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Is polymerase chain reaction fully reliable? A critical review in the light of published evidence from the golden decades of molecular genetics

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Simple Summary: Soon after its introduction in 1987, Polymerase Chain Reaction (PCR) has become a cycle-based technique widely employed in diagnostic medical devices and in forensic science with the intention of amplifying genetic information. The achievement of a sufficient degree of fidelity is mandatory for reliability in applications; however, PCR prescribes that each of its cycles must include a heating subprocess at 95°C or more, denominated DNA denaturation, which may entail a risk of disruption of the DNA molecule.

Understanding that the *conditio sine qua non* for the fidelity of PCR is to prevent such a disruption, a review is presented of the historical literature of the years 1950-1960 elucidating the effects of heating on possible DNA fragmentation. The main conclusion of this review is that the body of examined evidence consistently and redundantly confirms such disruption when DNA is heated at temperatures above 90°C, even for one minute. This appears to contradict the paradigm of PCR fidelity and leads the authors to raise the concern that PCR may amplify information, but, at least for long sequences, in an unreliable way. This should open a discussion on what the PCR paradigm could signify in various fields in which PCR is used.

Abstract: The purpose of this review is to verify the reliability of the PCR test in amplifying the genetic information contained in the samples to be tested. The fundamentals of PCR include, in each of its amplification cycles, a phase of denaturation of DNA by heating, which – according to the proponents – allows the two chains of the molecule to be separated, followed by a cooling phase intended to allow the formation of two equal molecular sequences of DNA. The repetition of the procedure for several times would allow to obtain huge quantities of the same sequence, thus allowing easier detection and analysis of a desired sequence. The possibility of fragmentation could however compromise the final result. While this is excluded by the inventor, scientific literature on the matter is far from being unanimous.

Tracing back to the seminal studies on DNA heating, proof of longitudinal sequence-breaking random thermal fragmentation of DNA molecules is found. To ascertain this phenomenon, three main measurement methodologies have been applied on native and heated DNA by independent group of researchers: sedimentation rate, viscometry, and light scattering. The examined experimental studies agree – all but a few exceptions – in finding important alterations of DNA even after one heating-cooling cycle: it would seem amplified DNA molecules could not maintain their original structure.

The real significance of PCR is questioned, and a thorough reevaluation suggested on this matter and its consequences.

Keywords: PCR; thermal depolymerization; amplification fidelity; diagnostic reliability; genetic information integrity.

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Sommario in italiano: Poco dopo la sua introduzione nel 1987, la Polymerase Chain Reaction (PCR), una tecnica basata sulla reiterazione di procedimenti ciclici di manipolazione del materiale genetico, ha trovato largo impiego, in ambito medico diagnostico e nelle scienze forensi, in dispositivi impiegati dagli utenti con l'intento di amplificare l'informazione genetica contenuta in campioni di materiale. Il raggiungimento di un grado di fedeltà di amplificazione sufficiente è fondamentale per garantire affidabilità nelle applicazioni; tuttavia, la PCR prescrive che in ciascuno dei suoi cicli sia incluso un processo di riscaldamento a temperature uguali o superiori a 95°C, denominato denaturazione del DNA, che può comportare il rischio di distruggere le molecole di DNA.

Procedendo dalla considerazione che la *conditio sine qua non* affinché il requisito di fedeltà della PCR sia garantito è che sia scongiurata una distruzione caotica della molecola oggetto di amplificazione, viene qui presentata una revisione della letteratura storica a cavallo degli anni 1950-1960 allo scopo di chiarire gli effetti del riscaldamento sulla possibile frammentazione del DNA. La conclusione principale di questa revisione è che il corpo complessivo di evidenze esaminate conferma consistentemente e ridondantemente una siffatta distruzione quando il DNA è riscaldato a temperature superiori ai 90°C anche per un solo minuto. Questa conclusione appare contraddire il paradigma della fedeltà della PCR e induce gli autori a sollevare la preoccupazione che la PCR possa amplificare l'informazione ma, almeno per sequenze di lunghezza maggiore, in un modo non affidabile e non controllabile. Questa conclusione dovrebbe sollecitare una discussione sul reale significato del paradigma della PCR nei vari campi in cui questa tecnica è impiegata.

1. Introduction

As narrated by himself in an autobiographic note [1], in 1983 Kary Mullis was struck by the idea of a procedure for synthesizing repeatedly Deoxyribonucleic Acid (DNA) in vitro, which he named *Polymerase Chain Reaction* (PCR). Beginning with a single target molecule of DNA, he envisaged he could find DNA fragments and “generate 100 billion similar molecules in an afternoon”. In 1985 he applied for a patent for PCR [2], then finished and substituted it by a subsequent 1987 application [3]. He described such procedure also in a scientific record [4] in the format of an experimental method. The Authors report [3,4] that PCR is based upon the reiteration of a reaction cycle which includes, in each cycle, a heating subprocess referred to as *denaturing* or *denaturation*. In the “Claims” section of the patent application [3], denaturation was so mentioned: “wherein said nucleic acid is double stranded and its strands are separated by denaturing”. Hereafter, we will refer to such heating subprocess intended for DNA strands separation as the ‘denaturation claim’ or, more briefly, as DNA denaturation.

The presence of a DNA heating step, in a conceivable theoretical scheme for nucleic acid amplification and sequencing, turns out to be a critical point for the sake of the fidelity of such an amplification procedure against the risk of thermal depolymerization, or disaggregation, of the DNA molecule. This could lead to a consequential loss of information in the sequence of nucleotides, due to the random breakage mechanisms similar to those explaining the molecular weight decay observed under the effect of gamma-rays on

DNA, described for instance by Cox et al. (1955) [5], and reported also for heating by many authors. However, concerning DNA stability to heating, there can be found relevant discrepancies between more recent studies dealing with the exploitation of DNA heating above 80°C for nucleic acid amplification purposes, and the results in the original early studies devoted to the analysis of the disruptive effects of heat on DNA. Such discrepancies are briefly sketched hereafter.

Innis et al. in 1988 [6], cited by Kary Mullis in 1990 [1], reported a remarkable fidelity of PCR by heating. It is stated indeed [6] that with PCR “*segments of single-copy genomic DNA can be amplified >10 million-fold with very high specificity and fidelity*” and (referring to a modified version of PCR therein proposed) that “*the fidelity of PCR [...] is quite respectable (approximately one mistake in 4000-5000 base pairs sequenced after 35 cycles of PCR and cloning of the products)*”. They write “*The mixture was heated to 90°C for 3 min, incubated at 42°C for 20 min, cooled to room temperature*”, but no specific considerations or references are reported concerning the problem of a thermal depolymerization. Similarly, in [3,4] it is reported the possibility to customarily perform heating steps from a temperature of about 80° up to temperatures as high as 100°C and even 105°C [3].

Conversely, the very early pioneering studies of historical relevance which were entirely dedicated to the physical, chemical and biological effects on DNA by several physical agents [7,8], provided compelling evidence that heating above 81°, even for few minutes, determines a sharp thermal DNA degradation. The observed degradation also consists in strands fragmentation into shorter molecular segments involving deterministically unpredictable sequence-breaking fractures of linkages along a single polynucleotide chain. This phenomenon was early shown by several authors. Among others, Shooter et al. carefully experimentally examined it [9], while Applequist in 1961 well described its bond scission kinetics by a random degradation model [10] achieving significant predictivity of several degradation processes. Also, the biological implications concomitant to DNA fragmentation with loss of biological activity were early shown as a first macroscopic effect by Zamenhof et al. (1953) [8].

It is worth anticipating that, in this respect, a watershed publication dealing with the denaturation claim is a 3 pages short communication by Doty and Rice published in 1955 [11]. With some emphasis, this paper can be said to represent a sort of “parting of the Red Sea” as it marked the birth of a new post-1955 paradigm on the preserved integrity of molecular information (after DNA is brought to temperatures capable to irreversibly reduce its viscosity, generally above 81°C, and consecutively cooled). Within a pre-1955 paradigm, the DNA molecule appears to be divided into many smaller fragments upon heating above 81°C while, within a post-1955 paradigm retrievable in part of the literature, it undergoes only a marginal alteration which, at least according to what has been stated by Doty in his 1955 paper, may also leave its molecular weight unchanged or even determine strand disassociation. Quite remarkably, heating above 81°C, under some *fast cooling* conditions requirements specified in a subsequent 1960 paper by Doty and coauthors [12], is deemed to be even capable of bringing neat strands separation with halving of molecular

weight. Given the discriminant role of the 1955 short communication, this paper is carefully scrutinized in a subsequent section of the present study.

The aim of the present review is to analyze such apparent conflict between what has been reported in the more recent papers dealing with the denaturation claim and what has been found in previous seminal scientific publications of the decade 1950-1960, which have first investigated the matter of thermal denaturation/degradation.

The overall focus of the present study is on answering the following main research question:

- *based on the available scientific literature concerning the investigation of thermal degradation of DNA, is it plausible to admit that an amplification of genetic information, stored in the sequence of DNA, can be achieved by consecutive heating/cooling cycles with temperatures above 90°C?*

To answer the question above a detailed scientific review is reported of publications which have provided qualitative and quantitative evidence elucidating if, how, and under which conditions, an onset of longitudinal sequence-breaking random fragmentation of DNA molecules is possible at the PCR heating temperatures above 90°C. Based on the critical scientific review and discussion of diriment issues relevant to the plausibility of achieving amplification of DNA information by consecutive heating/cooling cycles at temperatures above 90°C, this study is brought to a precise answer to the main research question above and implications on PCR fidelity are drawn in a conclusive section.

Given the ever-increasing diffusion of PCR in forensic science and in kits for diagnostic applications asseverated by the World Health Organization (some of which commonly known as “PCR swabs”), the purpose and significance of the questions posed in the current study are promptly understood to be of high relevance for public health policies as well as in forensic science.

1.1. Guide to reading subsections of this review

The present study is organized as follows. In Section 2 a scientific background is reported and discussed in three parts separately.

- Subsection 2.1 presents a retrospective bibliographic survey which starts from the account of the denaturation claim in Mullis’ 1987 patent and parses all evidence about the denaturation claim retrievable from the bibliographical references contained in the main papers which have presented PCR.
- Subsection 2.2 presents a first introductory review of three papers of Doty et al. of the years 1955–1960 which have introduced some controversial elements of interpretation of the effect of DNA heating at high temperatures.
- A third subsection, 2.3, reviews the pioneering studies from 1950 to 1960 on the effects of heating and of other physical and chemical agents, which mainly describe evidence against the

denaturation claim and consequently raise questions on the fidelity of PCR. 189
190

Focus of Section 2 is specifically kept over experiments capable to show whether or not DNA complementary strands neatly separate as a consequence of heating, and – even more – over the associated degree of preserved integrity of molecular information upon repeated heating-cooling cycles with heating above 80°C. 191
192
193
194
195

The organization of this review prosecutes as follows. Section 3 contains a closer scrutinization and critical review of the papers by Doty and coworkers in the years 1955-1960 and pinpoints key issues related to the denaturation claim which are identified to be pivotal elements of controversy when compared with contemporary results on DNA heating. In Section 4, such key issues are discussed in the light of experimental evidence collected in this study from the literature herein reviewed. Conclusions are finally drawn in Section 5. 196
197
198
199
200
201
202
203

2. Scientific background 208

2.1. The denaturation claim from PCR back to Doty and Rice (1955) 209

In Mullis and Faloona’s paper [4] few information is reported on the DNA denaturation step. Among five methods therein presented, for methods I and II the Authors state: “The solution is brought to 100°C for 1 min, and is cooled to 25 ° for 30 sec in a waterbath + 2 min” and the same heating time and temperature are used for method II. For methods III, IV, V and VI, heating is reported at a temperature of 95° with time varying from 2 to 5 minutes. The paper does not contain references which deal with the function of the DNA denaturation associated with the specific methodology employed, except for three footnote references [13–15]. No differences of results are mentioned concerning the use of the two different temperatures and times. 210
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214
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Since Mullis and coworkers in their first descriptions [3,4] do offer scarce bibliographical support to the denaturation claim, a dedicated background is reported in this section. Hereafter, excerpts from three references [13–15] mentioned in [3] and from those retrieved in Mullis’ patent [16–17] are reported, selected as far as they contain significant information concerning the issue under scrutiny, i.e. relevant or relatable to the denaturation claim as presented by K. Mullis. Following a philological rationale, these excerpts are presented by publication date in reverse order: 220
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- In (Innis, 1988) [6], mentioned by Kary Mullis in 1990 [1] an “annealing reaction” is described comprising a step in which “the mixture was heated to 90°C for 3 min”. Also, brief accounts of “PCR thermal cycling” and of “denaturation at 93°C for 30 sec” are reported as well as brief accounts of denaturing steps “at 80°C for 3 min” and “at 75°C for 5 min”. 228
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231
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- In (Kwok et al., 1987) [15], where the authors deal with the application of procedures related to PCR in search for identification of 234
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a virus deemed responsible for acquired immunodeficiency syndrome (AIDS), a denaturation step is mentioned, although no relevant information is provided on the denaturation procedure therein employed.

- In (Scharf et al., 1986) [14] it is reported: “2 minutes of denaturation at 95°C, 2 minutes of cooling at 37°C”.
- In Saiki et al. (1985) [13] the information relevant to heating that can be retrieved is the temperature of 95°C, and heating times of 2 minutes, 5 minutes or more, up to 10 minutes.
- In (Gaubatz and Paddock, 1982) [16] a “Heating and quick cool” step is reported in Fig. 5 therein which depicts an “Hypothetical approach for sequencing recombinant plasmid containing a cDNA”. In Section 4 therein it can be also read about “heat denaturation and quenching in ice”.
- The 1979 paper by Caton and Robertson [17] reports: “a novel technique is described for the production of pure, full-length influenza virus ds DNA’s corresponding to each segment of the influenza virus genome, and suitable for molecular cloning and restriction endonuclease” and, concerning heating, it is reported that “Corresponding cDNA’s were [...] boiled for 30 seconds and cooled on ice”.

The excerpts collected above constitute the almost entire body of textual information on the heating/cooling step that is readily retrievable after a *first level* bibliographic scan from [1–4]. For the sake of scientific reproducibility of materials and methods related to the PCR denaturation step, this information is not sufficient to allow prompt repetition of the same experimental methods by other researchers; even more, this information is not even adequate to permit a basic understanding of the elementary significance and purpose of temperature change in a 20°C-wide range of critical values, —i.e., from 80°C to 100°C — in this step of the PCR scheme. Times of heating also frequently differ without explanation (from 1 minute to 1 hour or even more).

This substantial lack of information may let the reader presume that the denaturation claim might have been considered established enough in the specialized scientific community, at the time of these publication (i.e., in the time frame 1979–1990), so as to not require additional descriptions or elucidations.

A second level bibliographic investigation of references cited in the papers just mentioned [6, 13–17], proceeding from 1979 backwards, offers a few additional elements about the denaturation claim.

Innis et al. [6] place PCR in the wake of refinements of DNA sequencing techniques, which followed the methodology reported in a 1977 paper by Sanger et al. [18]. Therein, concerning the denaturation claim, the only statement retrievable is that a solution of DNA is “heated to 100°C for 3 min”. This procedure refers to the DNA sequencing method described in a paper by Air et al. (1976) [19], which is actually based on heating at 100°C for 3 min and which, in turn, is reported to correspond, with slight modifications, to a previous DNA sequencing method described by Sanger and Coulson (1975) [20].

In [20] the denaturation step is reported “to separate the newly-synthesized strands from the template”. The importance of denaturing is remarked and, concerning heating, temperatures and time are indicated at 95°C as well as “at 95

to 100°C for 3 min". In presenting the principle of their synthesis method, Sanger and Coulson cite Wu and Kaiser (1968) [21] who also refer in their materials and methods section, when dealing with hydrolysis of DNA, of a heating process of DNA mixtures "to 100°C for 3 min". Therein reference is made to the enzymatic synthesis method of DNA proposed by Josse et al. (1961) [22]. In this last paper, denaturation by heating is described with a longer heating time: They specifically report "*Heating of calf thymus DNA for 30 minutes at 100° in a medium of low ionic strength results in collapse of the rigid helical structure to a randomly coiled configuration.*"

It can be concluded from this first literature parsing that the practice of heating steps along times from 1 to 5 minutes, reported by Mullis and Faloona (1987) and by Innis et al (1988), can be indirectly traced back to heating practices already accepted in 1961 as documented by the paper of Josse et al. who apply 100°C for the larger time of 30 minutes.

Josse et al. report that "*The pattern of sequence frequencies was the same in DNA synthesized with calf thymus DNA primer or with enzymatically prepared primer in which only traces of the native calf thymus DNA primer were present. The pattern was also unaltered when the primer used was denatured by heating.*"

A fundamental point to be remarked concerning the paper by Josse et al. (1961) is the notion of *unaltered pattern of frequencies*. In the first page of this paper it is clearly explained, however, that pattern of frequencies refers to the frequency of occurrence of nucleotides. Understanding that the notion of *unaltered pattern of frequencies of nucleotides* is completely different from the notion of *unaltered pattern of nucleotides*, a fundamental question to be formulated for the sake of integrity of genetic information becomes the following: "*To what extent the 'collapse' entailed by such heating denaturation practices is reversible for the sake of the integrity of the pattern of the sequences of nucleotides?*"

Geiduschek and Holtzer in their 1962 review on light-scattering studies for the characterization of DNA [24] also review the stability of DNA in aqueous solutions to acids and heat and summarize the findings achieved at their time into the following sentences (see pages 489-490 therein): "*No distinction between heat and acid denaturation is to be made. By suitable adjustment of these variables, it is possible to deform DNA without change of molecular weight although denaturation may under the proper circumstances be followed by degradation or even aggregation*". Also, they write: "*The native helical structure of DNA can be disrupted in a variety of ways not involving changes of its chemical constitution*" and specify that among other causes "*addition of acid [...], heating [...]* are all capable of producing denaturation". A list of 13 references supports this last assertion on heating denaturation. These references are examined according to their ability to provide diriment elements of evidence for answering to the key research question of the present study: if, and how, at the PCR heating temperatures, above 90°C, an onset of longitudinal sequence-breaking random fragmentation of DNA molecules is possible, and under which conditions. The works of this list of 13 references containing diriment elements for answering are reviewed in closer detail in Subsection 2.3 together with other fundamental publications. Four of these 13 works are, instead, more briefly reviewed hereafter (explaining the reason for such a briefer review).

- The scientific content of the one-page conference proceeding by Butler, Shooter, and Pain containing their report at the 1955 Third International Congress of Biochemistry in Bruxelles [25] is more broadly presented and discussed in the six-pages publication by Shooter Pain and Butler [9] on the journal *Biochimica et Biophysica Acta* submitted just one month after the conference, on September 1955. This last scientific publication is reviewed in detail in Subsections 2.3.6 and 3.1.
- From the 1955 book "The nucleic acids" edited by Davidson and Chargaff, and contained among the 13 references by Geidusheck, no specific conclusive information can be retrieved on the effect of heating, in terms of proof or disproof of the occurrence of sequence-breaking longitudinal fragmentation. In particular concerning heating at 100°C, in the fifth chapter on hydrolysis of nucleic acids by Loring [26] on page 196 it can be read: "*The purine bases of DNA are easily removed by mild acid treatment (heating the free nucleic acid in 2 % solution at boiling water bath temperature for 10 minutes) apparently without complete degradation of the original polynucleotide structure. The material remaining was early recognized as a complex substance*". In Chargaff's tenth chapter of the same book [27], the distinction between denaturation and degradation is discussed. Concerning the meaning of denaturation, it can be read: "*A mild, but persistent, mistreatment of a protein leads to a state of malaise known, vaguely, as denaturation.*" The Author writes: "*The line separating a denaturation product from a degradation product is not clearly drawn; but one could define as denaturation products those substances whose preparation caused interference with the physical properties, but not with the chemical composition, of the parent nucleic acid, while the latter change will form part of the description of a degradation product.*". This definition is understood to leave completely open the question on whether the word *denaturation* contemplates or not a disruption or a randomization of the genetic sequence by *sequence-breaking fragmentations* and *sequence-recombining aggregations*. However, no specific review or discussion of heating effects is reported in Chargaff's chapter and in the remainder of this book. Concerning molecular weight decay as a primary indicator of possible longitudinal sequence-breaking fragmentation, the reassuring conclusion achieved by Reichmann, Bunce and Doty (1953) [28] is reported by Chargaff that the adjustment of calf thymus DNA solutions (in 0.2 M NaCl), to pH 2.6 by dialysis "*did not affect the molecular weight (7,700,000), as determined by light scattering.*". This conclusion by Reichmann et al. quoted in reference [28] is closely reviewed in Subsection 3.2.2 together with the underlying experimental evidence. Concerning stability to heat, even in Jordan's chapter [29], devoted to the physical properties of nucleic acids, no decisive information is retrieved about the thermal stability to longitudinal fragmentation of the DNA molecule, apart from a mention of the protective effect of salt against heat denaturation, as found in Thomas' work [30-31]. It can be read in this chapter: "*A further protective effect of salts against heat denaturation of DNA solutions has been observed by Thomas. For calf thymus DNA in sodium chloride solutions of various concentrations, some denaturation occurs at room*

temperature at $<10^{-4}$ M and below 70° in 10^{-2} M. Denaturation is strongly inhibited even at 100° C. in 10^{-1} M and M solutions. It is claimed that heat denaturation occurs stepwise and that the critical temperature for each step varies for DNA from different sources. Thus DNA from starfish testis in 10^{-2} M sodium chloride undergoes the first step in denaturation at 55° C. compared with 70° C. for that from calf thymus." In particular, the statement that "Denaturation is strongly inhibited even at 100° C. in 10^{-1} M and M solutions" is a quotation of Thomas (1954) [29,31]. Concerning this last statement by Thomas, it should be remarked that Thomas detects denaturation by changes in optical density at 260 m μ and that this measure is not conclusive neither in proof nor in disproof of occurrence of sequence-breaking longitudinal fragmentation of the DNA molecule at 100° C in presence of these salt concentrations.

- Finally, the two papers by Zamenhof and coauthors [32,33], among the 13 cited by Geiduschek and Holtzer concerning heating degradation and denaturation, are also not reviewed in detail since these two works just extend measures of viscosity drop and bacterial transformation inactivation reported by Zamenhof et al. (1953) [8] and do not provide conclusive evidence in proof or disproof of the possibility of the onset of disruptive longitudinal sequence-breaking random fragmentation of DNA molecules in addition to the fundamental elements of evidence already reported by Zamenhof in [8]. For this reason, in Subsection 2.3.3 only the scientific publication by Zamenhof et al. (1953) is reviewed.
- The remaining 9 references quoted by Geiduschek and Holtzer are all reviewed in closer detail in the next subsections.

2.2. The pivotal papers of Doty et al. of the years 1955–1960

Continuing the literature review by keeping a reverse chronological order and a focus on possible evidences of molecular integrity of the sequences of nucleotides in a same strand at high temperature and/or of possible strands separation, the bibliographic scan runs across three papers by Doty and coworkers published between 1955 and 1960 [11,10,23], and already mentioned in the introduction. These papers report results of physical and chemical analyses specifically devised to follow the molecular weight changes of DNA during and after a heating/cooling cycle in order to attempt to understand how depolymerization and possible strand separation progress as a consequence of heating. The conclusions of these three papers by Doty regarding the consequences of DNA heating at 100° C deserve to be remarked since they entail significant elements of mutual disagreement (i.e., conflict among conclusions stated in these same three papers) and of conflict with the conclusions of studies on DNA heating published before 1955. Some of these elements of disagreement are reported hereafter.

In particular, Doty et al. (1960) [12] conclude their study reporting, in a final summary, a very general statement: "When solutions of bacterial DNA are denatured by heating and then cooled, two different molecular states can be obtained in essentially pure form depending on the choice of conditions, that is, rate of cooling, DNA concentration, and ionic strength". They continue: "One state corresponding

to fast cooling consists of single stranded DNA having about half the molecular weight of the original DNA. The other state corresponding to slow cooling consists of recombined strands united by complementary base pairing over most of their length. This form has as much as 50 per cent of its original transforming activity and is called renatured." The statement above is remarkably very general since, without specifying the heating temperature and the heating time, it depicts a single stranded product resulting from complete disassociation of all hydrogen bonds between complementary strands into molecular weight halving. We will refer to this picture as the *high temperature heating + fast cooling dissociation claim*. A second point of remark for the 1960 statement above is that it turns out to be in open conflict with the conclusions by Doty and Rice in two papers, one published in 1957 [23] and the other one published as a preliminary note in 1955 [11]. Their preliminary 1955 conclusion is that, upon heating a neutral saline DNA solution to 100°C for 15 minutes, the product is "found to have the same molecular weight as the native DNA" [11]. Such conclusion is further specified in 1957 to be inferred from light scattering studies, as the Authors write that, upon observing the effects of heating of four calf thymus DNA preparations along exposure times of one hour and more at temperatures from 89°C up to 100°C, "the molecular weight remains unchanged according to light scattering studies" [11]. We will refer to the paradigm introduced in these last 1955 and 1957 papers by Doty and Rice as the *100°C stable molecular weight claim*. Although the Authors include in [11] bibliographical references to previous studies, such as the study by Goldstein and Stern [7] and Zamenhof et al. [8], which bring instead to the opposite conclusion of DNA molecules thermally fragmenting in aqueous solutions already at temperatures not higher than 81°C, the *100°C stable molecular weight claim* is remarkable since they make no mention of the existence of such a macroscopic conflict with the previous literature. Even more remarkably, except for the 1955 preliminary note, no subsequent mention is made by Doty et al. in [10] and [23] of Goldstein and Stern's study among the referenced works, so that the trace of their fundamental contribution is lost.

Additional elements of perplexity rise from reading in Doty et al. (1960) [12] statements that appear to be in open conflict with both the *high temperature heating + fast cooling dissociation claim* and the *100°C stable molecular weight claim*. For instance, the Authors report in [12] that heating pneumococcal DNA of molecular weight 8.2 million at 100° for 10 minutes in standard saline citrate and subsequently quickly cooling this material, they find a molecular weight of 2.0 million (see page 471 therein). Even excluding, simplistically, any considerations of polydispersity, this evidence is neither compatible with the first claim (which would require a decrease of molecular weight by a factor of one half) nor with the second claim.

The literature showing evidence of thermally induced DNA fragmentation and the characteristic temperatures above which this phenomenon is observed is examined in the next subsection 2.3. The discrepancies determined by the 1955 and 1957 papers by Doty and Rice with previous findings are specifically examined and critically reviewed in the subsequent Section 3 of the present study.

2.3. Pioneering pre-1961 studies on DNA heating not authored by Doty 479

Prosecuting the literature scan before 1955, results are found implying significant elements of divergence related to the *high temperature heating + fast cooling dissociation claim* and to the 100°C stable molecular weight claim. This group of references are presented hereafter in progressive chronological order.

2.3.1. Evidence of viscosity drop and of heat turning fibrous material into flocculent precipitate 487

Strong and consistent evidence of DNA thermal degradation resulting in the fragmentation into much smaller molecular products (thus incompatible with a hypothesis of orderly disassociation or with one of unchanged molecular weight) had clearly emerged in several papers between 1950 and 1960. Milestone results of experiments on thermal depolymerization showing this fragmentation evidence are described by Goldstein and Stern in 1950 [7]. The Authors report the following diriment experimental observation "*Preliminary tests showed that a highly viscous aqueous solution of sodium desoxyribosenucleinate loses its viscosity when it is heated almost to the boiling point and then permitted to cool to room temperature. Upon adding the solution to 1.5 volumes of ethyl alcohol a flocculent precipitate is formed instead of the fibrous material yielded by "native" nucleic acid preparations.*" Concerning viscosity, Fig. 12 therein (reproduced in Fig. 11 of Section 4.1) shows that a 0.1% solution of purified calf thymus DNA in a barbital buffer at pH 7.2, heated just one minute at 100°C, experiments an irreversible drop of relative viscosity, from a value of 3.4 to a value below 1.4 (see Fig. 7TEMP in Section 4.1). Besides, the same figure shows a similar drop to a value, close to 1.4, is obtained by a 2 minutes heating at 90°C.

Notably, Goldstein and Stern warn that an experiment that fails in detecting evidences of depolymerization (produced either sonically or thermally) is ultraviolet absorption spectrophotometry. Specifically, concerning changes upon heating in ultraviolet spectrum absorption of solutions of tetrasodium salt of DNA (therein indicated by the acronym STN), they report: "*The examination in the Beckman spectrophotometer of a 0.048% STN solution in distilled water (after dilution to 0.008%) which had been heated for 15 minutes at 100° showed no change in the position of the maximum at 259 mμ or of the minimum at 231 mμ, although the relative viscosity had fallen from 3.11 to 1.35 as a result of this treatment. The rise in optical density from 1.28 to 1.46 at 259 mμ which was observed in this experiment may possibly be due to the fact that it was performed in the absence of a buffer system.*"

Goldstein and Stern also warn (see section therein "*Effect on Absorption Spectrum*") that when the optical density absorption spectrum is observed in the wave lengths range from 225 to 300 mμ, changes are detected only if the solvent is distilled water ("*unbuffered*" solution) while in the buffered solutions no changes are observed. Such findings find confirmation in a study by Blout and Asadourian (1954) [35] showing that the intensity of ultraviolet absorption of the sodium salt of DNA is dependent on the ionic strength. The intensity of absorption is highly pH dependent, in the absence of salt, in the pH range 3 to 12. These and other results therein reported concerning various

heating tests between 60 and 100°C lead the Authors to the conclusive observation: “The depolymerization of desoxyribosenucleic acid by thermal treatment is of special interest in view of the high temperature coefficient and, hence, large energy of activation, observed in the range between 60 and 100°C (see Fig. 12). While this phenomenon is similar to the heat inactivation and denaturation of proteins (cf. ref. 22), there exist important differences. Thus, the nucleic acid molecules appear to be split into many small fragments on heating while the thermal denaturation of proteins, as a rule, does not lead to a significant alteration of their molecular weight, although in some instances aggregation will occur under these conditions (cf. ref. 22). This would seem to indicate that the bond strength of the sugar-phosphate links in the polynucleotide chain is smaller than that of the peptide bonds in proteins.”

In the same year 1950, Miyaji and Price [34] find confirmation of the viscosity drop induced by heating. They also study the effect of NaCl addition coming to the following conclusion: “The viscosity of aqueous solutions of sodium thymonucleate is reduced both by heating and by addition of sodium chloride and other salts. If, however, the nucleate solution is heated in the presence of a sufficiently high concentration of salt, there is no further decrease in viscosity beyond that induced by the salt. The protective effect of the salt is reversible, for if the salt is removed by dialysis from the heated nucleate-salt mixture, the residual aqueous solution of nucleate again suffers a marked reduction in viscosity on heating”. They also find the singular phenomenon above 85°C that “At temperatures over 85°C, mixtures of nucleate and salt sometimes yielded a small amount of white precipitate.”

Miyaji and Price highlight the importance of discriminating whether the drop in viscosity is due to 1) either fragmentation of the DNA molecule or 2) to coiling of the DNA molecule with reduction of molecular radius yet in absence of fragmentation, or even to a combination of the two phenomena.

2.3.2. Evidence of thixotropy induced by heating

Also in 1950, Zamenhof and Chargaff [36] find that heating a 0.3 per cent solution of DNA at 86°C for 90 minutes makes the specific viscosity, η_{sp} , drop from 30.3 to 3.9. They find that the solution degraded by heating can repolymerize but show that a DNA preparation degraded by heat (similar to the degradation produced by other agents such as acid and alkali) acquires a marked thixotropy (i.e., reduction of viscosity determined by forced flow and viscosity regain as the forced flow is arrested). They also show, conversely, that an intact DNA preparation exhibits no thixotropy since its viscosity remains unaltered upon subjecting it to forced flow. They observe that this effect of induced thixotropy is irreversible since it cannot be removed by repolymerization. They conclude that their experiments show that “original preparations of desoxypentose nucleic acids possess unique physical properties (non-thixotropic viscosity, regular temperature-viscosity relationship) which, once lost, cannot be regained by repolymerization”. They specify that such phenomena of irreversible acquisition of thixotropy “were observed with desoxypentose nucleic acid preparations from calf thymus and from yeast, depolymerized not only by acid or alkali but also by heat”. Also, with Goldstein and Stern they are among the first who “extend the concept of irreversible denaturation from the field of proteins to that of

another group of macromolecules, the nucleic acids" and likewise remark the irreversible character of the transformations produced in DNA by heating.

2.3.3. Evidence of vanishing of transforming activity paired by viscosity drop

A biological confirmation of the simultaneous onset, at heating temperatures higher than $T_{td} = 81^{\circ}\text{C}$, of irreversible viscosity drop phenomena paired by biological inactivation phenomena is reported by Zamenhof, Alexander and Leidy in 1953 [8]. The Authors extract and purify DNA from a culture of Hemophilus influenzae that has resistance to streptomycin. They add dilutions of these aqueous solutions of purified DNA from streptomycin-resistant colonies to bacterial suspensions obtained from cultures derived from strains characterized by initial *absence* of resistance to streptomycin, under controlled 37°C temperature and time. Subsequently, they add streptomycin (SM) and detect, by observing the quantity of surviving bacteria, the degree of resistance to streptomycin induced in the non SM-resistant colonies by DNA addition. Thanks to dilutions they can also measure the degree of biological active the extracted DNA solutions.

As well known, biology interprets this phenomenon of induced resistance as a parasexual phenomenon, known as Griffith bacterial transformation activity, thanks to which the bacterium that initially does *not* have resistance to streptomycin, upon entering in contact with the purified DNA solution «acquires» the genetic code contained in the DNA molecules thus receiving the necessary instructions to build the polysaccharide bacterial capsule that gives the increase in resistance to streptomycin, and transmits it to its own lineage. They perform part of their experiments with H. Influenzae after subjecting the DNA extract to heating at a variable T temperature in a pH 7.4 buffer with 0.14 M NaCl and 0.015 M sodium citrate (upon reporting the temperature to 23°C before each activity assay). They find that viscosity and the activity are practically unaffected by 1 hour heating to temperatures (T) as high as $76\text{-}81^{\circ}\text{C}$ while, in a temperature range (T) between 81 and 90°C heating, induces a more than significant reduction and, by further increasing (T), they see a drop to practically zero of the phenomenon of bacterial transformation activity.

Points on the graph of Fig. 1 by the Authors on page 379 [8], concerning measures of relative bacterial transformative activity (in percentage), have been digitized; the resulting numerical values, converted from logarithmic scale, are reported in Table 1 herein. The values of Table 1 are plotted in Fig. 1 reporting, on the x horizontal axis, the heating temperature of DNA in $^{\circ}\text{C}$ and, on the ordinates, the percentage of residual bacterial transformation activity employing a linear scale in place of the logarithmic scale of the original figure.

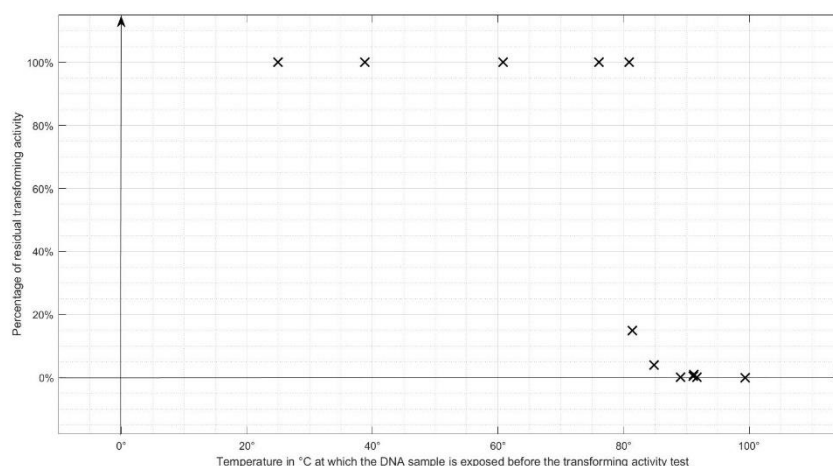
625

Table 1. Numerical values obtained by digitalization of points in Fig. 1 in [8] concerning the stability of the transforming principle preparation to heat. 626
627

Heating temperature	Activity
[°C]	[%]
24.92	100.000
38.84	100.000
60.85	100.000
76.05	100.000
80.91	100.000
81.39	14.908
84.84	4.040
91.12	0.392
91.68	0.146
89.06	0.100
99.38	0.001
99.35	0.001

¹ Values of activity have been converted from the logarithmic scale of the original figure. 628
629

630



631

Figure 2. Activity vs. heating temperature reproduced from Fig. 1 in [8]. Ordinates are represented by an ordinary linear scale in place of the logarithmic scale of the original figure. 632
633
634

The evidence found in [8], clearly represented by Fig. 2 herein, is that, upon subjecting a purified DNA aqueous solution to a preliminary heating/cooling cycle bringing it to a variable temperature (T) and back again to 23°C, the residual bacterial transformation activity is stable when T ranges between 25 and 80.9°C. When the heating temperature T reaches 81.4°C, the transforming activity is reduced to 15%, and, by increasing the heating temperature, the activity continues to decay rapidly to zero, so that 90°C it is basically absent. 635
636
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642

A further fundamental result in [8] concerns stability of DNA viscosity after heating. Upon measuring viscosity in the same pH 7.4 buffer at 23°C, they find that “the viscosity and the activity are practically unaffected by 1 hour heating to temperatures as high as 76-81°C.”

Concerning the possibility that heating or acidity may just induce molecular contraction and not depolymerization Zamenhof et al. also write: “It has recently been suggested (22, 26, 27) that the decrease in viscosity of DNA solution upon mild H⁺ treatment is due to the change in asymmetry caused by the contracting of the molecule rather than by actual depolymerization. This may also be true for the mild heating. The contraction may be made possible by the breakage of labile bonds (such as hydrogen bonds) under the action of thermal oscillations (40, 41). At higher temperatures, actual depolymerization may occur (32).”

2.3.4. Evidence of depolymerization by ultraviolet light measurements

Thomas in his 1954 publication [31] on the study of the ultraviolet absorption spectrum of neutral saline NaCl (unbuffered) solution of desoxyribonucleic acids (DNA) isolated from calf thymus, starfish (*Asterias Glacialis*) testicles and red frog (*Rana Temporaria*) testicle devotes an entire section, titled “Effect irreversibles de la temperature sur le spectre U.V”, on the effects of heating on changes of optical density measures. Besides finding that this measure is strongly sensitive to the NaCl concentration, he plots the graphs of the function relating the absorption at 260 mμ versus the preheating temperature and he finds sudden increases of the optical density at temperatures ranging between 55°C and 80°C with intermediate plateau regions in which the absorption remains constant. For calf thymus DNA he finds a maximum upper limit for stability against depolymerization corresponding to a temperature around 80°C, in agreement with Goldstein and Stern (although these authors are not quoted by Thomas).

It is worth observing that the detection by Thomas of changes in optical density as function of heating temperature are also in agreement with the explanation by Goldstein and Stern since Thomas employs unbuffered solutions.

2.3.5. Detection of depolymerization by staining

Measurements indicating depolymerization of DNA available in 1954 are summarized by Kurnick [39] and recalled in the following list:

- reduction in viscosity;
- reduction in rate of sedimentation in the ultracentrifuge;
- increase in ultraviolet absorption (detectable in unbuffered solutions);
- lowering of PH;
- formation of acid-soluble and dialyzable products.

Based on his previous study [40] where he could show that methyl green selectively stains only highly polymerized desoxyribonucleic acid, and fails

to stain, to any significant extent, depolymerized desoxyribonucleic acid and ribonucleic acid, Kurnick introduces a new methodology for studying depolymerization induced by heat [39]. From experiments on calf thymus DNA, among which ultracentrifugation patterns of DNA solutions, with and without 0.02% methyl green, heated to 80° for 7 hours, and to 100° for 1 hour or 2 hours as well as on unheated DNA, he finds that *“Heat and enzymatic depolymerization of DNA produce certain effects in common: both reduce the viscosity of the solution rapidly [...], increase the ultraviolet absorption [...] and reduce methyl green affinity”*. He remarks that *“These changes are maximal before any dialyzable, acid-soluble oligonucleotides are formed [...] and nearly maximal before reduction in sedimentation rate appears”*. He adds: *“The products of heat and initial enzymatic digestion (and of sonic depolymerization) are large molecules which, as judged by their sedimentation velocity, are probably still quite asymmetric rods. This hypothesis is in better accord with the unaltered sedimentation rate (despite marked reduction in viscosity) than the alternative suggestion [citation of Creeth Gulland and Jordan (1947) [41] and of Conway and Butler] that heat has produced collapsed molecules of the original molecular weights. Such collapsed molecules would show a great reduction in viscosity, but would also show considerable change in sedimentability.”*

He conclusively provides an explanation of the heating degradation at 100°C in terms of *“bombardment by the solvent molecules”*: *“Thus, at 100°C, a molecule with very low intrinsic viscosity, but of a considerable size as characterized by its non-dialyzability, sedimentability and affinity for methyl green [...] is stable. This suggests that the increased bombardment by the solvent molecules at elevated temperature snaps the rigid molecule into still large units, but of such lesser size as to be stable when subjected to the bombardment characteristic of this temperature.”*

From the background above on the studies on thermal depolymerization published between 1950 and 1954, it consistently emerges that heating at temperatures above 81°, or just above 81°, ordinarily produces depolymerization (or disaggregation) in purified DNA solutions. For completeness, it is worth adding that a residual possibly conceivable alternative justification explaining drop in viscosity could be the hypothesis considered by Creeth Gulland and Jordan (1947) [41] of a coiling of the DNA molecule capable of *“reducing the molecular asymmetry but not the molecular weight”*. It should be pointed out, however, that such a coiling hypothesis is drawn in [38] to explain decrease in viscosity observed after addition of NaCl, occurring without production of titratable groups and that in [41] no heating experiments are carried out.

2.3.6. Estimates of molecular weight from viscosity-sedimentation experiments

An important contribution comprehensively accounting for most of the previously published literature on the effect of DNA heating, confirming Goldstein and Stern’s conclusion on DNA fragmentation above 81°, is a physical chemical study by Dekker and Schachman (1954) [42]. This study, while essentially focused on the assessment of different investigated models for the possible macromolecular structure of DNA (among which also models differing from the Watson and Crick double helical chain [43]), is important for the

sake of understanding the effect of heating, since it reports a simple quantitative estimate of the molecular weight change before and after heating due to fragmentation, by a calculation from sedimentation and viscosity data before and after heating.

Dekker and Schachman do not exclude ab initio a possible coiling of the DNA molecule determined by heating and they are the first to point out that paired measurements of viscosity and sedimentation rate of dilute solutions of DNA and heated DNA could differentiate between a disorganization of the DNA molecule without a change in molecular weight (coiling), on one side, and a process in which there is a degradation into much smaller pieces, on the other side. Their key reasoning is that if, upon heating, the DNA molecule is divided into smaller fragments, the viscosity must decrease and the sedimentation coefficient must decrease as well, while, in case heating should produce just a coiling of the molecule, unlike viscosity the sedimentation must instead increase.

Based on their experimental measurements, the Authors make the pertinent observation which compels them to exclude a hypothesis of thermal coiling as they write: *“The reduced viscosity of a 0.005 per cent solution heated at pH 7 for 15 minutes at 100°C. decreased from 30 (gm/100 cc)⁻¹ to less than 1.0 (gm/100 cc)⁻¹. Instead of an increase in the sedimentation rate, which would be expected from the amount of coiling necessary to produce such a large drop in viscosity, we found that measurements at 0.005 per cent DNA showed that the heated material had a sedimentation coefficient of about 6S, whereas the unheated preparation had a value of nearly 20S. These experiments provide proof that the molecular weight of DNA changes from about 5×10⁶ to 5×10⁴ as a result of this mild heating procedure.”* In the previous quotation, uppercase S indicates the Svedberg unit which corresponds to $s \times 10^{-13}$ where s stands here for seconds. It is also worth recalling that the reduced viscosity, η_{red} , is related to the viscosity η through the following relations: $\eta_{red} = \eta_{sp}/c$ where c is the concentration and η_{sp} is the specific viscosity $\eta_{sp} = \eta_r - 1$, being $\eta_r = \eta/\eta_0$ the relative viscosity defined as the ratio of solution viscosity η to solvent viscosity η_0 . The intrinsic viscosity $[\eta]$ is obtained by extrapolation of reduced viscosity against concentration at infinite dilution, $c = 0$, viz.: $[\eta] = \lim_{c \rightarrow 0} \eta_{sp}/c$. [5,44].

The proportion behind this simple quantitative estimate by Dekker and Schachman can be presented as follows. Denoting by M the average weight of the DNA molecule, or of its fragment produced upon heating (assuming here introductively and simplistically that all molecules or fragments have the same weight and molecular hydrodynamic radius r_s) the following direct proportionality holds

$$M \propto (r_s \times s \times \eta), \tag{1}$$

where s is now the sedimentation constant, η the viscosity and \propto is the symbol of direct proportionality. References for the proportion above, which is obtained combining Svedberg’s equation [45] with Stokes-Einstein’s equation, can be found in treatises of physical chemistry of macromolecules such as Barrow’s treatise (see, e.g. [46], Chapter 20 and, in particular, page 659, Eq. (23)).

Fundamental more advanced investigations, contemporary to those years, based on careful consideration of the hydrodynamic Stokes's law, empirical data for long chain polymers, dimensionless analysis and investigation of universal constant for flexible long chain polymers such as DNA can be found in (Mandelkern et al., 1952) [47] and related references.

Dekker and Schachman's estimate stems from the simplistic consideration that the hydrodynamic radius remains unchanged upon heating so that, denoting by subscripts N and H the quantities relevant to native DNA and to DNA possibly fragmented by heating, respectively, one infers from Eq. (1) setting $r_{sN} = r_{sH}$:

$$\frac{M_N}{M_H} = \frac{r_{sN}}{r_{sH}} \times \frac{s_N}{s_H} \times \frac{\eta_N}{\eta_H} = 1 \times \frac{20}{6} \times \frac{30}{1} = 100. \quad (2)$$

Shooter, Pain and Butler (1956) [9] also dedicate a study to examining the effect of thermal degradation above 80°C on DNA. Based on their experiments they propose some simple graphical models suggesting the effects of heating on DNA (see the reproduction in current Fig.3).

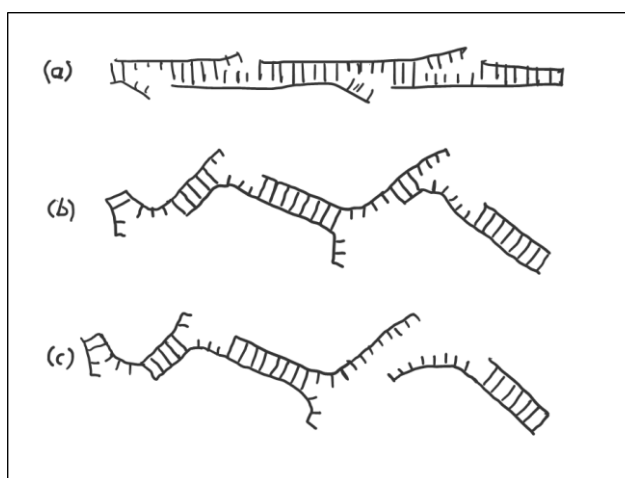


Figure 3. Handmade reproduction of Fig.4 by Shooter et al. (1956) [9] 'Suggested effect of heating'

They conclude their study stating that after 15 minutes heating at 100°C, DNA samples always produces thermal degradation. They remark that the extent of degradation and the distribution of the size and shape of the fragments produced depends on how DNA is prepared.

2.3.7. Evidence of irreversibility of heating degradation from titration analyses 820

821
822 Titration analyses although do not provide direct quantification of mo-
823 lecular weight decrease and direct evidence of possible onset of disruptive
824 longitudinal sequence-breaking random fragmentation of DNA molecules,
825 can detect however some irreversible features occurring during the heating
826 process and the quantity of broken hydrogen bonds and for these reasons
827 part of these works on titration relevant to DNA heating are hereafter re-
828 viewed.

829 The study by Cavalieri and Rosenberg (1957) [48] reports of potentiom-
830 etric titration analyses on DNA solutions using calomel and glass electrodes.
831 The study ascertains the conditions under which hydrogen bonds in DNA
832 can be cleaved reversibly by heat, and the conditions under which heat bonds
833 cleave spontaneously and irreversibly. Concerning the temperature at which
834 irreversible phenomena are observed the Authors report: “A temperature is
835 finally reached (about 70° in 0.017 M NaCl, for example) at which denaturation oc-
836 curs without titrating any bases, and cannot be prevented by heating in buffers of
837 any pH. In such cases, the original H-bonds of DNA must all be thermally and irre-
838 versibly cleaved.”

839 Cox and Peacocke (1956) [49] prior to presenting their results on electro-
840 metric titration well summarize the contemporary debate on the stability of
841 the molecular weight to heating: “When sodium deoxyribonucleate is heated in
842 neutral aqueous solution, irreversible changes occur above a critical temperature
843 which varies with the source of the deoxyribonucleate and its method of extraction.
844 These irreversible changes, which often take place over a temperature range of only a
845 few degrees, include: a drop in the viscosity; an increase in the ultraviolet absorption;
846 a displacement of the spectrophotometric titration curves; the appearance of new in-
847 frared absorption bands; changes in sedimentation constant; and displacement of the
848 titration curves. Some investigators deduced a decrease in molecular weight from
849 sedimentation and viscosity measurements after the nucleate had been heated in wa-
850 ter [(Dekker and Schachman, 1954) [42], (Sadron, 1955) [51], and in salt [(Shooter et
851 al., 1956) [9]], whereas others reported [(Doty and Rice, 1955) [11], (Sadron, 1955)
852 [51] no change in molecular weight after heating in the presence of sufficient sodium
853 chloride.” (notice that, in the quotation above, text references originally re-
854 ported in superscript format have been converted into the current squared
855 brackets format adding also author and year). They summarize in Fig.2
856 therein that the percentage of ruptured hydrogen bonds in herring-sperm in
857 a (0.15%, 0.05 M -NaCl) solution heated along 1 hour at 95°C is 100%. More-
858 over, on the basis these titration experiments the Authors infer evidence for
859 “the random nature of the heat-denaturation process”.

860 We incidentally remark that the statement by Cox and Peacocke (1956)
861 [49] according to which (Sadron, 1955) [51] would report no change in molec-
862 ular weight after heating in the presence of sufficient sodium chloride, ap-
863 pears to be incorrect as the data in this publication by Sadron show in all cases
864 a decrease of the molecular weight after heating except for just one case where
865 it is reported even an increment of molecular weight. This issue is examined
866 in detail in Subsection 2.3.9 dedicated to the light scattering measurements by
867 Sadron on heated DNA.

In 1957 Cox and Peacocke [52] specify the limit of titration in detecting changes in molecular weight: *“ionizing radiations invariably and heat, under certain conditions also cause changes in molecular weight but this degradative aspect of their action will not be considered further here, if only for the reason that the titration curves can only just detect a release of one secondary phosphoryl end group in 50 nucleotides.”*

Most importantly, Cox, Overend, Peacocke and Wilson in their publication on Nature (1955) [5], reaffirm *“it is only changes in the intrinsic viscosity which are of significance in the estimation of molecular size and shape”* and propose the employment of the following expression, usual for high weight polymers, relating intrinsic viscosity $[\eta]$ to the is the viscosity-average molecular weight, M_v :

$$[\eta] = K(M_v)^\alpha, \tag{3}$$

where K and α are constants for homologous polymers. Based on their experimental data the Authors compute $\alpha = 0.93$. Remarkably, agreement with this value is found by the same Doty and coworkers who later compute in 1960 [12] from two DNA preparations from Diplococcus Pneumoniae DNA and Escherichia Coli two determinations of α and K whose average value is $\alpha = 0.923$ and $K = 3.28$. The exponent close to unity signifies that the relation between viscosity-average molecular weight and intrinsic viscosity is almost linear.

Use of this formula with the intrinsic viscosity data, measured before and after a heating treatment, allows to compute the drops from the molecular weight of the unheated molecule M_N to the weight of the molecule after heating, M_H , by the following formula:

$$\frac{M_H}{M_N} = \left(\frac{[\eta]_H}{[\eta]_N}\right)^{1/0.923} = \left(\frac{[\eta]_H}{[\eta]_N}\right)^{1.083}, \tag{4*}$$

On the basis of light scattering experiments and viscosity measurements on DNA degraded by gamma rays, Peacocke and Preston (1958) [50] improve the precision of the determination of coefficients α and K in (3) they find a round value $\alpha = 1.0$ for the exponent and find $K = 3.28$. They find that the weight-average molecular weight M_w is related to intrinsic viscosity by:

$$[\eta] = 4.26 \times (M_w)^{1.00}, \tag{5}$$

Most importantly, they find a remarkable consistency of the value M_w obtained from (5) with countercheck values obtained by light scattering measures, so that (5) can be considered a reliable choice for determining molecular weight from intrinsic viscosity, substitutive of light scattering measurements.

Employing the data reported by Shooter et al. (1956) [9] concerning measurements before and after 15 minutes heating at 100°C on different samples and also in absence of salt (see Table I therein), the viscosity-based average weight of the heated DNA molecules is computed from (4) to range between

1.45% and 5.01% of the average weight before heating, what signifies a depolymerization in considerably shorter fragments.

2.3.8. Models for random degradation matching experimental data

Kinetic molecular models provide a valuable mean for computing the number of scissions (fragmentations in shorter segments) that the DNA molecule undergoes during a heating experiment and the rate of fragmentations, i.e. the number of fragmentations per unit of time, based on diagrams of molecular weight plotted vs. time.

Peacocke and Preston (1958) [50], proceeding from viscosity and light scattering experiments on DNA samples degraded after exposure at different doses of gamma rays, find that internucleotide phosphodiester bonds in DNA are ruptured by a random fragmentation process. Fragmentation is the process which divides the molecule into shorter segments of lower molecular weight. They experimentally find for DNA an almost quadratic relation (exponent 1.85) between the radiation dose R and the intrinsic viscosity $[\eta]$ instead of the linear relation characteristic of single strand polymers. From such fundamental difference they infer that in DNA (and in any other similar double stranded polymer) the fragmentation of the molecule in a fragmentation process must be generated by a double breakage mechanism. This mechanism consists of the breakage of two intra-chain longitudinal bonds each located at two facing nucleotides situated at the same longitudinal position of the molecule but belonging to opposite strands. This mechanism is mathematized in the context molecular weight distributions [53]. In this context distinction is made between the *number-average* molecular weight M_n which is the arithmetic mean of the weights, and the *weight-average* molecular weight M_w which is the weighted arithmetic mean in which the molecular weights themselves as the averaging weights. For a uniform distribution $M_w = M_n$ while for a random distribution (i.e., a distribution generated from random fracture of an infinite chain) $M_w = 2M_n$.

The considerations of Peacocke and Preston are conveniently summarized hereafter in the format of proportionality relations. The *weight-average* molecular weight M_w and *number-average* molecular weight M_n are related to the number of fragmentations F per original number-average molecule:

$$F = \frac{(M_w)_0}{(M_w)} - 1 = \frac{(M_n)_0}{(M_n)} - 1 \quad (6)$$

where the 0 subscript indicates the original molecular weight of the molecule before fragmentation starts. We recall that in special cases For a higher number of molecules and fractures ($F \gg 1$) relation above can be represented as the proportionality relation:

$$F \propto \frac{1}{M_n} \quad (7)$$

For degradation by radiation of single strand polymer chains it is ordinarily expected that the probability of single chain break p_s and the radiation dose R are related to fragmentation by:

$$F \propto p_s \propto R \tag{8}$$

what should imply from (7) that $\frac{1}{M_n} \propto R$. Peacocke and coworkers find instead, experimentally, that after a transitory range of exposition the proportionality holds: $\frac{1}{M_n} \propto R^2$. They explain in terms of probability distributions such evidence, unusual for single stranded polymers, to be the consequence of a fragmentation process in which a fragmentation is produced when two intrachain scissions at opposite strands in two facing nucleotides occur (or in two proximal nucleotides). The probability of this double-step process is shown to follow the proportionality holds: $p_D \propto p_s^2$, so that, owing to the previous proportionality relations, one has:

$$F \propto p_D \propto p_s^2 \propto R^2 \tag{9}$$

Owing to the previous equation and to (7) one finally has for DNA and for any double stranded polymer the theoretical prediction:

$$\frac{1}{M_n} \propto R^2 \quad \text{or equivalently} \quad \frac{1}{\sqrt{M_n}} \propto R \tag{10}$$

It is important to highlight the difference between the previous relation (10) and the corresponding relation for single-stranded polymers which reads instead:

$$\frac{1}{M_n} \propto R \tag{11}$$

Peacocke and Preston find that for gamma-rays degradation of DNA relation (10) is closely respected and consider this as evidence that the fragments ensuing from gamma-ray degradations are always double stranded.

The Authors also investigate the effect on molecular weight of heat degradation (100°C along 15 minutes in a 0.1 M NaCl solution) applied to fragments obtained by previous gamma ray degradation. Their data shows this thermal treatment yields a decrease in molecular weight, but the Authors underline that quantitative appreciation of the entity of molecular weight decrease and of number of fractures is made difficult by the *“the known tendency for the polynucleotide chains to remain entangled, in spite of the removal of hydrogen bonding, and for separated chains to re-aggregate on cooling”*.

A kinetic statistical model for random degradation of a two-stranded polymer model specifically devised by Applequist [10] to analyze DNA degradation provides a more detailed description of the kinetics of double fracture proposed by Peacocke and Preston which accounts for independent probabilities of intrachain fractures (termed P-bonds by the Author and denoted in our synthetic review) and crosslinking hydrogen bonds (H-bonds).

Denoting by p the fraction of broken P-bonds, by q the fraction of broken H-bonds, and by p' the probability that a pair of facing nucleotides is followed by a double chain break, they compute that at the beginning of the degradation process when almost all P-bonds are unbroken the relation holds:

$$p_D = \frac{(1 + q)p_s^2}{(1 - q)} \tag{12}$$

This relation is a more explicit determination of the proportionality relation highlighted by Peacocke and Preston and contained in (9). By further introducing the simplest assumption that P-bonds are broken by a first-order rate process, viz. $1 - p = (1 - p_0) \cdot e^{-kt}$ corresponding in the early stages of degradation when $kt \ll 1$ to the linearized law $p = p_0 + (1 - p_0) \cdot kt$, they find that the linear relation:

$$\left(\frac{M_{w0}}{M_w}\right)^{1/2} = 1 + \frac{1 - p_0}{p_0} kt \tag{13}$$

excellently fits the asymptotic trend in experimental data $M_w(t)$ obtained from enzymatic degradation [54], acidic degradation [55] and thermal degradation (Doty, Marmur, Eigner and Schildkraut, 1960) [12]. The data for thermal degradation is reported together with the fit provided by (13) (divided by $(M_{w0})^{1/2}$) in Figs. 4(a) and 4(b), respectively.

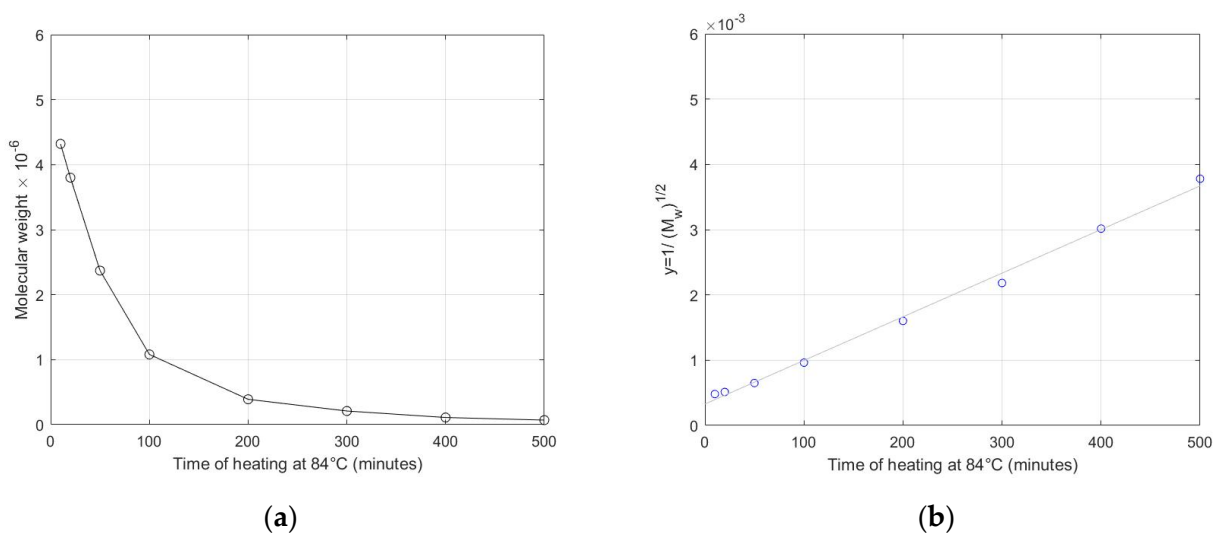


Figure 4. (a) thermal degradation of diplococcus pneumoniae DNA from Doty et al.[12]. (b) same data represented in the format of Eq. (13) and corresponding least-square linear fit.

This very close fit constitutes a strong proof that the DNA remains actually two-stranded during the thermal degradation process reported by Doty et al., (1960) [12]. This observation is pivotal for the focus of our study for three reasons:

- 1) Fragmentation rules out also the plausibility of the *100°C stable molecular weight claim*. 1019
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- 2) Doty et al., (1960) [12], based on the circumstance that one of their 1021
determination of the molecular weight of the DNA heated at 84° is 1022
approximately decreased by a factor of two (from 10.5 million to 5.0 1023
million), advance the claim that “*with aggregation eliminated and depolymerization taken into account it can be said that strand separation did occur in the very early stages of the exposure to the elevated temperature*”. Conversely, the fit in Fig. 4(b) shows the two-stranded character of the heated DNA and points to the clear evidence that the continuous fall of molecular weight is the result of progressive fragmentation into pieces that substantially remain two-stranded, so that the molecular weight halving recorded by Doty et al. is only fortuitously in agreement with the value of 1/2. In this respect it is worth acknowledging that also Doty et al. [12] in the statement above mention the necessity to account for “*depolymerization*”, thus duly excluding the possibility that halving of molecular weight might correspond to separation of intact single strands:. 1024
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- 3) The elucidation of the substantial two-stranded character of the material produced by heating, besides confuting the *100°C dissociation claim* as advanced by Doty et al. in the same paper [12], rules out any meaningfulness of heating denaturation for the purpose of strand separation. 1037
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A second important highlight in the contribution by Applequist is the role of the initial induction relatable to the presence of an initially uniform distribution of molecular weights and the determination based on the data by Shumaker et al. of the presence of initially broken P-bonds corresponding to $p_0 = 1.57 \times 10^{-2}$. This numerical determination will be recalled in the discussion of Section 4.2. 1043
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An examination of the type of intra-strand bond breakage is reported in Section 4 based on a closer review of the insightful analysis by Dekker and Schachmann (1954). 1049
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2.3.9. Evidence from light scattering of DNA fragmentation upon heating 1052 1053 1054

This subsection reviews determinations of DNA degradation by heating via light-scattering experiments reported in publications other than those by Doty and Rice of 1955 and 1957, which are instead more closely reviewed in the dedicated Section 3. 1055
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Peacocke and Preston (1958) [50] also perform light scattering measurements on DNA degraded by combined exposure to gamma rays and heating at 100°C for 15 minutes. They confirm observation of a decrease in molecular weight but highlight that the observation of the effect of heating degradation in larger DNA molecules is less pronounced and emphasize that the interpretation of the entity of this decrease is affected by “*the known tendency for the polynucleotide chains to remain entangled, in spite of the removal of hydrogen bonding, and for separated chains to re-aggregate on cooling*”. 1060
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Sadron performed measurements of molecular weights by light scattering after heating alone (in absence of gamma-rays exposure) and reported his findings in several publications between 1955 and 1959 [51], [60], [61]. In the 1955 biochemistry congress proceedings (see addendum on page 134) he reports the effect of heating at 100°C along 15 minutes on two DNA preparations of initial molecular weight $M_0 = 6.0 \times 10^6$. The weights he determines by light scattering on heated DNA at different NaCl concentrations show that DNA weight is always reduced as a confirmation that DNA is the more degraded the less is the NaCl content.

In particular, the table in his 1955 addendum on page 134 in [51] reports that just 15 minutes heating in absence of salt bring the molecular weight from 6×10^6 to 0.28×10^6 and 0.35×10^6 . In just one experiment with 1M NaCl he finds, in one of the two preparations, a weight increment to an average value $M = 7.0 \times 10^6$. Later, Freund, Pouyet and Sadron (1958) [61] report tests of calf thymus DNA (CV71) in 1M NaCl solutions and obtain by light scattering for the unheated preparation molecular weight $M_0 = 7.4 \times 10^6$. They measure by light scattering the molecular weight M in a 0.01 M NaCl solution after thermal degradation produced by heating at temperatures of 75°, 80°, 85° and 98°C during a variable time θ . They find that by heating at temperatures of 80°, 85° and 98°C the ratio $R = M/M_0$ as function of θ first increases, reaches a maximum and then drops to a residual value $R = 0.047$ corresponding to $M = M_2 = 350000$. The point data in the original figure have been digitized and those corresponding to temperatures of 85°C and 98°C are reported in Fig.5. The only correction applied to the digitized number was that minutes that were rounded to closest simple fractions of the hour of multiples of 5 minutes and to ordinates reached at the horizontal asymptote which were rounded to the value 0.047 explicitly reported by Freund et al.

The Authors find that the higher is the heating temperature the higher is the maximum, as shown in Fig. 5. This figure shows that heating at 98° brings this maximum above 1.4 and then makes R suddenly drop at approximately 10 minutes to a value below 0.35. It is also seen that at 15 minutes the interpolation reaches 10% of the native weight. This measure brings further quantitative straightforward evidence of the sudden fragmentation that the DNA molecule undergoes when heated at temperatures above 90°C.

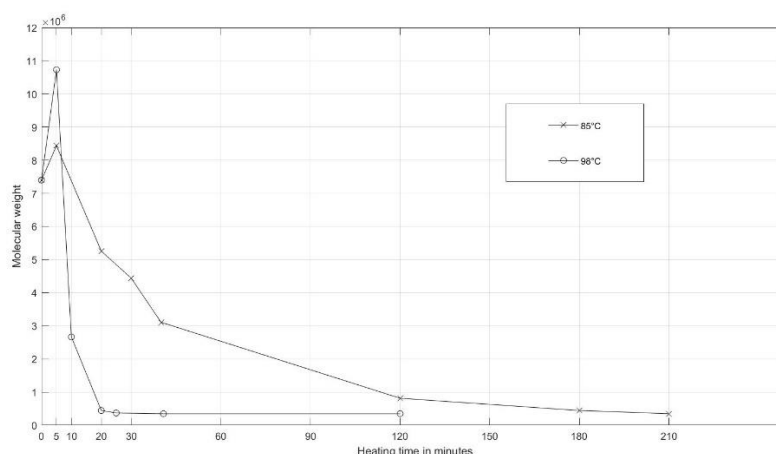


Figure 5. Digitized reproduction of Fig. 1 by Freund, Pouyet and Sadron (1958) [61]. Ratio $R = M/M_0$ vs. heating time θ . Only data for 98°C and 85°C are reported.

2.3.10. DNA Heating experiments in presence of different electrolytes

Hamaguchi and Geiduschek (1962) [56] perform a broad analysis of thermal stability of DNA in aqueous solution in presence of many different electrolytes and under many different pH conditions and temperature ranges. They confirm that thermal stability of DNA has a relatively broad maximum at pH 7-8 in 0.1 M NaCl and that in any concentration of any of the many electrolytes therein investigated the upper bounds of the temperature threshold of thermal stability can be never above 92.6°C (see Table I and Fig. 4 therein), irrespective of the electrolytes content and type.

3. Review of the experiments on DNA heating by Doty and Rice (1955, 1957)

The arguments relevant to the effects of DNA heating, and their interpretation, contained in the 1955 short communication by Doty and Rice “The denaturation of desoxyribose nucleic acid” [11] deserve the utmost attention since, to the authors’ knowledge, this paper is chronologically the first record where it can be read of the *100°C stable molecular weight claim* for DNA. This is in neat disagreement with the big picture consistently emerging from the studies analyzed in Sections 2.2, about DNA undergoing thermal depolymerization when the threshold of 81°C is surpassed, or even at lower temperatures in less stable conditions. In [11] it is reported indeed that, after heating a neutral saline solution to 100°C for 15 minutes the product is found “to have the same molecular weight as the native DNA”.

This third section enucleates the experimental evidence reported in [11] relevant to the effect of DNA heating and contains an analysis of the interpretation given by Doty and Rice to such experimental evidence aimed at understanding if, and how, new diriment measurements are reported by Doty and Rice in this 1955 study able to reverse the previous overall experimental-

theoretical picture on the phenomenon of DNA fragmentation on heating above 80°. 1141 1142

A critical scientific review is also contextually carried out of results and interpretations by Doty and Rice following the contemporary scientific debate on the consequences of DNA heating. To this end important contributions by K.V. Shooter, R.H. Pain, J.A.V. Butler, P. Alexander, K.A. Stacey and C. Sadron are also examined in this chapter in more detail. 1143 1144 1145 1146 1147

As [11] is a preliminary note, it is incomplete of exhaustive information on relevant materials and methods. The lacking information can be however sourced from the subsequent 1957 publication by Rice and Doty [23] referred as the continuation of such preliminary note. By joining the information contained in [23] and [11] it is known that the tested samples are of calf thymus DNA and that the results reported in [11] are obtained from the sample designated in [23] as SB-11 “prepared according to Simmons Method B”. 1148 1149 1150 1151 1152 1153 1154

The Authors inform that the relevant “preparative procedure involves multiple extractions of minced thymus in saline-citrate (0.015 M sodium citrate and 0.15 M NaCl) by blending and centrifuging to obtain a sediment containing the nucleoprotein.” Notably, the Authors specify that “30% sodium p-xylene sulfonate is used to deproteinize the nucleoprotein.” 1155 1156 1157 1158 1159

The three fundamental measurements employed by Doty and Rice for determining the molecular weight are the following: 1160 1161

- sedimentation measurements by ultracentrifuge; 1162
- viscosity measurements by a capillary viscometer; 1163
- light scattering measurements. 1164

Evaluation of molecular weight from this combined sedimentation and viscosity measurements by Doty and Rice is examined first in Subsection 3.1. Next, determinations of molecular weight from light scattering measurements by the same Authors are examined in subsection 3.2. 1165 1166 1167 1168 1169 1170

3.1. Molecular weight from combined sedimentation/viscosity measurements and the scientific debate among Doty, Shooter and coworkers 1171 1172

The data in [11] which Doty and Rice report in possible support the 100° stable molecular weight claim are collected in Table 2. 1173 1174

Table 2. Experimental sedimentation/viscosity measurements reported by Doty and Rice (1955) [11] employable to infer molecular weight in native and heated DNA. Data relevant to preparation coded “SB-11” in [23]. 1175 1176 1177

Quantity	Symbol	Units of measurement	Value measured on native DNA	Value measured on DNA after heating for 15 minutes to 100°
Reduced intrinsic Viscosity	$[\eta]$	$[0,1 \cdot \frac{m^3}{Kg}]$	72.0	4.3
Sedimentation constant referred to 20° and extrapolated to null dilution coefficient	S_{20}^0	S: Svedberg units [seconds per 10 ⁻¹³]	21.0	30.0

Concerning quantities in Table 2 and their units it is worth recalling what follows.

Quantity $[\eta]$ in Table 2 is the intrinsic viscosity: the specific increase in relative viscosity, $\Delta\eta/\eta_0$, determined by adding a quantity of DNA associated with a concentration increment $\Delta\rho$ to the solvent (of viscosity η_0), divided by $\Delta\rho$. Accordingly, $[\eta] = \frac{\Delta\eta}{\eta_0 \cdot \Delta\rho}$ and, as $\Delta\rho$ is in grams per deciliter, $[\eta]$ is in reciprocal units of deciliters/gram. The solvent of Doty and Rice is a saline-citrate solvent (0.015 M sodium citrate and 0.15 M NaCl).

The sedimentation constant s_{20}^0 is a rate of sedimentation measured by the ultracentrifuge experiment methodology developed by Svedberg [57]. Indicating by r the radial coordinate in the axial symmetric setup of the ultracentrifuge, the centripetal acceleration a is a radial field varying as $a = r\omega^2$, where ω is the angular velocity. The rate $\frac{dr}{dt}$ is measured by an optical system through so called *schlieren photography* which essentially returns diagrams of changes in refractive index that typically have a «striped» pattern (“schlieren” = strip from German) and that inform about the change in concentration as function of time and radial coordinate r . If the centrifugal field is strong enough to cause the molecules or particles to sediment with measurable velocity, $v = \frac{dr}{dt}$, the sedimentation constant is then $s = \frac{1}{r\omega^2} \frac{dr}{dt}$.

The principle of measurement in centrifuge is that the rate of sedimentation increases with the mass of the particle and with the intensity of acceleration. Tracing a parallel with a falling object of mass M immersed in atmosphere and in a uniform gravitational field, it is known that due to the presence of air friction, the object deviates from the Galilean prediction of uniformly accelerated motion with gravitational acceleration g and reaches after a transitory time a steady velocity. The frictional force F_f , directed upwards, in a simplest description, is proportional to the velocity v of the falling object so that $F_f = b \cdot v$. As theoretically predicted by the force balance (*gravitational force = frictional force; $F_a = F_f$; $M \cdot a = b \cdot v$*) and empirically confirmed, the final velocity reached by the object increases with the object mass M and with acceleration a which for the gravitational field is uniform in space and equal to the terrestrial gravitational acceleration constant g : $a = g$. The mass is accordingly computed as $M = \frac{b \cdot v}{a}$ or $M \propto \frac{v}{a}$. As recalled, the main difference between the acceleration field of the centrifuge set-up and the gravitational field is that rotation implies a circular relative motion field with acceleration increasing with the radius by $a = r\omega^2$ so that acceleration is no longer uniform as in the example of the falling object. The DNA molecules, heavier than the solvent, by inertia, will *appear* in the non-inertial rotor reference frame to be subject to a radial motion component directed towards the periphery of the centrifuge. This motion component may be causally interpreted as the consequence of not being sufficiently «pushed» in centripetal direction by the pressure gradient field that is generated in the solvent in response to the centripetal acceleration generated by the rotor. Through a special focus lenses measurements of the concentration along the radial direction are obtained from measurements of light absorption changes and/or refraction changes. Records are taken of the «strips» produced by the different optical properties of this moving «solute clouds». From these striped diagrams it is possible to

measure concentration changes along the radius and, in turn, evaluate the speed dr/dt . The higher the $\frac{dr}{dt}$ rate of sedimentation of this «dust», at the same acceleration $a = r\omega^2$ (and therefore at the same angular velocity of rotation ω and radial distance r in the blender), the greater is the mass M of the DNA molecule or molecule fragment by the proportion $M \propto \frac{dr/dt}{a}$. The final raw quantitative measurement obtained from this experiment is the *sedimentation constant* $s = \frac{1}{r\omega^2} \frac{dr}{dt}$ whereby the direct proportionality holds $M \propto s$. Experiments are performed to extrapolate the limit s^0 of s at zero concentration (zero superscript), so that direct interaction between molecules can be excluded. If account is also taken of temperature which can change the solvent viscosity and a reference temperature of 20°C is employed, the symbol s_{20}^0 is used to refer to such quantity. Ultracentrifuge sedimentation analysis of DNA containing a detailed report of observed sedimentation boundaries are presented by Cecil and Ogston (1948) [57,58]. Also, to the authors' knowledge, the first reported molecular weight measurements for DNA, based on the application of combined viscosity-sedimentation methods, are by Krejci, Sweeny and Hambleton (1949) [59].

Concerning the ultracentrifuge sedimentation experiments, Rice and Doty [23] report two important information:

- When DNA solutions are exposed to 100°C for 15 minutes the hyper-sharp sedimentation profile is lost and “two differently sedimenting species in solutions” appear.
- Such a polydisperse character of the heated solution, in the same words as Rice and Doty, “posed a difficult problem” and induced the Authors to erroneously attribute a sedimentation constant of 30 S. In fact they also report, “Dr. K. V. Shooter and Professor J. A. V. Butler have examined our sample SB-11 by ultraviolet optics in the ultracentrifuge and found that the distribution of sedimentation coefficients broadened but remained single-peaked at 21 S. when heated at 100°C for 15 min. at a concentration of 10 mg./dl.” (see Shooter et al. [9]).

The scientific debate between Doty and Rice and Shooter et al. deserves attention. Measurements by Shooter et al. [9] are conveniently reported in Table 3 since the Authors re-execute both sedimentation and viscosity measurements for sample SB-11 (in the acknowledgement the donation by Prof. Paul Doty is mentioned) investigating the effect of 15 mins heating at 100°C and achieve increased accuracy for sedimentation measurements. Increased accuracy is achieved since the Authors employ a preferable lower 0.01% concentration instead of 0.035% of the samples examined in [23], and operate optical measurements in the ultra-violet range where they had shown that heterogeneity with respect to sedimentation coefficient is more easily observed [62]). By comparing Tables 2 and 3 it is seen that reduced intrinsic viscosity values are the same in [23] and [62] while a value of 20.0 S is more accurately measured for the sedimentation constant after heating thus identifying and

correcting the experimental error recognized by Doty and Rice and recalled above. 1272
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Table 3. Sedimentation/viscosity measurements by Shooter et al. [9] in native and heated samples of 0.01% DNA preparation SB-11. Units are the same of Table 2. 1275
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Quantity	Value measured on native DNA	Value measured on DNA after heating for 15 minutes to 100°
Reduced intrinsic Viscosity $[\eta]$	72.0	4.3
Sedimentation constant referred to 20° and extrapolated to null dilution coefficient s_{20}^0	20.80	20.0

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Shooter et al. [9] finalize their study on the effect of DNA heating and their countercheck of Doty and Rice measurements by concluding that “The results given suggest that in all cases the samples of DNA are degraded on heating but that the extent of the degradation and the distribution of size and shape of the fragments produced depends upon the way in which the DNA has been prepared”. It is important to remark that this conclusion is achieved in [9] upon testing sedimentation constants and viscosities of four DNA preparations (TNA 7, TNA 15 , TNA 23 and SB-11) under multiple concentrations, in presence and in absence of the protective effect of NaCl . The Authors emphasize that sedimentation constant decreases in all samples except for samples TNA16 and SB-11 for which it remains unchanged. They remark that *despite the unchanged sedimentation constant the apparent molecular weight is reduced in all cases*. They have the merit of highlighting the possible fallacies in deducing the molecular weight from combined sedimentation and viscosity measurements. Fallacies may arise from reaggregation of the fragments when heating is performed at higher concentrations. Indeed, in one case they find that heating the TNA23 solution at 0.05% concentration at 100°C for 15 minutes makes the sedimentation constant increase from 27 to 36 Svedberg units. This measurement shows that particles appear to have possibly even increased their mass, an effect that may be explained only by reaggregation. They also observe for all DNA preparations that the “spread of sedimentation coefficients is changed, there being an increase of material with both high and low sedimentation coefficients”. Such result is readily interpreted as evidence of the production by 15 minutes heating of both fragments of lower molecular weight as well as of aggregations of fragments of higher molecular weight. Shooter et al. highlight that preparations TNA 23 and SB-11 are obtained, in particular, by detergent methods (it is worth recalling that SB-11, donated by 1279
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Doty, has been prepared employing *sodium p-xylene sulfonate*) pointing to the risk that higher sedimentation constants may be preparation artefacts due to the use of detergent methods while lower sedimentation constants might be artefacts due to purification procedures employing enzyme methods which have a depolymerizing effect.

Next, in their 1957 publication [23] containing the reply to Shooter et al. countercheck, Rice and Doty do not make considerations on the evidence that fragments reaggregation readily explains the increase in sedimentation constant from 21 *S* to 30 *S* of Table 2 that they had published in their 1955 preliminary note. On the other hand, Shooter et al. had shown that heating at 0.05% concentration, a value close to the 0.035% concentration employed by Rice and Doty, makes the sedimentation constant of TNA 23 increase from 27 *S* to 36 *S*.

Doty and Rice reference the empirically well-established relation of Krigbaum, Flory, Mandelkern and Sheraga [11] which is the following:

$$\frac{s^0 [\eta]^{\frac{1}{3}}}{M^{\frac{2}{3}}} = \frac{K(1-\bar{v}\rho)}{\eta_0 N}, \tag{14}$$

In Equation (14) *K* is a universal constant which has the same value irrespective of molecular weight, temperature and solvent and depends only on the flexibility of the molecular chain. Its value ranges between $K_{flex} = 2.5 \cdot 10^6$ for a flexible coiled molecule and $K_{stiff} = 3.6 \cdot 10^6$ for ellipsoids of increasing axial ratio, η_0 is the solvent viscosity, *N* is Avogadro's number, ρ is the density of the solution and \bar{v} is the partial specific volume. All quantities on the right hand side of Eq. (14) are constants, or can be treated as such, in the experiments under examination.

Equation (14) permits a more accurate determination of molecular mass. It can be applied to examine Doty and Rice data of Table 2 and to investigate the scientific substantiation of their key argument: i.e., to investigate whether changes induced by 15 mins heating at 100° can be explained exclusively by calling into question a possible coiling or change in the molecule flexibility, excluding any molecular weight change, or if compelling evidence exists of molecular fragmentation from data of Tables 2 and 3.

Subscripts N and H are used below in a way similar to Eq. (2) of Sect. 2.3.6 above to indicate values relevant to native and heated DNA. Assuming, first, that *K* is not influenced by heating, so that it takes the same value *K* for the heated and unheated material, one infers from Eq. (14):

$$\frac{M_N}{M_H} = \sqrt{\left(\frac{s_N^0}{s_H^0}\right)^3 \times \frac{\eta_N}{\eta_H}} \tag{15}$$

Hence from Doty e Rice 1955 data we compute under this assumption of unchanged flexibility:

$$\frac{M_N}{M_H} = \sqrt{\left(\frac{21}{30}\right)^3 \times \frac{72}{4,3}} = 2.39, \quad (16)$$

which states that after heating the molecules are more than 2 times smaller, on average. It is understood, however, that Shooter et al. have elucidated that the sedimentation $30 S$ appears to be an erroneous measurement artefact. Moreover, by taking into account the correction of the erroneous measurement evidenced by Shooter et al., one further computes:

$$\frac{M_N}{M_H} = \sqrt{\left(\frac{20}{20}\right)^3 \times \frac{72}{4,3}} = 4.09, \quad (17)$$

so that after heating the molecules are about 4 times smaller, on average. If one wishes to defend further the claim of Doty and Rice, appealing to the residual argument that the increase in sedimentation constant might only be due to coiling of the molecule (putting apart the already reasonably clear evidence of fragments reaggregation) one can employ $K_{stiff} = 3.6 \cdot 10^6$ for the native material and $K_{flex} = 2.5 \cdot 10^6$ for the heated material. One can thus write:

$$\frac{s_N^0[\eta_N]^{1/3}}{M_N^{2/3}} = \frac{K_{stiff}(1-\bar{v}\rho)}{\eta_0 N}, \quad (18)$$

$$\frac{s_H^0[\eta_H]^{1/3}}{M_H^{2/3}} = \frac{K_{flex}(1-\bar{v}\rho)}{\eta_0 N}, \quad (19)$$

so that from (18) and (19) one infers:

$$\frac{M_N}{M_H} = \sqrt{\left(\frac{s_N^0 K_{flex}}{s_H^0 K_{stiff}}\right)^3 \times \frac{\eta_N}{\eta_H}}. \quad (20)$$

Hence employing the available experimental data one would compute:

$$\frac{M_N}{M_H} = \sqrt{\left(\frac{20}{20} \times \frac{2.5}{3.6}\right)^3 \times \frac{72}{4,3}} = 2.368, \quad (21)$$

It is hence seen that, even appealing to such a coiling hypothesis, fragmentation remains a compelling evidence matching with the “two sedimenting species” recorded by Rice and Doty.

3.2. DNA Molecular weight from light scattering

Section 3.1 has shown the absence of any evidence from combined sedimentation and viscosity measurements supporting the possibility that native DNA molecules can avoid fragmentation and remain thermally stable above 85° with the specific buffer solutions subject to testing and have also shown the existence of consistent and redundant evidence of such fragmentation above 85°. The present section shows that the same conclusion is arrived at even when results coming from light scattering measurements are carefully examined.

Light scattering deserves attention for several reasons:

- it is the third and last measurement technique reported in [23] on the basis of which it is claimed therein that no fragmentation occurs above 85° in the DNA molecules (if not, admittedly, only after a heating period of 15 minutes and more) since it is claimed, from light scattering results therein reported, that no significant change in molecular weight occurs on thermal denaturation;
- among the methods available for the determination of the size and shape of macromolecules, light-scattering in 1954 was considered the method giving more nearly correct values for the molecular weight and the radius of gyration by several researchers [63-65] actively working on DNA molecular characterization, such as Reichmann, Doty and coworkers [63] and Alexander and Stacey [64] and by Sadron and coworkers;
- it is the tool which brought to the remarkable result that DNA purified from different biological sources such as calf thymus, herring sperm, bacteria and bacteriophage [63-65] has always the same molecular weight of about 6×10^6 when the extraction and purification methods are the same.

It is thus understood that if light scattering is properly employed for determining the molecular weights of native DNA and of molecules after potentially fragmenting treatments, such as heating, acid treatment or depolymerizing enzymes, it can be a mean for quantitative verification of different hypotheses on possible depolymerization or coiling of the molecule.

Clearly, we anticipate that as we have already remarked in the previous sections of this survey, in examining the effect of these treatments it is fundamental to properly discern four possible situations:

- s1) the possibility that the molecules remain with unchanged weight and unchanged size;
- s2) the case of a reduction in size of the molecule due to a *real fragmentation* into parts of lower molecular weight;
- s3) the case of a *merely ostensible reduction in size* which is due instead to just a coiling of the molecule with unchanged molecular weight;
- s4) the case specular to case 2) and elucidated by Shooter and coworkers [9] in which, a depolymerizing action (such as heating or acid treatment), yields, *in presence of a not sufficiently low*

concentration of DNA, a reaggregation of fragments producing aggregates showing an ostensible measurable effect which may be misinterpreted as the indication of the production of a material having higher molecular weight (this is the case, we recall, of the increase of the sedimentation coefficient for preparation TNA 23 at 0.005 % concentration rising from 27 Svedberg units (before heating) to 36 Svedberg units [9]).

3.2.1. Brief notes on the fundamentals of light scattering

Before examining Rice and Doty's light scattering results, a brief introduction of the measurement principle and measurement technique of the light scattering method and of the related turbidimetry method.

Fundamental advancements to optical methods of turbidity and light-scattering have been contributed by Debye [70-72] and by Zimm [75-76]. In brief, the essential physical principle of these optical techniques applied to molecules in solution, clearly described by Debye in 1944 [70], is based on the following relation expressing the turbidity τ of diluted solutions as function of the refractive index of the solvent, μ_0 , of the refractive index μ of the solution, of the wave-length λ , and of the number n of molecules per cm^3 :

$$\tau = \frac{32\pi^3}{3} \frac{\mu_0^2 (\mu - \mu_0)^2}{\lambda^4} \frac{1}{n} \quad (22)$$

If μ_0 for the solvent is known, n can thus be measured, in the first instance, on the basis of two experiments, a first one giving μ and a second one giving τ . Once n is determined, the mass of the particle u and its molecular weight c follow from the concentration c as $u = c/n$ and $M = cN/n$, being as usual N Avogadro's number. For practical purposes the quantity $K = \tau/(Mc)$ is introduced by Debye. This quantity, on account of (22) and of relation $nM = cN$, achieves the expression:

$$K = \frac{32\pi^3}{3} \frac{\mu_0^2 (\mu - \mu_0)^2}{N\lambda^4 c^2} \quad (23)$$

K is thus for turbidity, in a first approximation, a refraction constant which, once determined, permits to compute M as $M = \tau/Kc$ or, equivalently:

$$\frac{K \cdot c}{\tau} = \frac{1}{M} \quad (24)$$

However, Debye discusses in 1947 [72] that, for solutions of high-polymer substances, such as the case of DNA, even at high dilutions K cannot be treated as a constant with respect to concentration c but is found to be a function $K(c)$ of the concentration c , and the same occurs for turbidity τ which is similarly a function $\tau(c)$ of concentration. He advises to employ the linear interpolation:

$$\frac{K(c) \cdot c}{\tau(c)} = \frac{1}{M} + 2B \cdot c, \quad (25)$$

in which B is a constant depending on the solvent so that in the limit of zero concentration, $c \rightarrow 0$, Eq. (24) is recovered and M is computed. This corresponds to the following two-dimensional plot extrapolation:

- the refraction term K and the dilution τ are determined for different concentrations c_1, c_2, c_3, \dots obtaining several determinations $K(c_1), K(c_2), K(c_3), \dots$ and $\tau(c_1), \tau(c_2), \tau(c_3), \dots$;
- from these determinations the left-hand side of (25) as function of the independent variable c , $L(c) = K(c) \cdot c / \tau(c)$, is interpolated (in [71] he employs in particular the method of least squares);
- function $L(c)$ is extrapolated to zero concentration obtaining the limit $\lim_{c \rightarrow 0} L(c) = \lim_{c \rightarrow 0} \frac{K(c) \cdot c}{\tau(c)}$; this limit is equal to the limit of the right-hand side $\lim_{c \rightarrow 0} \left(\frac{1}{M} + 2B \cdot c \right) = 1/M$;
- eventually, this method graphically provides the molecular weight M as the intercept at $c = 0$ of curve $L(c)$.

The method described so far for determining M only involves measurement of the turbidity τ and not of light scattered at different angles. For larger thread-like polymerized molecules, such as DNA, a more refined measurement technique which is based on the additional information obtainable from measurement of the angular distribution of the scattered light is further devised by Debye [72]. Debye derives a fundamental analytical relation for the intensity I of scattered light as function of the angle θ between the primary beam and the scattered beam. The function $I(\theta, c)$, accounting for such dependency upon θ and upon the concentration c of solute in each experiment, is theoretically elaborated by Debye through an averaging procedure accounting for all conceivable particle configurations (since the particles are statistically free to rotate and fluctuate in the optical model). The expression of $I(\theta, c)$ resulting from this theoretical elaboration depends on the employed model for the particle. Debye presents specific analytical expressions for particles modeled as having spherical or ellipsoidal shape and, additionally, considers a third model (a so-called *random coil* model, essentially devised for schematizing the optical behavior of polymers) in which thread polymers are schematized as a flexible number of links, each one able to rotate freely. Debye finds that, for this set of molecular models (random coils, spherical shapes or ellipsoidal shapes) the relevant function $I(\theta, c)$ turns out to be conveniently expressed in a relatively simpler analytical closed form of the following type: $I(\sin^2(\theta/2), c)$, i.e., by an analytical expression in which $\sin^2(\theta/2)$ is the first independent variable. A convenient alternate series expansion expression of function $I(\theta, c)$, applicable to a variety of molecular shapes and typologies and under some respects analogous to (12), is contextually elaborated by Debye one year later. A more explicit expansion of this form is also presented by Zimm [75] (see Eq. (26) therein). Zimm's series expansion yields a determination of the molecular weight M from light

scattering measurements at different angles and is similarly applicable to different particle models. The first three terms of this expansion are:

$$\frac{K \cdot c}{I(\theta, c)} = \frac{1}{M} \cdot \frac{1}{P(\theta)} + 2A_2 \cdot c + f(\theta) \cdot c^2 + \dots, \quad (26)$$

In the previous relation $I(\theta, c)$ is the intensity of the excess scattering (over that of the solvent alone) at a given concentration, $P(\theta)$ is a probability function such that $P(0) = 1$, A_2 is a constant independent from θ and c , and $f(\theta)$ is a term independent from c . Owing to these properties, it is seen that extrapolation of the left hand term in (19) at zero concentration and zero angle θ gives again the reciprocal of the molecular weight:

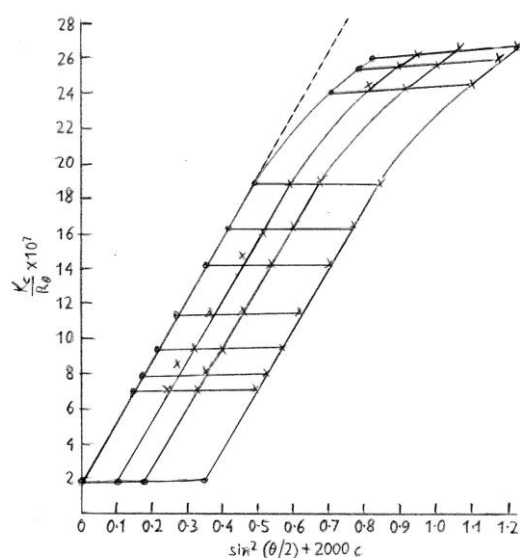
$$\left(\frac{K \cdot c}{I(\theta, c)} \right)_{\substack{c \rightarrow 0 \\ \theta \rightarrow 0}} = \frac{1}{M}. \quad (27)$$

Debye also shows [72] that the slope of the plots (as function of c) in proximity of $c = 0$ defined by coefficient $2A_2$ in (26) defines the square of the average size of the particle in terms of distance from its center of gravity (also termed radius of gyration by some authors [64],[69]). For clarity in the employed terminology, it is recalled that the function $\frac{K \cdot c}{I(\theta, c)}$ is also called in [64] and [69] reciprocal reduced intensity and the Zimm plots are also denominated *reciprocal reduced intensity plots*.

The accuracy of the extrapolation procedure stated by (27) is understood to be critical for the accuracy of the determination of the molecular weight. The extrapolation is operatively carried out by graphical construction of a grid-like plot (denominated Zimm plot) in the plane having the quantity $\sin^2(\theta/2)$ on the horizontal axis and the quantity $K \cdot c/I(\theta, c)$ on the vertical axis.

For the problem of interest, which is the determination of the weight of the DNA molecule by light scattering, the construction of a Zimm plot has been well exemplified and explained by Alexander and Stacey (1955) [64] who, aside to Doty and coworkers, have been among the first researchers performing such a measure. Fig.6 is a handmade copy of the plot performed by Alexander and Stacey [64] for determining the molecular weight of herring sperm DNA at PH 6.8. On the horizontal axis of the graph in Fig. 6 it can be read $\sin^2(\theta/2) + 2000 \cdot c$ since the addition of the term $2000 \cdot c$ is simply a mean for graphically shifting in the horizontal direction the curves, function of $\sin^2(\theta/2)$, obtained at each constant value for c . The intercept at $c = 0$ is seen to be $\frac{1}{M} = 1.7 \cdot 10^{-7}$ and from this value the result $M = 5.9 \cdot 10^6$ is computed by these Authors. Observe that the slope of the horizontal segments of the plot represent the sensitivity of this method to the solute concentration and that the property of these segments of being almost horizontal in the vicinity of the intercept with the vertical axis ensures the independence of evaluation of the molecular weight from the concentration employed in the experiments.

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Figure 6. Handmade reproduction of Fig. 1 in reference [64] by Alexander and Stacey. Zimm plot for determining the molecular weight of herring sperm DNA at PH 6.8.

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We briefly recall now the light scattering results that Doty and coworkers publish in the years 1953-1957. With these data Doty supports two claims:

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- the claim of unchanged DNA molecular weight upon acidification to pH 2.6 (Reichmann, Bunce and Doty, 1953) [28];
- the claim of unchanged DNA molecular weight after 15 minutes heating at 100°C (Rice and Doty, 1957) [23].

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3.2.2. A fundamental digression on the detection of DNA acidic degradation by light scattering with a confutation

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Although the central object of discussion of this subsection are possible evidences from the light scattering measurements by Doty and Rice of the molecular weight stability of DNA to *heating*, a brief digression on the close examination of the light scattering data relevant to the assessment of stability upon pH 2.6 *acidification* of DNA, presented by Reichmann, Bunce and Doty in (1953) [27] is carried out hereafter to duly highlight possible sources of fallacious, or diverging, interpretation of data from light scattering. Such a digression is revealed to be important by the existence of an ongoing debate in the thread of publications [27], [64], [23] concerning the question of whether the molecular weight of DNA changes or not after acidification at pH 2.6 in aqueous solution with 0.2 M NaCl. Such debate is very instructive for discerning in heating degradation the situations s1), s2), s3) and s4) enumerated at the beginning of Section 3.2.

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The apparatus employed by Reichmann et al., (1953) [28] for light-scattering experiments is not described in detail, as the reader is referred to (Doty and Bunce, 1952) [69] where it is specified that measurements were made by a Brice-Speiser photometer.

Reichmann et al. (1953) [28] perform two sets of light scattering experiments for determining the molecular weight of calf thymus DNA in 0.2 M NaCl, at pH 6.5 and at pH 2.6 obtaining the two Zimm plots in Fig. 7, reproducing Fig. 1 by Reichmann et alii. These Authors interpret these plots as the evidence of unchanged molecular weight, ostensibly given by the common intercept attained by these grids in the limit of zero angle and zero concentration.

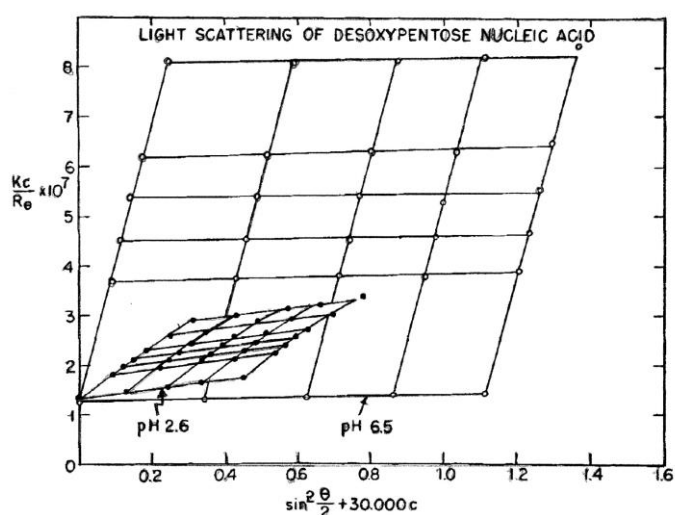


Figure 7. Zimm plots performed by Reichmann, Bunce and Doty, 1953) [27] (handmade reproduction of Fig. 1 therein) for determining the molecular weight of calf thymus DNA in 0.2 M NaCl at pH 6.5 and 2.6. As reported, tested concentrations vary from 0.005 to 0.05 mg./cc.

Reichmann et al. pair this possible evidence of stable molecular weight with the interpretation that the fall in viscosity would be the result of a contraction of the coiled molecule as the solution is acidified. They do not consider any possibility of the effect of disaggregation and reaggregation evidenced by Shooter, Pain and Butler (1956) [9]. However, this conclusion of stable molecular under pH 2.6 weight has been challenged by contrary evidence shown by Alexander and Stacey [64] who employed the same method of light scattering to analyze the behavior of this molecule in a broader set of similar experiments. The light-scattering analyses of DNA by these Authors are characterized by a specific view towards possible evidence of depolymerization and towards the delicate discernment of antagonist effects due to fragmentation and to fragments reaggregation highlighted in Section 3.1. In this study the Authors examine DNA depolymerization due to acid treatment under conditions similar to those of Reichmann et al. (1953) [44] while encompassing a countercheck of particle weight and particle size at different pH

values and even in presence of renaturalization. Their results relevant to the analysis of possible depolymerization due to acidification down below pH 2.6 are recalled hereafter.

The apparatus for the light scattering experiments by Alexander and Stacey experiments is described in [67] and is similar in design to the one used by Carr and Zimm [68]. Also, the experimental conditions examined by these Authors and by Reichmann et al. are readily comparable (solutions of 0.10 to 0.2 M NaCl).

In examining and confronting the experimental results, it is convenient to introductively recall that evidence of acid depolymerization below pH 5 in terms of viscosity reduction and decreased sedimentation has been already highlighted by Cecil and Ogston [58] who had reported that at pH 3.5 "Treatment with acid leads to the formation of two fractions, one of which is relatively homogeneous and resembles the original material except that it is altered by reprecipitation. The formation of the heterogeneous component is probably the result of disaggregation. The changes brought about at pH 3.5 proceed to a limit, and no further important change follows subsequent neutralisation. Reprecipitation of the neutralised solution causes a further large change, the homogeneous component disappearing and a gel-forming material appearing in its place : this change is likely to be due to a re-aggregation.". Evidence in terms of viscosity reduction and of decreased transformation activity in bacteria similarly pointing towards depolymerization has been also reported by Zamenhof et al. [8] (with a different buffer) and is synthesized in the following Fig. 8 taken from such reference.

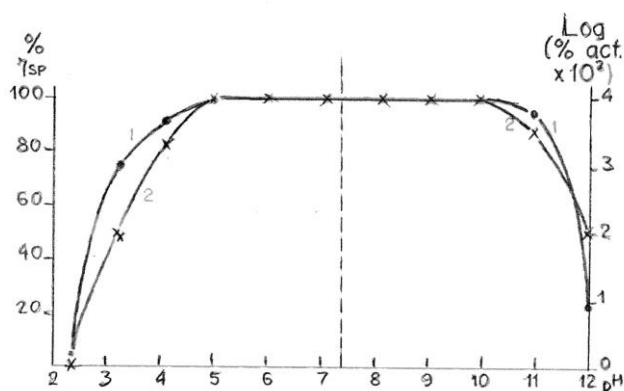


Figure 8. Handmade reproduction of Fig. 2 in Zamenhof et al. [8] showing effects of pH on viscosity and transforming activity. Dots: the measured viscosity; crosses: measured transforming activity; for further details on buffer composition refer to the original publication.

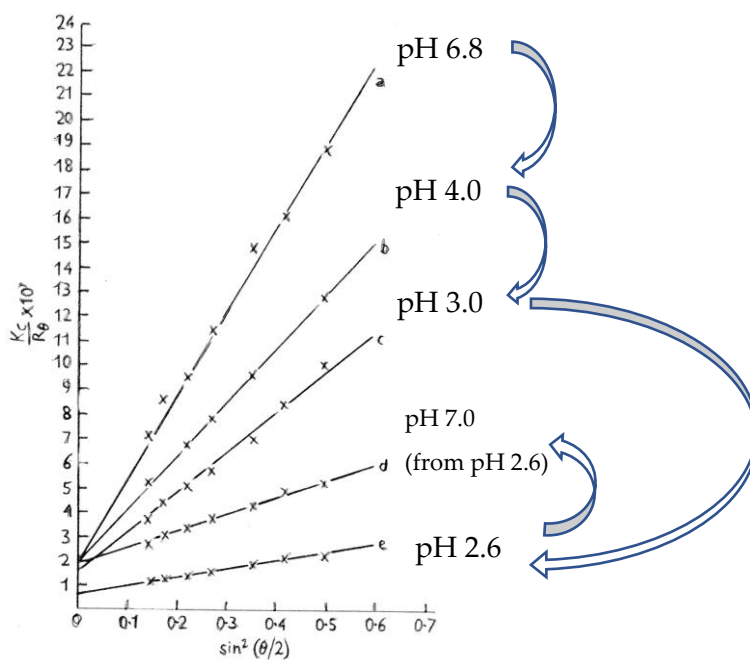
The data showing the effect of molecular weight decrease, as determined by the Zimm plot, and showing a spurious molecular weight increase due to fragments reaggregation are shown in Table 4 and Fig. 9, (data and image taken from [64]).

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Table 4. Influence of acid treatment on the weight and shape of DNA in solution as determined by light scattering measurements by Alexander and Stacey (1955) [64]

Treatment	$10^{-6} \times$ molecular weight	Radius of gyration [Å]	Marker in Fig. 9 graph (if present)
None at pH 6.8	5.9	1980	(a)
Addition of 0.01 M HCl to make solution pH 4.0	5.9	1610	(b)
Addition of acid to make solution pH 3.0 (measured after 15h)	6.6	1520	(c)
Re-neutralization to pH 7.0 from pH 2.6	5.9	920	(d)
Addition of acid to pH 2.6	15.9	1040	(e)
Re-neutralization to pH 7.0 from pH 2.2	2.45	800	not present in Fig. 9

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Figure 9. Hand-made reproduction of Fig. 3 by Alexander and Stacey [64]. Reciprocal reduced intensity plot after different acid and neutralization treatments. (a): None at pH 6.8; (b): pH 4.0; (c): pH 3.0; (d): pH 7.0 after re-neutralization from pH 2.6; (e): pH 2.6. See also corresponding values in Table 4

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herein. In the original source, only the curve corresponding to the extrapolation to $c \rightarrow 0$ are plotted and not the entire Zimm grids. pH labels and arrows on the right side are the only elaboration added to the figure obtained as the hand reproduction of the original figure.

We recall that, as the intercept gives $1/M$, when the intercepts of the curves in Fig. 9 are lifted up after a treatment, the molecular weight of the particle (or of the aggregate of particles) decreases. The opposite is true when the intercept decreases: the weight of the particle increases (as observed by the Authors this phenomenon can result from aggregation revealed by the observed precipitation). The curves are best examined following the sequence of incremental acidification (a) \rightarrow (b) \rightarrow (c) \rightarrow (e) and next considering the re-neutralization (e) \rightarrow (d) from pH 2.6 to pH 7.0. What is seen is that in step (a) \rightarrow (b) M remains the same. Next in step (b) \rightarrow (c), from pH 4.0 to pH 3.0, the lowering of the intercept corresponds to an increase of M from $5.9 \cdot 10^6$ to $6.6 \cdot 10^6$. This increase can be interpreted as a first sign of fragmentation and reaggregation. Such a picture is confirmed in step (c) \rightarrow (e) where pH is lowered from pH 3.0 to pH 2.6 and M rises from $6.6 \cdot 10^6$ to $15.9 \cdot 10^6$ showing a clear sign of reaggregation. The evidence that aggregation is reduced comes from step (e) \rightarrow (d) from pH 2.6 to pH 7.0 when M is lowered back again to 5.9, possibly giving the fallacious feeling of the molecule being undegraded since 5.9 is the same molecular weight of the best-preserved DNA samples. Any misinterpretation is ruled out by examining data in the last row of Table 4 showing that reneutralization to pH 7.0 from pH 2.2 yields a molecular weight 2.45.

Concerning the completeness of the dataset of measurements behind Fig.5, it is important to remark that the authors report to have performed the extrapolation by ordinarily testing the solution at different concentration (as required by the proper execution of the grid-plot methodology devised by Debie and Zimm) although only the curves corresponding to the extrapolation to $c \rightarrow 0$ are plotted by Alexander and Stacey in their figures and not the entire Zimm grids (this for avoiding data superposition and for permitting clear readability to the reader).

Altogether the data by in Table 4 and Fig. 9 by Alexander and Stacey [64] disprove the claim by Reichmann, Bunce and Doty (1953) [28] that the DNA molecule recovers its original size and shape upon the pH being returned to pH 6.5 from pH 2.6 and the fallacious explanation that the large decreases in viscosity observed by several researchers upon adding acid to DNA solutions are due primarily to the contraction of the DNA molecules, the contribution of degradation being only secondary.

Referring to media more acid than pH 3.0 the Authors explicitly report the following evidence: *"In more media there was a rapid aggregation until precipitation occurred. At pH 2.6 apparently stable aggregates with molecular weights of about 15×10^6 were produced (Fig. 3). This material is polydisperse and no significance can be attached to this numerical value since the weight-average molecular weight varies somewhat with slight changes of experimental conditions in this pH range."* [64].

It is thus recognized that the evidence obtained by Alexander and Stacey [64] 1718
 which has been herein recalled provides five elements of understanding: 1719

- it rules out the possibility of DNA not being fragmented at pH 2.6 1720
 under the experimental conditions considered by Reichmann et al.; 1721
- it confirms by the independent method of light scattering, also for 1722
 the acidic treatment (not yet for heating), the effect of disaggrega- 1723
 tion and reaggregation highlighted by Shooter, Pain and Butler 1724
 (1956) [9], among others, by ultracentrifuge and viscosity analysis 1725
 of heated DNA, previously recalled in Section 2.3.1; 1726
- it invalidates the hypothesis drawn by the group of Doty that the 1727
 decrease in viscosity can be justified by the sole effect of contraction 1728
 of the coiled DNA molecule; 1729
- it confirms the existing evidence for acidic degradation with dis- 1730
 aggregation and reaggregation already at pH 3.5 found by Cecil and 1731
 Ogston [58] recalled above; 1732
- most importantly, it points out the *tangible risk of possible fallacious* 1733
misdetetection of increased molecular weight, or even of unchanged molecu- 1734
lar weight, by light scattering in presence of a limited set of data. 1735

3.2.3. *Questioned light-scattering evidence by Rice and Doty of stable molecular* 1737
weight at 100° (with a second confutation) 1738

Once the elements of knowledge in the bullet list above are acquired, the 1741
 examination of the light scattering data by Rice and Doty (1957) [23] on DNA 1742
 subjected to heating at 94° and 100° can be carried out in an unbiased way 1743
 able to separate the “*wheat from the chaff*”, i.e., of separating a fallacious evi- 1744
 dence of increased or stable molecular weight in front of an incomplete set of 1745
 measurements. These results are presented hereafter. 1746

For information on the methods and apparatus employed for the light 1748
 scattering experiments, Rice and Doty refer to the experimental methods re- 1749
 ported in (Reichmann, Rice, Thomas and Doty, 1954) [63]. In this last publi- 1750
 cation the apparatus is not described in detail and outer reference is made 1751
 again to (Reichmann, Bunce, Doty, 1953) [27] and, transitively, to Doty and 1752
 Bunce (1952) [69]. In this last publication, as already recalled, it is specified 1753
 that the light scattering measurements were made by a Brice-Speiser photom- 1754
 eter. The Authors report that a saline-citrate solvent (0.015 M sodium citrate 1755
 and 0.15 M NaCl) had been used in all light scattering experiments. Concen- 1756
 tration of DNA solutions are reported to be of “*about 6 mg./dl.*” correspond- 1757
 ing to 0.06 mg/cc. The employed DNA sample is again the one coded SB-11. 1758

The study by Rice and Doty proceeds from the premise referred to the 1759
 publication by Reichmann, Bunce and Doty (1953) [27] that: “*When dis-* 1760
solved in 0.2 M NaCl, DNA can be exposed to PH values as low as 2.6 for several 1761

hours without significant chemical degradation". Such a premise has been just shown in Subsection 3.2.2 to be disproved by Alexander and Stacey [64] showing that what had been detected at pH 2.6 are joint phenomena of fragmentation and reaggregation, in confirmation of Cecil and Ogston's [58] early findings and of the multiplicity of evidences so far collected in Sect. 2.2 and 2.3.1.

The data that Rice and Doty claim would show that heating at 100°C during 15 minutes would leave the molecular weight unchanged are contained in Figs. 5 therein. Such figure has been digitized and reproduced in current Fig. 10(b).

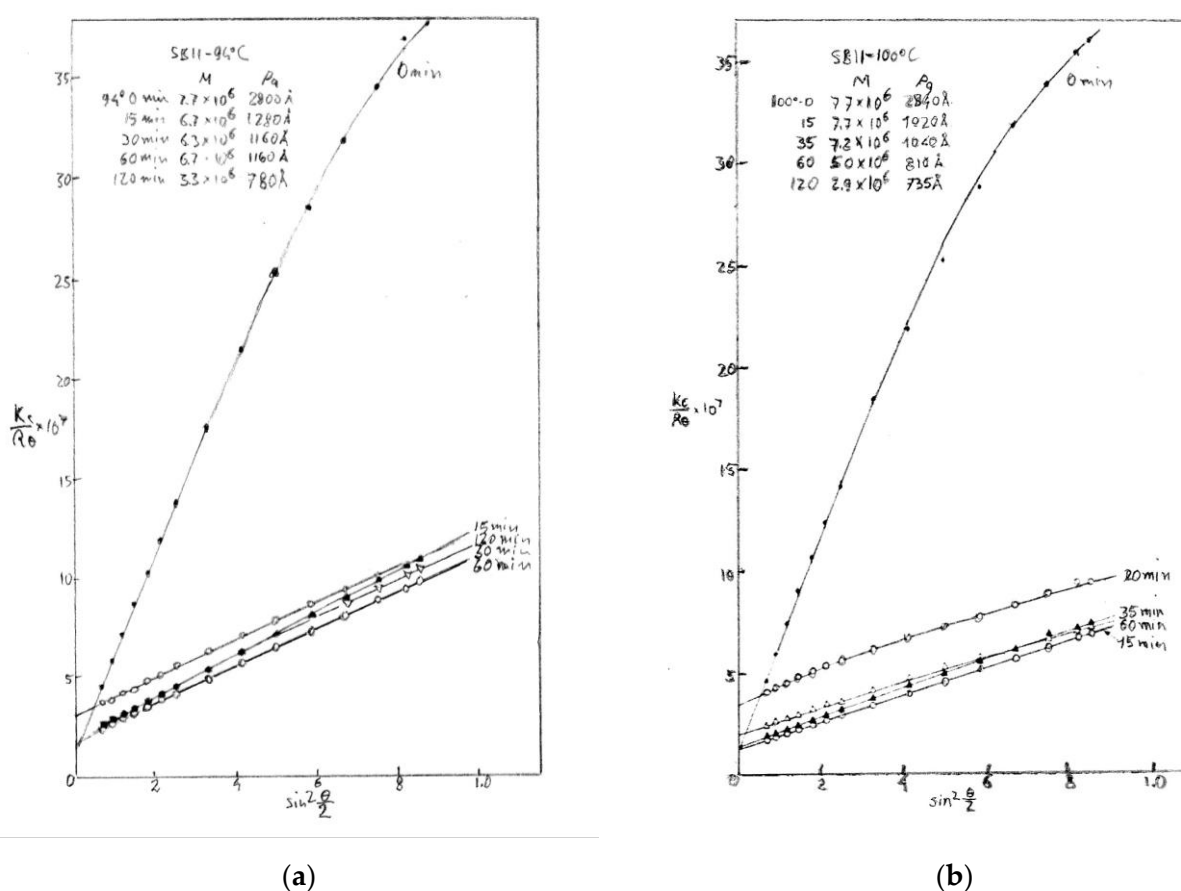


Figure 10. Hand-made reproduction of Figs. 4 and 5 by Rice and Doty (1957) [23]. Angular distribution of scattered light for calf thymus DNA sample SB-11: (a) measurements at 25° after the sample is heated at 94° during 0, 15, 30, 60 and 120 minutes. (b) measurements at 25°C after the sample is heated at 100°C for 0, 15, 35, 60 and 120 minutes.

Concerning the completeness of the dataset of measurements behind Fig.10, it is important to remark the following key circumstance. The Authors report that "the molecular weight can be obtained from measurements at a single concentration" and employ the value of 0.06 mg/cc, so that they do report of not having performed measurements at different solute concentrations and report of not having performed an ordinary extrapolation procedure with respect to concentration to obtain these curves. This choice appears to be non-

compliant with the standard Zimm-plot methodology (devised by Zimm and Debie and described in Section PIPPO) ordinarily applied, for instance, by Alexander and Stacey [64] with the graphical construction of Fig. 6 and ordinarily applied to obtain Figure 9. The non-compliant methodology deployed by Rice and Doty to obtain the plots and the molecular weight determination in Fig. 10 can be promptly criticized based on the following consideration. From Fig. 7 herein, which reproduces the complete Zimm plot extrapolation concerning a previous similar light scattering experiment by the same group of Doty over a similar DNA sample tested at pH 2.6 (Reichmann, Bunce and Doty, 1953) [27] (Fig. 1 therein), it is seen that the employment of one concentration, in place of the ordinary construction of the complete Zimm grids, is prone to considerable error. Actually, owing to the apparent variability of the curves upon concentration (see the subvertical curves therein intercepting the filled dots) by passing, for instance, from 0.005 to 0.05 mg./cc, the use of the single arbitrarily selected value of 0.06 mg/cc, indicated by the Authors, is prone to arbitrarily affect the particular point where the curve at the selected concentration intercepts the vertical axis. This approach is clearly prone to yielding a considerably large experimental error or even room for arbitrariness in selecting the concentration. Stated differently, if the curve reported by the Authors were to be determined at a different concentration – say of 0.005 mg/cc – it cannot be excluded that the intercept on the vertical axis and the corresponding determination for the molecular weight would have been considerably different. The criticality just highlighted deprives this measure of intersubjectivity and points to the necessity of rejecting this measure as a scientific evidence supporting the *100°C stable molecular weight claim*.

It is worth to point out that, after having recalled and remarked in Section 3.1 the absence of scientific probative value of the arguments by Rice and Doty from sedimentation-viscosity measures in support of their *100°C stable molecular weight claim*, the light scattering data reproduced in the plots of Figs 10(a) and 10(b) remained the last possible surviving evidence from those contributed by Rice and Doty (1957) [23] in support of such claim. It is also worth recalling that, as reviewed in Section 2.3.9, all light scattering experiments on DNA heated at elevated temperatures, from all retrievable scientific publications of the decade 1950-1960 on such argument, show consistent and coherent evidence disproving the *100°C stable molecular weight claim*. Also, to the authors' knowledge, there exist no other element of evidence from light scattering published in the literature of the decade 1950-1960 in possible support of the *100°C stable molecular weight claim*.

In the light of the considerations above and of the scientific background elucidated in Section 2.3, the confutation provided in the present subsection of a measurement of stable molecular weight from the light-scattering data of Doty and Rice brings to an end the review of the papers by Doty possibly supporting such claim and brings to zero the number of unconfuted elements of evidence in possible support of the *100°C stable molecular weight claim*.

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4. Discussion

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4.1. A summary of evidence of DNA thermal disruption

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Upon having disproved in Section 3 both claims by Doty and Rice of “*high temperature C heating + fast cooling dissociation*” and “*100°C stable molecular weight*”, we can summarize into the following statement the coherent picture emerging from the scope of the literature on thermal degradation of DNA (published between 1950 and 1960 comprehensively reviewed in Sections 2 and 3). Independent research, conducted by independent researchers using independent experimental methods, consistently and redundantly confirm Goldstein and Stern's first observation, both qualitatively and quantitatively: if an aqueous solution of intact DNA molecules is heated between 81°C and 100°C, even for few minutes, extensive sequence-breaking irreversible longitudinal fragmentation is produced and may be followed by reaggregation upon cooling. This evidence will be briefly referred to as *DNA thermal disruption evidence*.

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As reviewed, the most compelling qualitative and directly perceivable empirical evidence of polidispersity and degradation is detected by Goldstein and Stern by direct visual observation of the native fibrous material turning into a flocculent precipitate when DNA is heated almost to the boiling point and then permitted to cool to room temperature. Similarly, Zamenhof and Chargaff describe that heating at 86° for 90 minutes yields a material which is thixotropic whereas unheated DNA solutions are not and that heated DNA upon cooling does not return to preheating condition and becomes a gel-like material.

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Precise indication of degradation after heating times closer to those provided for by PCR (ranging between 1 and 5 minutes per cycle) is reported in both works by Dekker and Schachman and by Shooter, Pain and Butler: already after 15 minutes heating, irreversible drop of viscosity to a value close to that of the solvent alone occur associated with other evident phenomena such as spread of the sedimentation profiles with appearance of distinguishable species in solution and clear evidence of degradation and reaggregation of fragments.

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These perceptive and qualitative evidence of thermal disruption were quantitatively shown in Section 3.1 of this review to be an undeniable fact even for the most stable preparations obtained by detergent methods, such as SB-11 tested by Doty, Rice, Shooter, Pain and Butler, and even appealing to a possible ancillary influence of coiling contributing to the decrease in viscosity.

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A specific quantitative determination of the number of fragmentations undergone by DNA after one or few minutes heating can be obtained from the relative viscosity vs. time data and experimental conditions (in a barbital buffer) reported by Goldstein and Stern in Fig. 11 which is a handmade reproduction of the original 1950-dated Figure 12.

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Readily employable tools for approximately evaluating the average molecular weight of the fragments (customarily employed by Cox, Overend, Peacocke and Wilson in 1955 as well as by Doty, Marmur, Eigner and Schildkraut in 1960) are the almost linear relation with the intrinsic viscosity $[\eta]$: $M \propto [\eta]^{1.083}$ or the fully linear relation obtained by Peacocke and Preston (1958) [50] and reported in Equation (5).

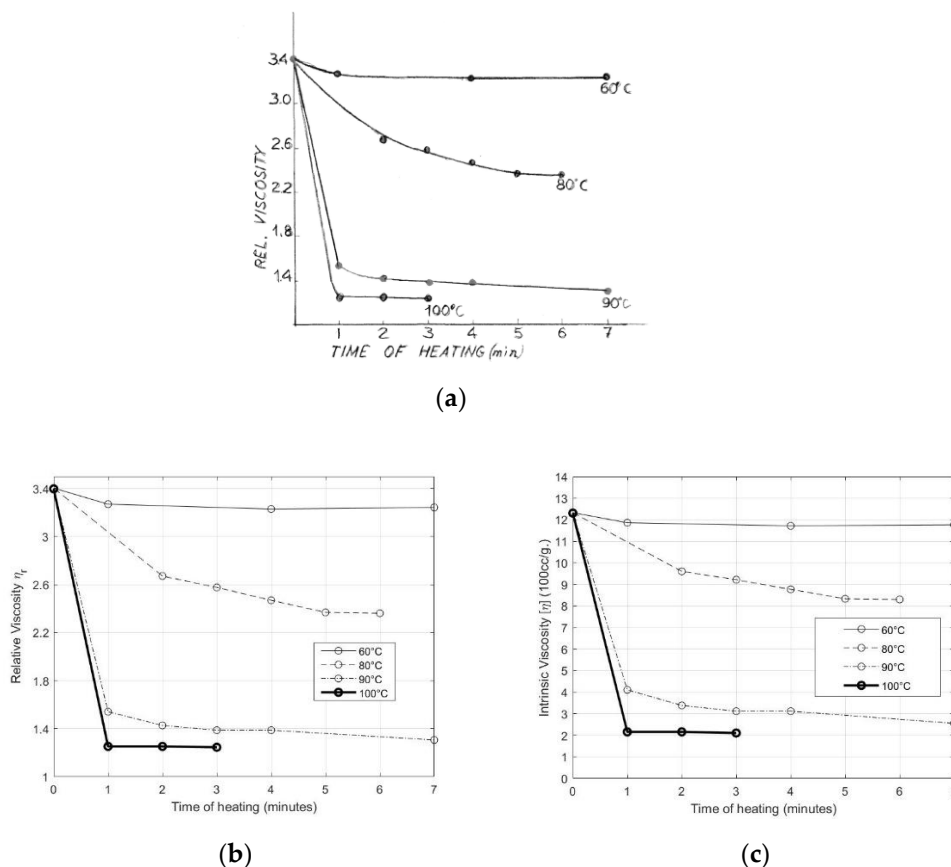


Figure 11. (a) Thermal depolymerization of 0.1% calf thymus DNA. Hand-made reproduction of Figure 12 by Goldstein and Stern (1950), see ref. [7]. (b) Digitization of Figure 12 by Goldstein and Stern (1950), see ref. [7]. (c) Corresponding plot of intrinsic viscosity vs. time of heating computed from Eq.(18).

From data on relative viscosity in Fig 11 (a,b) the intrinsic viscosity can be computed. In particular, Cox et al. [5] find, for a given DNA preparation from experiments at different concentrations, a linear relation between reduced viscosity and concentration (in g./ml.) representable in the following form:

$$\eta_{red}(c) = [\eta] + k' \cdot [\eta]^2 \cdot c , \tag{28}$$

in which the experimentally determined value $k' = 0.77$ consistently matches with the value proper to extended chain molecules obtained by Simha [66].

Relation (28) can be employed to compute the intrinsic viscosity from the relative viscosity. Actually, from (28) and recalling the relations among

reduced, specific and relative viscosity ($\eta_{red} = \eta_{sp}/c$ and $\eta_{sp} = \eta_r - 1$) we obtain:

$$\eta_{sp} = \eta_r - 1 = [\eta] \cdot c + k' \cdot [\eta]^2 \cdot c^2, \quad (29)$$

Terms in Eq. (29) can be ordered in the usual format for second order algebraic equations introducing the relative viscosity:

$$k'c^2 \cdot [\eta]^2 + c \cdot [\eta] - \eta_r + 1 = 0 \quad (30)$$

The intrinsic viscosity $[\eta]$ as related to η_r is thus computed as the positive solution of Eq.(30), viz.:

$$[\eta](\eta_r) = \frac{1}{2k'c^2} \left(-c + \sqrt{c^2 - 4 \cdot k'c^2 \cdot (1 - \eta_r)} \right) \quad (31)$$

The plots intrinsic viscosity vs. time resulting from elaboration of data in Fig. 11(b) via function (31) and corresponding to $c = 0.001$ and $k' = 0.77$ are reported in Figure 11(c).

The intrinsic viscosity of the unheated solution is $[\eta]_N = 12.31$ and the intrinsic viscosity of the solution after 1 minute heating at 100°C is $[\eta]_{100^\circ,1min} = 2.16$. These values can be used to compute from the most accurate viscosity-mass relation (5) of Section 2.3.7, obtained by Peacocke and Preston (1958) [50], the (weight-average) molecular weight drop based on viscosity measurements:

$$\frac{(M_w)_{100^\circ,1min}}{(M_w)_N} = \frac{[\eta]_{100^\circ,1min}}{[\eta]_N} = \frac{2.16}{12.31} = \frac{2.16}{12.31} = 0.17 \cong \frac{1}{6}. \quad (32)$$

The experiments by Goldstein and Stern thus permit to compute that one minute heating at 100°C determines the division of the initial molecule into segments whose average length can be closely rounded to the simple integer fraction of one-sixth and Equation (6) of Section 2.3.8 permits to compute the corresponding average number of sequence-breaking fragmentations

$$F = \frac{(M_w)_N}{(M_w)_{100^\circ,1min}} - 1 = \frac{(M_n)_N}{(M_n)_{100^\circ,1min}} - 1 \cong 5. \quad (33)$$

This number of fragmentations per one minute corresponds to $5 \times 15 = 75$ fragmentations during a heating time of 15 minutes by assuming the number of fragmentations per minute to be a constant (in reality, this assumption yields an optimistic lower bound of fragmentations since the reviewed statistical models by Peacocke and Preston and by Applequist show that the number of full fragmentations is a quadratic function of time). It can be readily controlled that such a number of 75 fragmentations well fits with the determinations obtained for the longer time of 15 minutes heating by Shooter, Pain and Butler and by Dekker and Schachman, as well as by Sadron (all results reviewed in the previous sections). The results of these authors show the

production of fragments whose average weight ranges between 5% and 1% of the molecular weight of molecule before heating (corresponding to 20 and 100 average number of fragmentations).

It is worth recalling that cooling after fragmentation is also a remarkable disordering factor since many works herein reviewed, describe, upon DNA cooling, evidence of fragment reaggregation and of formation of a gel-like dispersion (see in particular the works by Shooter and coworkers) or even the formation of aggregates of molecular weight even higher than the original one.

4.2. What really happens during DNA thermal fragmentation?

Before attempting an answer, in the light of the bulk of the available data herein reviewed on the physical and chemical behavior of DNA subject to heating, to the opening question of this review (i.e. *“To what extent the integrity of the sequential information of nucleotides is affected when the DNA solutions in the buffer conditions provided for by PCR is accordingly subjected to heating and cooling cycles of 95°C or 100°C as described by Mullis and Faloona?”*) it is convenient to gain few closer insights on the longitudinal sequence-breaking mechanism of DNA during thermal fragmentation. These insights will permit, based on simple arithmetical considerations, a computation of lower bounds of the number of fragments produced under PCR heating conditions in a sequence of nucleotides assumed initially intact.

Before attempting any calculation, it is however necessary to understand if the sequence-breaking longitudinal rupture by heating alone involves either phosphodiester covalent bond breakages or the opening of labile hydrogen bonds, or even if both the circumstances have to be contemplated. By paraphrasing the title of a 1968 review by Chargaff titled *“What Really Is DNA?”* [78], the further question can be hence formulated *“What really happens during DNA thermal fragmentation?”*.

Perhaps the simplest and most effective explanation of thermal disruption of DNA in aqueous solution was early well depicted by the words of Kur-nick as the disruptive effect of the *“bombardment by the solvent molecules”*.

A remarkable work which specifically and comprehensively examined this issue early in 1954 is the proceeding of the National Academy of Sciences of the United States of America authored by Dekker and Schachman [42]. These Authors, upon collecting a considerable body of evidence from light-scattering, ultracentrifugation, diffusion, viscosity, streaming birefringence, electron microscopy, titration, and other end-group studies such as dye binding, show sufficient evidence to cast doubt on the adequacy of a model made up of only two contiguous uninterrupted polynucleotide strands. Proceeding from the postulate that *phosphodiester bonds cannot be thermally disrupted below 100°C by heating alone*, Dekker and Schachman show that, if compliance with all data from experimental evidence on DNA degradation by several agents is sought, it is mandatory to consider a modification of the Watson and Crick model of the DNA macromolecular structure to explain its division in shorter

segments and, specifically, *it is necessary to introduce an hypothesis on the presence of some sparse weaker and thermally labile longitudinal bonds, perhaps of hydrogen type, so as to consider that the true length of uninterrupted phosphodiester chains contained in an individual strand does not coincide with the full length of the strand. Specifically, they investigate the presence of: "either secondary forces, like hydrogen bonds, holding smaller molecular entities into some well-organized structure, or easily hydrolyzed covalent bonds, such as deoxy-ribosyl-1-phosphates, spaced at roughly periodic intervals along the main chains".* They show that a model more compatible with all data therein reviewed on DNA degradation is the interrupted two-strand aggregate sketched in Fig.12 (b) corresponding to the Watson & Crick's double helical structure but composed of much smaller molecules held together in a specific configuration by hydrogen bonds between the purine and pyrimidine rings.

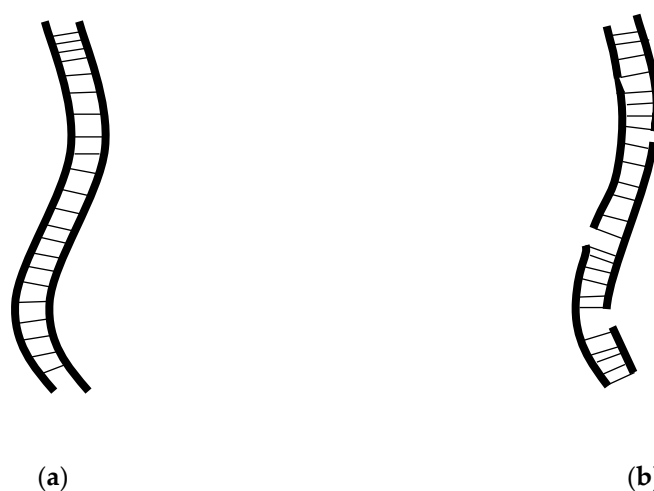


Figure 12. Models for the macromolecular structure of DNA investigated by Dekker and Schachman (1950), see ref. [42]. (a) Watson and Crick's model (helical structure is not represented in the sketch); (b) Dekker and Schachman's model (helical structure is not represented in the sketch).

Proceeding from the consideration that it is highly improbable that a 15 minutes heating at 100°C of DNA solution is capable of breaking phosphodiester bonds, Dekker and Schachman show that all evidence therein reviewed concur to bring to the conclusions that the observed features of thermal degradation can only be the result of the sole rupture of hydrogen bonds and that DNA is an aggregate of fragments of smaller molecular weight. They find corroboration of this model assumption from titration studies which can detect terminal phosphoryl groups giving good evidence for the existence of at least one terminal phosphate group for every 30 to 50 nucleotides [73]. On the same trend, other studies reviewed by Dekker and Schachman, based on rosaniline binding [74], lead to estimating one terminal phosphoryl group for every 300 to 100 phosphorous atoms. Further studies therein reviewed on the

action of magnesium ions on DNA bring to an analogous computation of one terminal group every 20 to 30 phosphorus atoms.

Interestingly enough, a further corroboration of this range of values can be now a-posteriori recognized from the statistical analysis of enzymatic degradation performed by Applequist in 1961 [10], seven years after the publication by Dekker and Schachman. Applequist computes the fraction p_0 of "seemingly already broken" bonds on account of his double-strand random degradation model (reviewed in Section 2.3.8) from the analysis of the kinetics of enzymatic degradation from data later published in 1956 by Schumaker, Richards and Schachman [54] to be $p_0 = 1.57 \times 10^{-2}$. This value corresponds to approximately 1 interrupted P-bond every 64 nucleotides and hence to 1 terminal group every 32 nucleotides thus fitting well the range of data reported above. It is worth noticing, however, that in [10] Dekker and Schachman [42] are not quoted.

If the macromolecular interrupted-chains model by Dekker and Schachman was correct, it would readily explain the observations early reported by Goldstein e Stern of fragmentation after just one-minute heating, albeit such rupture would be allowed not by thermal ruptures in the sugar-phosphate links of the polynucleotide chain, yet by hydrogen bonds or by some other thermally labile and electrophoretically labile bonds

In conclusion, to find a quantitative scientific answer (or at least a conservative estimate) on what is the effect in terms of molecular information integrity of the bombardment of the solvent molecule on the DNA molecule based on all empirical data collected in this study and proceeding from the undeniable evidence that the molecule is considerably segmented at high temperature even after just one minute, two hypotheses and two ensuing models must be comprehensive contemplated:

- (USH) standard Uninterrupted Strands Hypothesis: the molecule is made of two uninterrupted strands and the longitudinal sugar-phosphate bonds (P-bonds) can be ruptured at high temperatures;
- (ISH) Interrupted Strands Hypothesis by Dekker and Schachman: the P-bond cannot be ruptured even at high temperatures so that the segmentation is produced by initially weaker, or completely absent, longitudinal links which are found one every j nucleotides with j ranging between 40 to 600 and with 64 being a *qualified number* compatible with all estimation and range inferences from independent titration analyses, dye binding analyses, magnesium binding analyses and enzymatic degradation analyses.

To appreciate the repercussions of the previously computed probability datum of average 5 fragmentations per 1 min 100°C heating cycles (shortest heating time in PCR repeated a minimum of 10 times) on the integrity of a given sequence of nucleobases, it is convenient to identify a sample test molecule model of given molecular weight. To this end it is convenient to consider the same reference molecule of DNA considered by Dekker and Schachman of weight 5×10^6 which is the best molecular value reported in their study

and obtained from applications of the “mild” method by Signer and Schwander’s for extracting calf thymus DNA. Recalling that the average molecular weight of the four nucleobases is equal to $(G + A + C + T)/4 = (151 + 135 + 111 + 126)/4 \cong 130$ and that deoxyribose and phosphate group weights are, respectively and approximately, 134 and 95, a weight of 720 per pair of nucleotides is obtained which corresponds to approximately 7000 pairs.

In the USH hypothesis a molecule of weight 5×10^6 is thus composed of two strands of 7000 nucleotides each. In the ISH hypothesis, the evidence collected by Dekker and Schachman and the number of nucleotides above estimated on the basis of the number of detected end-groups lead these Authors to estimate between 300 to 500 terminal groups in a molecule of weight 5×10^6 , corresponding to a value ranging from 150 to 250 uninterrupted sub-chains (altogether in both strands) and, approximately, to an equal number of gaps/interruptions ranging from 150 to 250.

Table 5. Test molecule models USH, and ISH with equally spaced sub-chains of 56 and 93 nucleotides.

(USH): two uninterrupted strands		strands number $\widehat{2}$	nucleotides per strand $\widehat{7000}$	nucleotide weight $\frac{[g]}{[mol]}$ $\widehat{360}$	avg. nucleotide weight $\frac{[g]}{[mol]}$ $\widehat{360}$	molecular weight $\frac{[g]}{[mol]}$ $\widehat{5040000}$	total molecular weight $\frac{[g]}{[mol]}$ $\widehat{5040000}$
(ISH): interrupted strands	lower bound for sub-chain length: 56 nucleotides	strands number $\widehat{2}$	number of sub-chains $\widehat{125}$	nucleotides per sub-chain $\widehat{56}$	avg. nucleotide weight $\frac{[g]}{[mol]}$ $\widehat{360}$	nucleotide weight $\frac{[g]}{[mol]}$ $\widehat{360}$	total molecular weight $\frac{[g]}{[mol]}$ $\widehat{5022000}$
	upper bound for sub-chain length: 93 nucleotides	strands number $\widehat{2}$	number of sub-chains $\widehat{75}$	nucleotides per sub-chain $\widehat{93}$	avg. nucleotide weight $\frac{[g]}{[mol]}$ $\widehat{360}$	nucleotide weight $\frac{[g]}{[mol]}$ $\widehat{360}$	total molecular weight $\frac{[g]}{[mol]}$ $\widehat{5040000}$

Since in the ISH model the most experimentally qualified number of nucleotides per sub-chain on the basis on the evidences reviewed herein and by Dekker and Schachman is 64, a most qualified single strand model, taking integer numbers, can be selected as being composed of 110 sub-chains ($110 \times 64 = 7040$ nucleotides). We consider here, however, the example most favorable for the sake of molecular integrity corresponding to the lowest number of 75 ‘thermally unbreakable’ sub-chains made of 93 nucleotides each. This model is denominated *interrupted-strand model ISH-93*.

Evidence shows that a cycle of heating followed by rapid cooling entails molecular fragmentation followed by possible reaggregation of fragments. A quantitative optimistic lower-bound estimate of the increment of the degree of disorder produced by such a *fragmentation + reaggregation cycle* can be obtained by a 1-dimensional sequential model in which the only information maintained is the sequence of nucleotides in the individual strands at the beginning and at the end of the heating/cooling cycle. Accordingly, any other degree of freedom of the molecule fragments (included rotation and translation) is excluded as well as the possibility of the formation, upon reaggregation, of a more topologically complex 3-dimensionally disordered arrangement, typical of colloidal aggregates, not describable by a sequential scheme. In such a simplest model, the datum of a lower bound of 5 complete double scissions with molecular segmentations per one minute heating at 100°C (experimentally confirmed from Goldsten and Stern's data), is taken as the basis for computation. In the abstraction considered, the sequence of nucleotides in an individual strand undergoes a *shuffling* rearrangement process characterized by 5 sequence openings.

For the USH model this shuffling process consists of the following two steps:

- Step 1. A scission step represented as the random division of the initial ordered sequence of 7000 nucleotides into five sub-sequences each preserving the order of the contained nucleotide sub-sequences. This step can be also thought of as the cutting of a deck of 7000 cards into 5 subdecks.
- Step 2. A permutation step in which the sequence of 5 sub-sequences resulting from Step 1 is subjected to a permutation which reassembles a new full sequence in which the order of each nested subsequence is left unchanged. Following the card deck analogy, this step corresponds to a permutation of the 5 subdecks obtained from cutting in Step 1 of the initial deck, and to stacking the permuted subdecks to obtain a new deck of cards.

For the *ISH-93 model*, the shuffling model is the same above, although uninterrupted phosphodiester sub-chains now replace the role of the individual cards of the USH shuffling model. Accordingly, shuffling consequent to 5 fragmentations consists of the following steps:

- Step 1. A scission step represented as the random division of the initial ordered sequence of 75 atomic (not divisible) sub-chains into five sub-sequences of sub-chains each preserving unaltered the order of the sub-chains; this step can be illustratively depicted as the ordered division of a deck of 75 cards into 5 subdecks.
- Step 2. A permutation step in which the sequence of subsequences is re-ordered leaving the order of each nested subsequence unchanged.

The two models above provide an 'optimistic' quantitative estimate of the probability, for *model USH*, that a given sequence (or subsequence) of nucleotides of a certain length can preserve its integrity after a shuffling process or, analogously for the *ISH-93 model*, the probability that a given sequence (or

subsequence) of sub-chains of nucleotides can preserve its integrity after a shuffling process representative of heating degradation.

These shuffling models provide a – very simplistic, yet very optimistic – quantitative answer to the question of what ‘really’ happens, for the sake of the integrity of molecular information, during DNA thermal fragmentation.

Considerations and computations on the basis of the specific number and duration of PCR heating cycles are carried out in Section 4.3.

4.3. Implications of DNA thermal disruption evidence on PCR fidelity

Considerations on PCR buffers

Even if one resorts to appealing to a possibly salvific protective action of the electrolytes in the PCR buffer reported by Mullis and Faloona, the data reported by Hamaguchi and Geiduschek (1962) [56] indicate that conditions provided for in the PCR buffer are prone to thermal instability at the PCR temperatures (of 95°C and 100°C) and that are less stable to thermal disruption than those of most of the DNA solutions employed in the many works herein reviewed. Actually, data reported by Hamaguchi and Geiduschek (1962) show that at any concentration of any of the many electrolytes therein investigated, upper bounds of thermal instability onset temperatures (defined as midpoints of thermal denaturation curves) cannot be, in any case, above 92.6°C so that a protective action of some electrolyte cannot be argued at 95°C and 100°C. To make matters worse, these data also show that thermal stability in sodium acetate (which is the prevalent solute in the PCR buffers) is even lower than in sodium chloride (see Table I therein) as the midpoint temperature of irreversible thermal denaturation curves viscometrically determined by these authors (which turns out to be always an upper bound of the temperature of thermal depolymerization onset) is lowered from 90.0°C to 83.5°C.

Most remarkably, the absence of NaCl in the PCR buffers deprives these solutions of the protective effect of salt against thermal degradation which the reviewed literature has shown to be fundamental.

Considerations on the known degrading effect of depurination by heating are not carried out but could be also taken into account. The addition of dithiothreitol (DTT, the threo isomer of 2,3-dihydroxy-1,4-dithiolbutane), which is a strong reducing agent, provided for in two of PCR buffers (methods I and II) can be considered anything but a stabilizing component considering the capability of thiols of generating mercaptylated derivatives of DNA, as shown for instance by Tamm & Chargaff (1953) [77] employing phenylmethanethiol (benzyl mercaptan). The effect of addition of DTT is also not contemplated in the subsequent analyses.

Optimistic computations of number of DNA fragmentations consequent to PCR heating

PCR heating temperatures are of 95°C and 100°C. The number of heating cycles in PCR multiplied by the duration of each heating process ranges approximately from an *overall* time of exposure to heating of $10 \times 1 = 10$ minutes (in methods I and II prescribing addition of dithiothreitol) to overall heating times higher than 40 minutes (in methods III, IV, V and VI and in absence of DTT) and as high as $27 \times 2 = 54$ minutes in particular in method V.

The most promptly employable information for estimating the entity of fragmentation of PCR short heating cycles, among those herein reviewed, is represented by the 5 sequence-breaking fragmentations computed in Section 4.1 from Goldstein and Stern's data on the viscosity drop consequent to one minute heating at 100°C followed by cooling. We recall from the reviewed literature that the *optimistical significance* of this estimate from viscosity data in representing a reliable *lower bound* to the weight-average number of fragmentations and number-average number of fragmentations experimented by the distribution of DNA molecules during 1 minute heating at 100° is supported, among many others, by the following elements:

- “*the random nature of the heat-denaturation process*”. Cox and Peacocke (1956) [49];
- the knowledge that “*it is only changes in the intrinsic viscosity which are of significance in the estimation of molecular size and shape*” reaffirmed, for instance, on Nature by Cox, Overend, Peacocke and Wilson [5];
- the partial fragments reaggregation on cooling which determines a regain in viscosity (in part of thixotropic nature) so that what is actually determined by measuring a lower bound of the viscosity drop upon cooling of the heated solution is just a lower bound of the number of fragmentations really experimented by the molecule;
- the evidence, shown by Peacocke and Preston (1958) and ny Applequist (1961), that single-strand fragmentation is a first-order rate process and that the number of single-strand sequence-breaking ruptures by heating is linear with time all through the heating degradation.
- the evidence, shown by the same Authors, that the number of single strand sequence-breaking ruptures by heating is much higher than the number of complete fragmentations of the molecule (requiring each a double longitudinal rupture), since it is a quadratic function of time.

Lower bound estimate of the probability of survival of a sequence of nucleotides after repeated heating/cooling 1 minute, 100°C cycles

Based on the two models described in Section 4.2, *USH* and *ISH-93*, and on the conservative assumption that one heating/cooling 1 minute, 100°C produces 5 fragmentations, a highly optimistic computation of the probability of survival (integrity preservation of molecular information) of a selected sequence of nucleotides contained in the main single-strand sequence after repeated cycles is carried out. As discussed above such a computation provides

a lower bound of the degree of disordering and rupture experimented by a nucleotide sequence during such a cycle. We recall that:

- the *USH* model consists of the abstraction of uninterrupted strands of 7000 nucleotides assumed having thermally labile longitudinal bonds;
- the *ISH-93* model is an abstraction in which each strand is made of 75 sub-chains whose internal intranucleotide bonds are not thermally labile.

To perform a computation, the length of the sequence intended for PCR “amplification” must be introduced. Mullis and Faloona write: “*The sequence to be synthesized can be present initially as a discrete molecule or it can be part of a larger molecule.*”

As far as the integrity of the full sequence is concerned, the computation is the same for the *USH* model and for the *ISH-93* model. Five random thermal longitudinal fragmentations occurring in one minute heating correspond to randomly dividing the main sequence into 5 subsequences each preserving its internal order of nucleotides. In the optimistic abstraction herein followed of cooling always being able to restore a sequential reaggregation of fragments, cooling corresponds to randomly reassembling these five ordered subsequences into one out of $(5 + 1)! = 720$ new possible main sequences each obtainable as a random permutation, so that the probability of reobtaining the original full sequence is $1/720$. Already after only 3 cycles this probability becomes very low dropping to $\frac{1}{(6!)^3} = 1/(2.6874 \times 10^{11})$. After the total number of 10 cycles provided for by PCR, corresponding to $n_{Tfrag} = 10 \times 5 = 50$ overall fragmentations, the optimistic estimate of the probability of preserving the integrity of the molecule is 3.1097×10^{-32} .

The integrity of smaller contained sequences after 10 heating cycles is now examined. This issue is important, for instance, when the possible employment of PCR is intended by its users for searching for information possibly relatable to gene expression of a protein. The length of the subsequence is indicated by n_{sub} . A documented representative value of n_{sub} can be set to $n_{sub} = 1000$ adopting, for instance, an optimistic lower bound of the typical lengths retrievable in public databases for the so-called spike protein (US National Center for Biotechnology Information, GenBank: QGV12784.1, $n_{sub} = 1386$ [80]; European Bioinformatics Institute (EMBL-EBI), Universal Protein Resource UniProtKB - P59594 (SPIKE_SARS), $n_{sub} = 1255$ [81]).

For the *USH* model, denoting by $n_T = 7000$ the total number of nucleotides, by $n_{sub} = 1000$ the number of consecutive nucleotides of a subsequence, and by $n_{Tfrag} = 10 \times 5 = 50$ the total number of fragmentations after 10 cycles, the number of combinations by which the main sequence can be fragmented in 50 subsequences is computed as:

$$N_m = \frac{n_T!}{(n_T - n_{Tfrag})! n_{Tfrag}!} = \frac{7000!}{(7000 - 50)! 50!} = \frac{7000 \cdot 6999 \cdot \dots \cdot 6952 \cdot 6951}{50!},$$

where $n!$ indicates the factorial of number n . The number of combinations by which the main sequence can be fragmented in 50 subsequences preserving the integrity of the main sequence is

$$N_{int} = \frac{(n_T - n_{sub})!}{(n_T - n_{sub} - n_{Tfrag})! n_{Tfrag}!} = \frac{(7000 - 1000)!}{(7000 - 1000 - 50)! 50!}$$

$$= \frac{(6000)!}{(5950)! 50!} = \frac{6000 \cdot 5999 \cdot \dots \cdot 5952 \cdot 5951}{50!}.$$

The probability of preserving the integrity of the main sequence is thus computed to be:

$$P_{integr} = \frac{N_{int}}{N_m} = \frac{6000 \cdot 5999 \cdot \dots \cdot 5952 \cdot 5951}{7000 \cdot 6999 \cdot \dots \cdot 6952 \cdot 6951} \cong 0.00044$$

The same computation can be carried out with the *ISH-93* model in the same way by just converting in number of sub-chains elements (we recall, each sub-chain being composed of 93 nucleotides, see Table 5) the lengths of 7000 and 1000 in sub-tokens of 93 while the number of total fragmentations remains $n_{Tfrag} = 10 \times 5 = 50$. Accordingly, one computes:

$$n_T := \frac{n_T}{93} = \frac{7000}{93} \cong 75 \quad \text{and} \quad n_{sub} := \frac{n_{sub}}{93} = \frac{1000}{93} \cong 11$$

The probability of preserving the integrity of the main sequence is thus computed to be for the *ISH-93* model:

$$P_{integr} = \frac{N_{int}}{N_m} = \frac{64 \cdot 63 \cdot \dots \cdot 16 \cdot 15}{75 \cdot 74 \cdot \dots \cdot 27 \cdot 26} \cong 0.00000091$$

so that computations elucidate that the ‘*ISH-93* model’ at hand, although composed of longer sub-chains (hypothesized thermally unbreakable), turns out to be, as a matter of fact, less thermally stable, as far as the integrity of a sequence of 1000 nucleobases is concerned.

The above computations well exemplify and illustrate the remarkable proneness of PCR to entropy-related error determined by the presence of heating steps in such procedure, even more so if it is considered that all simplifying hypothesis introduced to arrive at such computations do have a lower-bound optimistic character, i.e., they all contribute to the benefit of reducing the computed probability of disruption produced by the PCR heating cycles. The considerations above raise a serious concern towards longitudinal sequence-breaking degradation, even more so when the action of the randomizing effect of fragments reaggregation upon cooling is contemplated in addition to the randomization effect produced by thermal degradation at the end of each PCR heating cycle (ranging between 1 and 5 minutes). A critical factor related to this statement is the length of the sequence to be amplified and whether this sequence is larger than the sub-chain elements with uninterrupted phosphodiester bonds identified by Dekker and Schachman. If the sequence to be amplified or to be detected is larger than the average length of the Dekker & Schachman sub-chains (93 in our simplified computations), as it is the case of a sequence of more than 100 nucleobases such as the length

reported by NIH under the denomination spike protein, the probability of preserving integrity is thus computed to be very low.

In this regard, it is worth recalling that the abstraction of the physical phenomena of heating and cooling leading the computational models adopted for the calculations above have speculatively excluded the possibility of translation and rotation of the molecular fragments and the possibility of formation, upon reaggregation, of a more topologically complex-and more disordered nonsequential arrangement, like, for instance, a net.

Perhaps fewer words and fewer computations are necessary to represent to the reader the degree of disorder which can be achieved after just one heating-cooling cycle at 60°C , by showing here in Fig. 13 the electron micrograph taken by Williams (1952) [79] of a sprayed 0.01% water solution of DNA prepared by the method by Schwander and Signer subjected to a process consisting of 1) freezing at -78°C, 2) 60°C heating for drying, 3) rapid cooling.

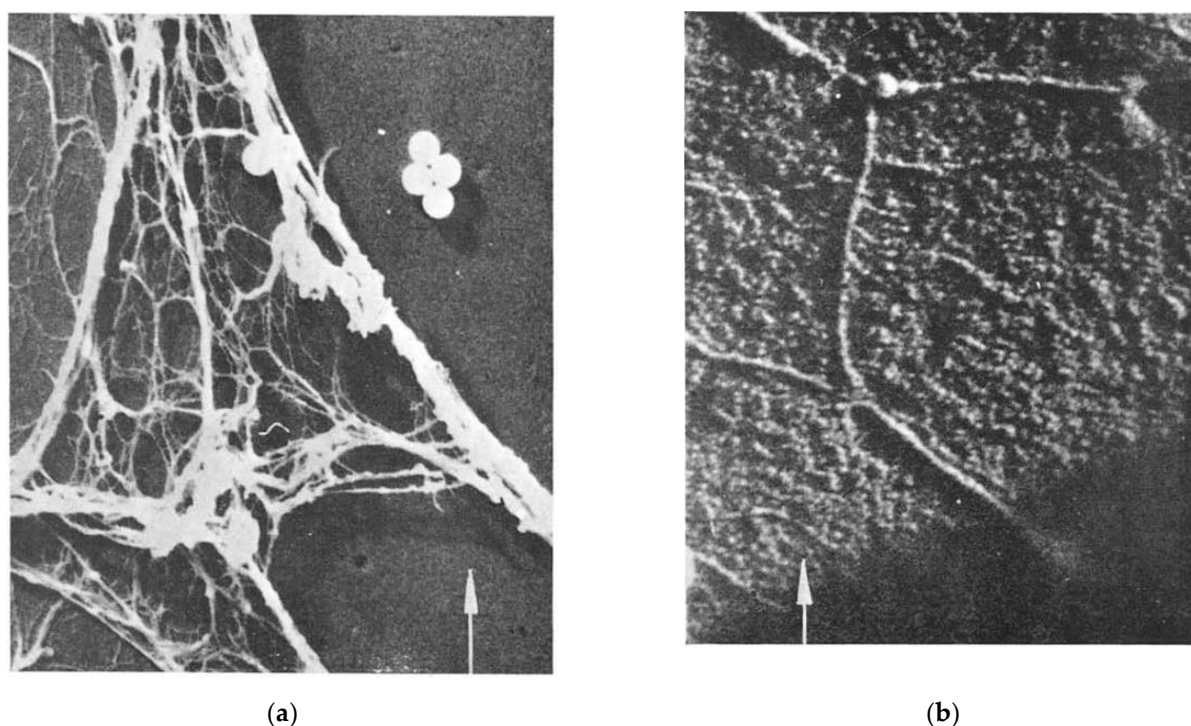


Figure 13. Reproduction of Fig.1 by Williams (1952) [79]. (a) “Low-magnification ($\times 13,500$) electron micrograph of a freeze-dried water solution of DNA. Three-dimensional character of specimen is well shown by the separation of fibers and their shadows in the region marked with an arrow. The horizontal fibers here are about 0.5μ above substrate. Spherical particles are of polystyrene latex”. (b) “High magnification ($\times 100,000$) electron micrograph of DNA. Region indicated with an arrow shows fibril about 15 A in diameter.” Reprinted from ‘*Biochimica et Biophysica Acta*, 9, Williams Robley C., *Electron microscopy of sodium desoxyribonucleate by use of a new freeze-drying method*, Pages 237-239, Copyright (1952), with permission from Elsevier.

It may be argued that Figure 13 may not be representative of the degree of disorder induced by PCR cycles because the DNA freeze-drying process, with 60°C heating, leading to the micrograph of Figure 13 may have induced disordering factors that cannot be comparable with the effect in PCR of

multiple 95°C and 100°C heating steps followed by quick cooling. It is undeniable that, while the milder 60°C heating must have induced a significantly lower degradation, concentration and drying have a significant role in determining the picture of densely agglomerated fibers in Figure 13.

On the other hand, micrographs of the molecules in the material produced after DNA heating at 100°C have been taken by Doty, Marmur, Eigner and Schildkraut (1960) [12]. Figure 3 in [12] shows the electron micrograph with a magnification factor ($\times 95,000$) of DNA molecules from a solution heated in saline-citrate at 100°C for 10 minutes, and then diluted, *slowly cooled* and dialyzed. Such micrograph shows longer molecules having all, with no exceptions, a cross-linked, non-sequential, topologically complex and involuted structure, appearing in large part quite intricately coiled and, as observed by the Authors, with *“irregular patches at the ends of cylindrical threads”*. Figure 4 in [12] shows a micrograph obtained with exactly the same methodology with the only difference that the cooling step was instead performed *quickly*. The description given by the Authors is that the figure *“shows only irregularly coiled molecules with clustered regions”*. As this second micrograph has the same magnification factor of the first Figure 3, relevant to slowly cooled DNA, the lengths of the observable molecular threads in the two figures can be compared. By comparing the two figures, it is evident that the visible aggregates in Figure 4 besides being in some part clustered are also much smaller in length and few individual thread-like fragments, even up to 20 times shorter than the thread-like structures of Figure 3, can be almost clearly discerned.

Summarizing, as elucidated by the elements of empirical evidence and by the conservative quantitative lower-bound predictions collected in the present Section 4.3, it would not be an exaggeration to argue that achieving a faithful amplification, and even before detection, of a long nucleobase sequence after reiterated cooling and uncooling cycles of PCR, may appear to be as much plausible as pretending to faithfully reconstitute at the Ångströms scale a complete long celery stalk of the size of few thousands Ångströms from a soup of randomly aggregated and very similar vegetable fragments, with each fragment being dozens of times smaller in size. Such a duty would be burdensome enough for a nanoscale Maxwell’s demon with tweezers capable of seeing and reconstructing the searched nucleobase sequence, even more so in absence of such a demon and by just reiterating the thermal bombardment by heating/cooling cycles.

5. Conclusions

The present study has examined the fundamental scientific research published across the decade 1950-1960 concerning the effect of DNA heating under the conditions planned prescribed for every PCR cycle. This review has shown consistent and redundant evidence, emerging from all experimental results in the available scientific documents, that the heating step determines the onset of longitudinal sequence-breaking random fragmentation of DNA

molecules. On the basis of the reviewed experimental data and by adopting conservative modelling hypotheses, prediction of lower bounds of the induced degree of disorganization in molecular sequences is possible. The extent of the computed disruption of nucleobase sequences raises the concern towards a high probability that, under the heating conditions provided by PCR, this methodology may amplify chaotically randomized sequences of nucleotides in an unreliable way. Even if these unwanted fragmentations were fewer than those observed at the PCR temperatures, the chain reaction would inevitably amplify the errors in a geometric progression. The scenario that for larger genetic sequences reliability might not be far from the condition of unfeasibility should be considered, as well as the scenario that PCR may tout court amplify informational noise. Concerns towards molecular integrity of sequences of any length may also be raised from the use in PCR of buffers of reducing agents such as DTT and from possible thermal depurination, although these issues have not been herein investigated and are left for possible future work.

In the light of the strong elements of qualitative and quantitative evidence collected in the present study, a preliminary, but very precise, answer may be given to the opening question: "To what extent is Polymerase Chain Reaction fully reliable?" The conclusion is that reliability appears out of reach of all those methodologies, intended for DNA amplification, which are based on heating at high temperatures under conditions equal or similar to those prescribed by PCR. The authors of this study believe that a discussion should be opened on what this unreliability scenario could signify in various fields in which PCR is used, especially in medical diagnostic applications and in forensic sciences.

5. Conclusioni (*in Italian*)

Il presente studio ha sistematicamente esaminato le ricerche scientifiche fondamentali, pubblicate a cavallo della decade 1950-1960, riguardanti gli effetti del riscaldamento del DNA nelle condizioni previste nei cicli della PCR. Questa revisione ha mostrato consistenti e ridondanti evidenze, provenienti dalla totalità dei risultati sperimentali reperibili nei documenti scientifici presi in esame, che tale processo di riscaldamento determina frammentazioni delle molecole di DNA interrompendo in modo non controllabile, per bombardamento termico, la sequenza di nucleotidi. Sulla base dei risultati sperimentali oggetto della revisione e sulla base dell'adozione di ipotesi modellistiche ottimistiche, in sottostima dell'entità del danno prodotto dalla suddetta frammentazione, è stato possibile ricavare minoranti del grado di disorganizzazione molecolare indotto da tale frammentazione. L'entità della distruzione della sequenza di nucleotidi così computata solleva una fondata e considerevole preoccupazione rispetto alla elevata probabilità che, nelle condizioni di riscaldamento previste dalla PCR, questa metodologia possa amplificare sequenze caoticamente randomizzate di nucleotidi in maniera non affidabile e non controllabile. Anche laddove l'entità di queste indesiderate

frammentazioni potesse essere, per ipotesi, minore rispetto a quanto ordina- 2463
riamente osservato alle temperature di riscaldamento caratteristiche della 2464
PCR, il carattere reiterativo della ‘reazione a catena’ amplificherebbe l’errore 2465
secondo una progressione geometrica. Lo scenario in cui, per sequenze gene- 2466
tiche di maggiore lunghezza, il requisito di affidabilità possa essere non lon- 2467
tano da una vera e propria condizione di non conseguibilità dovrebbe essere 2468
preso in considerazione, al pari di uno scenario in cui l’amplificazione della 2469
PCR possa generare semplicemente del tutto *rumore informativo*. Ulteriori 2470
preoccupazioni rispetto all’integrità molecolare di sequenze di qualsiasi lun- 2471
ghezza derivano altresì dall’uso di agenti riducenti come il DTT impiegati in 2472
alcune soluzioni tampone previste dalla PCR, e da possibili effetti di depuri- 2473
nazione per azione termica, sebbene queste tematiche non siano state oggetto 2474
di ricerca specifica in questo studio e sono rimandate a un possibile lavoro 2475
futuro. 2476

Alla luce dei considerevoli elementi di evidenza, di carattere sia qualita- 2477
tivo che quantitativo, raccolti nel presente studio, una risposta preliminare, 2478
ma molto precisa, può essere fornita alla domanda di apertura: “*In quale mi- 2479
sura la Polymerase Chain Reaction è affidabile?*”. La conclusione a cui perviene 2480
questo studio è che la caratteristica di affidabilità appare fuori della portata 2481
di tutte quelle metodologie, intese per l’amplificazione del DNA, che siano 2482
basate su cicli di riscaldamento a elevate temperature uguali o prossime alle 2483
temperature previste dalla PCR. Gli autori di questo studio ritengono che una 2484
seria discussione debba essere aperta nella comunità scientifica sulle implica- 2485
zioni che questo scenario di inaffidabilità può comportare sulla validità, e sul 2486
significato, dei risultati forniti dalla PCR nelle sue applicazioni in ambito me- 2487
dico-diagnostico e nell’ambito delle scienze forensi. 2488

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