The “scientific catastrophe” in nucleic acids research that boosted molecular biology

DOI 10.1074/jbc.CL119.007397

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The distinctive profile of the double-helix DNA molecule is today, along with Rutherford’s depiction of the atom as a miniature planetary system, a worldwide-recognized symbol of twentieth-century science. The complex story of how DNA’s tertiary structure was determined is also well-known. Surprisingly, however, far less is commonly known about how the structural subunits of the nucleic acids—i.e. nucleotides, nucleosides, and the specific carbohydrates that distinguish DNA and RNA—were first identified and their connectivity ascertained. This comparative oblivion seems due, at least in part, to the conceptual association of those key findings with an erroneous model of nucleic acid structure, which postulated that these macromolecules would consist of repeating sets of four nucleotides. This model came to be known as the “tetranucleotide hypothesis” and prevailed as the dominant paradigm through almost 4 decades of arduous research in the field. When debunked—with researchers referring by then to this hypothesis as an “effort to force nature into a straitjacket of puerile approximations” (1), a “scientific catastrophe” (2), and an “absurd” instance of oversimplification (3)—the whole idea receded into the sidelines of the literature along with its insightful analyses of how the nucleic acids are built in the first place.

Early historians have provided more nuanced records (4–6), but later accounts still ignore or greatly deplore the tetranucleotide hypothesis (7, 8) or mention it just as needed without due references (9, 10), and even when giving favorable views they come to the usual conclusion that it became a distraction or an obstacle to further advance in the field (11, 12). Nevertheless, as shown below, the data contained in the tetranucleotide hypothesis—leading up to the first accurate description of DNA polymeric structure (13), here recognized as a Classic paper—immediately paved the way toward the basic framework of molecular biology as we know it today.

Precedents

The apparent first observation of a substance later identified as nucleic acid was made by Justus Liebig, who in 1847 reported the presence of an acidic material in a filtrate obtained from beef muscle, which he therefore named “inosinic acid” (from the Greek word inos, fiber, and more specifically those of muscle) (Ref. 14, p. 187). Over 20 years later, Friedrich Miescher discovered in nuclei of leukocytes a phosphorus-rich substance remarkably resistant to protein digestion, which he labeled as “nuclein” (15). Finally, in 1889, Richard Altmann succeeded in removing, by pepsin treatment and alkaline hydrolysis, most or all of the protein associated with nucleins obtained from either yeasts or some animal organs, hence referring for the first time to the remaining acidic material as “nucleic acid” (Nucleinsäure) (16). This technical achievement opened the way to investigate nucleic acids apart from their accompanying proteins.

Over the following years, Albrecht Kossel, a future Nobel Prize laureate in medicine for his contributions to cell chemistry “including the nucleic substances” (17), applied Altmann’s method plus other procedures devised by his own research team to determine the main chemical components of the nucleic acids (18). Kossel’s laboratory found that hydrolysis of nucleic acids obtained from various sources produced, along with large quantities of phosphoric acid as Miescher had described, specific sets of other molecules depending on the original material used as the source. Such compounds included several purine and pyrimidine bases—guanine, adenine, thymine, uracil, and cytosine—and some kind of carbohydrate suspected to be a pentose (19).

By the turn of the century, a variety of nucleic acids with different elemental compositions had been reported, depending on the primary material used, the particular methods applied, and the research groups involved (20). Eventually, two main kinds of nucleic acids were distinguished, each kind labeled after the example preferred as an experimental system by the biochemists of the period and therefore better characterized: the yeast nucleic acid, believed to be typical of plants (also called “phytonucleic”), and that obtained from the thymus gland (“thymonucleic”, or occasionally “zoonucleic”), which was seen as peculiar to animals. The main differences detected between these two sorts of nucleic acids—actually RNA and DNA—were in composition regarding one of the pyrimidine bases (thymine in thymonucleic versus uracil in yeast) and the sugar in each case (a pentose in yeast versus what was by then supposed to be a hexose in animal tissues). Despite these distinctions, however, “it was found that in nearly all the acids the bases were present in approximately equimolecular proportions, that the number of molecules of phosphoric acid corresponded to that of the bases, and the number of molecules of carbohydrate was equal to that of phosphoric acid” (20).

There was no hint at the time as to how these various constituents of a nucleic acid would join with one another. Still, Kossel included the nucleic acids, together with proteins and huge carbohydrate molecules like starch and cellulose, in a group of cell substances that would likely be composed of specific building stones (Bausteine), explaining “that these units

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Furthermore, they suggested that those tripartite modules
had identified the base “thymine.” They used the new gradual
cosidic form” (adenine and guanine, right) coupled to a carbohydrate (center) constitutes a “nucleoside.” The carbohydrate of each nucleoside is also linked in turn to a phosphate group (left), thus making up a “nucleotide.” The two nucleotides, linked through their respective phosphate groups, form a dinucleotide. Although the linkage of the nucleotides is wrong in this still merely conjectural formula, the structure shows the first approximation to a correct representation of how the primary components of a nucleic acid are assembled, thus making up a “nucleotide.”

Figure 1. Structure of a dinucleotide in thymus nucleic acid. Single figure in Ref. 27 (reproduced here with permission). This research was originally published in Berichte der Deutschen Chemischen Gesellschaft. Levene, P. A., and Mandel, J. A. Über die Konstitution der Thymo-nucleinsäure. Ber. Deut. Chem. Ges. 1908; 41:1905–1909. © John Wiley and Sons. Each of two bases (adenine and guanine, right) coupled to a carbohydrate (center) constitutes a “nucleoside.” The carbohydrate of each nucleoside is also linked in turn to a phosphate group (left), thus making up a “nucleotide.”

may be united to form larger structures and that their union takes place according to a determined plan or architectural idea” (21). He was thinking, obviously, of the notion known today as “monomers,” and apparently unaware that in the case of nucleic acids such building blocks had been tentatively identified and named just 3 years earlier by a one-time visitor to his own laboratory (see below).

Nucleosides, nucleotides, and polynucleotides

Phoebus Levene, a Russian physician who at only 22 emigrated to the United States, got his start in science in New York and as a visitor in Germany at the laboratories of both Kossel and Emil Fischer (22, 23). In 1905, he was appointed a member of the recently created Rockefeller Institute for Medical Research, and then head there of a department that reportedly became “the center of bioorganic chemistry in America” (24).

Levene’s interest in nucleic acids was evident right away. First, he devised a novel method for their preparation (25) and continued from here with a number of findings on the subject, including a new more elaborate and sequential rather than simultaneous hydrolysis technique (26). This procedure involved milder pH levels and avoided the use of alcohol for precipitating the liberated parts, as commonly done by his competitors, thus obtaining intermediate products of hydrolysis with enough purity for a consistent and therefore more reliable quantification of their relative amounts.

In 1908, Levene communicated results of work carried out in collaboration with his student J. A. Mandel on “thymo-nucleic acid” (27)—i.e. DNA extracted from the thymus, where Kossel had identified the base “thymine.” They used the new gradual decomposition method described in the former paper, leading them to the preliminary conclusion that linear complexes “consisting each one of a phosphoric acid, a carbohydrate, and a base . . . bind in such a way that they form a polyphosphoric acid. The base is probably bound to the sugar moiety in glycosidic form” (Fig. 1; see Ref. 14, pp. 266–275, for alternative structures of nucleic acids in the contemporary literature). Furthermore, they suggested that those tripartite modules might build also nucleic acids of a higher order, and coined the German words Mononucleotiden and Polynucleotiden to distinguish two levels of integration, although it was not clear to them at the time just how the latter would be integrated.

The following year, Levene and Walter A. Jacobs—an assistant and later associate researcher at the Rockefeller Institute—found this primary nucleotide constitution again in the yeast nucleic acid (i.e. RNA) (28). Here, they also announced to have succeeded in purifying the freed glycosidic components of yeast guanylic acid down to their crystalline form and proposed for these subunits “the general term ‘nucleosides’.” Moreover, this same work provided evidence that the pentose contained in yeast nucleic acid, so far believed to be l-xylose, was in fact “d-ribose” (i.e. a sugar synthesized almost 20 years earlier in Emil Fischer’s laboratory (29) but scarcely characterized thereafter).

Perhaps overconfident after these brilliant successes, in a subsequent paper that year Levene and Jacobs tentatively argued, based upon various considerations, that “Since the pentoses of guanylic acid and of yeast nucleic acid are identical with [that of] inosinic acid, the nature of [the] pentose in this nucleic acid may now be regarded also as d-ribose” (30). This hasty ruling, which simply ignored a critical hydroxyl group in the sugar of inosinic acid (i.e. DNA), was seemingly confirmed a few weeks later as they claimed that in yeast nucleic acid, “the pentose proved to be identical to that of inosine or guanosine, namely to d-ribose,” according to results obtained by hydrolysis with picrate (31).

Correcting this major error took Levene and his associates many more years of hard work (see below). The assumption that d-ribose would be the common sugar in all nucleic acids soon became problematic, as the chemical properties of the sugars in each of the two acids proved to be markedly different. Specifically, whereas yeast nucleic acid (RNA) would readily degrade when heated with ammonia, thymus nucleic acid (DNA) “remained apparently unchanged” under these alkaline conditions (32). At higher temperatures or neutral pH, free purines and pyrimidines were released and no intact nucleosides were obtained, so the authors attributed their results to some instability of the sugar residue. Then World War I intruded, heavily affecting scientific activity and communication.

Meanwhile, the identity of the sugar moiety in thymus nucleic acid remained a nagging central doubt. All attempts to obtain this carbohydrate following hydrolysis under various conditions had so far been unsuccessful. In contrast to the yeast nucleic acid, which regularly produced d-ribose, the thymus nucleic acid hydrolysates contained only levulinic acid, a common product of cellulose degradation. Hence, given the above mentioned general consensus that concerning nucleic acids the yeast was representative of plants whereas the thymus stood for animals, the view that the sugar in thymus nucleic acid was likely a hexose became prevalent, as stated in an influential treatise published in 1914 by Walter Jones, a former Kossel student: “Plant nucleic acids contain a pentose group, and all animal nucleic acids a hexose group in their molecule” (33). Clearing up this issue had to wait for a novel experimental approach made possible by a unique chain of incidents.
**A new sugar for nucleosides**

As it happened, in 1923 Levene was urgently contacted by the Russian neuroscientist and Nobel Prize laureate Ivan Pavlov, who had once been his physiology teacher while studying medicine at St. Petersburg. The now elderly professor was at the moment in New York on his way to Paris, and in quite a difficult situation after being robbed of both his money and passport at Grand Central Station. Levene promptly managed to obtain financial help from the Rockefeller Institute and also assisted Pavlov in getting a new visa. This unexpected encounter after so many years allowed both scientists to talk at length about their respective research. Levene became highly interested in the technical details about Pavlov’s world-famous demonstration of a conditioned reflex, by which gastric juice was secreted in a dog’s stomach at the sound of a bell or other specific sensory stimulus previously associated with food offer. The fresh secretion was collected for analysis through an implanted fistula, straight from the animal’s stomach.

Levene surely recalled an experiment he had carried out with Jacobs years earlier, using “a dog with an intestinal fistula which permitted feeding the dog on nucleic acid and collecting the nucleic acid impregnated with enzyme through the fistula” (see the experimental section of Ref. 34). They were then attempting once again to obtain free nucleosides from thymus nucleic acid, but the test had produced only an impure substance unsuitable for definitive chemical identification. Now, however, Pavlov’s experimental setup suggested to Levene that he could try to digest the nucleic acid with fresh gastric juice in a test tube. The following year, Levene visited Pavlov’s laboratory in Russia to perform this study, but these attempts were again unsuccessful, “undoubtedly for the reason that the juice was very poor in enzymes” according to Levene. Nevertheless, it was agreed that an expert member of Pavlov’s research team would go to the Rockefeller Institute to carry out additional trials.

That visit was delayed until 1928, but this time the results were quite interesting. In order to work with an empty and clean gastro-intestinal segment, Russian professor Efim S. Lon-don prepared dogs with two fistulas, one for passing nucleic acids into the stomach and the other for collecting the products of digestion from the upper intestine, after the process had occurred within the animal in a fairly natural way. Early the next year, they published a two-page note announcing the isolation and subsequent hydrolysis of a guaninedesoxypentoside [sic] from thymus nucleic acid (35), and shortly after they reported, at a meeting of the National Academy of Sciences, the successful isolation of nucleosides from “thymonucleic acid” (i.e. DNA) as well as their sugar component which, “contrary to expectation, is not a hexose but a deoxypentose” (36). By the time this meeting was held, Levene and London had been able to show the sugar moiety to be also a deoxypentose in three other nucleosides of this same acid (see the experimental section of Ref. 34).

The finding by Levene’s team of a possible third sugar ring (37) presented one additional puzzle, but further inspection revealed some errors in this study. Therefore, soon they were able to declare: “Hence, the carbohydrate of thymonucleic acid is d-2-ribodesose” (38) (i.e. 2-deoxy-D-ribose). And then they used this assignment to anchor their—and our—thinking about the fundamental nature of nucleic acid polymers, saying (Ref. 14, pp. 261–262):

Thus, comparing the components of each nucleic acid, it is seen that they differ from each other in the structure of one pyrimidine base and in that of the carbohydrates. The striking differences in the chemical and also in the physical properties of the acids are determined principally by the carbohydrate which enters into their structures. […] It is therefore logical to classify nucleic acids according to their component carbohydrate into ribonucleic acids and ribodesose [i.e. deoxyribose] nucleic acids. In each group the nucleic acid can then be classified by the number of nucleotides contained in it into mononucleotides and polynucleotides. Among the latter, tetra-, penta-, and hexanucleotides have been described. It must be stated, however, that whereas the existence of tetrnucleotides is established beyond doubt, that of the higher order is a question in need of further investigation.

Next, Levene and co-workers concentrated on the detailed characterization of the junctions of the phosphate groups with their respective bases in the two nucleic acids. In a paper co-authored with R. Stuart Tipson a few years later, they argued as “evident that in desoxy-ribose nucleic acid the positions of the phosphoric acid radicles are carbon atoms (3) and (5) of the desoxy-ribose,” whereas in the “ribose nucleic acid . . . the phosphoryl residues are attached at positions (2) and (3)” (13) (Figs. 2A and 3A here). And upon these distinctions, they explained the contrasting responses of the two nucleic acids to alkaline and acidic conditions.

It took almost 20 more years to realize, through extended digestion with ribonucleases and other novel procedures, that in fact the internucleotide phosphodiester linkages in RNA are attached at carbons 3' and 5' of the pentose, just as in DNA (39, 40). Other than this, however, Figures I and II in Levene and Tipson’s paper of 1935 (13) showed for the first time the correct—for DNA—and nearly correct—for RNA—chemical structures of the two nucleic acids. And this triumph of modern science opened the map toward a whole new age in biology.

**From tetrnucleotides to polymeric macromolecules**

As described above, the idea that nucleic acids could be constituted by nucleotides linked up in a series was first conceived by Levene and Mandel in 1908 (27), and it was in that work that they also mentioned the possibility that a tetrnucleotide or perhaps a pentanucleotide might be involved. This supposition ultimately led to the notion that nucleic acids would consist of several repeats of a set of four nucleotides, with each of the latter corresponding to one of the respective bases.

The actual origin of such a view, which became known in the literature as the “tetrnucleotide hypothesis,” can be traced back to Kossel and Neumann’s 1893 speculation that “It is highly probable that there are four nucleic acids, each of which contains only one of the nucleobases” (41). This last statement clearly referred to only one base being present in a particular nucleic acid, understood then as a small molecule, whatever the
In the meantime, however, evidence had accumulated showing that most of the nucleic acids so far examined from many sources, including wheat, yeast, calf thymus, and fish sperm (42–44), contained roughly equal ratios of the four bases corresponding to their particular type, either yeast-like or thymus-like. Hence, there was wide experimental support to sus-

Figure 2. Structure of deoxyribose nucleic acid. A. Fig. I in Ref. 13 (reproduced here with permission). This research was originally published in the Journal of Biological Chemistry. Levene, P. A., and Tipson, R. S. The ring structure of thymidine. J. Biol. Chem. 1935; 109:623–630. © the American Society for Biochemistry and Molecular Biology. Consecutive deoxyribose rings, each coupled to one of four specific bases (left), are linked in series by phosphate groups (right), thus constituting a tetranucleotide. The phosphate groups join carbons 5' and 3' of the successive deoxyriboses, though condensed formulae of the bases do not show the position at which they link to carbon 1 of the corresponding deoxyribose. Dotted lines are meant to indicate the limits of each nucleotide, but the unexplained angled second dotted line leaves the adenine part reduced to a simple nucleoside while the cytosine one becomes a diphosphate. Several copies of this basic chain were assumed to be similarly connected by phosphodiester bonds at their ends to make up longer sequences. This figure is the first correct depiction of the actual DNA structure. B, current model. Comparative graphic drawn according to the general design and labeling of Fig. I in Levene and Tipson (13). The only differences between both interpretations are the elemental compositions of the nucleobases, which are here updated. Dotted lines indicate the actual limits between adjacent nucleotides.

Figure 3. Structure of RNA. A. Fig. II in Ref. 13 (reproduced here with permission). This research was originally published in the Journal of Biological Chemistry. Levene, P. A., and Tipson, R. S. The ring structure of thymidine. J. Biol. Chem. 1935; 109:623–630. © the American Society for Biochemistry and Molecular Biology. Four ribose rings, each coupled to one of the four specific bases, are linked in series through phosphate groups, thus constituting a tetranucleotide. The phosphate groups join carbons 3' and (incorrectly) 2' of the successive riboses, but condensed formulae of the bases do not show the position of their linkage to carbon 1 of the corresponding ribose. Dotted lines indicate the limits of each nucleotide. This figure is the first approximately correct depiction of the RNA structure. B, current model. Comparative graphic drawn according to the general design and labeling of Fig. II in Levene and Tipson (13). The only differences between both interpretations, apart from the correct connectivity between carbons 3' and 5' of adjoining nucleotides, are the elemental compositions of the nucleobases, which are here updated. Dotted lines indicate the limits between adjacent nucleotides.
tain the notion of a four-base regularity in the constitution of nucleic acids, by then already suspected by Levene and others to be chains of subunits. Still, although Levene continued arguing in favor of the basic tetranucleotide model up to the end of his days, he also kept in mind the possibility that the tetranucleotide unit was “the minimum molecular weight and the nucleic acid may as well be a multiple of it” (Ref. 14, p. 289; see also last remark in the above-quoted paragraph of p. 262).

This suspicion was strengthened when various methods showed that at least the thymus nucleic acid could have a molecular weight of up to about one million (45), and Levene and his team immediately took this as a fact for their own experimental approaches (46). Moreover, Levene and Gerhard Schmidt offered evidence that this novel viewpoint applied also to the yeast nucleic acid, because pancreatin was found to act as “a depolymerizing agent, limited to the dissociation of the tetranucleotides of high molecular weight into those of lower molecular weight,” so the “native RNA is a polymer of the tetranucleotide” (47). They even pioneered one of the innovative techniques for gauging the relative sizes of large molecules—sedimentation by ultracentrifugation—to examine the effects of enzymatic treatment on DNA of various “degrees of polymerization” (i.e., chain lengths) (48). These data contributed to the emerging conception of the nucleic acids as large linear macromolecules.

**Assessment**

At the dawn of the twentieth century, most of the molecular components of the nucleic acids had been already identified and grossly quantified, but only faint guesses had appeared as to their connectivity. The modular basic structure of both nucleic acid types, each module consisting of a nitrogenous base associated with a specific pentose, which is in turn linked through phosphate groups to adjoining similar subunits in a linear series, was first elucidated by Levene and his co-workers starting from 1908. Already by the end of the following year, Jacobs and Levene summarized their early but relevant views in the abstract of a paper presented at a meeting of the American Society of Biological Chemists: “We believe it very likely that the nucleic acids are built up of groups, nucleotides [sic], similar in composition to the inosinic acid, which are joined together as the phosphoric acid radicals in the polyphosphoric acids” (49).

That work was to culminate in Levene and Tipson’s 1935 report showing accurately for the first time the actual molecular structure of DNA, as well as a nearly correct depiction of the RNA structure. This achievement merits the distinction of this paper as a Classic in molecular biology literature.

In addition, Levene introduced, “for the sake of convenience” (20), distinct names to designate the molecular components of the nucleic acids—i.e., nucleosides, nucleotides, and polynucleotides, plus the terms ribonucleic acids and deoxyribonucleic acids—which since then and up to present time over a century later remain as the standard nomenclature in the field. It is therefore a historical conundrum that, instead of these seminal contributions to our present knowledge in such a major topic of biology, it is the erroneous tetranucleotide hypothesis that is usually recalled in relation to speculations on the structure of the nucleic acids over the first half of the twentieth century.

There is no question that this hypothesis was flawed on several counts. The real question is why its various correct parts had not been widely recognized as classic in the literature. It has been surmised that “Personal factors have probably played some part in this” (11), alluding to Levene’s autocratic and unforgiving style in both science and daily life. Another view suggests that such temper, perhaps combined with “the enormous respect his contemporaries wielded toward him [may have resulted in the situation that] at some point the tetranucleotide hypothesis started playing the role of hindering progress” as regards the function of the nucleic acids (12). One may question, however, whether these interpretations are indeed supported by the facts.

A common criticism is that the tetranucleotide hypothesis “contributed to the view that nucleic acids were ‘dull’ and even ‘idiotic’ molecules” (12). Yet its main promoter prudently reserved any premature judgment about the function of the nucleic acids in cells, to the point of declaring at a 1916 meeting of the American Association for the Advancement of Science: “They are indispensable for life, but carry no individuality, no specificity, and it may be just to accept the conclusion of the biologist that they do not determine species specificity, nor are they carriers of the Mendelian characters” (50). And in accordance with this statement, Levene left all pronouncements on the functions of these cell substances to biologists, limiting himself for the rest of his work on this subject just to the chemical characterization of the organic compounds proper.

Biologists, in turn, for the most part were also unconcerned about the role of the nucleic acids in cells, concentrating instead their attention for potential bearers of genetic information on the highly multifarious proteins. That is until, a few years after Levene’s death, solid evidence suddenly appeared indicating that at least the deoxyribonucleic type seemed to be, or to carry, a “transforming principle” (51). Then John M. Gulland (52, 53), Erwin Chargaff (1, 3, 54, 55), and many other biochemists then leading the field, prompted by the exciting news, wrote profusely about nucleosides, nucleotides, and polynucleotides with the original technical meanings of these terms since 1908, and called both types of nucleic acids according to the specific pentose involved in their particular structures. Yet little or no mention was made of the earlier papers by Levene and his team where such concepts, and the experimental findings behind them, were first presented. For example, just a couple of papers by this group are cited in a 20-page 1953 review on nucleotides by Alexander Todd (56)—who, a few years later, was awarded the Nobel Prize in chemistry for his work on this and related subjects.

As regards charges of obstructing further progress, these are even harder to correlate with the actual developments. The immediate general appropriation, steady usage, and long durability of both the knowledge about the chemical structure of the nucleic acids, and the distinctive nomenclature associated with it, attest to the overall validity of the previous research unfortunately linked with the malign tetranucleotide hypothesis. In other words, despite its various drawbacks, the worthiness of such work has been amply recognized in practice, if not in credit. Thus, instead of hampering scientific advance, these key precedents actually paved the way for establishing our
current understanding about the nucleic acids. Downplaying and even neglecting the original and largely correct theoretical framework upon which the field was being reformulated, from the mid-1940s onward, may have been a consequence of the extremely competitive atmosphere on the matter at a time when its main author had already passed away.

Kossel’s speculation that major biomolecules are constructed from building stones (Bausteine) disposed “according to a determined plan or architectural idea” (21), a conception championed and extended a decade later by Hermann Staudinger (57), was demonstrated as true by Levene’s team for the most complex and significant of all biomolecules: the nucleic acids. Due credit for this achievement could be easily acknowledged by just substituting the numerical prefix in the name of his hypothesis on this matter, so as to read “the poly-nucleotide theory.”

Acknowledgments—We are deeply indebted to Jeffrey Seeman and Eusebio Juaristi for critical comments and suggestions on a previous version of the manuscript, and to Enrique Ramírez de Arellano for help in the correct interpretation of texts in German. We also thank Selene Rangel for obtaining a number of key original papers. Special thanks are given to Wiley Online Library, current owner of copyright for the Berichte der deutschen chemischen Gesellschaft, and to the Journal of Biological Chemistry, for granting permissions to reproduce Fig. 1 and Figs. 2A and 3A, respectively.

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