Science Vision www.sciencevision.org *Science Vision* www.sciencevision.org *Science Vision* www.sciencevision.org *Science Vision* www.sciencevision.org

Historical Review

Science Vision 16 (1)
January-March
ISSN (print) 0975-6175
ISSN (online) 2229-6026

The making of modern biotechnology: how ethidium bromide made fame

K. Lalchhandama

Department of Zoology, Pachhunga University College, Aizawl 796001, India

Received 7 January 2016 | Accepted 21 January 2016

Abstract

Ethidium bromide is a household name in biology, and is arguably the most popular stain in molecular research. It was discovered as a consequence of the growth spurt in chemical synthesis towards the very end of 19th century and in the first half of the 20th century. The precursor compound phenanthridine aroused its potential as a drug, basically because of its quinoline ring, which is interestingly the basis of the medicinal properties of important drugs, such as quinine, known at the time. A medical breakthrough was made in 1938 when some derivatives of phenanthridine were experimented to effectively kill *Trypanosoma congolense* and *T. vivax*, the protozoan parasites causing trypanosomiasis (sleeping sickness) in cattle. In 1946, the most effective compound was identified, and with slight chemical modification, it was mass produced as a trypanocidal drug, dimidium bromide, or trypadine. It was the principal veterinary drug in Africa until another chemical modification in 1952 yielded a more potent and less toxic compound, the now-famed ethidium bromide. Manufactured by Boots Pure Drug Co., Ltd. as homidium (Ethidium®), it served as the drug of choice in cattle trypanosomiasis for three decades. Its pharmacological property lies on its ability to intercalate between base pairs in the nucleic acids. It was a serendipitous moment when Piet Borst and Cees Aaij, dismayed at their broken ultracentrifuge, began to use the compound for staining DNA in gel electrophoresis. And the rest, as they say, is history.

Key words: Dimidium bromide; ethidium bromide; electrophoresis; phenanthridine; trypanosome.

Introduction

Ethidium bromide (EtBr) is chemically phenanthridinium, or 3,8-diarnino-5-ethyl-6-phenyl-bromide, or 2,7-diamino-10-ethyl-9-phenanthridinium bromide, or 2,7-diamino-9-phenylphenanthridium ethobromide. Under the pharmaceutical name homidium (sometimes bromide), it is marketed as an antiprotozoal drug, a drug of choice for veterinary trypanosomiasis for several decades. It is most widely used as an orange-red cationic fluorescent dye such as for visualising nucleic acid as it binds to both RNA and DNA.[1] In this respect, it has an advantage over other

Corresponding author: Lalchhandama
Phone: : +91-9436198718
E-mail: chhandama@gmail.com

CC BY-SA 4.0 International 2016

nucleic acid stains, which are often specific for a specific type of nucleic acid. Under an ultraviolet light, the EtBr-stained nucleic acids fluoresce, allowing convenient identification and visualisation of nucleic acid bands. As both a DNA-dependent intercalating agent and a DNA-independent protein inhibitor, it is being used in a variety of biochemical and molecular techniques as a generic and specific marker, most importantly for analytical and preparative gel electrophoresis.[2] As an indispensable molecular stain, there are many variations on the use of EtBr. Inaddition, different biotechniques employ EtBr, including capillary electrophoresis, fluorometry, spectrophotometry, flow cytometry, polymerase chain reaction (PCR) amplification.

Further, EtBr is good not only for staining of nucleic acids but also for staining of proteins. Similar to nucleic acid bands, proteins are stained with EtBr after polyacrylamide gel soaking in trichloroacetic acid solution.[3] EtBr also has a large-scale industrial application. A most sophisticated and standard quality test of milk is done by treating milk sample with EtBr. EtBr easily reveals cellular impurities such as pathogens, and by this the hygiene and safety of the milk as well as that of the cow itself is easily assessed.[4]

Genesis

EtBr belongs to a class of tricyclic aromatic heterocyclic compounds called phenanthridine (Fig. 1). Phenanthridine was originally obtained from destructive distillation coal. It was specifically produced from the pyrolytic condensation of benzaldehyde and aniline at bright red flame. The discovery was reported by Swiss chemists Amé Pictet and H. J. Ankersmit from the Chemisches Laboratorium der Universität Genf in Geneva, Switzerland, in 1891.[5] Pictet and A. Hubert gave a more efficient reaction in 1896 by dehydrating acyl-o-xenylamines in fusion with zinc chloride at high temperatures.[6] This is known as the Pictet-Hubert reaction (Fig. 2). Driven by chemistry such as this, and discoveries of the causative agents of important diseases, the turn of the 20th century brought an unprecedented gusto in drug research. After decades of latency, phenanthridine also began to receive research attention.

In the early 1930s, a number of chemical derivatives of phenanthridine were synthesised, and some of them were tested indicating that they possessed some pharmacologial properties.[7] A pioneering and inspirational work was done by two British chemists (Sir) Gilbert Morgan

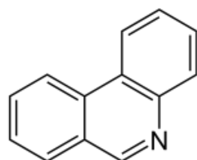

Figure 1. Phenanthridine structure.

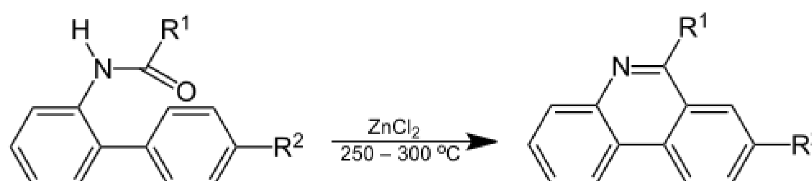

Figure 2. Pictet-Hubert reaction.

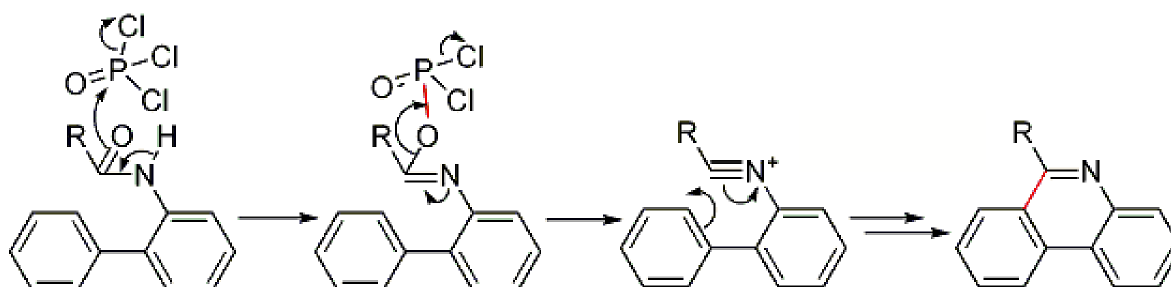

Figure 3. Morgan-Walls reaction.

and Leslie P. Walls. In 1931, they published a paper in which a more efficient method of phenanthridine synthesis was described,[8] which is now known as Morgan-Walls reaction (Fig. 3), and remains the most popular method for phenanthridine sysnthesis. In 1934, Walter G. Christiansen, Glen Ridge, and William Braker of the E. R. Squibb & Sons in New York filed for patent for inventing the methods for preparing "Phenanthridine derivatives". The patent was granted in 1939 under US patent number US2176889 A.

The landmark discovery was made in 1938 by Morgan and Walls, teaming up with Carl Hamilton Browning and J. V. M. Robb, based at the Western Infirmary in the University Glasgow. They found that some phenanthridines were highly effective against the major protozoan parasites of cattle, *Trypanosoma congolense* and *T. vivax* (Fig. 4).[9] These trypanosomes are causative pathogens of a fatal parasitic disease called trypanosomiasis, or more popularly, sleeping sickness, or still more popularly in Africa, nagana (a Zulu word for "to be depressed"), which is responsible for heavy economic losses in cattle farming. In 1946, they found out that one compound in particular, 9-phenyl-phenanthridinium (or simply, phenidium) that contained two amino groups, was the most potent on *T. cruzi.*[10] Walls filed for patent in 1945, on behalf of The Imperial Trust for the Encouragement of Scientific and Industrial Research, specifically for the method of preparation of 2:7-diamino-9-phenylphenanthridine, and was granted in 1948 under US patent number US2437869 A. In 1947, he showed that substitution of the 9-phenyl group with different amino and *o*- and *p*-nitro groups greatly enhanced the trypanocidal potency.[11,12] Among the various derivatives he synthesised, 2:7-diamino-10-methyl-9-phenyl-phenanthridinium bromide was the most potent of all (eventually to be called dimidium bromide, and for its trypanocidal activity, trypadine; Fig. 5). The improved method for the chemical synthesis was patented (US2495051 A) by Harry James Barber for May & Baker Limited, Essex, England, in 1950. Barber's claim was the ability

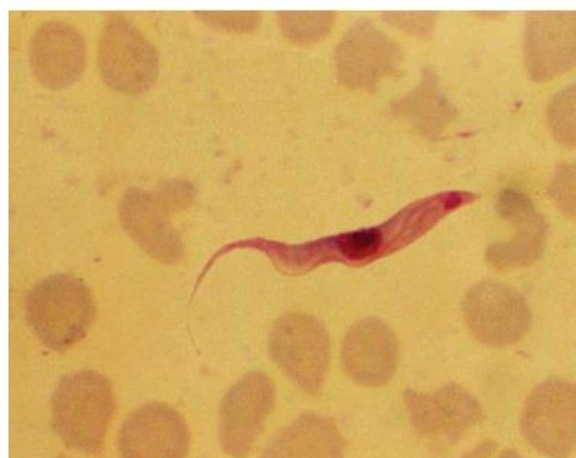

Figure 4. Blood smear of a cow infected with *Trypanosoma vivax*, from Brazil. [Osório *et al*. (2008). *Mem Inst Oswaldo Cruz*, **103**, 1-13.]

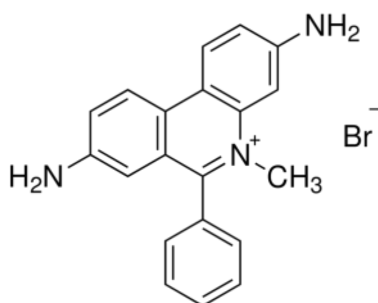

Figure 5. Dimidium bromide.

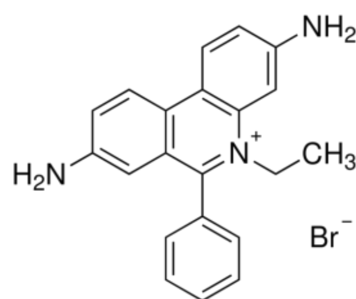

Figure 6. Ethidium bromide.

to obtain phenanthridine analogues by reacting with n-butyl or amyl alcohol the product obtainable by treating a quaternary salt of 2:7-diamino-9-phenylphenanthridine with an excess of alkali in a solvent medium.

It was from this moment that Walls apprehended, and stated:

> It seemed to me that the study of phenanthridine series might lead to results of interest in chemotherapy for the following reasons. Phenanthridine contains a quinoline ring system, which is present also in quinine and the first satisfactory antimalarial, pamaquine, and also an isoquinoline ring system present in many physiologically active alkaloids; it is isomeric with and analogous to acridine, the mother substance of many powerful antiseptics of which acriflavine is the best known.[12]

Rightly so, phenathridines became the mother substance of several drugs to come – homidium, prothidium, isometamidium, including the latest pro-cancer drug, phenanthriplatin.[13]

The bovine saviour but a futile one

Field trials of dimidium bromide for practical use in veterinary medicine were conducted from the late 1940s. The first reports were on the effective treatments of *T. simiae* infection of pigs in Uganda,[14] and *T. congolense* infection of cattle in Uganda,[15] and Sudan.[16] It soon earned its reputation by becoming the drug of choice for cattle trypanosomiasis in Africa. The drug is given by subcutaneous or intravenous injection at 1 mg/kg of body weight. But it was soon realised that the drug was highly toxic as indicated by liver failure and symptoms of photosensitization in cattle, in addition to the compound being unstable when prepared in solution.[17]

In 1952, T. I. Watkins and G. Woolfe at the Boots Pure Drug Co., Ltd. in Nottingham, UK, synthesised a modified compound by replacing the methyl group with an ethyl group.[18] Watkins further demonstrated that the novel compound was much more efficacious (10 times more potent, which is attributed to higher permeability through cell membrane), stable and less toxic than the original compound in laboratory test and field trials.[19] The new drug became ethidium bromide (Fig. 6), and marketed as homidium (Ethidium®), and it remained the principal drug for mass treatment of cattle trypanosomiasis for three decades. The Boots Pure Drug Co., Ltd. was the principal manufacturer of the drug for several decades. The drug is still used today, but to lesser extent as a result of drug resistance and development of new drugs.[20] The possibility of EtBr resistance in *T. congolense* in experimental mice was demonstrated by F. Hawking in 1963.[21] The first report of reduced efficacy or resistance for the recommended dose (1 mg/kg) came from Nigeria,[22] and West Africa in 1966.[23] By the next decade it became an unbridled phenomenon throughout cattle farms in Africa.[24]

Mechanism of action

Soon after the discovery of the DNA structure and function, and development of molecular techniques, EtBr binding to and effects on DNA was immediately investigated. In 1957, B.A. Newton of the University of Cambridge reported that EtBr rapidly inhibits DNA synthesis in a parasitic flagellate *Strigomonas oncopelti*.[25] The specific binding of EtBr on DNA became known in the early 1960s. A British microbiologist Michael John Waring at the University of Cambridge, UK, reported in 1964 the molecular complex formed by EtBr with DNA, and its inhibition of RNA polymerase in the bacterium *Escherichia coli*.[26] At the same time, French chemist J.-B. Lepecq and his team at the Gustave-Roussy Institute in Villejuif also reported similar DNA-interaction.[27] In the same year, Waring, with Watson Fuller at the University of London King's College, produced an X-ray diffraction image and molecular model depicting the EtBr-DNA interaction. They specifically showed that the planar ring system of the drug is inserted between successive base pairs of the DNA.[28] This was the discovery of the DNA-intercalating property of EtBr (Fig. 7).

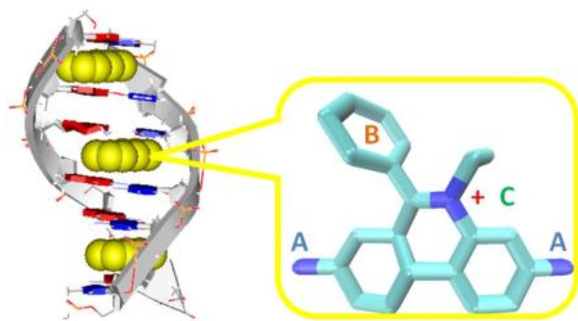

Figure 7. EtBr intercalating in DNA strand. [Tumir *et al.* (2014). *Beilstein J Org Chem*, **10**, 2930–2954.]

Protozoans belonging to the group Kinetoplastida possess a mass of cytoplasmic circular DNA, much like mitochondrial DNA, called kinetoplast (the DNA being called kDNA), in addition to their nuclear DNA. EtBr specifically targets cytoplasmic DNA. A French pharmacologist at the Gustave-Roussy Institute in Villejuif, Guy Riou was one of the first to show (in 1967) that it is by interfering with DNA function that kDNA in trypanosomes are inhibited by EtBr, thereby resulting in trypanocidal activity.[29] Yet, the exact mechanism of kDNA damage remained a mystery until 2010 when a team of researchers from the Johns Hopkins Medical School and University of North Carolina, Chapel Hill, USA, revealed that EtBr is responsible for helix distortion of free minicircles and inhibiting replication initiation, and accumulated effects of these activities lead to kDNA loss and ultimate cell death.[30]

Other uses

Several *in vitro* studies have supported the potential use of EtBr as an antitumorigenic chemotherapeutic agent. One of the earliest reports on the antitumour activity was in 1962. T. S. Kandaswamy and J. Frank Henderson at the Department of Pharmacology, George Washington University School of Medicine, reported that it completely inhibited the growth of Ehrlich ascites tumour cells. The antitumour activity was specifically by the incorporation of preformed purines (adenine, guanine and hypoxanthine) into nucleic acids. They even suggested by comparative test that a combination with antibiotic azaserine as anticancer drug.[31] It is effective against mammary adenocarcinoma EO-771, sarcoma 180, and Flexner-Jobling carcinoma solid tumors, ascitic tumors in mice, and in the leukemia L-1210 ascites system.[33]

EtBr has also been used as a drug or drug precursor, for example as a parasitotoxic, antiprotozoal drug for the treatment of another parasitic disease called leishmaniasis (caused by a another trypanosomatid parasite *Leishmania*), in combination therapeutic regimens using DNA reactive agents, and in the development of anionic and hydrophobic derivatives as trypanocides.[34]

Making straight the way of biotechnology

"[The] well-being of mankind which I promised yesterday may, alas, have to wait several more years still. It is, however, fortunate, although sometimes a little embarrassing, that people nowadays understand that research, especially fundamental research, will have to take its time before it pays its dividend," recapitulated Arne Wilhelm Kaurin Tiselius,[35] a Swiss chemist who single-handedly discovered the principle of electrophoresis and invented a working device for it. In 1925, Tiselius joined the laboratory of a renowned Swiss chemist Theodor Svedberg (recipient of the 1926 Nobel Prize in Chemistry *"for his work on disperse systems"*) at the Uppsala University in Sweden. He was assigned a project on the application of electricity for analytical tools of proteins. He soon developed an equipment for free electrophoresis which was about five meters in length, large enough to occupy a typical laboratory of the time. In 1930, he was able to report in the Swiss Royal Society journal *Nova Acta Regiae Societatis Scientiarum Upsaliensis* that purified proteins migrated as homogenic bands in his new equipment (Tiselius 30).[36] A thesis titled *The Moving Boundary Method of Studying the Electrophoresis of Proteins* in the same year eventually earned him a doctorate. Thus, moving boundary electrophoresis was invented (Fig. 8). But there was a major defect with this electrophoresis, heat convection due to electric supply caused protein bands to blur. He made a series of modifications to achieve a desired result, but was successful after six years only. By replacing the round tubes with flat vessels (Fig. 9) and cooling the instrument to +4°C, he could effectually separate normal serum into albumin, and distinct α-, β-, and γ-globulin bands. The seminal paper was originally submitted to a chemical journal in 1936 but rejected on the ground that it was "too physical". It was published in the 1 February 1937 issue of *Biochemical Journal*.[37] The paper was immediately followed by a series of follow-up publications in 1937.[38,39]

Yet another improvement in 1950, with Herman Haglund, led him to an invention of zone electrophoresis using a glass capillary system with glass powder as the stabilizing agent (Fig. 10).[40] The apparatus was based on the device developed by Swedish chemists Harry Svensson

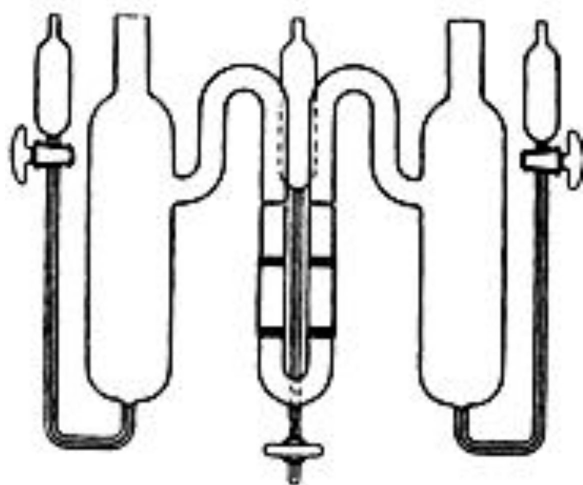

Figure 8. Schematic diagram of moving boundary electrophoresis from Tiselius (1930).

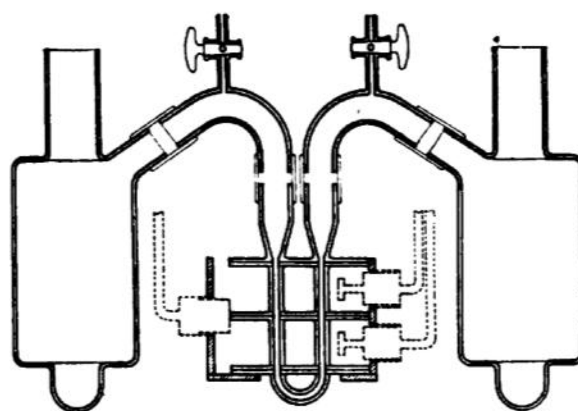

Figure 9. Schematic diagram of an improved electrophoresis from Tiselius (1937).

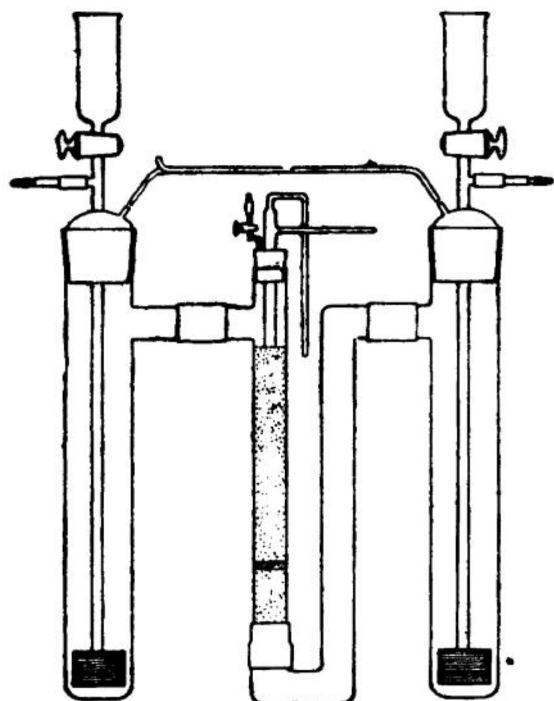

Figure 10. Schematic diagram of zone electrophoresis from Haglund and Tiselius (1950).

and Inger Brattsten at the Laboratories of LKB-Produkter Fabriksaktiebolag in Stockholm, early in the same year.[41] Then, in 1951, Tiselius, in collaboration with an American chemist Henry G. Kunkel at the Rockefeller Institute for Medical Research in New York, developed an electrophoresis using filter paper. The novel method was superior to existing methods as it could achieve complete separation of the components, analyse smaller sample concentrations, and isolation of the components.[42] Tiselius eventually won the Nobel Prize in Chemistry 1948 "*for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins*", and earned a lasting epithet as the "father of electrophoresis."[43]

The initial invention was taken to the next level by a British biochemist Oliver Smithies while working at the Connaught Medical Research Laboratories in Toronto, Canada. A fresh graduate from the University of Oxford in 1953, he was planning to work at the University of Wisconsin–Madison in Wisconsin, US. But visa problem landed him to work as a research associate to David A. Scott, "an insulin specialist", as he called him, in Toronto. He was encouraged, he recalled, "...[To] work on anything I liked, as long as it had something to do with insulin."[44] He chose to investigate on insulin precursor, and was instantaneously kept at bay by the limitations of electrophoresis available then for his purposes. This made him created a lasting fame, not in insulin, but in electrophoresis.[45] He noticed from the works of Tiselius, particularly the filter-paper electrophoresis, and the work of Kunkel and Robert J. Slater. The latter researchers reported in 1951 that starch provided a good supporting media for protein separation because of its low adsorption in aqueous buffers.[46] Smithies thought of combining the zone, filter-paper and starch electrophoreses. From the filter-paper method, he would use the method of loading the sample in a narrow zone but in the zone electrophoresis. Then he used starch gel as a supporting medium, and to his utter satisfaction, found that the method was useful for separation of plant enzymes, and serum protein. His invention and its results were published in 1955 in *Biochemical Journal*.[47] This led to an improved two-dimensional electrophoretic system in 1956.[48] In 1959, he published a further improvement in which a supporting media could be avoided.[49] He called the new accessory device "removable slot former," which was the beginning of making wells by gel combs. Indeed, Smithies' works paved an easy way for the development of the now more popular agarose gel electrophoresis, introduced in 1956 by an Indian biochemist Kramadhati Venkata Giri at the Indian Institute of Science in Bangalore,[50,51] and polyacrylamide gel electrophoresis, introduced in 1959 independently by two American teams, Baruch J. Davis and Leonard Ornstein at the Mount Sinai Hospital in New York,[52] and Samuel Raymond and Lewis Weintraub at the University of Pennsylvania in Philadelphia.[53] It is not a profligate digression to mention that

Smithies shared the Nobel Prize in Physiology or Medicine 2007 with Mario R. Capecchi and Sir Martin J. Evans "*for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells*."

Back then a variety of stains already existed for protein, such as amido black, bromophenol blue, Coomassie blue, to name a few. The chemistry and biology of nucleic acids were just starting to enter the limelight of research.

The fluky orange-men

EtBr was the first and has been the most widely used nucleic acid dye in molecular biology. Its unwarranted infamy as an ill-fated chemical because of its toxicity and tumour-causing effects, compounded by the development of novel stains in recent years have given rise to its diminished popularity, and what is more, an unprecedented criticism and denunciation of its practical use in terms of safety.

The birth of EtBr-based gel electrophoresis was a pure stroke of serendipity, a tale that affirmed Louis Pasteur's maxim: chance favoured the prepared mind. The first invention was reported in 1972 by a Dutch physician Piet Borst and his student Cees Aaij, while working in the Department of Medical Enzymology at the University of Amsterdam. Borst research focused on the structure and replication of the circular DNAs from vertebrate mitochondria. By that period, the conventional technique of assessing the DNA quality was by ultracentrifugation. One day their work was thwarted by the centrifuge that just would not kick off to run its purpose.[54] While perspiring for the possible cause and solution, Borst realised that back in 1966, an American microbiologist H.V. Thorne at the University of Minnesota Medical School had used agarose gel electrophoresis to separate cellular DNA from polyoma viral DNA.[55] With that hindsight, he was convinced that it would just might also work for his rat mtDNA. Using only 0.6% agarose (instead of 1% as used by Thorne), he found that DNA separation a relatively simple process. But the immediate problem he encountered was how to visualise the DNA bands. Borst realised that cesium chloride-EtBr mixture they used in ultracentrifugation to produce bright orange fluorescence might do the trick. So he tried staining the gel only with EtBr, and to his anticipated amazement, the fractionated DNA showed distinct orange bands. The colour had no particular connection with the Dutch national colour, but it was a beautiful coincidence. The novel technique proved to be much more efficient than any other method for very small DNA molecules. The finding was published in the 10 May 1972 issue of *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis*.[56] After certain modifications, Borst pronounced:

> In the end, ethidium-agarose electrophoresis worked like a charm for quantitating topoisomers of mtDNAs and we never went back to our analytical ultracentrifuge for DNA quality control.[54]

The luckier Americans

But the Dutch were not alone. Unbeknown to the Amsterdam researchers, American molecular biologist Philip A. Sharp and his team at the Cold Spring Harbor Laboratory had successfully tested EtBr to stain electrophoretic product. Sharp had previously used EtBr for staining whole DNA samples while working as postdoctoral student at the California Institute of Technology. In 1971, he joined the Cold Spring Harbor Laboratory to investigate on the genetics of tumour DNA viruses, particularly SV40. At the time, electrophoretic gel products were assessed using a laborious autoradiography. With his experience with EtBr, Sharp stained the gel and quickly noticed distinct DNA bands.[57] Sharp and his team eventually discovered that EtBr-stain gel was very efficient for isolating DNA fragments, specifically two restriction endonucleases, in the bacterium *Haemophilus parainfluenzae*. They used a very low concentration of 0.5 μg/ml in 1.4% agarose gel, which was sufficient to stain DNA as small as 0.005 μg. This tech-

nique led them to identify two new endonucleases, *Hpa*I and *Hpa*II. They reported their work in the July issue of *Biochemistry* in 1973.[58]

These two biotechnological innovations raised EtBr-based gel electrophoresis as a gold standard in nucleic acid research. It is a bit unfortunate that the original publication by the Dutch workers is largely forgotten, while Sharp *et al*. is a more frequent, and identified "classic" citation. For instance, as of March 2016, the number of Google Scholar citation for Borst and Aaij is 372, while the number is 1,463 for Sharp *et al.*

References

1. Williams LR (2001). Staining nucleic acids and proteins in electrophoresis gels. *Biotech Histochem*, **76**, 127–132.
2. Lai JS & Herr W (1992). Ethidium bromide provides a simple tool for identifying genuine DNA-independent protein associations. *Proc Natl Acad Sci*, **89**, 6958–6962.
3. Dvortsov IA, Lunina NA, Chekanovskaya LA, Shedova EN, Gening LV & Velikodvorskaya GA (2006). Ethidium bromide is good not only for staining of nucleic acids but also for staining of proteins after polyacrylamide gel soaking in trichloroacetic acid solution. *Anal Biochem*, **353**, 293 –295.
4. Tabit FT (2016). Advantages and limitations of potential methods for the analysis of bacteria in milk: a review. *J Food Sci Technol*, **53**, 42–49.
5. Pictet A & Ankersmit HJ (1891). Ueber das Phenanthridin. *Justus Liebigs Ann Chem*, **266**, 138–153.
6. Pictet A & Hubert A (1896). Ueber eine neue Synthese der Phenanthridinbasen. *Ber Dtsch Chem Ges*, **29**, 1182–1189.
7. Theobald RS & Schofield K (1950). The chemistry of phenanthridine and its derivatives. *Chem Rev*, **46**, 170–189.
8. Morgan GT & Walls LP (1931). Researches in the phenanthridine series. Part I. A new synthesis of phenanthridine homologues and derivatives. *J Chem Soc*, **1931**, 2447–2456.
9. Browning CH, Morgan GT, Robb JVM & Walls LP (1938). The trypanocidal action of certain phenanthridinium compounds. *J Pathol*, **46**, 203–204.
10. Browning CH, Calver KM, Leckie MW & Walls LP (1946). Phenanthridine compounds as chemotherapeutic agents in experimental *T. cruzi* Infections. *Nature*, **157**, 263 –264.
11. Walls LP (1947). Researches in the phenanthridine series. Part VIII. Further investigation of trypanocidal types. *J Chem Soc*, **1947**, 67–74.
12. Walls LP (1947). The chemotherapy of phenanthridine compounds. *J Soc Chem Industry*, **66**, 182–187.
13. Wainwright M (2010). Dyes, trypanosomiasis and DNA: a historical and critical review. *Biotech Histochem*, **85**, 341–354.
14. Wilson SG (1948). The trypanocidal effects of dimidium bromide (phenanthridinium 1553) on *Trypanosoma simiae* in domestic pigs. *Vet Rec*, **60**, 474–477.
15. Wilson SG (1948). Further observations on the curative value of dimidium bromide in *Trypanosoma congolense* infections in bovines in Uganda. *J Comp Pathol Ther*, **58**, 94–106.
16. Evans JT (1948). *Trypanosoma congolense* infection in cattle in the Sudan; treatment with dimidium bromide. *Vet Rec*, **60**, 418–420.
17. Wilde JKH (1949). Experimental use of dimidium bromide (1553) in the treatment of *T. congolense* infection in zebu cattle. *J Comp Pathol Ther*, **59**, 48–53.
18. Watkins TI & Woolfe G (1952). Effect of changing the quaternizing group on the trypanocidal activity of dimidium bromide. *Nature*, **169**, 506–507.
19. Watkins TI (1952). Trypanocides of the phenanthridine series. Part 1. The effect of changing the quaternary grouping in dimidium bromide. *J Chem Soc*, **1952**, 3059–3064.
20. Holmes PH, Eisler MC & Geerts S (2004). Current chemotherapy of animal trypanosomiasis. In: *The trypanosomiases* (I Maudlin, PH Holmes, MA Miles, eds). CABI Publishing, Wallingford, Oxfordshire, U.K., pp. 411–444.
21. Hawking F (1963). Drug-Resistance of *Trypanosoma congolense* and other trypanosomes to quinapyramine, phenanthridines, berenil and other compounds in mice. *Ann Trop Med Parasit*, **57**, 262–82.
22. Jones-Davies WJ & Folkers C (1966). The prevalence of homidium-resistant strains of trypanosomes in cattle in northern Nigeria. *Bull Epizoot Dis Afr*, **14**, 65–72.
23. Stephen LE (1966). Observations on the resistance of West African N'dama and Zebu cattle to trypanosomiasis following challenge by wild *Glossina morsitans* from an early age. *Ann Trop Med Parasit*, **60**, 230–246.
24. Geerts S, Holmes PH, Eisler MC & Diall O (2001). African bovine trypanosomiasis: the problem of drug resistance. *Trends Parasitol*, **17**, 25–28.
25. Newton BA (1957). The mode of action of phenanthridines: the effect of ethidium bromide on cell division and nucleic acid synthesis. *J Gen Microbiol*, **17**, 718–730.
26. Waring MJ (1964). Complex formation with DNA and inhibition of *Escherichia coli* RNA polymerase by ethidium

bromide. *Biochim Biophys Acta*, **87**,358–361.

27. Lepecq J, Yot P & Paoletti C (1964). Interaction of ethidium bromhydrate (BET) with nucleic acids (NA) spectrofluorimetric study. *C R Hebd Seances Acad Sci*, **259**, 1786–1789.
28. Fuller W & Waring MJ (1964). A molecular model for the interaction of ethidium bromide with deoxyribonucleic acid. *Ber Bunsenges Phys Chem*, **68**, 805–808.
29. Riou G (1967). Induction of a "dyskinetoplast" by ethidium bromide in *Trypanosoma cruzi* cultivated in vitro. *C R Hebd Seances Acad Sci*, **265**, 2004–2007.
30. Roy Chowdhury A, Bakshi R, Wang J, Yildirir G, Liu B, Pappas-Brown V, Tolun G, Griffith JD, Shapiro TA, Jensen RE & Englund PT (2010). The killing of African trypanosomes by ethidium bromide. *PLoS Pathog*, **6**, e1001226.
31. Kandaswamy TS & Henderson JF (1962). Inhibition of ascites tumour growth by the trypanocide, ethidium bromide, in combination with azaserine. *Nature,* **195**, 85.
32. Kramer MJ & Grunberg E (1973) Effect of ethidium bromide against transplantable tumors in mice and rats. *Chemotherapy*, **19**, 254–258.
33. Pavelic K, Beerman TA & Bernacki RJ (1985). An evaluation of the effects of combination chemotherapy in vitro using DNA-reactive agents. *Cancer Drug Delivery*, **2**, 255–270.
34. Tiselius A (1968). Reflections from both sides of the counter. *Annu Rev Biochem*, **37**, 1–23.
35. Tiselius A (1930). The moving boundary method of studying the electrophoreses of proteins. *Nova Acta Soc Sci Upsal,* **7**, 4.
36. Tiselius A (1937). Electrophoresis of serum globulin. I. *Biochem J*, **31**, 313–317.
37. Tiselius A (1937). Electrophoresis of serum globulin: Electrophoretic analysis of normal and immune sera. *Biochem J*, **31**, 1464.
38. Tiselius A (1937). A new apparatus for electrophoretic analysis of colloidal mixtures. *Trans Faraday Soc*, **33**, 524–531.
39. Tiselius A (1937). Electrophoresis of purified antibody preparations. *J Exp Med*, **65**, 641–646.
40. Haglund H & Tiselius A (1950). Zone electrophoresis in a glass powder column. *Acta Chem* Scand, **4**, 957–962.
41. Svensson H & Brattsten I (1950). An apparatus for continuous electrophoretic separation in flowing liquids. *Arkiv Kemi*, **1**, 401–411.
42. Kunkel HG & Tiselius A (1951). Electrophoresis of proteins on filter paper. *J Gen Physiol*, **35**, 89–118.
43. Kyle RA & Shampo MA (2005). Arne Tiselius – father of electrophoresis. *Mayo Clinic Proc*, **80**, 302.
44. Williams R (2011). Oliver Smithies: born inventor. *Circ Res*, **108**, 650–652.
45. Kunkel HG & Slater RJ (1952). Zone electrophoresis in a starch supporting medium. *Exp Biol Med*, **80**, 42–44.
46. Smithies O (2012). How it all began: A personal history of gel electrophoresis. In: *Protein Electrophoresis: Methods and Protocols* (BT Kurien, RH Scofield, eds). Humana Press, Springer Science+Business Media, LLC, pp.1–21.
47. Smithies O (1955). Zone electrophoresis in starch gels: group variations in the serum proteins of normal human adults. *Biochemical J*, **61**, 629–641.
48. Smithies O & Poulik MD (1956). Two-dimensional electrophoresis of serum proteins. *Nature,* **177**, 1033.
49. Smithies O (1959). An improved procedure for starch-gel electrophoresis: further variations in the serum proteins of normal individuals. *Biochem J*, **71**, 585.
50. Giri KV (1956). Zone electrophoresis of serum proteins in agar gel. *Naturwissenschaften*, **43**, 36.
51. Giri KV (1956). Agar electrophoresis on cellophane and polyester films. *Naturwissenschaften*, **43**, 226.
52. Davis BJ & Ornstein L (1959). A new high resolution electrophoresis method. [Delivered at the Society for the Study of Blood at the New York Academy of Medicine.]
53. Raymond S & Weintraub L (1959). Acrylamide gel as a supporting medium for zone electrophoresis. *Science*, **130**, 711.
54. Borst P (2005). Ethidium DNA agarose gel electrophoresis: how it started. *IUBMB Life*, **57**, 745–747.
55. Thorne HV (1966). Electrophoretic separation of polyoma virus DNA from host cell DNA. *Virology*, **29**, 234–239.
56. Aaij C & Borst P (1972). The gel electrophoresis of DNA. *Biochim Biophys Acta*, **269**, 192–200.
57. Sharp PA (1981). This week's citation classic. *Curr Cont*, **CC 3**, 18.
58. Sharp PA, Sugden B & Sambrook J (1973). Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. *Biochemistry*, **12**, 3055–3063.