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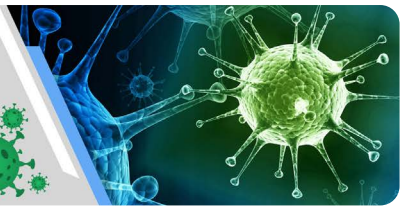

Alwin Max Pappenheimer Jr. and the Elucidation of the Structure and Mechanism of Toxicity of the Diphtheria Toxin

Michael Fry

Abstract

Diphtheria, a potentially fatal human disease, is caused by the infective bacillus *Corynebacterium diphtheriae* which releases a cytotoxic diphtheria toxin (DT). DT damages various host tissues and organs, causing much of the pathology of the disease. Universal implementation of effective vaccination practically eradicated diphtheria by the 1940s. Thus, pre-vaccination era research that engaged in clinical and immunological aspects of diphtheria evolved after its eradication into curiosity-driven studies of the structure and mechanism of toxicity of DT. In a career that spanned almost 50 years, the American biologist Alwin Max Pappenheimer Jr., (1908-1995) has made significant contributions to the elucidation of the synthesis, structure, and mechanism of the toxic action of DT. This paper traces Pappenheimer's pursuits of DT within the broader context of the advancement of DT research in the second half of the 20th century. Beginning with brief overview of the history and historiography of diphtheria, the paper next recounts Pappenheimer's personal and professional biography and outlines his scientific accomplishments. Last, I argue that Pappenheimer's scientific work is a representative case of a class of biomedical studies that while originally seeking solution to medical problems, ended up uncovering fundamental biological entities and principles.

Keywords: Diphtheria, Corynebacterium diphtheriae, Diphtheria Toxin, Alwin Max Pappenheimer Jr., History of toxinology, Bacterial toxinology

Introduction

Diphtheria, a potentially deadly epidemic human disease is caused by virulent strains of the infective bacillus *Corynebacterium diphtheriae*. Normally confined to the upper respiratory tract, *C. diphtheriae* bacilli induce formation of a leathery pseudomembrane in the throat and release Diphtheria Toxin, (DT) that spreads and damages various uninfected organs such as the heart, lungs, kidneys, and adrenals. Although the disease was known under various other names since antiquity, it was only in 1826 that the French physician Pierre Bretonneau described the pathological characteristics of diphtheria and defined it as a distinct clinical entity: 'une affection morbide sui generis'.

Diphtheria posed a grave threat to the health and life of predominantly children, until the introduction in the 1930-40s of highly efficacious universal vaccination that practically eradicated the disease. Studies in the pre-vaccination era focused on clinically relevant bacteriological, pathological, and immunological aspects of diphtheria. This phase of medically oriented research came to an end in the wake of the successful obliteration of the

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disease in the Western world (left arm of the timeline in **Figure 1**). Remarkably, however, just as diphtheria ceased to be a consequential medical problem, researchers launched vigorous long-term basic research programs that were divorced from clinical concerns. Begun in the 1930s and continuing today, (right arm of **Figure 1**) these efforts advanced the understanding at the cellular and molecular levels of the nature, structure, and mode of toxic action of diphtheria toxin, (DT). This paper chronicles the history of the experimental elucidation of the structure and activity of DT, focusing on major contributions to this endeavor by the American biologist Alwin Max Pappenheimer Jr., (1908-1995). Dedicating nearly 50 years to studies of DT, Pappenheimer had made major contributions to the elucidation of its synthesis, structure, and mechanism of toxic action. This paper traces Pappenheimer's pursuits of DT in the broader context of the general advancement of DT research in the second half of the 20th century. The work opens with a brief overview of the history of diphtheria, its clinical features, DT, and anti-diphtheria vaccination. It next surveys historiographic studies of diphtheria, recounts the personal and professional biography of Pappenheimer, outlines his scientific accomplishments, and places his work in the context of trends of 20th century biomedical research.

Diphtheria: History, Clinical Features, Diphtheria Toxin, Immunotherapy, and Vaccination

Bacteriology and Clinical Presentation

Diphtheria is an acute, infective disease caused by exotoxin-producing club-shaped bacillus *Corynebacterium diphtheriae*. This bacterium, which infects and resides in the pharyngeal mucosa, was first isolated by the German Swiss physician Edwin Klebs [1]. Rigorously applying the criteria of Koch's postulates, (see ***3.1*** below), the German bacteriologist Friedrich Loeffler established *C. diphtheriae* as the causative agent of diphtheria [2]. Prior to vaccination, transmission of the bacillus by direct contact, coughing, or sneezing, had spread diphtheria in Europe with an average annual mortality of 6-7 per 10,000 persons in the general population and up to 80% among affected children under 10 years of age. *C. diphtheriae* multiplies and ulcerates the mucosa of the upper respiratory tract, inducing formation of a white to black pseudomembrane of inflammatory cells, necrotic tissue, and bacteria. In severe cases, amassed pseudomembrane caused suffocation which was treated in some cases by life-saving tracheotomy. Although the *C. diphtheriae* bacilli usually remain in the pharyngeal mucosa, they release an exceptionally potent exotoxin, DT, which is spread to remote organs *via* the blood stream. Attacking heart, lungs, kidneys, and nerve cells, DT may evoke severe systemic pathology. Klebs was the first to propose that a 'venom' produced at the site of infection circulates in the blood stream and exerts 'a severe alterating effect on the vascular wall' [1]. Noting that the bacillus was present only in the pseudomembrane but not in deeper tissues, Loeffler explained the remote effect of *C. diphtheriae* by postulating, (without experimental proof), that it releases a toxin that damages uninfected distant tissues [2].

The Diphtheria Toxin

By demonstrating in 1888-90 that injected cell-free filtrates of *C. diphtheriae* cultures caused death in guinea pigs, Alexandre Yersin and Émile Roux of the Pasteur

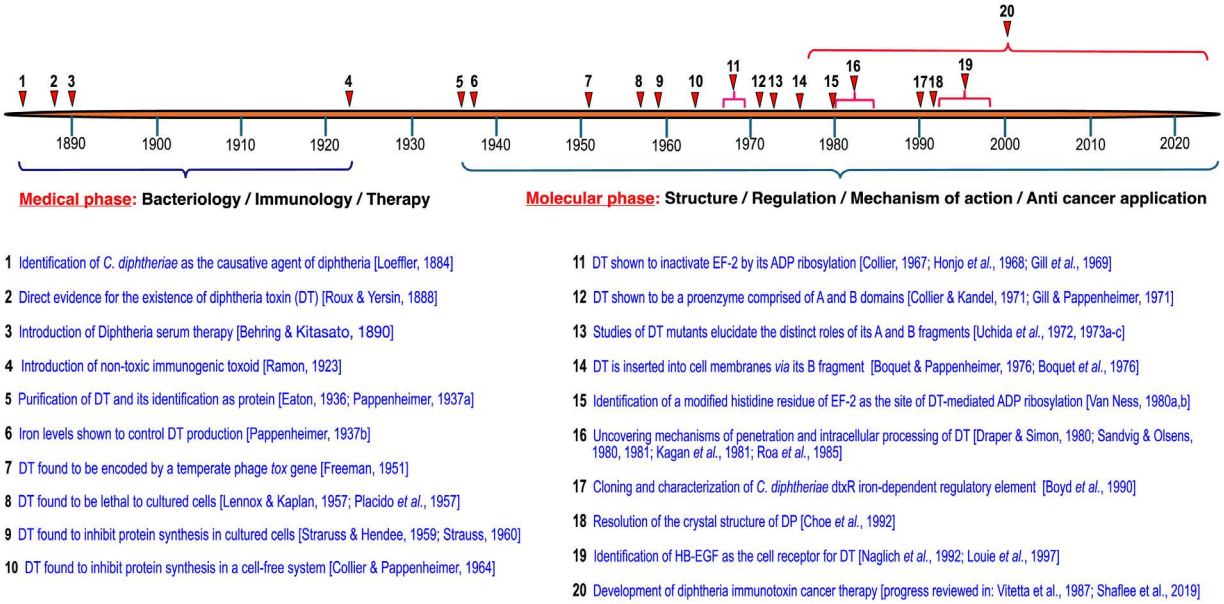

Figure 1: Timeline of research on *C. diphtheriae* and its toxin (DT). Research activity until the 1930s focused on clinical, bacteriological and immunological aspects of diphtheria. Introduction of effective universal vaccination in the 1930-1940s eradicated the disease and researchers turned to basic questions of DT structure and mechanism of toxicity.

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Institute demonstrated the existence in filtrates of *C. diphtheriae* of 'venom' or 'toxin' that was until then only a hypothetical entity. They also showed that rabbits and pigeons that were naturally more resistant to diphtheria than guinea pigs, were also more resistant to the injected filtrate. Furthermore, Yersin and Roux detected DT in urine that was collected shortly before the demise of sick children. Addressing in 1891 a Hygiene and Demography Congress in London, Roux proclaimed (English translation by [3]): "It is natural to conclude that microbes act through their chemical products, true poisons specific to each of them, and which determine the symptoms of the disease in man and in animals […] The infectious disease is therefore a poisoning: the source of the poison is the microbe settled in the tissues; it elaborates his toxin there at the expense of the living being that it is going to kill" . Roux also foresaw the use of filtrates of *C. diphtheriae* cultures for the induction of protective immunity. Distinguishing such procedure from vaccination with attenuated whole bacilli, he named it "chemical vaccination":

"It is therefore not necessary for the refractory state to be acquired, that the microbes penetrate into the body, it suffices that the substances prepared in the artificial cultures be introduced into it. If, therefore, by swarming in the organism, microbes give immunity, it is undoubtedly because they produce the same chemicals that we find in in vitro cultures."

Immunotherapy and Vaccination

Emil von Behring and Shibasaburō Kitasato discovered that exposure of naïve guinea pigs to serum from animals that were injected with immunizing sub-lethal amounts of tetanus toxin, rendered the exposed animals resistant to live tetanus bacilli [4]. Behring, (without Kitasato) next showed that antiserum ("antitoxin") that guinea pigs produced against filtrates of *C. diphtheriae* cultures imparted naïve animals with passive immunity against DT [5]. Large-scale clinical trials in Berlin in 1894 [6], and in Copenhagen in 1896-97 [7], established that treatment of human patients with anti-diphtheria antisera, (serum therapy; serotherapy) significantly lowered the proportions of fatal cases. In a first attempt to induce permanent immunity, guinea pigs that were injected with a mixture of DT and its antitoxin were found to develop immunity against diphtheria [8]. Subsequently, similar toxin-antitoxin treatment successfully immunized susceptible children against diphtheria [9]. Gaston Ramon of the Pasteur Institute next provided the means for effective general vaccination. Treating DT with formaldehyde and heat, he produced nontoxic but immunogenic 'toxoid' that effectively induced production of anti-diphtheria antibodies in injected individuals [10]. Vaccination with the diphtheria toxoid, which was first launched in the 1930s, became universal in industrialized countries by the late 1940s. As a result, incidence and mortality in North America of ~200 and 15 cases per 100,000 population in 1920, were correspondingly lowered in the 1990s to ~0.001 and 0.0001[11].

The Molecular Mechanism of DT Toxicity

After vaccination had practically eradicated diphtheria, research into the disease lost its medical exigency. Yet Pappenheimer and other scientists launched in the late 1930s research programs aimed at elucidating the structure and mechanisms of the toxicity of DT and of related bacterial toxins. As detailed below, it was found that DT exerted its cytotoxic activity by inhibiting cellular protein synthesis through modification and inactivation of elongation factor 2, (EF-2) an essential component of the cellular protein synthesis machinery.

Historiography of Bacteriological and Immunological Facets of Diphtheria

In studying diphtheria, historians of biology and medicine assessed the discovery of its causative agent, *C. diphtheriae*, in the context of German bacteriology of the late 19th century whereas other historians studied various aspects of the development of passive immune treatment of the disease, (serum therapy) and of anti-diphtheria vaccination.

Loeffler and the Koch School of Bacteriology

Loeffler's discovery that diphtheria was caused by *C. diphtheriae* must be viewed in a wider context of the Robert Koch school of medical bacteriology. Loeffler and Georg Gaffky, (who later identified *Bacillus salmonella typhi* as the causative agent of typhoid disease), were the first trainees to join in 1880 Koch's newly established Berlin laboratory. This laboratory soon became the epicenter of German bacteriology that side-by-side with the laboratory of Koch's adversary Louis Pasteur, had shaped medical microbiology in the late 19th century. Koch's introduction of the technique of growing bacteria on solid media opened the way to his subsequent triumphant cloning and isolation of stable bacterial species [12, 13]. Employing this method, Koch and his associates identified and isolated between 1877 and 1906 twenty different bacterial pathogens [14] p. 290. Loeffler concocted in this context a solid medium, ('Loeffler's medium') specifically suited for the growth and isolation of pure cultures of *C. diphtheriae*. Once obtaining the isolated bacteria, he meticulously followed the edicts of Koch's postulates to unequivocally demonstrate that this bacterial species was the causative agent of diphtheria. Loeffler professed that his eight years in the Koch laboratory were his happiest after which he was appointed in 1888 professor of hygiene at the University of Greifswald [15]. There he conducted studies on the foot and mouth virus which pioneered virological research in Germany [15, 16].

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Passive (Serotherapy) Treatment of Diphtheria and Active (Vaccination) Prevention of the Disease

Historians of diphtheria mostly focused on the development and application of passive (serotherapy) and active (vaccination) for the respective treatment and prevention of the disease. Historiographies were mainly concerned with scientific, social, political, and economic aspects of passive anti-diphtheria immunization in the late 19th century. Derek Linton comprehensively examined Von Behring's life and scientific work, both successful, (serotherapy of tetanus and diphtheria) and failed, (development of anti-tuberculosis vaccine) [17]. Linking Behring's introduction of serum therapy to his prior military-related studies of disinfectants, Jonathan Simon argued that he originally considered serotherapy to be a disinfection procedure and he only later came by more intelligible theories of immunity [18]. Historians paid considerable attention to the standardization and quality control of diphtheria antisera, delineating the major contribution of Paul Ehrlich to this endeavor, [19-21] and describing parallel efforts at the Pasteur Institute [22, 23]. Other studies charted the administrative regulation of the potency and quality of diphtheria antitoxin in late 19th century Germany and France [24-26, 20] and the conflict between philanthropy and business in the administration of serotherapy in *fin-de-siècle* France and Germany [24, 26, 25].

As to active immunization (vaccination), some authors investigated the life and scientific contributions of Gaston Ramon, the deviser of diphtheria toxoid [27, 28]. Others addressed the subsequent history of the development of diphtheria vaccination [29, 30]. Also, attention was paid to local histories of the production, administration, and failures of vaccination programs in countries such as the United States (Philadelphia, and New York) [31, 32], England [33], and Iran [34]. Unlike the well-researched histories of medically oriented studies of the bacteriology and immunology of diphtheria in the late 19th and very early 20th century, there are virtually no historiographies of the non-medical phase of diphtheria research that, starting in the 1930s, that was directed at the elucidation of the structure and mode of action of the diphtheria toxin, (DT). In this paper I survey the history of pivotal 20th century developments in DT research, focusing on the life and work of Alwin Max Pappenheimer Jr., a major contributor to the clarification of the structure of DT and its mechanism of toxicity.

Alwin Max Pappenheimer, Jr., Biographical Notes

The following concise biographical notes are based on Pappenheimer's own short memoir [35] and on his portrayals by others: [36-40]; [41], 71-78. Born in 1908 in Cedarhurst, New York, Alwin Pappenheimer Jr., (**Figure 2**) was the eldest child of Alwin Max Pappenheimer Sr., a Columbia University professor of pathology. His younger siblings were Anne Pappenheimer Forbes, a Radcliff and Harvard educated endocrinologist and a clinical professor at the Harvard Medical School, and John Richard Pappenheimer, a Cambridge University educated physiologist and Harvard professor. Pappenheimer's grandfather, who arrived in New York as a penniless Jewish immigrant from southern Germany later prospered in the tobacco business. His son Alwin Max Pappenheimer Sr. was educated in Harvard College and in the Columbia University College of Physicians and Surgeons, becoming a prominent professor of pathology in Columbia whose research interests encompassed bacteriology, serology, endocrinology, virology and vitamin deficiencies.

Figure 2: Alwin Max Pappenheimer Jr. in 1954. Image courtesy of The Lillian and Clarence de la Chapelle Medical Archives at New York University.

Alwin Max Pappenheimer Jr., (named 'Pappenheimer' in this paper) grew up in a highly cultured and musical household. After concluding his studies in the long since defunct Lincoln School of Teachers College in New York, Pappenheimer graduated in 1929 from Harvard College. Against expectations, he chose not to study medicine but rather to pursue PhD research in organic chemistry under the guidance of James B. Conant who later served as the President of Harvard University. Although his PhD thesis was in organic chemistry, Pappenheimer, who was attracted to biological questions, also worked on the oxidation potential of the hemoglobin-methemoglobin system. After obtaining a PhD degree in 1932, he was awarded a National Research Council Fellowship and worked in 1932-1934 in the National Institute for Medical Research in London under the neurophysiologist and 1936 Nobel laureate Sir Henry Dale. There he unsuccessfully attempted to isolate a hypothetical bacterial growth factor. Back in America, Pappenheimer spent the years 1935-1937 in Harvard, serving as research fellow and instructor in immunology. In parallel he also worked as a senior chemist in the Antitoxin and Vaccine Laboratory in Jamaica Plain, Massachusetts. The original mission of this

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Institute which was established in 1894 by the Massachusetts Board of Health, was the production of diphtheria antitoxin. It later undertook preparation of vaccines against smallpox, typhoid, tetanus, and pertussis. During World War II the Laboratory produced transfusable human albumin and immune globulins and in 1950 it was the first manufacturing site of Diphtheria-Tetanus-Pertussis (DTP) combination vaccine. Renamed MassBiologics, the Laboratory is operated since 1997 by the University of Massachusetts Chan Medical School. It was in this laboratory the Pappenheimer launched his life-long pursuit of DT by successfully isolating a highly purified form of DT and discovering that DT production in *C. diphtheriae* was regulated by iron (see section **5**).

Despite his productive sojourn in the Antitoxin Laboratory, Pappenheimer's missed a university environment and thus joined in 1939 the Department of Bacteriology at the University of Pennsylvania. Inopportunely, however, heavy teaching duties and limited research prospects left him unsatisfied in Pennsylvania. He thus willingly accepted in 1941 an offer from Colin MacLeod, the designated Chair of a new Department of Bacteriology in New York University (NYU), to join its faculty as an assistant professor. Other recruits to the department included the biochemist Efraim Racker, the bacteriophage expert Mark Adams, the epidemiologist Royal Christianson, and the bacterial toxinologist Alan Bernheimer. Just six months after joining NYU, the United States entered World War II and Pappenheimer put aside his scientific work to serve in the in the US Army Medical Corps in the Pacific theatre. Discharged in 1945, Pappenheimer returned to NYU and soon recruited his first graduate student, an acquaintance from the army, Melvin Cohn, who became in time a leading immunologist and cofounder of the Salk Institute for Biological Studies in La Jolla. Shortly thereafter his laboratory attracted additional mentees: Sherwood Lawrence, a later expert on lymphocyte function and head of the NYU Department of Infectious Diseases and Immunology, Lane Barksdale, a later professor of microbiology at NYU, Jonathan Uhr who became a leading expert on toxins (including DT) as potential cancer therapeutics, and Matthew Scharff, a later Distinguished Professor of immunology at the Albert Einstein College of Medicine. Pappenheimer's productive research work at NYU established him as a leader in the DT area. During that period, he expanded his 1936-37 studies on the effect of iron on DT production, investigated relationship of a lysogenic β phage that carries the DT gene into its *C. diphtheriae* host, and examined the effect of DT on the metabolism of silkworm. Importantly, he urged Norman Strauss and Edelmira Hendee in his laboratory to conduct experiments that revealed that DT inhibited protein synthesis in cultured HeLa cells. This finding that was a first critical step in the consequent unraveling of the mechanism of DT toxicity (see Section **5** below).

After Colin MacLeod has left NYU, Pappenheimer replaced him in 1956 as chair of the NYU Department of Microbiology. Shortly thereafter, however, the Harvard biochemist John Edsall invited Pappenheimer to serve as professor of biology at Harvard and to chair its Board of Tutors in Biochemical Sciences, a tutorial program for students of cellular and molecular biology. In 1961 he was also appointed Master of Harvard's Dunster House. Beginning in 1958 and lasting till the 1980s Pappenheimer conducted in Harvard experimental studies on the structure of DT and its toxic activity. There he also acted as an influential mentor to students and fellows, many of whom later became prominent scientists on their own. As detailed in the next section, Pappenheimer's main achievements during the Harvard period included the discovery of the molecular mechanism of inhibition of protein synthesis by DT, studies on DT structure-function relationships, and investigations of mechanisms of DT entry into target cells and its subsequent intracellular processing. Elected in 1973 to the National Academy of Sciences USA, Pappenheimer also served as president of the American Association of Immunologists. He died in 1995 at the age of 86.

The Elucidation of the Structure and Toxic Action of DT

Once diphtheria ceased to be a threat to public health with the introduction of effective vaccination in the 1940s, research on this disease lost its medical impetus and became instead curiosity driven. Pappenheimer delved in the late 1930s into basic studies of the diphtheria toxin (DT). These early experiments presaged his next fifty years of quest after DT structure and mechanism of toxicity. Pappenheimer's own research work and that of his mentees augured the emergence the presently expansive field of microbial toxinology. The historical case of Pappenheimer's career exemplifies the basic and potential practical merits of biomedical research with no direct clinical exigency. This section describes Pappenheimer's contributions to the study of DT in a broader context of a collective endeavor of many researchers to determine the structure of DT and explain the mechanism of its toxic action.

Pappenheimer Purified DT and Established its Proteinaceous Nature

Pappenheimer launched in 1936-37 his career-long pursuit of DT by isolating a highly purified form of toxically potent DT from filtrates of *C. diphtheriae* cultures. This was not a trivial undertaking as up to that time *C. diphtheriae* cells were cultivated in complex growth media that contained proteins and peptides which masked the minute amounts of toxin that the bacteria released into the medium. Pappenheimer overcame this problem by formulating a growth medium that contained free amino

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acids but no proteins or peptides [42, 43]. Growing *C. diphtheriae* in this medium he isolated from cell-free filtrate of the medium a ~70,000 Da potent proteinaceous toxin at a 95-98% purity [42, 44, 45]. Precise measurements later determined that the actual molecular weight of DT is 58,350 Da. Pappenheimer speculated already in the 1930s that a few or a single molecule of DT sufficed to kill one host cell [35]. Decades later an Osaka University research team confirmed this prediction by experimentally demonstrating that a single DT molecule induced death in one cell [46]. Parallel to Pappenheimer, the Harvard microbiologist Monroe Eaton devised a stepwise purification procedure that allowed isolation of minuscule amounts of highly purified DT from filtrates of protein-containing growth medium [47-49]. Eaton's and Pappenheimer's independent similar findings resolutely established that DT was a protein in its entirety. This was a turning point since Pappenheimer initially thought that the DT protein moiety served as mere carrier for a poisonous cofactor such as strychnine or curare [35]. However, when his analysis of acid hydrolysate of the highly purified DT revealed that it contained only amino acids, he realized that DT was wholly a protein. This raised a question how a proteinaceous DT exerted its toxicity, a riddle that Pappenheimer and others pursued in subsequent decades.

Studies on the Regulation of DT Production by Iron

As they were preparing a protein-deficient *C. diphtheriae* growth medium, Pappenheimer and his assistant Sylvia Johnson noticed that concentration of iron salt in the medium determined the levels of DT that the bacteria produced [50, 51]. Iron at low concentration was required for the synthesis and release of maximum amounts of DT but higher levels of the metal strongly inhibited toxin production. This discovery, which heralded the birth of a fertile area of research on regulation of DT biosynthesis by iron, earned Pappenheimer the 1941 Eli Lilly award. As Pappenheimer later attested, (cited in [39]) that this modest beginning had set the course for his career-long scientific endeavor: "As a graduate student and even as a postgraduate fellow, it worried me a great deal that I did not have an important problem in mind. However, with the discovery of the importance of iron in controlling the production of diphtheria toxin, this insecurity left me and I have never since had to worry about finding something to work on. One thing always leads to another". Pappenheimer returned in the late 1940s to the question of the mechanism of iron control of DT production. Perhaps because of his past doctoral work on hemoglobin, he focused on cytochromes, which like hemoglobin, also contain an essential heme cofactor with an iron atom at its core, as potential keys to the regulation of DT synthesis by iron. Work in the next several years misled him to believe that DT toxicity was due to its interference with the synthesis or action of the cytochrome system in infected cells.

Pappenheimer and his NYU associate Edelmira Hendee measured release from *C. diphtheriae* of DT and of the heme precursor coproporphyrin III, measured iron content in the bacteria, and determined activities of their iron-containing enzymes cytochromes and catalase [52]. They found that DT and coproporphyrin III were secreted only during the terminal stages of bacterial growth after all the iron in the growth medium was exhausted. Also, reduction of the bacterial iron enzymes was positively correlated with the secretion of DT and coproporphyrin III. Based on these results, Pappenheimer speculated that DT represented the protein moiety of *C. diphtheriae* cytochrome and that infected cells died because this DT/cytochrome protein interfered with the synthesis or activity of their own cytochrome system. Subsequent experiments on the *Platysamia cecropia* silkworm appeared to support to this speculation. Collaborating with the Harvard zoologist Carrol Williams, Pappenheimer found that DT inhibited cytochrome synthesis in developing pupae [53, 54]. However, the cytochrome hypothesis of DT toxicity collapsed when later experiments in Pappenheimer's own laboratory showed that DT killed host cells by inhibiting their protein synthesis and that the interference with the cytochrome system was a secondary outcome of this inhibition (see below).

Considerable parts of the true mechanism of the control of DT production by iron were ultimately elucidated by two of Pappenheimer's former mentees, John Murphy of Boston University and Randall Holmes at the Uniformed Services University. Essentially, it was found that transcription of the DT encoding gene tox was controlled by an iron-activated repressor protein, DtxR. Once higher concentrations of the metal activated DtxR, it bound to a specific regulatory sequence in the tox gene to repress its transcription [55, 56] reviewed in [56]. In sum, Pappenheimer's key contribution was his initial discovery that DT production was controlled by iron. However, his attempt to link this phenomenon to the mechanism of DT toxicity proved to be incorrect.

Studies of Immunological Aspects of DT

Much of Pappenheimer's research work from the late 1930s to the 1950s was devoted to immunological facets of DT. Diverging from his later focus on cellular and biochemical features of DT, this direction of research was taken because DT toxoid vaccination had still left some questions unanswered. Also, the foci of interest of Pappenheimer's MD mentees at the time, Sherwood Lawrence and Jonathan Uhr, were mainly immunological and medical. An early line of Pappenheimer's immunological investigations dealt with the interaction of DT with its antitoxin [57-60]. Lawrence and Pappenheimer later attempted to minimize adverse allergic reactions to immunization among adults by modifying the preparation protocol and mode of injection of the diphtheria toxoid [61-63]. A third line of studies dealt with delayed

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inflammatory hypersensitivity reaction to diphtheria toxin, toxoid, and toxin-antitoxin complexes. Presaging today's understanding that delayed hypersensitivity is mediated by monocytes/macrophages and not by circulating antibodies, Pappenheimer, Lawrence, and Uhr found that the delayed allergic reactions to injected diphtheria toxin or toxoid occurred in the absence of circulating anti DT antibodies [64-67].

Pappenheimer Used Phage Encoded DT to Study the Toxin Protein

DT research took an unexpected turn when it was discovered that the toxin was encoded by a gene of a *C. diphtheriae* specific bacteriophage and not by a bacterial gene. Victor Freeman, then at the University of Washington, fortuitously observed in 1951 that infection with a specific bacteriophage transformed avirulent strains of *C. diphtheriae* into DT producing bacteria [68, 69]. This initial observation was followed by a demonstration that corynephage b was solely responsible for the conversion of avirulent strain of *C. diphtheriae* into DT producing virulent bacteria, implying that DT was encoded by a structural gene in the prophage genome [70-73]. A later work in Pappenheimer's laboratory strengthened this idea [74]. Finally, this notion was validated when the toxin encoding gene, tox, was mapped within the prophage genome [75]. Further, the amount of produced DT was directly proportional to the number of integrated prophage genomes in the host bacteria genome [76].

Pappenheimer was mostly interested in studying the normal and mutant protein products of the corynephage tox gene. He first investigated with Barksdale ultraviolet light (UV) induction of lysis of prophage-hosting bacteria [77]. More than a decade later he returned to corynephages by studying morphologies of B and b corynephage strains [78]. A concurrent study of DT protein products of normal and mutant corynephage tox genes left Pappenheimer uncertain whether the toxin encoding gene resided in the bacterial or the phage genomes [79]. However, subsequent studies of mutated tox gene that encoded a nontoxic but immunologically cross-reacting protein, (CRM), and purification and analysis of the CRM, strongly suggested that tox dwelt in the phage genome [74, 80]. This was validated by a demonstration that purified DNA from normal tox^+ and mutant tox^- phage strains directed the respective *in vitro* syntheses of DT and its related nontoxic CRM [81]. Pappenheimer later employed CRM proteins to probe structure-function relationships in the DT protein molecule (see 'DT structure-function' below).

Discovery of the Mechanism of DT Toxicity

Pappenheimer's career-long pursuit of the diphtheria toxin reached its apex with his discovery of key elements of its mechanism of its toxic action. A first step toward uncovering of the molecular basis of DT toxicity was a seemingly modest observation that DT was lethal to cultured cells from toxin sensitive organisms [82, 83]. Norman Stauss and Edelmira Hendee of the Pappenheimer NYU laboratory next observed that DT arrested incorporation of the amino acid methionine into HeLa cell proteins, indicating that the toxin inhibited synthesis of cellular protein [84]. Furthermore, because repression of protein synthesis preceded the interruption of DNA and RNA syntheses and of energy metabolism, the blocking of protein synthesis appeared to be the primary cause of cell death [85-87]. Later in Harvard. Pappenheimer collaborated with his then doctoral student John Collier who later went on to independently study DT, first in the University of California, Los Angeles (UCLA) and later in Harvard. His contributions to this area were recognized by several prestigious awards. Collier and Pappenheimer reported that DT inhibited synthesis of proteins in a HeLa-derived cell-free system [88]. This *in vitro* system proved to be a vital platform for subsequent experimental discoveries of molecular details of DT toxicity. First, during his postdoctoral fellowship in Geneva, Collier used this system to find that DT inhibited cellular protein biosynthesis by inactivating elongation factor 2 (EF-2), an essential component of the protein synthesis apparatus that acts as a polypeptidyl-tRNA translocase that mediates a translocation step in protein synthesis [89]. In parallel, Pappenheimer and his doctoral student Ronald Goor showed that Nicotinamide Adenine Dinucleotide (NAD), (**Figure 3A**) was a required cofactor for DT inhibition of protein synthesis in the cell-free system [90-92].

Soon, experiments performed by Tasuku Honjo and associates in Kyoto University, and by Michael Gill in the Pappenheimer laboratory, linked the requirement for NAD to the mechanism of EF-2 inactivation by DT [93-95]. Part of the DT protein, ('fragment A', see next section) was found to act as an enzyme that hydrolyzed NAD, releasing its nicotinamide moiety and covalently bonding the remaining ADP-ribose part to EF-2 [96-98], (**Figure 3A**). ADP ribosylation inactivated EF-2 thus blocking protein synthesis. The contribution of Pappenheimer and his mentees to the elucidation of the molecular basis of the toxic action of DT heralded succession of discoveries on analogue and dissimilar toxic mechanisms of many other bacterial toxins [99-101].

Investigations of DT Structure-Function Relationships

In the 1970s Pappenheimer's attention turned to the relationship between the structure of DT and its function and to related questions on the mode of entry of the toxin into cells and its subsequent intracellular processing. A distinct domain in DT was found to catalyze ADP ribosylation of EF-2. Two of Pappenheimer's former mentees, John Collier and Michael Gill separately showed that DT was synthesized as a single intact polypeptide chain that contained two disulfide

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bridges and no free sulfhydryl groups [102, 103] (illustrated at the top of **Figure 3B**). Work in the laboratories of Collier, Gill, Pappenheimer, and others, [104, 105, 103, 106-110] revealed that *in vitro* nicking of DT by a protease such as trypsin followed by reductive breakage of its disulfide bridges, yielded two distinct polypeptide chains. One chain, ('fragment A') which corresponded to the amino terminal part of DT catalyzed ADP ribosylation of EF-2. The other chain, ('fragment B') represented the carboxy terminal portion of the toxin and was devoid of enzymatic activity (see **Figure 3B**).

Experiments showed that isolated fragment A was incapable of entering the cells and that the fragment B portion of DT mediated the penetration of the intact toxin molecule into the cell.

In a genetic line of inquiry, Tsuyoshi Uchida and Pappenheimer generated different nontoxic but serologically cross-reacting mutant CRM proteins of DT. Analysis of different mutant CRMs revealed that mutations in the fragment A region of DT nullified its ADP ribosylation enzymatic activity whereas mutations in the fragment B domain impeded entry of the toxin into cells [80, 111]. Also, by using normal DT and nontoxic CRM mutant proteins that he synthesized in *E. coli*-derived cell-free system, John Murphy, Pappenheimer's postdoctoral fellow at the time, obtained first hints for the existence of a host-encoded iron binding repressor of toxin synthesis [81]. Later studies identified and characterized a *C. diphtheriae* encoded iron-binding protein, (DTxR) that repressed transcription of the DT tox gene in the presence of excess iron: [112-114].

Studies on the Entry of DT into Target Cells and its Subsequent Intracellular Processing

Taking a biochemical approach, Pappenheimer and his then postdoctoral fellow Patrice Boquet, (later at the Pasteur Institute) studied the interaction of DT with cell membranes. Based on their experimental results, they offered an early model of the interaction of DT with the cell membrane under which the DT fragment B region initially associates reversibly with a hydrophobic locale in the cell membrane [115, 116]. This early interaction is followed by a major irreversible conformational change in the toxin molecule that capacitates its insertion *via* the fragment B domain into the lipid bilayer of the cell membrane. Once DT reaches the inner surface of the membrane, a cellular protease nicks the link between its A and B domains, and the disulfide bridges are reductively broken. A freed fragment A then catalyzes in the cytoplasm ADP ribosylation of EF-2 whereas fragment B is degraded [116]. Pappenheimer modified this model when a third intermediate hydrophobic domain was identified as the first DT segment that penetrates the plasma membrane [117]. In 1982 Pappenheimer presented a refined final model of the entry of DT into target cells and its subsequent intracellular processing [118]. Details of the complex processes of DT entry into cells and its intracellular processing were elucidated after Pappenheimer retired from active research. DT was found to bind to a specific proHB-EGF cell membrane receptor and to be cleaved intracellularly by the protease furin, [119, 120]. Yet, although Pappenheimer could not be aware of these subsequent discoveries, the contours of his model remain valid today.

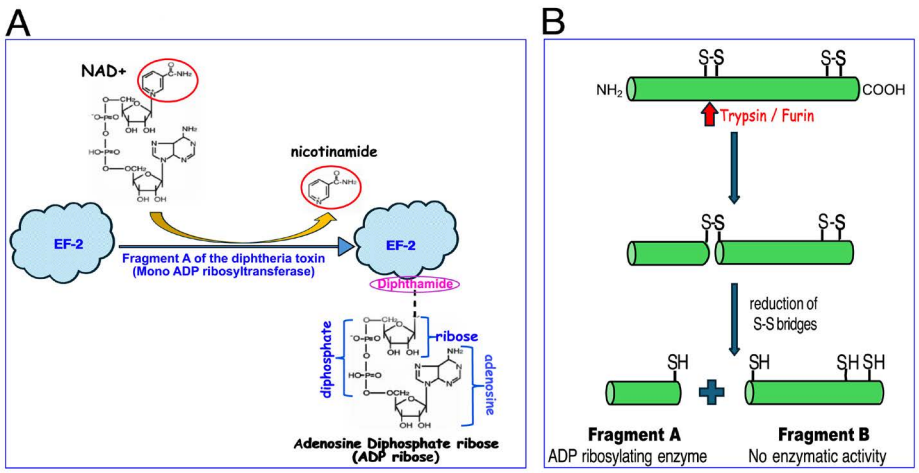

Figure 3: ADP ribosylation of EF-2 by fragment A of DT and intracellular processing of the toxin. **A.** Fragment A of DT acts as an NAD hydrolyzing mono ADP ribosyltransferase enzyme that releases nicotinamide and covalently binds the ADP ribose moiety to a specific modified histidine residue, (diphthamide) in the EF-2 molecule. As a result, EF-2 is inactivated and cellular protein synthesis is inhibited. **B.** Processing of the intact diphtheria toxin molecule. The full DT polypeptide is internally nicked by a protease such as trypsin (*in vitro*) or furin (*in vivo*), and inter-segment disulfide bridges are reduced. The DT molecule is subsequently split into two free polypeptide segments, an enzymatically active N-terminal fragment A and a C-terminal non-enzymatic fragment B.

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From Pappenheimer's Basic Research to Deployment of DT in Cancer Therapy

Pappenheimer's scientific oeuvre steadfastly adhered to investigations of foundational aspects of DT with no attention to possible practical applications. Other investigators, however, were alert to the potential of using DT and other toxins to selectively target and destroy cancer cells. The idea was to render a toxin selective for malignant cells by its conjugation to tumor-specific antibody, forming a so-called 'immunotoxin'. As the antibody component of the immunotoxin binds specifically to the tumor cell, its toxin part becomes internalized and kills the cell. A first proof of this principle was provided when a DT conjugate with antibody against a surface antigen on mumps virus-infected cells killed mumps infected but not normal cultured cells [121]. This initial report was promptly followed by similar works [122, 123] that heralded emergence of a new field of immunotoxin-mediated anti-tumor therapy [124-128]. Multiple clinical trials of various immunotoxins have already yielded a few FDA approved anti-cancer immunotoxins [129-132]. Thus, although Pappenheimer himself adhered to basic research on DT, his work contributed to the use of DT and other toxins as cancer therapeutics.

Pappenheimer's Science as a Representative Case of 20th Century Medical Studies that Yielded Discoveries of Fundamental Biological Entities and Principles

A widely held belief among biomedical researchers of the middle to late 20th century was that progress in medicine is best served by curiosity-driven basic investigations. The biochemist and 1959 Nobel laureate Arthur Kornberg, lucidly articulated this view [133]. Broaching representative historical cases, he argued that historically, curiosity motivated investigations proved to be generators of major practical advances in medicine. For instance, basic bacteriological investigations yielded penicillin, the fundamental discovery of X-rays furnished medicine with potent diagnostic tools and with radiation therapy, basic virological studies produced anti-viral vaccines, and techniques of genetic engineering yielded multifarious medicinal and pharmaceutical applications. Overall, Kornberg asserted [133]:

The pursuit of curiosity about the basic facts of nature has proven, with few exceptions throughout the history of medical science, to be the route by which the successful drugs and devices of modern medicine were discovered. Though it seemed unreasonable and impractical, counterintuitive even to scientists, to solve an urgent problem of disease by exploring apparently unrelated questions in biology, chemistry and physics, these basic studies proved time and again to be utterly practical and cost-effective.

A closer look at the history of biomedical science in the second half of the 20th century reveals, however, a parallel reverse direction of research. In some instances, studies that were originally aimed at solving specific medical problems, evolved to yield new foundational biological insights. Pappenheimer's work followed such a pattern. His initial efforts to answer medically relevant immunological questions concerning diphtheria vaccination evolved into curiosity-driven experimentation that produced new information on the structure of DT and its mechanism of toxicity, opening a basic field of bacterial toxinology. In full circle, however, gained basic insights contributed to development of immunotoxin cancer therapy. Pappenheimer's route of medical to basic research is not a solitary episode as two other representative cases illustrate.

The Identification of DNA as the Genetic Material

This landmark discovery represents an archetypical instance of medically motivated study that transformed into pursuit of a key basic question. The seed for this discovery was sown by the British clinical bacteriologist Frederick Griffith. Conducting epidemiological study of different serotypes of *Streptococcus pneumoniae* (*pneumococcus*) that were isolated from pneumonia patients, he unexpectedly discovered that mixing a killed and thus non-lethal virulent serotype with a live avirulent serotype resulted in transformation of the latter into virulent bacteria [134], narrated in [135], pp. 49-59. This observation was later taken up by Oswald Avery, a Rockefeller Institute bacteriologist whose whole career was dedicated to (unsuccessful) pursuit of effective vaccination against bacterial pneumonia. Based on Griffith's observations, Avery launched in the 1930s a search for a "transforming principle" i.e., the constituent in the killed virulent bacteria that mediated the transformation of avirulent into virulent bacteria. Painstaking careful experiments culminated in the isolation from virulent *pneumococci* of purified transforming material that was chemically defined as DNA,[136]. For a history of this discovery and its mute or negative initial reception, see [135], pp. 46-49 and 62-105. Avery, Macleod, and McCarty, guardedly suggested, therefore, that DNA represented the hereditary material of cells. Thus, what began as medically oriented pursuit of anti-pneumonia vaccine ended up in the discovery that DNA was the genetic material. This watershed revelation rapidly engendered countless basic insights and practical applications that revolutionized the life sciences.

The Discovery of transfer RNA

Work of Paul Zamecnik and Mahlon Hoagland in the 1950s offers another example of medical research that evolved into cardinal basic discovery. Trained as a physician, Zamecnik launched in the late 1940s a medically motivated research program aimed at identifying putative differences in protein

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biosynthesis by normal and malignant cells [137]. To build experimental platform for these studies, Philip Siekevitz, then a postdoctoral fellow in the Zamecnik laboratory, constructed liver-derived cell-free protein synthesis system [138]. Realizing the potential power of this *in vitro* system, Zamecnik gave up his original medical objective and instead undertook with Hoagland to identify components of the cell-free protein synthesis system. Stepwise experimentation revealed that each amino acid was carried to the site of protein synthesis, (ribosomes) by specific molecules of transfer RNA (tRNA) that were charged with corresponding amino acids by specialized enzymes, aminoacyl tRNA synthetases [139, 140]. For historical and philosophical analyses of these discoveries see [141, 142]. This fundamental discovery explained how the nucleic acid 'language' of the genetic code is translated into the 'language' of amino acids and translated protein.

Statements

Ethical Approval Statement:

Human or animal studies: Not applicable

Patient consent: Not applicable

Informed consent: Not applicable

Clinical trials: Not applicable

Data availability: Not applicable

Conflict of Interest:

None declared

Permission for reused copyright material:

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