Penicillins and Staphylococci: A Historical Interaction

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PENICILLINS AND STAPHYLOCOCCI: A HISTORICAL INTERACTION

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The development of beta-lactam antibiotics (penicillin and its contemporary cousins) is intertwined with the history of disease-producing staphylococci. Strains of staphylococci have been employed as laboratory models for the study of the biochemistry, mechanism of action, in vitro activity, and in vivo efficacy of beta-lactams since the "discovery" of penicillin in 1928. After the clinical introduction of penicillin in the early 1940s and its extensive use as a treatment for staphylococcal and other bacterial infections, the emergence of bacterial enzyme-mediated resistance in staphylococci inspired the chemical modification of penicillin to regain activity against resistant strains. The introduction of these new agents in the early 1960s again met with the development of resistant staphylococci. In the last two decades, the prominent role of *Staphylococcus aureus* in clinical infections has been supplemented by the emergence of related staphylococcal species (coagulase-negative staphylococci) as significant pathogens often highly resistant to antimicrobial therapy.

In 1883, Sir Alexander Ogston described cluster-forming cocci whose resemblance to a cluster of grapes suggested the name "staphylo-" (Greek for "bunch of grapes") cocci [1]. Rosenbach isolated staphylococci in pure culture the subsequent year and distinguished between two colony types. Organisms appearing as orange-yellow colonies were termed *Staphylococcus pyogenes aureus,* and those growing as white colonies were named *Staphylococcus pyogenes albus* [2]. The *aureus* variant, recognized immediately as a major pathogen, is indeed a true species (*Staphylococcus aureus*), but the *albus* variant is actually a collection of

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species collectively known as "coagulase-negative staphylococci." At least 21 species have been distinguished within this group [3], but relatively few (primarily *S. epidermidis*) are isolated from human infections.

The first reference to an in vitro bacterial-fungal interaction was made in 1871 by Sir John Burden-Sanderson, who observed that whereas liquid culture media exposed to air became turbid with growing bacteria, if a penicillium mold happened to grow on the liquid surface no turbidity ensued [4]. Joseph Lister noted this observation and planned immediate clinical application, writing in 1872 that "should a suitable case present, I shall endeavor to employ *Penicillium glaucum* and observe if the growth of the organisms be inhibited in the human tissues" [5]. There is some evidence that he did successfully treat the stubbornly infected wound of a young woman with an extract of "penicillium," but he did not publish the results. Unpublished accounts from other investigators describe the ability of penicillium culture extracts to inhibit the growth of virulent bacteria and to ameliorate infections in experimental animals and humans [4], but these trials were virtually ignored. Alexander Fleming, who is credited with the discovery of penicillin, was therefore not the first to observe interaction between residents of a culture dish, but he was the first to conduct extensive laboratory characterization of the substance he termed "penicillin" before attempting its clinical application.

The events surrounding Alexander Fleming's discovery of penicillin in September 1928 were described to historians by a colleague, D. M. Pryce. Fleming was studying variations in the coloration of staphylococcal colonies that seemed to be related to virulence, color changes that only became apparent after several days of room-temperature incubation. In the process of describing his current experiments to Pryce, Fleming selected several culture plates from a Lysol-filled basin into which they had been discarded. Fleming had worked with many cultures that day, thus a few culture plates were situated above the level of the liquid antiseptic [4]. One such plate contained a contaminating mold whose presence seemed to be influencing the morphology of the surrounding colonies of staphylococci: colonies in proximity to the mold became transparent and seemed to be undergoing lysis [6].

This discovery has been described by MacFarlane as a remarkable conspiracy of several discrete chance events. Fleming happened to be studying staphylococcal colony morphology characteristics that required room-temperature incubation (conditions that permit the growth of molds such as *Penicillium*) rather than the usual 37° Celsius environment used in bacteriologic work. The mold which grew on the contaminated plate happened to be not only capable of producing penicillin, but was in fact a rare penicillin hyperproducing strain whose effect on the bacterial colonies was grossly visible. No other isolate of this mold species has

been found to possess such high penicillin-production capacity. On the day of the discovery, Pryce chanced to walk into Fleming's lab to share research notes. Therefore chance also conspired to allow Fleming a fateful second observation of a culture plate which had apparently escaped special notice during his first observation. The *Penicillium*-contaminated plate happened to escape sterilization in the Lysol bath and to be selected at random by Fleming as a demonstration of colony morphology [4].

Initial studies of penicillium extracts established that, unlike contemporary antiseptics such as carbolic acid, penicillin was nontoxic to animals and it exerted no observable effect on leukocyte function [7]. Fleming speculated that the mechanism of action of this substance might be fundamentally different than that of antiseptics, since antiseptics killed bacteria within minutes but the "mould filtrate" required several hours of contact for a detectable effect [4]. He noted in his original paper describing penicillin that "penicillin's action is very marked on the pyogenic cocci," and he documented activity (in order of decreasing sensitivity) against streptococci, staphylococci, pneumococci, meningococci, gonococci, and *Corynebacterium diphtheriae* [6, 8].

Fleming's reports of the inhibitory activity of penicillium extracts initially met with little interest, and he emphasized the utility of penicillin in the isolation of *Bacillus influenzae (Haemophilus influenzae),* which like most other Gram-negative species is relatively insensitive to penicillin. The principal clinical interest in penicillin, however, concerned its potential utility in staphylococcal infections, for which there was no adequate therapy at that time. Penicillin, unlike sulfonamides, was found to retain antibacterial activity in sites of extensive tissue destruction [9], and this was seen as a marked advantage over sulfa drugs for staphylococcal infections. Ernst Chain and Howard Florey demonstrated penicillin's in vivo efficacy in 1940 through a series of experimental staphylococcal infections in mice. Whereas 21 of 24 animals were cured by repeated subcutaneous injections of the antibiotic, all 24 untreated animals died of sepsis [10].

The first patient to receive parenterally administered penicillin was a woman dying of breast cancer, in whom it was hoped to demonstrate the safety of the drug. Contaminants in the penicillin preparation caused her to develop a high fever, but fortunately this setback did not discourage Florey and his coworkers from further patient trials. The procedure of penicillin preparation was modified, and a policeman suffering from a mixed staphylococcal-streptococcal cellulitis and septicemia was chosen as the next recipient. Penicillin could not be produced rapidly enough in the laboratory to complete a course of therapy, however, and the disseminated infection proved to be fatal. At this time, Florey decided to proceed with a pediatric patient, since a child would

require smaller doses more suited to the limited penicillin production capacity. A four-year-old boy with a severe staphylococcal facial infection experienced a marked clinical improvement with penicillin therapy, but he died unexpectedly of noninfectious complications. Autopsy revealed that healing of the infection was proceeding well at the time of death, and the efficacy of parenteral penicillin therapy had thus been demonstrated [7].

A series of 10 successes with staphylococcal and streptococcal infections followed, and penicillin soon enjoyed a proven role in the treatment of sepsis, conjunctivitis, and pneumonia. Penicillin was hailed as an effective therapy for staphylococcal infections in particular, but its action against "the pneumococcus" and other streptococci was also immediately appreciated. The agent's spectrum of use soon extended to include meningococcal, gonococcal, and syphilitic disease. In 1945, a Nobel Prize was awarded to Alexander Fleming, Howard Florey, and Ernst Chain in recognition of their efforts toward the development of penicillin as a desperately needed chemotherapeutic agent.

The first study of penicillin-resistant strains of staphylococci was made before the application of parenteral penicillin in clinical trials. During the mid-1930s, Craddock found that staphylococci could become resistant to penicillin after a brief exposure in vitro [11]. Resistance was not described in a publication until 1941, when Abraham and Chain reported that the sensitivity of *Staphylococcus* to penicillin could be decreased one thousandfold by sequentially subculturing organisms to media containing higher concentrations of penicillin [12]. Other workers found that passage of sensitive staphylococci in vivo with penicillin administered to the experimental animal failed to generate a penicillin-resistant strain, and the likelihood of emergence arising during a course of therapy was questioned. Furthermore, a reduction of virulence and of maximal growth rate were typically associated with the *in vitro* acquisition of resistance by a laboratory strain, and sensitivity to penicillin was restored upon subculturing to penicillin-free culture media [13]. Some interpreted this constellation of findings to mean that the emergence of resistant strains was of little clinical significance.

In 1940, Abraham and Chain had extracted a "penicillin-inactivating substance" from the Gram-negative *Bacillus coli (Escherichia coli),* which was shown to be a protein by virtue of its destruction by heating or papain treatment. Since *B. coli* was not killed by penicillin, it was proposed that the enzyme, dubbed penicillinase, could be a sole determinant of resistance in many species of bacteria. Enzyme activity was not detected in penicillin-sensitive strains of *S. aureus,* but activity somewhat less than that obtained in the *B. coli* preparation was detected in *Micrococcus lysodeikticus,* a species which was indeed less sensitive to penicillin than staphylococci [14]. Therefore it seemed that the amount of this

enzyme present in the bacterial cell had a major impact on the susceptibility of that organism to penicillin.

Further research, however, showed that the level of penicillin resistance expressed in *B. coli* strains could not be explained by the ability of the organisms to destroy the antibiotic. Moreover, strains of *S. aureus* made resistant to penicillin by subculturing into penicillin-containing media possessed no demonstrable enzyme activity [12]. The contribution of penicillinase to resistance in *S. aureus* was not established until 1944, when Kirby found that extracellular penicillinase produced by clinical isolates of *S. aureus* correlated with their degree of penicillin resistance [15]. The correlation was strengthened by the work of Spink and Ferris in 1945 [16]. Ironically, these investigators expressed skepticism as to the potential clinical significance of staphylococcal penicillin resistance. Bloomfield and Kirby wrote in 1944 that "the development of penicillin fastness probably plays little part in therapeutic failures," and they implicated inadequate dosage regimens, lack of adequate surgical drainage, and the hopelessness of overwhelming sepsis as proximate causes of failed therapy [17]. The next year, Spink and Ferris wrote that "the dissemination of occasional strains of staphylococci with permanent resistance may be associated with clinical failures . . . but thus far it does not appear to have much clinical significance" [18].

Nonetheless, evidence of clinically significant penicillin resistance rapidly accumulated. In 1943, Florey had reported that in 2 of 5 cases of disseminated *S. aureus* infection, sensitivity of the organism to penicillin was decreased after penicillin therapy [19]. Rammelkamp and Maxon reported that initial isolates were more susceptible than posttherapy isolates in 3 of 4 patients studied, and that poor clinical outcome in one case could be attributed to the resistance of the organism [20]. A 1946 study of *S. aureus* osteomyelitis treated with penicillin revealed that in 22 percent of cases, isolates obtained after antibiotic therapy demonstrated increased in vitro resistance to penicillin. The removal of penicillin from the bacterial environment (termination of pharmacologic therapy) did not result in a restoration of sensitivity: penicillin-resistant staphylococci were isolated from patients as long as a year after the discontinuation of penicillin [13]. This alarming discovery raised the spectre of resistant strains becoming endemic in hospitals and entrenched in individual patients. The authors emphasized the importance of employing adequate therapeutic dosages of penicillin in all infections, since prolonged exposure to inadequate levels of penicillin in slowly healing, poorly vascularized sinus tracts seemed to predispose to the emergence of resistant strains.

Reports of resistance were presented in academically-oriented specialty journals rather than medical publications of a broader scope, and this information did not generally reach the community practitioner in

the years immediately following World War II, according to Manson Meads, MD (personal communication). The lack of continuing education regarding the potential for resistance, coupled with reports of miraculous cures disseminated by the popular media and the spectacular successes physicians themselves witnessed upon the institution of penicillin therapy for staphylococcal and streptococcal infections, led to the application of penicillin to a wide spectrum of infectious and even noninfectious disease and therefore to intense selection pressure on the bacterial population for penicillin resistance. The prevalence of penicillinase-producing *S. aureus* rose rapidly with the widespread use of penicillin G after the Second World War, first in nosocomial bacterial populations and subsequently among community isolates. The frequency of fully penicillin-resistant strains of *S. aureus* in one British hospital climbed from 12.5 percent in 1946 to 36 percent in 1947 and 59 percent in 1948 [21].

The nature of genetic elements controlling penicillinase expression was explored during the 1960s. Several workers noted that penicillinase-positive strains of *S. aureus* frequently gave rise to penicillinase-negative variants, but that penicillin-sensitive strains did not seem to acquire the enzyme in vitro [22, 23]. The loss of penicillinase from resistant clinical strains appeared to be permanent, in that these strains did not spontaneously revert to their previous state of enzyme expression [24]. This conflicted with the observed behavior of other staphylococcal traits that had been subjected to genetic analysis. Novick, in his 1963 study of mutants of *S. aureus*, proposed an extrachromosomal mode of inheritance for the penicillinase genes, presumably involving the genetic element termed a "plasmid" by Lederberg in 1957. Novick suggested that the explosive increase in the prevalence of penicillinase-producing *Staphaureus* was not solely due to selection of *de novo* mutant strains by penicillin, but was accelerated by phage-mediated transduction of penicillinase genes and perhaps by "cell-to-cell contact" (plasmid transfer) analogous to that observed in Gram-negative bacterial conjugation [25]. The residence of the penicillinase gene on a plasmid also helped to explain the frequent development of multiple resistance, since plasmids had been found to carry and transmit several resistance determinants granting the organism improved survival in the presence of antibiotics of different classes. Indeed, a staphylococcal plasmid encoding penicillinase was isolated in 1969 [26]. Subsequent work has revealed over 20 different plasmids in *S. aureus* that encode both penicillinase and at least one resistance determinant to an unrelated antimicrobial agent [27].

Soon after the recognition of penicillinase as the major determinant of staphylococcal penicillin resistance, a search for specific inhibitors of this enzyme began. Housewright and Henry explored the use of

antipenicillinase serum in 1947, but found that this did not lead to increased in vitro penicillin sensitivity [28]. In 1950, Behrens and Garrison tested a number of compounds related to penicillin and found that some inhibited penicillinase in vitro, but none had any demonstrable effect on the susceptibility of penicillin-resistant bacteria [29]. In 1978, Neu described a beta-lactam derivative that irreversibly inhibited staphylococcal beta-lactamase in intact cells as well as in purified enzyme preparations. This agent, clavulanic acid, acts synergistically with penicillins and cephalosporins to inhibit and kill beta-lactamase–producing *S. aureus* [30]. In 1980 a second beta-lactamase inhibitor (sulbactam) was found to be effective, and it was noted that multiple in vitro passages in media containing ampicillin-sulbactam did not appear to select strains resistant to the combination [31]. As adjuvant therapy with ampicillin or amoxicillin respectively, sulbactam and clavulanic acid are employed today as antistaphylococcal chemotherapy.

Well before the successful application of penicillinase inhibitors, however, the development of semisynthetic penicillin derivatives provided a means of evading enzyme-mediated resistance. *Penicillium notatum* naturally produces a family of beta-lactam molecules (penicillins G, F, K, and X) that differ in the nature of an acyl group attached to the beta-lactam ring. Penicillin G was initially selected for intensive commercial production since it could be produced almost exclusively in large-scale fermentations by the addition of an appropriate side-chain precursor (phenylacetic acid) to the fermentation chamber [32]. The addition of alternate side-chain precursors to the fermentation chamber formed the basis of early pharmacologic exploration of the penicillin family, with the purpose of identifying semisynthetic members that might possess desirable pharmacologic properties. By this method penicillin V was obtained in 1948, although it was not fully appreciated as an oral agent until 1954.

It was hoped that these new beta-lactam compounds would demonstrate activity against penicillin-resistant staphylococci by serving as inferior substrates for beta-lactamase compared to penicillin G. Among the penicillin derivatives, it was found that antimicrobial activity (potency), affinity for penicillinase (enzyme inhibitory activity), and the ability to induce penicillinase varied independently. Since the mechanism of penicillin action was not well characterized in this era, it was not clear how best to modify the beta-lactam side chain to favor binding to targets while discouraging penicillinase access. Successful agents employed steric hindrance to protect the beta-lactam ring from hydrolysis, but used groups small enough to maintain the compound's bactericidal activity.

The chemical modification of penicillin G itself by in vitro substitution reactions began in 1949. Some new compounds of increased potency were soon obtained, but none expanded the bacterial spectrum of activ-

ity of penicillin G [32]. The 1957 isolation of the penicillin "nucleus" (6-aminopenicillanic acid) in quantity from fermentations provided a more convenient and chemically flexible starting material, and this substrate allowed the development of a large series of useful semisynthetic penicillins. The development of 2,6-dimethoxyphenyl penicillin (methicillin) in 1959 represented the most useful early product of semisynthetic penicillin research, for although methicillin possessed somewhat lower intrinsic antibacterial activity than penicillin G and strongly induced penicillinase synthesis, it was very weakly hydrolyzed by the enzyme. Synthesis of nafcillin and oxacillin soon followed, offering oral administration potential. The introduction of a halogen onto the acyl side chain (cloxacillin, dicloxacillin, flucloxacillin) was found to yield even higher blood levels after oral administration. The primary clinical utility of this group of penicillin derivatives is reflected in the term commonly applied to them: antistaphylococcal penicillins.

The first report of a clinical *S. aureus* isolate resistant to methicillin appeared in 1961, almost immediately after the introduction of the drug [33]. Several authors commented that this was an anecdotal finding, and in fact they expressed surprise that "naturally" occurring methicillin-resistant strains were not more common. A cluster of methicillin-resistant *S. aureus* (MRSA) was described in 1965 [34], but throughout the remainder of that decade methicillin resistance seemed to be rare and unrelated to prior use of the drug in individual patients or hospitals [35]. A sudden increase in the frequency of MRSA infections occurred in the United Kingdom in 1968, and MRSA epidemics began sweeping U.S. hospitals in the late 1970s. The spectrum of disease caused by MRSA came to resemble that of sensitive strains in terms of infection site and prognosis, and the lack of clinical response to beta-lactam antibiotics made such infections all the more ominous.

A mechanism for methicillin resistance was not elucidated as quickly after its emergence as was the penicillinase mechanism of penicillin resistance. The broad-spectrum beta-lactam resistance seen in methicillin resistance resembled the resistance created in vitro that was described before World War II, and the mechanism underlying this phenomenon was thrust into the spotlight of active research.

Understanding of methicillin resistance was achieved through diligent study of penicillin's mechanism of action on staphylococci. Early investigators encountered a bewildering array of metabolic and morphologic changes resulting from penicillin exposure, and confusion surrounded the fundamental nature of the drug. Ensuing study of the bacterial cell wall led to the 1965 conclusion that beta-lactam antibiotics inhibit transpeptidase reactions, which generate cross-links in the peptidoglycan component of the cell wall. This appears to be the result of the stereochemical resemblance of beta-lactams to D-alanyl D-alanine, a

component of these peptide bridges and, accordingly, a substrate of the transpeptidase [36, 37]. Four cell membrane proteins that bind penicillin have been identified in staphylococci, and evidence suggests that the reactions catalyzed by two of these "penicillin-binding proteins" (PBP-2 and PBP-3) are critical to the maintenance of cell wall stability [38]. Inhibition of these targets by beta-lactam drugs disrupts the synthesis-breakdown cycle of the cell wall. The result is a leakage of molecules believed to inhibit autolytic enzymes [39]. The uninhibited action of "autolysins" then weakens the cell wall to the point of osmotic lysis.

Since nearly all MRSA strains expressed high levels of penicillinase, many investigators originally felt that methicillin resistance was due to a second factor acting synergistically with the weak enzyme-mediated hydrolysis of methicillin. However, methicillin resistance was subsequently shown to be wholly independent of penicillinase activity. Furthermore, the resistance determinant responsible for methicillin resistance was localized to the bacterial chromosome, which contrasted with the plasmid-borne nature of staphylococcal penicillinase. Subsequently, attention was focused on the cell wall and the possibility that in methicillin-resistant strains, a fundamental change had occurred in the interaction of beta-lactam drugs with the bacterium. Bruns and Keppeler found in 1976 that methicillin-resistant strains bound less benzylpenicillin than did sensitive strains [40]. In 1981, Hartman and Tomasz demonstrated that altered penicillin-binding characteristics were due to the presence of a modified PBP-2 (termed PBP-2a or PBP-2′) in methicillin-resistant strains [41]. It appears that the reduced affinity of PBP-2a creates broad-spectrum beta-lactam resistance in MRSA by depriving these drugs of their major targets.

Currently, penicillin-sensitive strains comprise only 10 percent of *S. aureus* clinical isolates. The remaining isolates are resistant to penicillins due to the production of extracellular beta-lactamase. Typically, 30 to 50 percent of penicillin-resistant organisms originating from tertiary-care centers also express resistance to methicillin and other "penicillinase-resistant" penicillins and to cephalosporins. Nosocomial methicillin-resistant strains remain the most threatening representatives of the species. As with most nosocomial pathogens, simultaneous resistance involving two or more major classes of antimicrobial agents is common in these strains. Nearly all MRSA strains are susceptible to vancomycin, but this agent is much more expensive and somewhat more toxic to the patient than are most beta-lactams.

Serious problems with MRSA are unusual in small community hospitals and appear to be restricted to the physical facilities and patient populations of large referral centers. Colonized or infected patients serve as the major reservoir of MRSA strains, and colonization of health-care personnel provides a minor reservoir. Accordingly, transient

carriage on the skin (especially in lesions of dermatitis) and in the anterior nares of health-care personnel is the dominant mode of transmission among patients. Airborne spread also occurs and is of particular importance in burn units [42]. In some regions (e.g., Detroit) MRSA is isolated from community-acquired infections, particularly among intravenous drug users [43]. The patient population of a tertiary-care center possesses multiple risk factors for MRSA colonization that are not shared by most patients in community hospitals. These factors include high-dose or prolonged antimicrobial therapy, physical clustering of patients in special-care units (e.g., intensive care, burn), more frequent contact with health-care workers who may transmit the organism, and the generally more debilitated, immuno-compromised, infection-prone state of these patients [42]. Colonization carries a 30 to 60 percent risk of developing clinical infection, and patients may remain colonized for up to one year [44].

For nearly a century after their initial isolation, coagulase-negative staphylococci (CNS) were considered to be harmless saprophytes that were ubiquitous on human skin, the antithesis of *S. aureus*. Recognized infections caused by CNS prior to 1958 were few, and mostly comprised rheumatic heart disease–associated endocarditis, but the frequency of isolation rose in the 1970s as CNS infections became an increasingly common complication of foreign-body placement. It appears that the increased use of temporary or permanently implanted devices (central venous catheters for parenteral nutrition, central nervous system shunts, prosthetic cardiac valves, vascular grafts, and such) has created a pathogenic niche for CNS, perhaps facilitated by bacterial glycoproteins mediating adherence to synthetic materials [44].

Coagulase-negative staphylococci emerged to become a leading pathogen of nosocomial bacteremia by the mid-1980s. In immuno-compromised patients, the vast majority of intravenous catheters become colonized with *S. epidermidis* [45], and it is this group of patients who risk the greatest morbidity from nosocomial infections. Distinguishing mere colonization from true bacteremia is difficult, due to frequent contamination of blood cultures by skin microflora during venipuncture. Clinical judgment therefore remains critical in this diagnosis.

Penicillin resistance in CNS evolved in parallel with that of its more virulent relative, *S. aureus*. All staphylococci are believed to freely exchange plasmids carrying, among other information, antiobiotic-resistance determinants. Methicillin resistance is as common among nosocomial CNS populations as in coexisting *S. aureus*. Maki and Stevens presented data in 1982 suggesting that preoperative beta-lactam prophylaxis displaces the "normal" antibiotic-sensitive flora from the skin of patients and increases colonization by beta-lactam–resistant nosocomial strains. This effectively increases these patients' risk of developing CNS

infection, since these organisms are such successful residents of human skin [46].

The lessons learned from the evolution of staphylococcal penicillin resistance have been rigorously applied to other pathogens and to other antimicrobial agents, and a change of attitude toward new antibiotics has taken place since the early years of penicillin use. Prescription of several broad-spectrum antimicrobials (e.g., imipenem) is tightly restricted in the large medical facilities where nosocomial resistant strains are most prevalent, and physicians in community hospitals are also encouraged to practice restraint in their use. Policies which reserve the application of newer agents for life-threatening infections are designed to limit exposure of the resident microflora to the new agents, which in turn minimizes the selection pressure for resistance. In striking contrast to the dismissal of penicillin-resistant strains as anecdotal or clinically insignificant by early researchers, we now assume that new antimicrobial agents will only be useful for the relatively short period of time required for mutation and natural selection to forge subpopulations of resistant organisms. Staphylococci have taught us that the emergence of clinically significant antibiotic resistance must be considered not a question of whether, but of when.

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