointingly low yield, less than half that described.

2 : 7-*Diamino*-10-3'-*diethylaminopropyl*-9-*phenylphenanthridinium Iodide Methiodide*.—A solution of the foregoing compound in sulphuric acid (10 c.c.) and water (5 c.c.) was heated at 130° for 30 minutes and the product isolated as a *di-iodide* by the usual procedure. It crystallised from water containing potassium iodide, in red needles, m. p. 210—230° (decomp.), depressed on admixture with 2 : 7-diamino-10-3'-diethylaminopropyl-9-phenylphenanthridinium iodide hydriodide (3·6 g., 92%) (Found : C, 48·1; H, 5·0; N, 8·3; I, 37·2. $C_{27}H_{31}N_4I_2$ requires C, 48·5; H, 5·1; N, 8·4; I, 38·0%).

This procedure again went much as Watkins described except for a lower yield. From 5 grams of starting material I obtained about 2 grams in batches of varying purity. One, of .2 grams, had exactly Watkins' melting point.

Conclusions

At first examination, the results seem to be rather negative. The synthesis is long and difficult, and produced only a small amount of product. Unless a particular

use is seen, it is likely the labworker's time could be spent better elsewhere. The synthesis took me about seventy hours.

However, it is obvious that I did not do so well as Watkins. Had my yields equalled his, I might have produced ten or fifteen grams of material. I am basically unable to explain this, other than to say that the polished, practiced technique of the professional is no doubt superior to that of the student.

The ideal solution would be to convince a chemical company to produce it commercially. Some experiments do need it, and if all experiments used it, it might be that previously unnoticed phenomena would come to light because of the increased resolution of the bands.

Furthur work was done on other possible improvements. Although initial attempts were not successful, the ideas seem to merit more work:

A dimeric dye--if two dye molecules were connected by a relatively long chain, the DNA molecules might tend to form loose aggragates on binding. The buoyant density would not be affected, but diffusion would be slowed. Much sharper bands might result.

A dye bound to a column material--such a material could be used to separate large quantities of DNA quickly

and efficiently.

A dye with a meta- or ortho-substituted group on the phenyl ring--a bulky group might cause either a more restrictive flattening of the ring, enhancing fluorescence, or a greater spreading of the base-pairs, improving unwinding.

The foregoing is reported with the work of others in a paper to be published in March, "The Use of an Ethidium Analogue in the Dye-Buoyant-Density Process for the Isolation of Closed Circular DNA: The Variation of the Superhelix density of Mitochondrial DNA," B. Hudson, W. B. Upholt, J. Devinny, J. Vinograd, P.N.A.S., Vol. 59, March, 1969.

Thanks are due the California Institute of Technology, for its facilities, and Dr. Jerome Vinograd and Bruce Hudson for direction.

1. Trypanocides of the Phenanthridine Series, Part I: The Effect of Changing the Quaternary Grouping In Dimidium Bromide, T. I. Watkins, J. Chem. Soc., 1952, 3059

2. A Dye-Buoyant-Density Method for the Detection and Isolation of Closed Circular Duplex DNA: The Closed Circular DNA in HELA Cells., R. Radloff, W. Bauer, J. Vinograd, P.N.A.S., Vol. 57, No. 5, 1514, May, 1967

ABSTRACT

The dye-buoyant-density method for separation of circular and linear DNA is reviewed, and an improved dye proposed. The synthesis of the dye is described, and its feasibility as a general method discussed.