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Historical Opinion: Erwin Chargaff and his 'rules' for the base composition of DNA: why did he fail to see the possibility of complementarity?

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Erwin Chargaff was one of the more interesting and colourful figures of the historic decade that heralded the proposal of the double helical structure of DNA by Watson and Crick in 1953. In describing Chargaff's important contribution to the study of DNA, particularly its base composition, this article seeks to suggest why, despite his substantial achievements, he failed to anticipate some of the key features of the Watson–Crick model, particularly complementarity between bases – a failure that left him deeply embittered for the rest of his life.

Work on DNA before the Watson–Crick double helix

In DNA the purines in one chain are hydrogen bonded to pyrimidines in the other chain; more precisely, adenine (A) links to thymine (T) and guanine (G) to cytosine (C). DNA therefore contains equal quantities of purines and pyrimidines and in addition, the amount of adenine equals that of thymine and the amount of guanine equals that of cytosine, but the amount of guanine does not usually equal that of thymine, nor adenine that of cytosine.

These equivalences and non-equivalences were first discovered, unexpectedly, between 1948 and 1951 [1,2] by Chargaff and his colleagues who were seeking to determine whether DNA really did contain equal amounts of the four bases as required by the tetranucleotide hypothesis for the structure of nucleic acids put forward earlier by Levene and Bass [3]. Although the answer to this question was decisively negative, Chargaff did not offer any explanation for the curious relationships that he and his colleagues had found, despite valuable clues already in the literature with which he was familiar. Had they been able to relate these observations to their own work, the history of the period might have been quite different.

The chemistry of the nucleic acids was initially investigated by (among others) Albrecht Kossel in Heidelberg and later by Phoebus Levene in New York, after the identification of DNA by Friedrich Miescher in 1869. For many years it was believed, mainly because of studies of RNA from yeast, that the nucleic acids were relatively small molecules consisting of one copy of each type of the canonical nucleotides, forming a tetranucleotide, although in 1931 Levene and Bass, in a comprehensive monograph summarizing the available evidence, stated that "It must be borne in mind that the true molecular weight of nucleic acid [note use of the singular, not plural] is as yet unknown" [3]. Around this time RNA, containing the pentose sugar ribose, was differentiated from DNA, which was resistant to hydrolysis by alkali and in which the sugar was deoxyribose.

In the late 1930s it became apparent that the DNA molecule was much larger than a tetranucleotide. From birefringence of flow, Signer *et al.* [4] had found evidence for the presence of long chains of nucleotides joined together in a regular repetitive manner giving molecules with a molecular mass of 0.5–1 million Da. In the same year (1938), W.T. Astbury and Florence Bell [5] obtained evidence for a similar M_r using X-ray diffraction, as did Schmidt and Levene using ultracentrifugation [6].

These molecules could in principle still be consistent with the tetranucleotide concept if they were polytetranucleotides (that is, a polymer as opposed to a colloid), which somehow on degradation produced monotetranucleotide units. But, as J. Masson Gulland and colleagues [7] speculated in 1945, the macromolecules might be merely 'statistical' tetranucleotides, that is, structures containing equal amounts of each of the four bases, without them being in any specific repetitive sequence; they would, in fact, be sequence isomers. In this case there could be a large number of different DNA molecules of identical general chemical properties, but different from each other by virtue of the specific sequence of the bases. Although at the time little was known about the role of nucleic acids in cells, this proposal marked a significant advance in the understanding of the possible properties of these molecules. More specific refutation of the tetranucleotide hypothesis was one of Chargaff's principal achievements.

Chargaff's early career

Erwin Chargaff was born in 1905 in Czernowitz, then part of the Austro-Hungarian Empire, now the Ukraine, and trained as a chemist at the University of Vienna where he obtained his PhD in 1928. As described in his unusual autobiography [8] *Heraclitean Fire*[*], because of the dearth

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[*] Heraclitus of Ephesus (540–480 BC) an exponent of the concept of universal flux and of fire as the primary material. In his writings he adopted a contemptuous tone towards other people.

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of opportunities in Europe he went to Yale University where he stayed for two years studying the chemical composition of the avian tubercle bacilli (*Mycobacterium avium*).

Chargaff's arrival in the US was not without incident because the immigration officials in New York could not understand how someone whose passport described him as Dr Phil. would be coming to work on a 'student visa'. They therefore consigned him to Ellis Island, where he was rescued by Treat Johnson, the head of the Yale chemistry department. Chargaff returned from the US to Europe in the summer of 1930. He then spent two and a half years in what he claims to have been the happiest period in his life working with Martin Hahn at the Institute of Hygiene and Bacteriology in the University of Berlin, working again on bacterial lipids, this time from *Bacillus Calmette-Guérin* (*Mycobacterium bovis)* and the phosphatide fraction of diphtheria bacteria.

Reading the signs of the times in Germany and at the invitation of Albert Calmette, deputy director of the Pasteur Institute, Chargaff moved to Paris in 1933, but found working conditions difficult. At the end of 1934 he returned to the US, helped by Harry Sobotka who was in charge of biochemistry at Mount Sinai Hospital. In 1935 he moved to Hans Clarke's Department of Biochemistry at the College of Physicians and Surgeons at Columbia University. Here he remained for the remainder of his working life. He became full professor at the age of 47 and served as Department Chair from 1970–1974, retiring in 1974, embittered at the amount of his pension and at not having been made a single offer to go elsewhere in 50 years! Over the years he travelled widely and received many honours and awards. He died in 2002, aged 96.

Between 1936 and 1948 he published a large number of papers on various aspects of blood coagulation and on the biological use of labelled phosphorus in tracing metabolic

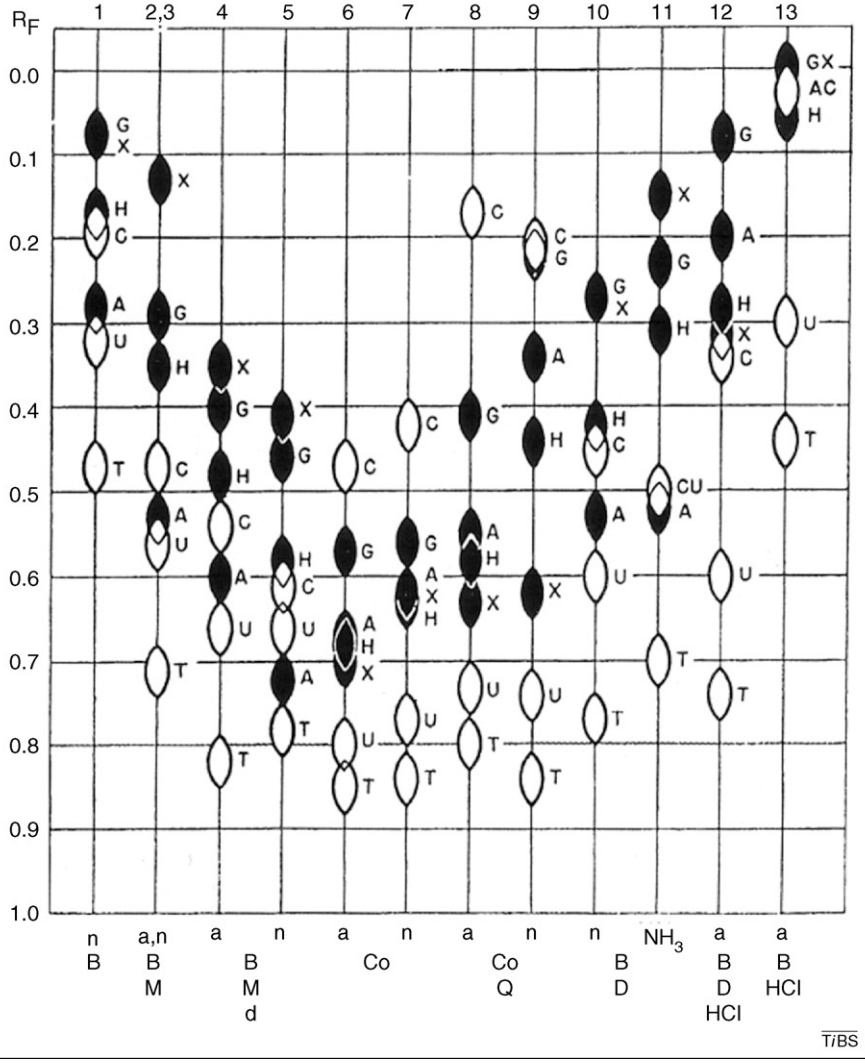

Figure 1. Schematic representation of the position of the purines and pyrimidines on a paper chromatogram, following the separation of a mixture. What is shown here is a composite figure summarizing analyses performed under different running buffer conditions. In each instance the same mixture of bases was applied at the horizontal position corresponding to R_F (distance moved by sample) 0.00 and solvent was allowed to pass from the top of the sheet to the bottom. Under these conditions the different bases move down the sheet at different rates, but it can be seen that thymine (T) always moves faster than adenine (A), which moves faster than cytosine (C), which moves faster than guanine (G). H stands for hypoxanthine, X for xanthine, and U for uracil which is present in RNA in place of thymine; the pyrimidines are represented by open spots and the purines by closed spots. When the run is finished, the position of the bases can be recognized from their ultraviolet absorbance. The bases can be individually eluted and their concentration determined. The letters beneath the figure indicate the conditions and solvents used in the separations: *a*, acidic; *n*, neutral; *B*, *n*-butanol; *M*, morpholine; *D*, diethylene glycol; *Co*, collidine; *Q*, quinoline. Reproduced from Ref. [12].

pathways. However, during World War II because of a contract with the Army Medical Service, he worked on the chemistry of the nucleic acid of *Rickettsia*. In 1944 his attention was drawn to the famous paper of Oswald Avery and colleagues [9], which was to transform our thinking about the role and importance of nucleic acids in cells, and in which the authors concluded that a nucleic acid of the desoxyribose[†] type (that is, DNA) is the fundamental unit of the transforming principle of *Pneumococcus* Type III. Around the same time Chargaff had been reading Erwin Schrödinger's seminal book *What is Life?*, a work that seems to have been inspirational to many physical scientists of the period who were interested in biology, and in which Schrödinger suggested that chromosomes contain some kind of code-script [10].

Hereditary code-script, chromosomes, genes composed of DNA which, if not a rigid tetranucleotide, was able to exist in extremely large numbers of different sequences: the stage was set. If DNA were a crucial part of this assemblage, it was essential that more be learned about its chemistry. As Chargaff later stated, 'Avery gave us the first text of a new language, or rather he showed us where to look for it. I resolved to search for this text' [8].

Analysis of DNA

In 1944 Consden *et al.* [11] showed that it was possible to separate individual amino acids and to determine the amino acid composition of protein hydrolysates by partition chromatography on paper strips. The method was, in principle, readily adapted for the separation and identification of a large number of other substances, including the purines and pyrimidines of the nucleic acids (Figure 1), a task carried out in Chargaff's laboratory by the Swiss post-doctoral fellow Ernst Vischer [12], and independently at the Rockefeller Institute by Rollin Hotchkiss [13].

Chargaff's group published numerous papers on the base composition of DNA isolated from different species and, where appropriate, made comparisons of the base composition of DNA from different tissues of the same species. The data generally showed that the bases were not present in equimolar proportions in the various DNA preparations as required by the tetranucleotide hypothesis [3]. Initial results from animal tissues, which showed similar amounts of the four bases in different tissues from the same species [14], all demonstrated an excess of A and T over G and C (Table 1).

To widen the range of these findings, the authors reasoned that "if as appears probable, certain nucleic acids are endowed with a specific biological activity (Avery *et al.*'s discovery [9]), a search for chemical differences in nucleic acids derived from taxonomically different species should be conducted, and microorganisms would appear to be one of the most promising sources" [15]. This is highlighted in Table 1. *Saccharomyces cerevisiae* and *Haemophilus influenzae* contained an even higher excess of A and T over G and C [15], whereas *Serratia marcescens* and *Bacillus Schatz* revealed considerably lower amounts of A and T than of G and C [16]. *Escherichia coli* contained roughly equal amounts of the four bases [17]. Perversely, had it been the only microorganism studied, it would have offered support for the tetranucleotide hypothesis, which might thereby have gained a new lease on life.

Table 1. Molar proportions of purines and pyrimidines in DNA from different species and organisms[a]

Species	A/G	T/C	Pu/Py
Ox *Bos taurus*	1.29	1.43	1.1
Man *Homo sapiens*	1.56	1.75	1.0
Wheatgerm *Triticum vulgare*	1.22	1.18	1.0
Yeast *Saccharomyces cerevsiae*	1.72	1.9	1.0
Avian tubercle bacillus (*Mycobacterium avium*)	0.4	0.4	1.1
Haemophilus influenzae, Type C	1.74	1.54	1.0
Escherichia coli, K-12	1.05	0.95	1.0
Serratia *Marcescens*	0.7	0.7	0.9
Hydrogen organism *Bacillus Schatz*	0.7	0.6	1.0

[a]Adapted from Tables 4 from [2] and [21]. The third column is the ratio of purines to pyrimidines.

Apart from not demonstrating equal amounts of the four bases and thus casting doubt on the validity of the tetranucleotide hypothesis, certain other unexpected patterns also emerged: the amounts of purines seemed always to equal those of pyrimidines (that is, A + G = C + T, or (A + G)/(C + T) = 1). This had been found by Alfred Mirsky [18] in 1943, but seems to have been overlooked by the Chargaff laboratory. More curiously, the ratios of A:G and T:C were always similar to each other whether they were greater or less than 1 (Table 1).

The significance of these relationships was puzzling and a constant source of comment. At the end of 1949 Chargaff noted that "A comparison of the molar proportions [of the bases] reveals certain striking, but perhaps meaningless, regularities" [15]. Early in 1950, he wrote "It is noteworthy, although possibly no more than accidental, that in all desoxypentose nucleic acids examined thus far the molar ratios of total purines to total pyrimidines were not far from 1. More should not be read into these figures." [19] Later in 1950, apparently as a last-minute insertion in the paper, Chargaff wrote "It is noteworthy – whether this is more than accidental, cannot yet be said – that in all desoxypentose nucleic acids examined thus far the molar ratios of total purines and total pyrimidines, and also of adenine to thymine and of guanine to cytosine [ratios curiously not actually presented], were not far from 1" [2]. The following year, he wrote "As the number of examples of such regularity increases, the question will become pertinent whether it is merely accidental or whether it is an expression of certain structural principles that are shared by many desoxypentose nucleic acids, despite far-reaching differences in their individual composition and the absence of a recognizable periodicity in their nucleotide sequence" [20]. He then added "It is believed that the time has not yet come to attempt an answer" [20], although clearly the subject was very much on his mind.

Chargaff, unlike Astbury [5], seemed to be afraid of becoming immersed in theories based on numerology. However, by the time of the Federation Meetings of 1951, a table in Chargaff's paper [21] containing the results for 11 different species shows inescapably that the ratios of purines to pyrimidines, A to T and G to C are all close to

[†] Note, in the period under discussion some journals preferred the use of the term 'desoxyribose' as opposed to the accepted usage today of 'deoxyribose'.

unity, whereas for only one organism (*E. coli*) can the same be said for A to G and T to C. As he stated 'it is almost impossible to decide at present whether these regularities are entirely fortuitous or whether they reflect the existence in all DNA preparations *of certain common structural principles* (my emphasis) irrespective of far-reaching differences in the individual composition and the absence of an easily recognizable periodicity' [21]. Later, in 1953, he remarked, "Another relationship that again proved remarkably constant was the ratio of amino groups to enolic hydroxyl groups" [22]. However, the ratio here is approximately 1.4 depending on base composition of the DNA, not 1, because G possesses an amino as well as an enolic hydroxyl group, resulting in an excess of the former.

Just down the road from Chargaff at the Rockefeller Institute, Mirsky and associates (who, as already mentioned, had previously found that DNA from plants and animals contained equal quantities of purines and pyrimidines [18]), in 1950 published a detailed analysis of DNA from numerous animal sources, wheat germ and *Pneumococcus* Type III [23], finding similar compositions for each species. As they pointed out, because A was clearly not equal to G, and T to C, the results rendered the tetranucleotide hypothesis untenable, but they did not comment on the now obvious 1:1 ratios of A:T and G:C that were, if anything, clearer in their figures than in many of the Chargaff papers. It was most unfortunate that Mirsky's studies did not include the organisms that Chargaff found to possess such markedly different compositions to mammalian and other species (Table 1) because it would be interesting to know what conclusions would then have been drawn.

What could base equivalencies mean?

Given his significant achievements, how was it that Chargaff did not make the conceptual jump to realize that the equality of amounts of A and T, a purine and a pyrimidine, and G and C, also a purine and a pyrimidine – equalities that seemed to hold in all different organisms despite large variations in ratios of A to G and C to T – had its origin in some fundamental feature of the general structure of DNA? Surely he did, but as Horace Judson suggests, "it was not easy to see how at the time he could have understood the significance of the equivalence rule or taken it any further... it remains that he did not take it further" [24]. Wilkins suggested that it was the negative influence of Levene and Levene's conviction that DNA was too simple a compound to contain genetic information that made Chargaff over-cautious about considering that the bases might be paired [25]. This is possible, but a chemist would have been keen to solve a structure for its own sake. Moreover, because Chargaff regarded Avery's work as the inspiration for his own studies, Wilkins's comment falls away.

In 1947, Gulland, professor of chemistry in Nottingham, carefully extracted DNA from nucleoprotein under conditions in which neither acid nor alkali were used [26]. Electrometric titration of material so prepared suggested the presence of many hydrogen bonds in the native DNA molecule between the amino groups of A and C, and the enolic groups of G and T. Careful titration with acid or alkali disrupted these bonds and the pH of the most rapid uptake or loss of protons coincided with a dramatic decrease in the viscosity of the solution [27], a phenomenon suggesting that the hydrogen bonding lay between bases in different chains. Had Chargaff taken more cognizance of the little-appreciated but highly relevant findings of Gulland *et al.* in 1947 [26,27] regarding the probable existence of hydrogen bonding between bases in DNA in solution, he might have had a major intuitive realization of the origin of, and reason for, his observed base ratios.

Gulland's work was well known to Chargaff. They had both attended the Cold Spring Harbor conference in 1947 where Gulland made an extensive presentation describing inter-chain hydrogen bonding [28]. It is very strange that Chargaff never sought to relate his own findings concerning the quantitative relationships of base composition to those of the hydrogen bonding between chains. If Gulland's work was to be believed, the hydrogen bonds existed between the bases, thus suggesting bonding between A and T and between G and C, a situation that would also be consistent with a purine always bonding to a pyrimidine. If such bonding were between bases in different chains it would suggest a degree of complementarity in base sequence – a finding with exciting implications. He might indeed have been the first to write that "it has not escaped our notice that the specific pairing we have postulated (Chargaff might have used the word 'observed') immediately suggests a possible copying mechanism for the genetic material" [29].

In 1948 Chargaff wrote a chapter on nucleic acids in the *Annual Reviews of Biochemistry* [30], surveying the new information published between 1945 and 1947. Although Chargaff commences "with a tribute of the memory of J.M. Gulland – his death means a sad and irreparable loss to us all" [30] (Gulland tragically died in a railway accident in 1947), his seminal work published in 1947 of the existence of hydrogen bonds between bases in DNA in solution received only cursory mention. It is as though Chargaff had a specific mental block towards this work and its implications, which were so relevant to his own findings. Unlike Watson a few years later [31], Chargaff did not seem to have experienced any revelatory insight from the reading or re-reading of Gulland's papers.

Chains and base-pairing

Before Watson and Crick's paper in 1953 [29] there was no definitive evidence for a specific number of chains in a DNA molecule. If one considers a single polynucleotide strand it is difficult to think what significance equal quantities of A and T, on the one hand, and G and C on the other, could have. With hindsight it is so easy to see the connection between base pairing and a double helix, and how complementarity arises, that we now have difficulty in recognizing the initial feat of realizing this. As Linus Pauling commented regarding the many years during which he pondered the folding of polypeptide chains, 'even rather simple ideas are sometimes very elusive' [32]. Indeed Pauling and Corey perceived nothing in Chargaff's data (or in the results of Gulland *et al.* on hydrogen bonding, for that matter) relevant to their impossible proposed structure for the DNA molecule [33] in which they had led

themselves astray by implicitly assuming that DNA would possess similar structural features to their protein α helix. Curiously for a chemist with biochemical interests, the only biochemistry cited by Pauling and Corey was Alexander Todd's elucidation of the phosphodiester linkage of nucleotides. Until 1953 the only published X-ray diffraction work on DNA was that of Astbury [34], and this provided no indication of the number of chains.

In 1952 Chargaff visited Europe, including Cambridge, where John Kendrew arranged a meeting for him with Watson and Crick. According to Robert Olby's account [35], when Chargaff described the 1:1 ratios between A and T and G and C, the effect on Crick was 'electric'. Crick suddenly realized that if there is complementary pairing there was sure to be a 1:1 ratio. At this point Crick had forgotten the names of the bases, which did not impress Chargaff, who arrogantly considered that he was wasting his time talking to a couple of 'pitchmen'[‡]. In fact at the time Crick was thinking less about the structure of DNA than about mechanisms of DNA replication. He had recently been told by the young mathematician John Griffith that if the bases are flat and they stack on top of each other, under these conditions there is an (oddly) preferential attraction between A and T and between G and C. But at the moment of meeting Chargaff, Crick apparently had forgotten the details of what Griffith had told him. Presumably, had Crick remembered what Griffith had found and had related it to Chargaff, the interest would have been mutual. But in point of fact the concept of preferential associations in base stacking parallel to the fibre axis was unlikely to have led either Chargaff or Watson and Crick to any useful insights.

Watson's realization of the possibility of specific hydrogen-bonded purine–pyrimidine base pairing explained the Chargaff rules rather than vice-versa because Chargaff failed to relate Gulland's findings of the existence of hydrogen bonding between bases with the base ratios he was observing. This seems to be the crux of the matter.

Chargaff's achievements

In *Heraclitean Fire* [8] Chargaff summarized his findings. 'The regularities of the composition of DNAs – some friendly people later called them the 'Chargaff rules' – are as follows: (a) the sum of the purines (adenine and guanine) equals that of the pyrimidines (cytosine and thymine); (b) the molar ratio of adenine to thymine equals 1; (c) the molar ratio of guanine to cytosine equals 1. And, as a direct consequence of these relationships, (d) the number of 6-amino groups (adenine and cytosine) is the same as that of 6-keto groups (guanine and thymine)' [8]. Of course (a) is a direct consequence of (b) and (c). Curiously, in the tables of Chargaff's papers the only ratios usually presented are A:G, T:C and A:C, rarely the crucial A:T and G:C. In the preface to his book *Essays on Nucleic Acids* [36], Chargaff states, "It will surprise many readers... to learn that the first announcement of base-pairing was made in 1950", but where it was made is not indicated. Moreover, there is a major distinction between base pairing of unknown significance and a structural interaction between specific bases such as Watson and Crick proposed.

Heraclitian Fire, written when Chargaff was in his 70s, is enjoyable reading in the earlier chapters describing his youth and early life in Austria, then at Yale, Berlin and Paris, but it later degenerates into a monotonous polemic against the modern world, particularly the scientific realm, made famous by the nonsensical phrase that molecular biology is "practicing biochemistry without a licence" [8]. Chargaff also reflected on his style of science – "In some ways I was the wrong man to make these discoveries: imaginative rather than analytical; apocalyptic rather than dogmatic; brought up to despise publicity; uncomfortable in scientific gatherings; fleeing all contacts; always happier with my youngers than with my betters; more afraid of an absurd world than trying to understand it; but ever conscious, day and night, that there is more to see than I can see, more to say than I can say, and even more to be silent about" [8]. Does all this merely attempt to mask the bitterness he felt that he was never able to make a conceptual leap to the significance of the various base ratios he had observed?

Conclusion

Chargaff's failure to 'see' base pairing overshadows his contributions to nucleic acid chemistry. He was the first to develop micro-methods for the accurate analysis of purines and pyrimidines and hence the base composition of nucleic acids. He recognized that DNA in its base composition is characteristic of the species from which it is derived and that different tissues of the same species yield the same DNA. He also showed that lack of equality of the four bases in most samples of DNA invalidated the tetranucleotide hypothesis and opened the way to the realization that different DNA molecules could have specific base sequences, a possibility already anticipated by Gulland and colleagues with their statistical tetranucleotide concept [7].

Could it be that, as was said of Avery, 'constant modesty and deep humility..(and) high regard for the printed word deterred him from theorizing in print' [37], or might it have been an arrogance born of the belief that nobody else could possibly know more on the subject than he? Unfortunately and sadly we shall never know, but somehow Chargaff succeeded in excluding himself from one of the great scientific breakthroughs of the 20th century.

[‡] 'Pitchmen' – a double entendre relating to salesmen (who pitch) and a preoccupation of Watson and Crick in their discussion with Chargaff of the pitch of a helix.

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