Mycophenolic Acid: A One Hundred Year Odyssey from Antibiotic to Immunosuppressant

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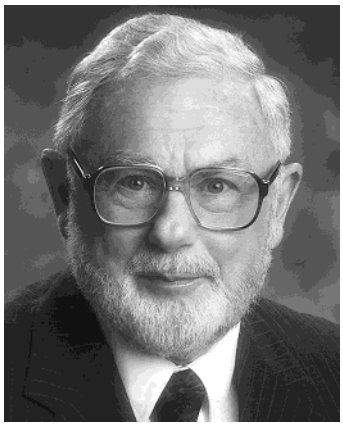
I. Introduction

Among natural products, one microbial metabolite—penicillin—stands above all others for its profound influence on human lives. The development of penicillin by Howard Florey and his colleagues opened the door to the golden age of antibiotics that has dominated medical practice for more than five decades. Penicillin is now acclaimed as a miracle drug for combating bacterial infections, although in rare cases it causes severe allergic reactions. A post-penicillin hunt for further antibiotics began in the 1940s and resulted eventually in the discovery of thousands of microbial metabolites with a wide array of biological properties. Some of these metabolites were useful, for instance, in medicine, but a few were mycotoxins with unfortunate consequences. One early discovery in this search was that a strain of *Penicillium brevicompactum* produced a material inhibiting growth of *Staphylococcus aureus*.[1] The active agent was identified as a known compound, mycophenolic



Ronald Bentley, born in England, received his Ph.D. degree from Imperial College, London, for work on penicillin chemistry and attempted synthesis (1943–1946). He was a Commonwealth Fund Fellow at Columbia University's College of Physicians and Surgeons, 1946–1947, studying stable isotope tracer techniques and from 1948 to 1952 was responsible for a mass spectrometer facility for stable isotope analysis at The National Institute for Medical Research, London. Emigrating to the United States, he joined the faculty of the University of Pittsburgh, originally as Assistant Professor in the Department of Biochemistry and Nutrition, Graduate School of Public Health, eventually becoming Professor of Biochemistry in the Department of Biological Sciences in 1960. He was retired (mandatorily at age 70) as Professor Emeritus in 1992. He was a Public Health Service Special Fellow at Hopkins Marine Station, Stanford University, in the summer of 1960 and held a John Simon Guggenheim Memorial Foundation Fellowship at the Institute of Biochemistry, University of Lund, Sweden, in 1963–1964. His research has focused on the biosynthesis of various microbial secondary metabolites and on the biosynthesis of ubiquinone and menaquinone (vitamin K_2). Other interests have included carbohydrate conformations and stereochemical implications in biology. Since retiring, he has written on a variety of topics and was one of six general editors for *The Oxford Dictionary of Biochemistry and Molecular Biology* (1997 and revised edition, 2000).

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acid (MPA),[2,3] that had been discovered as early as 1893 by an Italian physician, Bartolomeo Gosio.[4−6] Moreover, Gosio had observed that his fungal metabolite, a purified, crystalline compound, inhibited the growth of the anthrax bacillus. In consequence, Florey stated that "mycophenolic acid enjoys the distinction of being the first antibiotic produced by a mould to be crystallised".[2] In fact, it is the first purified antibiotic from any source.

In the more than 100 years since Gosio's serendipitous discovery (he was looking for a metabolite causing pellagra) and especially in the last half-century, MPA has become something of a cure-all and has been used in the treatment of various disorders. In a wondrous turn of events, an ester of mycophenolic acid, mycophenolate mofetil (MMF, brand name CellCept), has been widely used as an immunosuppressant in kidney, heart, and liver transplantation and as a medication to treat other ailments.

This review attempts to trace the century of work leading from an initially obscure fungal metabolite to a powerful member of the physician's armamentarium. In addition, recent research on MPA and related compounds and on their molecular target, inosine 5′-monophosphate dehydrogenase (IMPDH), will be reviewed. An eclectic approach will be necessary in view of the vast literature that has accumulated in recent years, and the emphasis will be on chemical and biochemical investigations with only brief mentions of clinical developments. A general review of MMF appeared in 1994,[7] and very recently several articles have focused on IMPDH and medical implications.[8−14]

II. Antibiosis and Antibiotic

Just prior to Gosio's work, the French word *antibiose* had been coined to describe antagonistic effects between microorganisms; it was the opposite of symbiosis ("C'est exactement l'inverse qui se passe dans la *symbiose*").[15] There had been many early investigations of this phenomenon, particularly of the antibiosis between fungi and bacteria; Gosio's observation was a further example, uniquely with a pure compound. Therapeutic possibilities were considered and, to a limited extent, implemented.[16] Strangely, neither Fleming's classic penicillin paper[17] nor the first Oxford penicillin publication[18] used the word antibiosis.

Selman Waksman, beginning in 1941, defined an antibiotic as "a chemical substance, produced by microorganisms, which has the capacity to inhibit the growth and even to destroy bacteria and other microorganisms, in dilute solutions".[19] An expanded definition now includes sources other than microorganisms as well as chemically synthesized materials. Surprisingly, workers in 1894 had used the modern meaning when they wrote "The eosinophile cells... with glandular powers directed to the production of a bactericidal, or at least antibiotic, substance".[20]

More rigidly, the definition of an antibiotic should include the stipulation that the substance had been isolated and tested in a pure state.[21] This stipulation was not met by penicillin until it was fully purified as a crystalline sodium salt in 1943. However, Gosio's material certainly merits the antibiotic classification; it was isolated as a pure crystalline material and at the same time was shown to inhibit the growth of the anthrax bacillus. Florey's gracious accolade is very well justified. Unhappily, it came too late for Gosio to have been aware of it since he died in 1944.

III. Mycophenolic Acid

A. Discovery

Before the role of vitamins in human nutrition was clarified, pellagra was associated with diets relying heavily on corn (maize). Corn was subject to fungal deterioration, and the suitability of deteriorated corn for human consumption was much debated. Writing in 1910, Black and Alsberg noted that the poisonous nature of spoiled corn was of little concern in the United States.[22] They continued, "That it is unsafe and the cause of pellagra is so firmly believed in Italy and the Austrian province of the Tyrol that the governments of these countries have enacted stringent laws regulating the quality of corn and corn meal which may be sold or imported". It was believed that toxic metabolites (possibly alkaloids) produced by fungal deterioration of corn might have an injurious effect in animals and humans and thus account for the symptoms of pellagra. Many investigators studied this possibility using mixtures of fungi under varying and ill-defined conditions, particularly with respect to the growth media (corn, wheat, bread). This early and confusing research is described in papers that are now difficult to access, but the work from 1870 to 1910 has been documented.[22,23]

To determine the extent of corn spoilage, Alsberg and Black recommended the determination of acidity under carefully controlled conditions—spoilage was associated with high levels of acid. It is a measure of societal change that they stated that any physician should be able to carry out their test with accuracy since "it was far easier than to determine the acidity of gastric juice, a determination with which every physician is familiar". As the 21st century opens, few physicians in general practice would qualify to determine corn acidity! In Italy, Gosio noted that extracts of deteriorated corn samples gave blue to violet colorations with ferric chloride solutions and proposed this as a convenient test for spoilage. Both the determination of acidity and the ferric chloride test had important roles in the MPA story.

Gosio began to study the possible relationship between deteriorated corn and pellagra in 1892. In November of 1896, he published in two parts a detailed account ("Memorie Originali") of his work with the title "Bacteriological and chemical studies on the adulteration of maize"; the work was described as a contribution to the etiology of pellagra.[5,6] A third paper, coauthored with E. Ferrati, was published in December of the same year.[24] These papers contain extensive discussion of the pellagra problem and of the toxic properties of adulterated maize as well as an account of original research.

The 1896 papers of Gosio are usually cited for the initial discovery of the material that was later termed mycophenolic acid. However, there is a clear descrip-

tion of the phenolic acid and its purification by fractional crystallization in a little known preliminary account by Gosio given at a session on May 16, 1893, of the Reale Accademia Medicina di Torino.[4] This account also records the antibiotic activity of the phenolic acid—"Sperimentati su culture pure di bacilli del carbonchio, dimostrarono notevole potere antisettica". Gosio also refers somewhat vaguely to another preliminary communication ("una nota preventiva apparsa") apparently to ward off possible competition. This appears misdated in Gosio's publication but may have been given in September of 1892; it has not been possible to locate it. In any event, the discovery of mycophenolic acid can be more correctly dated to 1893 rather than 1896.

Gosio's big step forward was to investigate pure fungal cultures growing on Raulin's solution, a simple, well-defined medium. The fungus, a green species of *Penicillium*, was said to be *penicillium (sic) glaucum*. Unfortunately, this name was then used indiscriminately for several green or blue-green fungi and much confusion has been caused;[25] it is no longer considered as an "accepted species". Thus, although Gosio clearly used a pure *Penicillium* culture it is not possible to be completely sure of which species (see later). Culture filtrates of this organism yielded a crystalline material, mp 143−144[5] or 145 °C;[6] both culture filtrates and isolated material gave a blue to violet color with ferric chloride solutions, indicative of phenolic properties. Moreover, the culture medium produced symptoms resembling phenol poisoning in various animals.

Gosio lamented that he had insufficient material to determine whether the isolated and purified material also produced poisoning in animals. However, he provided evidence for an "azione antisettica".[4,5] When 50 mg of his compound was dissolved in a little soda ("piccola quantità di soda") and then added to 5 mL of Löffler's broth, there was an inhibition of the growth of the anthrax bacillus; as just indicated, the latter was very expressively named as "bacilli del carbonchio" (carbuncle bacillus).

Biographical information on Gosio in English is scarce; however, he made another distinguished contribution to microbiology by observing the formation of a volatile material, "Gosio gas", when a fungus (now known as *Scopulariopsis brevicaulis)* grew in the presence of arsenic; the gas was later shown to be trimethylarsine.[26] He participated extensively in Italian anti-malarial campaigns with Robert Koch.

Alsberg and Black, working for the Bureau of Plant Industry, United States Department of Agriculture, in the curiously named "Office of Drug-Plant, Poisonous-Plant, Physiological, and Fermentations", were described as "Chemical Biologists". Following their studies of acidity in spoiled corn, they used corn-derived *Penicillium puberulum* and isolated a new acidic metabolite named penicillic acid.[23] This material gave a brownish-red color with ferric chloride, was toxic to various animals, and inhibited the growth of "*Bacillus coli*". It was clearly not Gosio's material; it is now recognized as the first well-characterized mycotoxin.

Fungi from spoiled U.S. corn samples tended to give a red to red-brown coloration with ferric chloride rather than the blue-violet described by Gosio. However, from deteriorated Italian corn, a colleague of Alsberg and Black isolated a fungus identified as *Penicillium stoloniferum*. Culture filtrates of this organism, grown in Raulin's solution, did give the characteristic blue to violet coloration with ferric chloride. Alsberg and Black then isolated a second acidic metabolite which they named as mycophenolic acid (i.e., an acidic phenol from a fungus).[23] The average yield after 2 weeks incubation at room temperature was about 0.3 g L^{-1}.

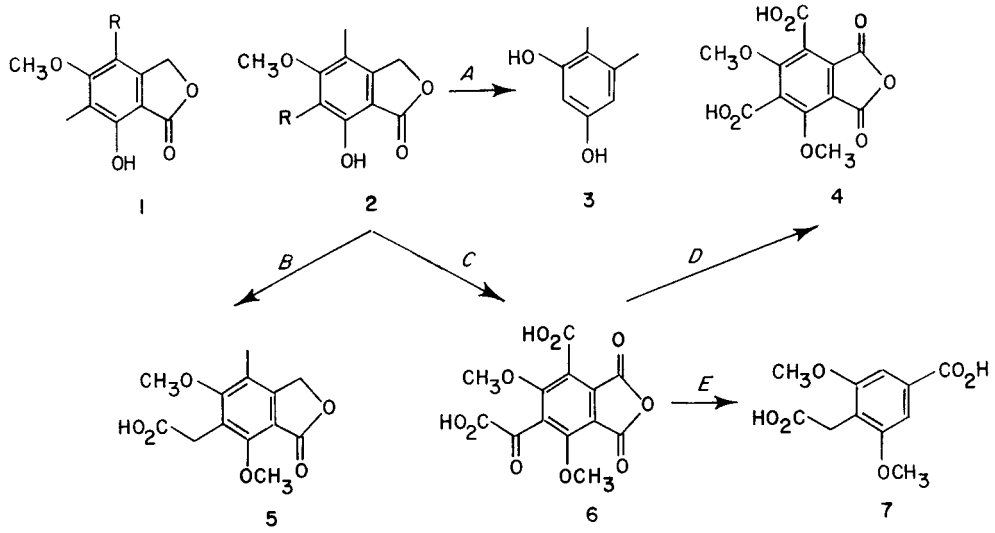
Alsberg and Black stated that MPA could not be identified with "any known compound" and that Gosio's substance "very greatly resembles it (though) these substances are probably not identical".[23] Surprisingly, they did not note the close correspondence of the melting points of the two materials—MPA, mp 140 °C, uncorrected, and Gosio's substance, mp 143−144 °C; modern sources give the mp of MPA as 141 °C. The difference in these values could well have been due to the use of different equipment and the possible use of uncorrected thermometers. Alsberg and Black focused their attention on the differences in the analytical data (see later) and ferric chloride color reactions. Gosio reported an intense blue color in ethanol, whereas Alsberg and Black observed a violet color when MPA was treated with a trace of ferric chloride; the color became green with excess reagent. Their unwillingness to accept that Gosio's substance was MPA may have stemmed from concern with priority issues.

In 1932, Raistrick and colleagues examined a number of species or strains of *Penicillium* belonging to the *P. brevicompactum* series as part of their extensive investigations of fungal metabolism.[27] From several of these they isolated MPA, mp 141 °C, and stated "There is, in our opinion, no doubt that Gosio's acid and Alsberg and Black's mycophenolic acid are one and the same substance". While there has never been a direct comparison, Raistrick's conclusion is now generally accepted. With ferric chloride they found "a pure blue colour" in alcoholic solution, noting parenthetically that "Alsberg and Black say a green color"; in aqueous solution with ferric chloride the color was blue-violet. Probably the supposed differences quoted by Alsberg and Black arose from the use of different concentrations of MPA and the ferric chloride reagent and the presence of possible impurities. In view of Raistrick's work, it is most likely that Gosio's organism was a strain of *Penicillium brevicompactum*. In the early literature and in the classic *Manual of the Penicillia*,[25] the species name is hyphenated as *brevi-compactum*. In the more recent literature and in a monograph,[28] the hyphen is omitted. A further complexity is that *Penicillium stoloniferum* has at various times been regarded as different from or synonymous with *Penicillium brevicompactum*. In 1979 it was stated that "*Penicillium stoloniferum* has again been reduced to synonymy".[28]

B. Chemistry

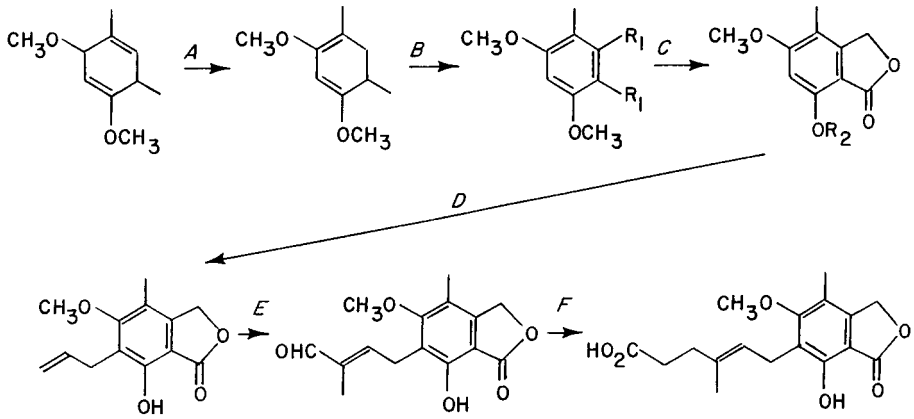
Gosio carefully reported a variety of properties for his material; it was slightly soluble in cold water but

Scheme 1. Structure[a] of Mycophenolic Acid[31,32]



[a] R = $-CH_2-CH=C(CH_3)-(CH_2)_2-COOH$. Reagents are as follows: *A* = KOH; *B* = O_3 on monomethyl ether; *C* = $KMnO_4$ on monomethyl ether; *D* = H_2O_2; *E* = a, HI, P; b, $(CH_3O)_2SO_2$; c, alkaline hydrolysis.

Scheme 2. Synthesis[a] of Mycophenolic Acid[35]



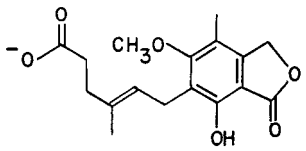
[a] $R_1 = -COOCH_3$; $R_2 = -CH_2-CH=CH_2$. Reagents are as follows: *A*, potassium *tert*-butoxide in dimethyl sulfoxide; *B*, dimethyl acetylenedicarboxylate; *C*, boron trichloride (selective demethylation) to yield *o*-hydroxyphthalic ester, form anhydride, Zn/HCl/AcOH, allyl bromide, potassium carbonate; *D*, thermal rearrangement; *E*, O_3, $(C_6H_5)_3-P=C(CH_3)CHO$; *F*, triethyl phosphonoacetate carbanion, hydrolysis, diimide.

freely soluble in hot water, ethanol, and ether, did not contain nitrogen, gave a negative Millon reaction, did not react with phenylhydrazine, and did not reduce Fehling's solution. From a single C, H combustion, Gosio concluded that his material had the empirical formula $C^9H^{10}O^3$ (*sic*): found, C, 65.21%; H, 6.34%; calculated for $C_9H_{10}O_3$, C, 65.06%; H, 6.02%. Despite a discrepancy with melting points, Gosio believed his material was "acido paraidrocumarico". Alsberg and Black translated this name as *p*-hydrocumaric (*sic*) acid but it is more appropriately termed 3-(4-hydroxyphenyl)propanoic acid (*p*-hydroxyhydrocinnamic acid, dihydro-*p*-coumaric acid, phloretic acid). However, this was not the correct structure for Gosio's material.

Alsberg and Black established the correct molecular formula for MPA as $C_{17}H_{20}O_6$, obtaining a molecular mass of 345 Da (titration), 328 Da (barium content of salt), and 314.5 Da (boiling-point elevation); the calculated value is 320 Da.[23] The material behaved as a dibasic acid. The slow progress to structure determination has been described,[29] and only a summary will be given here. Raistrick's group confirmed the molecular formula and found one CH_3O group, a single phenolic hydroxyl, a COOH group which could be esterified, a lactone group (hence accounting for the second titratable group), and the presence of a double bond.[27,30] Potassium hydroxide fusion gave 1,5-dihydroxy-3,4-dimethylbenzene (**3**). Although a tentative structure was proposed, further work several years later necessitated a revision leading to the two possibilities (**1, 2**).[31] In 1952, more than five and a half decades after Gosio's original isolation, Birkinshaw et al.[32] reinterpreted the structures of oxidation products (Scheme 1). From the monomethyl ether of MPA was obtained the phthalide (**5**) and the phthalic anhydride (**6**); the latter gave variously a second phthalic anhydride (**4**) and the benzoic acid (**7**). This work led to the now accepted structure for MPA, (*E*)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoic acid (**2**).

The conclusion was confirmed by synthesis of the degradation product 6-carboxymethyl-5,7-dimethoxy-4-methylphthalide (**5**),[33] by infrared spectroscopy,[34] and by total synthesis of MPA itself, the first being

in 1969 (Scheme 2).[35] There have been several other chemical syntheses; the most recent descriptions in 1997 and 1998 provide citations to the earlier work.[36,37] Moreover, the overall structure for MPA and the fact that the side chain double bond had the *E* (trans) configuration (**8**) were substantiated by a determination of the crystal structure.[38]



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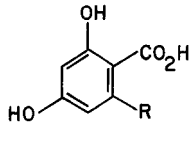

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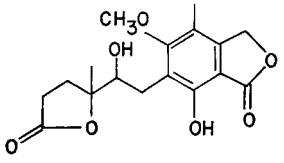

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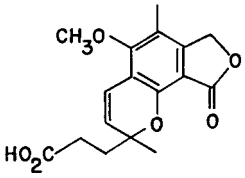

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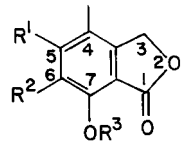
In addition to MPA, *Penicillium brevicompactum* produces other secondary metabolites in varying amounts.[39] In early work, Raistrick and colleagues isolated 3,5-dihydroxyphthalic acid (**9**, R = COOH), 2-carboxy-3,5-dihydroxybenzyl methyl ketone (**9**, R = $CH_2-CO-CH_3$), 2-carboxy-3,5-dihydroxyphenyl acetyl carbinol (**9**, R = $CHOH-CO-CH_3$), and 2,4-dihydroxy-6-pyruvylbenzoic acid (**9**, R = $CO-CO-CH_3$). Many years later, three further secondary metabolites more closely related to the MPA structure were isolated; they were the ethyl ester of MPA, its diol lactone (**10**), and a material named mycochromenic acid (**11**).[40] These metabolites are presumably derived from MPA itself. It is of interest to note the march of progress. While Raistrick's group had used tedious "classical" techniques for structure determination, the later investigators took advantage of newer technologies such as mass spectrometry, nuclear magnetic resonance spectroscopy, and infrared spectroscopy. Demethylmycophenolic acid (nor-*O*-methylmycophenolic acid) is also present in *P. brevicompactum* fermentations.[41] Other metabolites in the MPA biosynthetic pathway are discussed under Biosynthesis.

Several secondary metabolites containing a phthalide moiety, without the characteristic C_7 side chain, but with other substituents similar to those of MPA have been isolated from various organisms. They are as follows, using phthalide numbering (**12**): unidentified fungus, 6-formyl-7-hydroxy-5-methoxy-4-methylphthalide (**12**, $R^1 = CH_3O$, $R^2 = CHO$, $R^3 = H$); *Aspergillus flavus*, 5,7-dihydroxy-4-methylphthalide (**12**, $R^1 = OH$, $R^2 = R^3 = H$); *Penicillium* sp., 4,6-dimethyl-7-methoxyphthalide (**12**, $R^1 = H$, $R^2 = R^3 = CH_3$); *Penicillium gladioli*, 4,6-dimethyl-7-hydroxyphthalide (**12**, $R^1 = R^3 = H$, $R^2 = CH_3$); *Alectoria nigricans*, 5,7-dihydroxy-6-methylphthalide (**13**, $R^1 = H$, $R^2 = CH_3$); *Aspergillus terreus*, 4,5,7-trihydroxy-6-methylphthalide (**13**, $R^1 = OH$, $R^2 = CH_3$). Another phthalide structure, involved in MPA biosynthesis, is discussed later. Moreover, there are related benzenoid structures containing a COOH group and usually a second one-carbon group (e.g., CH_3, CH_2OH, CHO) in the *ortho* position. They are as follows: *Penicillium cyclopium*, cyclopaldic acid (**14**, $R^1 = R^3 = CHO$, $R^2 = CH_3$) and cyclopolic acid (**14**, $R^1 = CHO$, $R^2 = CH_3$, $R^3 = CH_2OH$); *Penicillium* sp., 2,3,5-trimethyl-4,6-dihydroxybenzoic acid (**14**, $R^1 = R^3 = CH_3$, $R^2 = H$); *Penicillium* sp., 3,5-dihydroxy-4-methylphthalaldehydic acid (**15**, $R^1 = H$, $R^2 = CHO$); *Aspergillus terreus* and *Penicillium spinulosum*, 3-methylorsellinic acid (**15**, $R^1 = H$, $R^2 = CH_3$); *Myrothecium* sp., rhizonic acid (**15**, $R^1 = CH_3$, $R^2 = H$).



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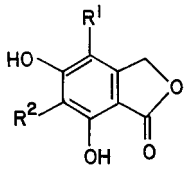

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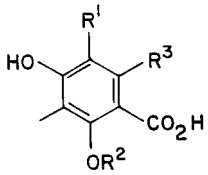

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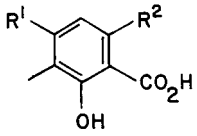

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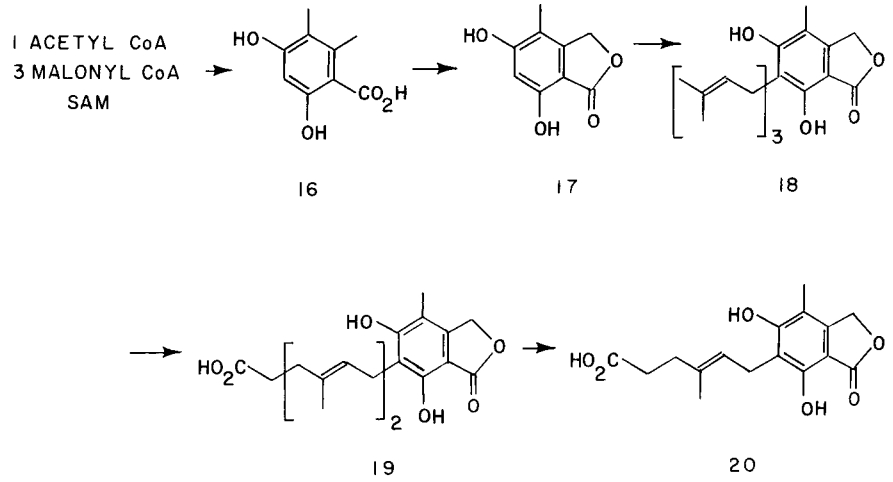
C. Biosynthesis

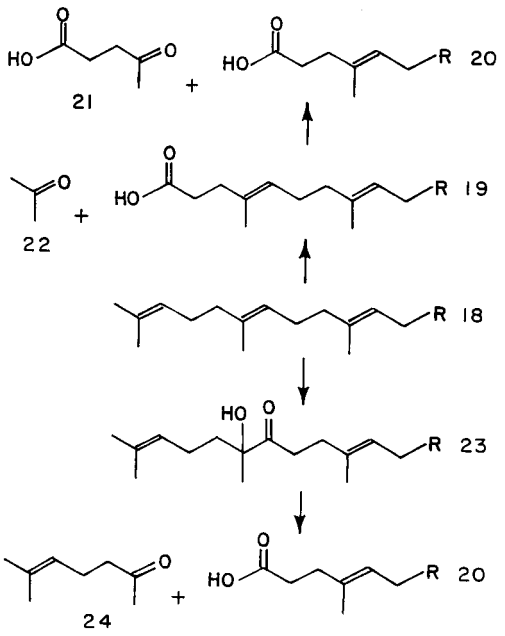
MPA biosynthesis involves two of the major pathways for secondary metabolite formation—the polyketide and isoprenoid pathways as well as methylation reactions on oxygen and carbon atoms (Scheme 3). A major role for acetate as a precursor was established in 1957; the labeling pattern from [1-^{14}C]-acetate was an alternating one with three labeled atoms each in the side chain and benzene ring and one in the carbonyl of the lactone group.[42] This observation was consistent with a polyketide origin for the aromatic nucleus and attached lactone and a mevalonoid origin for the side chain. A study of mevalonic acid utilization initially suggested that the seven carbon atom side chain derived by degradation of a C_{10} geranyl unit.[34] Subsequently, it was shown that the C_{15} farnesyl diphosphate provides these atoms and 6-farnesyl-5,7-dihydroxy-4-methylphthalide (**18**) is well established as an intermediate.[41] To proceed from this material to the penultimate intermediate, demethylmycophenolic acid (**20**), eight carbon atoms must be removed. There are apparently two side by side mechanisms to achieve this chain shortening (Scheme 4).[41] One involves two oxidative cleavages at the two side chain double bonds remote from the aromatic ring with levulinic acid (**21**) and acetone (**22**) being identified in *Penicillium brevicompactum* cultures as the two expected degradation products.[43] Compound **19** was shown to be a natural metabolite of *P. brevicompactum* and a biosynthetic intermediate to MPA.[44] Although described as "a prenylogue of mycophenolic acid", it lacks the necessary methyl on oxygen. The second process is a direct oxidation at the central double bond of the farnesyl side chain. Evidence for this reaction is that com-

Scheme 3. Biosynthesis of Mycophenolic Acid[a]



[a] SAM = *S*-adenosylmethionine. The tetraketide intermediate is methylated prior to cyclization to **16**. Following phthalide formation to **17**, reaction with farnesyl diphosphate yields **18**. Degradation of the side chain (see Scheme 4) yields demethylmycophenolic acid, **20**. The final methylation of **20** with a second molecule of SAM to yield MPA, **2**, is not shown.

Scheme 4. Degradation of the C_{15} Farnesyl Side Chain in Mycophenolic Acid Biosynthesis[a]



[a] R = phthalide nucleus. The two possible degradations are discussed in the text.

pounds with oxygen functions at this double bond ($-CH_2-CO-$ or $-CHOH-CO-$) were converted to MPA and could be recovered by isotope trapping experiments. The failure to recover the hydroxyketone (**23**) or the expected 6-methylhept-5-en-2-one (**24**)[43] in fermentation broths was explained by the low level of the endogenous pools of these materials since they presumably undergo further rapid transformations. The two pathways were regarded as being of equal importance. The initial step in the oxidations was postulated to be an epoxidation. The origin of the two methyl groups, one attached to oxygen and the other to carbon, was from the transferable methyl group of *S*-adenosylmethionine. The methylation on carbon was the first observation of this process.[34]

In summary (Scheme 3), a typical acetate−polymalonate condensation with a methylation prior to cyclization leads to the aromatic structure, 5-methylorsellinic acid (**16**); orsellinic acid itself is not utilized as a precursor. Lactone formation to **17** is followed by addition of the C_{15} farnesyl unit with formation of **18**. Oxidation of this unit by the two degradative pathways leads to the C_7 side chain found in **20**. Addition of methyl at the appropriate phenolic hydroxyl group of **20** is the final step. In basic terms, MPA is a simple tetraketide structure, modified by two methyl additions (one on C, one on O) and by addition of an isoprenoid derived C_7 side chain. The "Raistrick phenols" accompanying MPA are either the simple tetraketide, 3,5-dihydroxyphthalic acid (**9**, R = COOH), presumably derived by oxidation of orsellinic acid, or pentaketides (**9**, R = $CH_2-CO-CH_3$, $CHOH-CO-CH_3$, or $CO-CO-CH_3$). The other naturally occurring phthalide structures (**12, 13**) are also tetraketides modified by addition of either one or two methyls on C and sometimes with methylation of an OH group. The related, non-phthalide structures (**14, 15**) are derived similarly. The predicted labeling pattern from [1-^{14}C]acetate was demonstrated for cyclopaldic acid (**14**, $R^1 = R^3 =$ CHO, $R^2 = CH_3$).[45] In one respect, MPA biosynthesis is atypical. While many secondary metabolites are formed in the so-called idiophase after balanced growth (trophophase) is completed, MPA was produced in parallel with growth in both batch and continuous flow cultures.[43]

Addition of farnesyl groups to aromatic polyketides, as in MPA biosynthesis, is well documented. For siccanin biosynthesis by *Helminthosporium siccans*, the addition is to orsellinic acid,[46] and for austalide D formation by *Aspergillus ustis*, the addition is to 5-methylorsellinic acid; the same intermediate (**18**) is formed as in MPA biosynthesis.[47] For both cases, complex terpenoid rearrangements are subsequently involved.

D. General Biological Properties

MPA has been found to affect many physiological processes, and it is convenient to describe early

investigations prior to about 1970 at this time. More recent observations are discussed later. In view of Gosio's observations,[4,5] it is ironic that MPA has apparently never been retested against the anthrax organism. When MPA was rediscovered as an antibiotic,[1−3] detailed studies indicated that it was more active against Gram-positive than Gram-negative bacteria and that some resistance to the antibiotic developed with *Staphylococcus aureus*.[2,3] In mice (weighing 18−21 g) intravenous injection of 1 mg of sodium mycophenolate in water (0.1 mL) had no toxic effect, 5 mg gave "prolonged sickness", and about 10 mg was a lethal dose.[2,3] MPA has sometimes been classified as a mycotoxin since it does have some animal toxicity. Relatively high doses, however, are required as the following data for LD_{50} values (mg kg^{-1}) indicate.[29]

animal	oral administration	intravenous administration
rat	700	450
mouse	2500	550

However, as will be seen, MPA can be used in long-term human therapy, typically with dosage of 2 or 3 g day^{-1} of the 2-morpholinoethyl ester, MMF. It is interesting to compare the data for MPA with that for penicillic acid, the other compound discovered as a metabolite of *Penicillium puberulum* in the study of moldy corn.[23] For mice, penicillic acid shows an LD_{50} of 100 mg kg^{-1} for subcutaneous administration; moreover, unlike MPA, this material is also a carcinogen.

In tests of MPA against saprophytic fungi, development of the organisms generally tended to be retarded; with fungi pathogenic to humans significant growth inhibitions were observed.[2] Much later, in 1968, *Cryptococcus neoformans* and *Blastomyces dermatitidis* were shown to be inhibited by a low level of MPA (minimum inhibitory concentration, 12.5 μg ml^{-1}), *Candida albicans* and *Coccidiodes immitis* being less susceptible, and *Histoplasma capsulatum* being least susceptible.[48] Activity was also observed against several *Trichophyton* species, and the symptoms caused by *Tricophyton asteroides* infections in guinea pigs were suppressed by MPA.[49]

Beginning in 1968, biological activities were discovered in addition to the antibacterial and antifungal actions. Broth from growth of a strain of *Penicillium stoloniferum* had antiviral and antitumor properties, and the responsible agent was identified as MPA.[48,50,51] The antiviral properties were observed at low concentrations in a monkey kidney cell line against vaccinia, measles, Herpes simplex, and Newcastle disease viruses, but there was no significant in vivo activity against vaccinia, Herpes simplex, and influenza virus in mice. Oral administration of MPA to chickens inhibited development of the Rous sarcoma virus (94% inhibition with 0.125% MPA in the diet) and to a lesser extent splenomegaly in Friend virus infected DBA_2 mice. Similarly, MPA from an unidentified *Penicillium* sp. also had significant antiviral activity.[52] The in vitro antiviral activity was reversed by guanine, guanosine, GMP, and deoxy-GMP.[48,51]

Moreover, MPA inhibited growth of several transplantable murine solid tumors. Of particular note was marked inhibition of the rapidly growing and metastasizing Mecca-lymphosarcoma tumor.[48,53] The antitumor activity of MPA was confirmed in 1969;[54,55] however, there was no in vivo activity against neurovaccinia, Semliki Forest, encephalomyocarditis, and mouse sarcoma viruses.[55] More extensive animal testing and preclinical toxicology were carried out in 1972.[56,57] At the same time clinical trials against various cancers with a small group of patients showed only a poor response.[58]

Planterose (1969) made the significant observation that with mouse sarcoma virus, MPA appeared to act as an immunosuppressant.[55] Thus, mean spleen weights (mg after 3 weeks) in 2 week old TO mice (10−12 g) when the drug dose was started for 2 days before infection with virus and continued for 2 weeks were as follows (daily dose in parentheses): controls, 119; MPA, 215 (3 mg); mercaptopurine, 200 (300 μg); methotrexate, 330 (10 μg). Similarly, in the same year, Mitsui and Suzuki reported that in mice, MPA depressed the immune response to sheep erythrocytes and suggested that it "might be a useful immunosuppressive agent" (presumably having human use in mind).[59] Their expectations have been amply fulfilled. As to the possible mechanism of action of MPA, it was said in 1968 to be an inhibitor of inosine 5′-monophosphate dehydrogenase (IMPDH), an enzyme involved in biosynthesis of guanine. Before discussing this inhibition, some properties of IMPDH will be described.

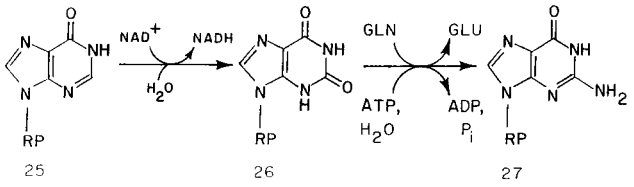
IV. Inosine 5′-Monophosphate Dehydrogenase

A. Role in DNA Synthesis

Despite the fact that the human immune system is geared to reject transplants by the action of "killer T-lymphocytes", organ transplantation has extended life for many people. A scenario to reduce the rejection problem is to inhibit lymphocyte proliferation. Since proliferation requires DNA synthesis, it might be reduced by controlling the formation of purine monophosphates that are necessary for DNA formation. Purine monophosphate formation is achieved in two ways. (A) By a de novo biosynthetic pathway in which simple metabolites (e.g., amino acids, CO_2, "one-carbon units", carbohydrate derivatives) form a key intermediate, inosine 5′-monophosphate (IMP). In turn, IMP can be converted to both adenosine 5′-monophosphate (AMP) and guanosine 5′-monophosphate (GMP). (B) By a "salvage" pathway in which free purines formed in catabolic (degradation) processes are reconverted to nucleoside monophosphates by reaction with 5-phospho-α-D-ribofuranosyl diphosphate. A key salvage enzyme is EC 2.4.2.8, variously named as hypoxanthine phosphoribosyltransferase or hypoxanthine-guanine-xanthine phosphoribosyltransferase.

It has transpired that lymphocytes are uniquely dependent on the de novo pathway almost entirely lacking salvage capability. Of the two major purine components of DNA (adenine, guanine), the guanine nucleotides have particularly important metabolic and/or regulatory functions. Hence, to cut a long and simplified summary short, a material inhibiting de

Scheme 5. Biosynthesis of Guanosine 5'-Monophosphate, GMP (27)[a]



[a] RP = β-D-ribofuranosyl 5'-phosphate. This abbreviation and R = ribose are used generally throughout this review. GLN = L-glutamine, GLU = L-glutamate, P_i = inorganic phosphate.

novo GMP biosynthesis could be a powerful immunosuppressant.

During the classical investigations nearly five decades ago on purine biosynthesis, an NAD (then DPN) requiring dehydrogenase activity necessary for the conversion of IMP (**25**) to xanthosine 5′-monophosphate (XMP, **26**) was first described in rabbit bone marrow extracts[60−62] and pigeon liver extracts[63,64] (Scheme 5). IMPDH was also observed in extracts of *Aerobacter aerogenes*, and a 60-fold purification was obtained.[65] In some early papers, IMPDH was listed as EC 1.2.1.14 but is now numbered as EC 1.1.1.205. In mammalian extracts, XMP was converted to GMP (**27**) with glutamine as the amino donor.[66−68] For the same reaction in bacteria, the amino donor was NH_3. These two enzymes are termed GMP synthase (glutamine hydrolyzing), EC 6.3.5.2, and GMP synthase, EC 6.3.4.1. Thus, the two reactions necessary for GMP synthesis (and in turn for GTP and DNA formation) in mammals are as follows

$$IMP + NAD^+ + H_2O = XMP + NADH \quad \text{IMPDH}$$

$$XMP + \text{L-gln} + H_2O + ATP = GMP + \text{L-glu} + AMP + PP \quad \text{GMP synthase}$$

B. Inhibition of Inosine Monophosphate Dehydrogenase

Beginning in 1968, it had been observed that the antiviral activity of MPA was reversed by compounds such as guanine, guanosine, and GMP.[48,51] It was suggested that MPA might inhibit cellular function by affecting "an essential guanosine metabolic process".[48] Early in 1969, MPA was shown to be a potent inhibitor of nucleic acid biosynthesis in the "L" strain of fibroblasts; the inhibition was reversed by guanine but not hypoxanthine, adenine, or xanthine.[69,70] Hence, it was believed that the action of MPA was related to an interference with the biosynthesis of guanine nucleotides—a conclusion similar to that derived from the work on the antiviral properties. In fact, MPA inhibited incorporation of ^{14}C-labeled hypoxanthine into xanthine and guanine nucleotides but not into adenine nucleotides in "L" cells and Landschütz ascites cells. Furthermore, IMPDH prepared from Landschütz ascites, calf thymus, and LS cells was strongly inhibited by MPA. Somewhat later, MPA administered orally to rats inoculated with the Yoshida ascites tumor was found to inhibit incorporation of ^{14}C-labeled hypoxanthine into nucleic acid guanine.[71] The very important observation that MPA inhibited IMPDH was apparently first described in a 1968 U.K. patent application (application no. 26562/68), the complete specification of which was given in 1970.[72] Although it was stated in 1972 that MPA also inhibited GMP synthase,[57] other work indicated that this inhibition was much less than that with IMPDH.[73] MPA inhibition of GMP synthase is now discredited.[74] MPA as well as ribavirin (Virazole) and 1-amino guanosine was shown to inhibit IMPDH in Ehrlich ascites tumor cells in 1974. MPA was the most efficient of these three materials giving 50% inhibition at <0.1 μM.[73]

Many other inhibitors of IMPDH are now known, and their chemical structures are described later. Inhibition of IMPDH activity clearly leads to a reduction in the supply of XMP and in turn to the curtailment of the formation of GMP and other guanine derivatives. Hence, one major consequence of inhibition of IMPDH with MPA is an overall reduction of DNA synthesis in organisms or cells dependent on the de novo pathway for purine monophosphate synthesis (e.g., lymphocytes). In other words, this enzyme inhibition leads to inhibition of cell proliferation in these circumstances. While it is clear that MPA induces intracellular GTP depletion, the actual mechanism by which this effect leads to inhibition of DNA synthesis is uncertain. While restriction of DNA synthesis could be attributed to the simple bulk depletion of dGTP or GTP, experiments with human CEM leukemia cells suggest that the GTP depletion by MPA is responsible for the suppression of the synthesis of RNA-primed DNA intermediates.[75]

The components of the "guanylate pool", GMP, GDP, GTP, and dGTP, are involved in many important metabolic processes. They are substrates for some reactions, play important roles as activators and/or regulators, and are concerned in transmembrane signaling.[76] Depletion of the supplies of these molecules, therefore, can account for some of the effects of MPA in addition to that on cell proliferation. The cellular GMP pool is actually the smallest purine (or pyrimidine) ribonucleotide pool and is possibly the most sensitive to modulation. Proliferating cells preferentially expand the guanosine nucleotide pool to supply limiting precursors for nucleic acid synthesis.[76]

As already indicated, early work had shown antiviral activities for MPA and there has been much investigation of this phenomenon. For Sindbis virus (SV) in cells of the mosquito, *Aedes albopictus*, there was a correlation between the inhibition of replication by MPA (and also by ribavirin and 2-aminothiadiazole) and a reduction in cellular GTP levels. By serial passage of the virus in the presence of 25 μM MPA, mutants resistant to the action of MPA and the other inhibitors were obtained.[77] Since GTP was a substrate for the RNA guanylyltransferase or the RNA polymerase, impairment of either function following from GTP depletion could account for the inhibition of viral replication. That the mutant coded for an altered RNA guanylyltransferase with increased affinity for GTP was later established by genetic methods. The mutations responsible for the MPA-

resistant phenotype were localized within the coding region of nsP1 (nsP = nonstructural viral protein). The nsP1 gene product also had methyltransferase activity as well as guanylyltransferase activity.[78] Moreover, the antiviral activity of MPA and ribavirin could be reversed by depriving cells of methionine or isoleucine or by treatment with fluorodeoxyuridine or actinomycin D. All of these effects have in common the property of inhibiting cellular growth and were apparently mediated by changes in the GTP pools of the treated mosquito cells.[79]

In investigations of B-lymphocyte cell lines transformed with Epstein−Barr virus infections, MPA did not inhibit events associated with cell transformation or the Epstein−Barr lytic cycle gene expression. It did block proliferation of newly infected or established Epstein−Barr virus-transformed cell lines. Hence, the use of MMF as a source of MPA in vivo would be predicted to decrease the load of transformed B cells in immunosuppressed patients.[80]

A somewhat different effect of IMPDH inhibitors is found in the enhancement of the antiviral activity of purine 2′,3′-dideoxynucleosides. Ribavirin was known to increase the effectiveness of 2′,3′-dideoxyadenosine (ddAdo) and 2′,3′-dideoxy-guanosine (ddGuo) as anti-HIV agents by as much as 5-fold. In a T cell culture system (Molt-4 cells) the conversion ddGuo → ddGDP was stimulated up to 20-fold in the presence of MPA, ribavirin, and tiazofurin.[81] At the same time, there were increases of up to 35-fold in the levels of IMP, the phosphate donor for ddGDP formation, and the usual decrease in GTP levels. The further conversion of ddGDP to the active metabolite, ddGTP, was also increased. In other words, the increased level of IMP occasioned by the inhibitory action of MPA, etc., on IMPDH increased the level of the anti-HIV agent.

The mechanism for the overall immunosuppressive effect of MPA has several components and is not simply the effect on cell proliferation; however, it is important that the cytostatic action is more potent with lymphocytes than with other cell types.[82] MPA also inhibits synthesis of fucose- and mannose-containing components of membrane glycoproteins by depletion of the GTP pool. This could decrease recruitment of lymphocytes and monocytes into sites of graft rejection. These and other important effects related to the use of MPA as a clinically useful immunosuppressive drug will not be further discussed here but have been reviewed in detail.[82−84]

MPA is known to induce differentiation in many cell types (e.g., MCF-7 breast cancer cells, K 562 erythroid leukemia cells, HL-60 promyelocytic leukemia cells, T-lymphoid CEM-2 leukemia cells). In HL-60 cells, MPA, 3-deazaguanosine, and tiazofurin induced cells to mature morphologically and functionally at micromolar concentrations.[85] Impaired cellular proliferation alone was not associated with the induction of HL-60. Detailed studies of IMPDH expression in differentiating HL-60 cells were undertaken. The decrease in cellular IMPDH activity observed in the presence of MPA was probably a determining factor in the acquisition of a mature phenotype.[86]

With T-lymphoid CEM-2 cells, treatment with MPA led to the acquisition of a suppressor/cytotoxic T-lymphocyte-like phenotype, similar to that observed with other inducers of CEM-2 differentiation. However, the pattern of IMPDH expression was not that expected for induction of cell differentiation. One pattern common to all of the inducers was that the reduction in IMPDH activity resulted from MPA (or tiazofurin) mediated inhibition of the cellular enzyme and that there was a decline in the level of cellular guanine ribonucleotides. It was noteworthy that 0.1 μM MPA (and 100 μM tiazofurin) did not arrest cell growth but did induce a differentiated phenotype.[87] It was suggested that the use of IMPDH inhibitors might provide a way to circumvent the differentiation block in some tumor cells.

The complex mechanisms leading to T cell activation have been analyzed with the aid of immunosuppressant molecules and conclusions have been recently reviewed.[88] While cyclosporin A and tacrolimus (FK-506) share the same mode of action, rapamycin and MPA act at different steps in the T cell activation process.

C. Isoforms of Inosine Monophosphate Dehydrogenase

IMPDH is now a much-studied enzyme, and only a brief account focusing on the more recent work can be given here. Most of the information to about 1994 has been summarized.[7] IMPDH was purified to homogeneity by conventional enzymological methods from Chinese hamster VM2 cells; this variant was selected for resistance to MPA and overproduced IMPDH 16-fold. The purified protein was apparently a mix of two charge isoforms in the ratio, 1:10, both with apparent molecular mass of 56 kDa.[89] With the availability of purified enzyme, a polyclonal antibody was developed and cDNA clones for IMPDH were characterized in 1988 from human and rodent cDNA libraries. In 1990, two distinct cDNAs for human IMPDH were isolated.[90] The encoded isoform proteins were termed IMPDH−I and IMPDH−II or alternatively IMPDH-hI and IMPDH-hII to indicate human (h) origin; the latter nomenclature will be used here. The two proteins each contained 514 amino acid residues showing 84% sequence identity. There were 84 amino acid changes between the two isoforms, with 52 being conservative substitutions and 32 diverging with respect to chemical properties. IMPDH isoforms are also known in the mouse, hamster, and *Saccharomyces cerevisiae*.

The two mRNA transcripts were initially thought to be regulated differently. IMPDH-hI was constitutively expressed and was the preponderant isoform in normal lymphocytes. IMPDH-hII was up-regulated in neoplastic and replicating cells and then became the major isoform. When neoplastic cells differentiated, the type II transcript was down-regulated to a level less than that of type I.[91,92] Therefore, it was suggested that IMPDH-hII was an inducible enzyme, closely linked to cell differentiation and neoplastic transformation. On the other hand, stimulation of isolated T cells with ionomycin and phorbol 12-myristate 13-acetate (PMA) gave a 15-fold increase

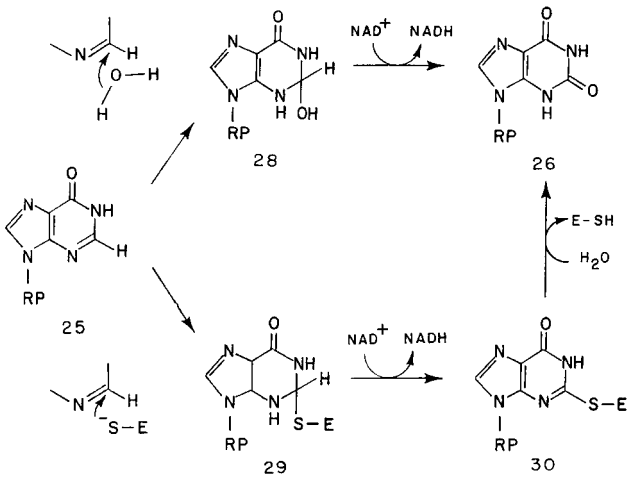
in IMPDH activity over a 72 h period.[93] There was a 10-fold increase in type II mRNA at 48 h; in resting cells type I mRNA was expressed at very low levels but also increased 10-fold at 24 h after the stimulation. The expression of type II mRNA was induced by either phorbol esters or ionomycin alone but to a lesser extent than when these agents were used together. Moreover, a second RNA (4.0 kb) was found in response to the type I cDNA probe; it was not present in normal tissues or leukemic cell lines. This second species of IMPDH-hI mRNA possibly resulted from an alternate splicing or use of an alternative polyadenylation site. Since, in contrast to the earlier studies, this work indicated that induction of both type I and type II IMPDH contributes significantly to the T cell proliferation response, the relevance of the two isoform proteins is not yet clear.

Overexpression plasmids were constructed in *Escherichia coli* for both human isoforms; under appropriate conditions, the IMPDH proteins accounted for 30% of the total soluble protein and substantial amounts of the enzymes were purified to homogenity by chromatographic techniques. The proteins were tetrameric with four 56 kDa monomeric units.[94] MPA was an uncompetitive inhibitor of mammalian IMPDH isoforms with respect to IMP and NAD^+. The K_i values for the two isoforms differed, being either 2-fold[94] or 4.8-fold larger for the type I enzyme.[95] The regulation of the two genes has been studied[96,97] but will not be discussed here. It cannot be emphasized too strongly that the comprehensive biochemical and kinetic analyses of the proteins and associated inhibitors were greatly facilitated by the availability of the recombinant IMPDH proteins.

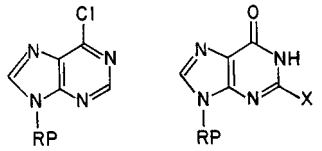
D. Reaction Mechanism

Two reaction mechanisms have been considered for the conversion of IMP (**25**) to XMP (**26**) by IMPDH. One requires a general base-assisted addition of OH^- at C-2 of IMP (**25**) to form (**28**) followed by oxidation; this is termed the noncovalent mechanism (Scheme 6). The alternative covalent mechanism requires formation of a tetrahedral intermediate (**29**) with a covalent linkage with the enzyme protein; hydride transfer to NAD^+ produces a trigonal intermediate (**30**) still with covalently bound enzyme, and finally, hydrolysis yields XMP (**26**). Experimental evidence strongly favors the covalent mechanism. Thus, the nucleotide analogue, 6-chloro-9-β-D-ribofuranosylpurine 5′-monophosphate (**31**), loosely termed 6-Cl−IMP and also 6-Cl−PRT, was found to inactivate bacterial IMPDHs irreversibly.[98,99] A likely target was a highly conserved cysteine residue in the amino acid sequence of IMPDHs from various sources (see later). Similarly, IMPDH-hII was also inhibited by 6-Cl−IMP; the presence of substrate IMP, but not NAD^+ alone, protected the enzyme.[100]

Scheme 6. Possible Reaction Mechanism for IMPDH[a]



[a] The enzyme, IMPDH, is represented as E-SH.



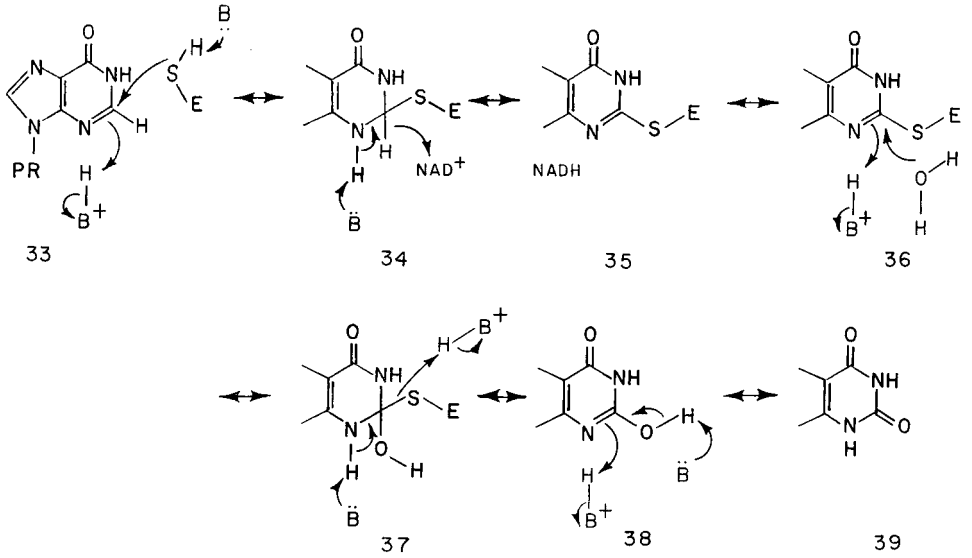
31 32

Iodoacetamide, another −SH reagent, was also inhibitory. In an elegant experiment, IMPDH-hII was first reacted (labeled) with 6-Cl−IMP and the remaining 7 cysteine residues were alkylated with iodoacetamide. Analysis of this preparation by HPLC−mass spectrometry showed that only the one residue, cys^{331}, was covalently modified by 6-Cl−IMP.[100] It was apparent that the analogue was bound at the usual IMP binding site in a covalent linkage with cys^{331} to form a purine riboside 5′-monophosphate−enzyme adduct. In another experiment, IMPDH was incubated in the presence of (saturating concentrations of) NAD and [8-^{14}C]IMP and an inhibitory amount of MPA.[101] Following denaturation and trypsinolysis, a radioactive peptide was obtained, not present in control experiments with IMPDH alone. Electrospray mass spectral analysis indicated that the peptide was derived from an enzyme−substrate adduct with a covalent bond between the sulfur atom of cys^{331} and C-2 of IMP. It was apparent that MPA had prevented the hydrolysis of the IMPDH−substrate covalent intermediate (**30**); more detail is presented in Scheme 7. In other words, MPA "trapped" the covalent intermediate. Enzyme turnover events accompanying MPA inhibition also support the covalent mechanism and trapping role of MPA.[102] Similar work, described later in connection with protozoan parasites, implicated cys^{319} for the *Tritrichomonas foetus* IMPDH enzyme.

It was of interest that IMPDH catalyzed the dehalogenation of two other IMP analogues, in this case with halogen at the 2 position of the purine nucleus (**32**, X = Cl or F).[103] These 2-halo analogues did not produce irreversible enzyme inactivation. Instead, both 2-chloro- and 2-fluoroinosine 5′-monophosphates were converted to XMP by IMPDH-hII in a process not requiring NAD^+. These results were also interpreted to require formation of a tetrahedral intermediate.

Extensive experimental observations, particularly from the X-ray diffraction analysis of various crystalline IMPDH preparations and complexes, and kinetic data (see later) fully confirm the covalent reaction

Scheme 7. Detailed Covalent Reaction Mechanisms for IMPDH[a]



[a] In this scheme, IMP is shown in association with the IMPDH protein (E-SH) as **33**. In subsequent structures, the five-membered imidazole ring, which remains unaltered, is abbreviated. Basic groups are drawn as B: or in protonated form as BH^+. For detailed description, see text. Note that **35** and **36** are the same intermediate (and compare **30**) but in association with either NADH (**35**) or H_2O (**36**).

mechanism. For convenience, the detailed reaction steps required for the IMP → XMP conversion are presented at this point as Scheme 7. This reaction mechanism, therefore, expands the simple representation given in Scheme 6. Two tetrahedral intermediates with covalently bound enzyme (**34**, **37**) and one trigonal intermediate also with covalently bound enzyme are postulated; the latter occurs in two forms, either in an association with NADH (**35**) or with H_2O (**36**). The abbreviations commonly used to define the intermediates (also used elsewhere in this review) and an overall view of the reaction are as follows. Initial association of the IMPDH enzyme (E-SH) with IMP forms a noncovalent association or adduct, E·IMP (**33**). Enzymatic thiolation yields a tetrahedral covalent intermediate, E-IMP (**34**; compare **29**). With hydride transfer, E-IMP yields a trigonal intermediate between E and XMP, abbreviated as E-XMP*. Initially, E-XMP* is associated with NADH (**35**). By a substrate exchange, NADH is released and replaced by a water molecule giving a second E-XMP* arrangement (**36**). Hydration of E-XMP* (**36**) yields a tetrahedral intermediate, E-XMP (**37**). Dethiolation of **37** yields XMP in the enol structure (**38**) and keto−enol tautomerism gives XMP in the (usual) keto structure (**39**). Some of the experimental results providing this information are discussed later.

E. Amino Acid Sequences

In view of the large volume of material, this present description will focus on the amino acid sequences at the active site of various IMPDHs. For more detail, a review by Zhang et al. is available.[9] Remarkably, at least 30 IMPDH enzymes have been sequenced. In general, all are homotetrameric with the monomer subunit containing from 387 (a *Synechocystis* sp.) to 529 amino acid residues (*Mycobacterium leprae, M. tuberculosis*). The median length of the monomers is 507−508 residues. The region surrounding the active site is highly conserved in all of these enzymes (Table 1). With few exceptions, the active site cysteine is accompanied by three other residues to form a G−S−I−C pattern (exceptions, see Table 1, are G−S−T−C in *M. leprae*, G−A−A−C in a *Synechocystis* sp., G−S−A−C in *Schizosaccharomyces pombe*). The critical cysteine varies in position from residue 222 (*Synechocystis* sp.) to 350 (*Drosophila melanogaster*) with the median position being 323−324. The residue following the cysteine is either T or I (exception, S in *Aeropyrum pernix*). There are many homologies in the 9 residues that precede and the 10 that follow the cysteine (Table 1). At 7 residues before the cysteine, the amino acid is always G as it is at 9 residues after the cysteine (exception, A in *Aeropyrum pernix*). The regularities observed at and near the active site cysteine are remarkable considering the overall variability in the size of the enzymes.

F. Crystal Structures

While an ester of MPA has been very useful in medical practice, there are limitations on its use (see later). Knowledge of the three-dimensional structure of IMPDH from various organisms might allow the design of improved pharmaceuticals for organ transplantation and in treating other disorders. It is indicative of the great interest in IMPDHs that there have been five studies by X-ray diffraction of the structure, both as apoprotein and containing bound components. A preliminary account of the apo IMPDH from the parasite, *Tritrichomonas foetus*, was given in 1995,[104] and full details were published in1997;[105,106] the apoprotein was observed at 2.3 Å resolution and complexed with XMP at 2.6 Å resolution. Analysis of a mammalian IMPDH complexed with an IMP reaction intermediate (2.6 Å resolution) followed in 1996.[107] The protein was a recombinant hamster IMPDH−II expressed in a mutant *E. coli* strain deficient in IMPDH. Two more structures were

Table 1. Inosine Monophosphate Dehydrogenase: Active Site Sequences[a]

Organism[b]	Sequence[c]	cys[d]	total[e]
Archaea			
A. pernix	R M G I S S G S I C S T G E V A G A A V	271	444
M. jannaschii	K V G I G P G S I C T T R V V A G V G V	306	496
P. abyssi	K V G I G P G S I C T T R I V A G V G V	301	485
P. furiosus	K V G I G P G S I C T T R I V A G V G V	301	485
P. horikoshi	K V G I G P G S I C T T R I V A G V G V	301	486
Bacteria			
A. calcoaceticus	K V G I G P G S I C T T R I V A G I G M	304	488
Aq. aeolicus	K V G V G P G S I C T T R I V A G V G V	309	490
Ba. subtilis	K V G I G P G S I C T T R V V A G V G V	308	513
Bo. burgdorferi	K V G I G P G S I C T T R I V A G V G V	229	404
C. vibriforme	K V G I G P G S I C T T R V V A G V G M	336	521
E. coli	K V G I G P G S I C T T R I V T G V G V	305	488
Hae. influenzae	K V G I G P G S I C T T R I V T G V G V	306	488
Hel. pylori	K V G I G P G S I C T T R I V A G V G M	300	481
M. leprae	K V G V G P G S T C T T R V V A G V G A	341	529
M. tuberculosis	K V G V G P G S I C T T R V V A G V G A	341	529
S. pyogenes	K V G I G P G S I C T T R V V A G V G V	310	493
Synechocystis sp	L V G I G P G A A C T S R G V L G V G V	222	387
Fungi			
P. carinii	R V G M G S G S I C I T Q E I M A V G R	263	454
Yeasts[e]			
C. albicans	R I G M G S G S I C I T Q E V M A C G R	333	521
S. cerevisiae 1	R I G M G T G S I C I T Q E V M A C G R	335	523
2	R I G M G S G S I C I T Q E V M A C G R	335	523
3	R I G M G S G S I C I T Q E V M A C G R	336	524
4	R I G M G T G S I C I T Q K V M A C G R	335	403
Sch. pombe	R V G M G S G S A C I T Q E V M A C G R	337	524
Protozoa			
L. donovani	R I G M G S G S I C I T Q E V L A C G R	327	514
Tri. foetus	K I G I G G G S I C I T R E Q K G I G R	319	503
Try. brucei	R I G M G S G S I C I T Q E V L A C G R	325	512
Plant			
A. thaliana	R V G M G S G S I C T T Q E V C A V G R	322	503
Insect			
D. melanogaster	R V G M G S G S I C I T Q E V M A C G R	350	537
Mammals			
Hamster	R V G M G C G S I C I T Q E V L A C G R	331	514
Human I	R V G M G C G S I C I T Q E V M A C G R	331	514
Human II	R V G M G S G S I C I T Q E V L A C G R	331	514
Mouse I	R V G M G C G S I C I T Q E V M A C G R	331	514
Mouse II	R V G M G S G S I C I T Q E V L A C G R	331	514

[a] Individual citations for the sequences are not given. The information was readily available from the standard data banks, GenBank or Swiss Prot. [b] The abbreviations used for the organisms are as follows. Archaea: *A.* = *Aeropyrum; M.* = *Methanococcus; P.* = *Pyrococcus.* Bacteria: *A.* = *Acinetobacter; Aq.* = *Aquifex; Ba.* = *Bacillus; Bo.* = *Borrelia; C.* = *Chlorobium; E.* = *Escherichia; Hae.* = *Haemophilus; Hel.* = *Helicobacter; M.* = *Mycobacterium; S.* = *Streptococcus.* Fungi: *P.* = *Pneumocystis.* Yeast: *C.* = *Candida; S.* = *Saccharomyces; Sch.* = *Schizosaccharomyces.* Protozoa: *L.* = *Leishmania; Tri.* = *Tritrichomonas; Try.* = *Trypanosoma.* Plant: *A.* = *Arabidopsis.* Insect: *D.* = *Drosophila.* [c] The sequences shown are the nine amino acid residues before the catalytically active cysteine and the 10 residues that follow. [d] The numbers indicate the position of the cysteine residue in the polypeptide chain. [e] The total number of amino acid residues in the IMP dehydrogenase protein. [f] *S. cerevisiae* contains four isoforms, referred to as IMH1, IMH2, IMH3, and IMH4.

described in 1999. One was of recombinant IMPDH-hII as a ternary complex with 6-Cl−IMP and the NAD analogue, SAD (see later).[108] The other was of *Streptococcus pyogenes* IMPDH, modified by inclusion of selenomethionine, and complexed with IMP.[109] The IMPDH from *Borrelia burgdorferi* was studied at 2.4 Å, and a preliminary account was available.[8] The full details are now published.[110] Only a brief description of these structures is possible here. Particularly for pictorial representation of structural details, often elegantly printed in color, the original papers should be consulted.

The basic IMPDH structure is a tetramer with the crystal lattice composed of groups of octamers result-

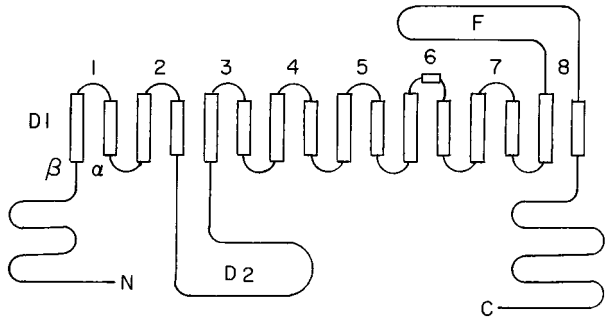


Figure 1. Eight-stranded β/α barrel arrangement in crystal structures of IMPDH. The drawing is simply designed to present a broad view of the various components and is not to scale. N indicates the amino terminal of the polypeptide chain and C the carboxy terminal. The units labeled β are β sheet arrangements and those labeled α are α helices. The barrel structure provides the catalytic domain. The active site includes a further α helical arrangement, "loop 6", that is shown between β6 and α6. The CBS domain is labeled D2, and the flap is labeled F.

ing from an association of two tetramers; the octamer pattern is variable. The monomeric units have a molecular mass of about 55 kDa and for the enzymes so far studied by X-ray diffraction from 404 to 514 amino acid residues (see also Table 1). An important feature in each case is that the proteins have two major domains and a number of other structural features.

The principal domain is a parallel eight-stranded β/α barrel (Figure 1) that provides a core framework for the positioning of structural and catalytic regions. The barrel has approximate dimensions of 40 × 40 × 50 Å^3. The β strands and α helices forming the core of the barrel are labeled β1−β8 and α1−α8. This barrel structure is also termed a TIM barrel[109] from its resemblance to the barrel structure in triose phosphate isomerase (TIM). In IMPDH-hII there is a second domain (see below); one group of workers indicates 120 residues in this domain, thus giving 394 residues (514−120) in the barrel; other workers include 135 residues in it leading to 379 (514−135) in the barrel. Other IMPDHs examined by X-ray diffraction are generally similar (Table 2).

The active site, with the crucial cysteine residue, is located on the C-terminal face of the barrel in a highly mobile loop, "Loop 6", extending from β6 to a helix, αD, and then back to α6. In the human and hamster enzymes, this loop contains 18 amino acids (residues 325−342), with the crucial cysteine at position 331. In the *Borrelia burgdorferi* enzyme, the loop has 13 amino acids (residues 225−237) with the catalytic cysteine at position 229. In the *Tritrichomonas foetus* enzyme, there are only 11 amino acids (residues 314−324) with the catalytic cysteine at position 319. The best defined loop 6 structure is that observed in the X-ray crystal structure of the *Borrelia burgdorferi* enzyme where it has been observed for the first time in the substrate-free state.[110] Comparison of loop 6 conformations shows that it is capable of large, hinged, rigid-body motion. In the apoprotein, loop 6 is in an open position, exposing the IMP binding site to both solvent and substrate. In this position, cys^{229} is so located that IMP could not be bound in such a way as to be susceptible to nucleophilic attack. The loop functions as a "lid" after substrate has been bound and the catalytic cys^{229} has been recruited into its proper position.

The active site is a long cleft with a binding pocket for nucleotide (IMP) and a binding groove for NAD. A stacking interaction between IMP and NAD facilitates the necessary hydride transfer. The stereochemistry of hydride transfer has been determined for IMPDH from murine lymphoblasts, *E. coli* and for IMPDH-hII.[111] In all cases, the transferred hydride ion becomes H_S (H_B) of NADH. The stacking pattern facilitates this stereochemical mechanism. In contrast to the binding sites for IMP, the binding areas for NAD are not conserved among the various IMPDHs. The adenosine component of NAD binds in the subunit interface with interactions with amino acid residues of a flap (see below). The interactions of the E-XMP* trigonal intermediate with amino acid residues of hamster IMPDH are indicated in Figure 2. It is noteworthy that the structure analyses clearly confirm a covalent link from the C-2 position of the purine ring to the S atom of a cysteine residue, e.g., cys^{331} in hamster.

A further feature is that the active site cleft is covered by a "flap", formed by a loop extending between the β8 and α8 units; this flap is also mobile. Several of the binding amino acids for E-XMP* are contained in this flap (Figure 2). Its length is from residues 400−450 (hamster) or 398−451 (IMPDH-hII). In the *Borrelia burgdorferi* enzyme the flap is much smaller with 36 amino acids (residues 309−344) and smaller still in *Streptococcus pyogenes* which has 24 amino acids (residues 364−419).

The binding of MPA to IMPDH-hII is uncompetitive with respect to both substrates (IMP, NAD), indicating that MPA binds after addition of both substrates. The binding is believed to be to the trigonal intermediate, E-XMP*. MPA binds in the nicotinamide subsite of the NAD site; specific interactions are shown in Figure 3. As already noted, the

Table 2. Composition of Structural Units in IMPDH from Various Organisms[a]

organism	total[b]	barrel[c]	cbs[d]	flap[e]	loop[f]
human	514	1−112 + 232−514 (394)	113−232 (120)	398−451 (54)	325−342 (18)[108]
hamster, human	514	1−109 + 245−514 (378)	110−244 (135)	400−450 (51)	325−342 (18)[112]
T. foetus	503	2−101 + 221−503 (383)	102−220 (119)		314−324 (11)[106]
S. pyo.	493	2−92 + 224−492 (360)	92−223 (131)	396−419 (24)[109]	
B. burg.	404	(366)	(38)	309−344 (36)	205−237 (13)[110]

[a] The number of amino acid residues in the two domains (barrel, CBS) and in the flap and loop are given in parentheses. [b] Total number of residues in the specified species. *T* = *Tritrichomonas*, *S. pyo.* = *Streptococcus pyogenes*, *B. burg.* = *Borrelia burgdorferi*. [c] Residues forming the main barrel domain. [d] Residues forming the CBS domain. [e] Residues forming the flap structure. [f] Residues forming the loop structure.

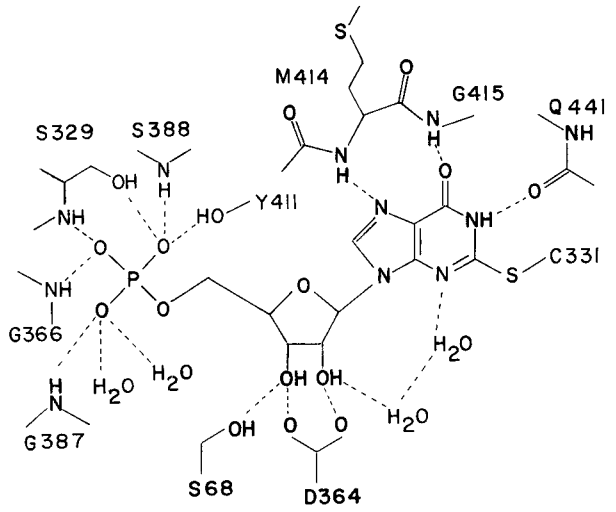


Figure 2. E-XMP* and its interactions with amino acid residues at the active site of hamster IMPDH (a redrawing of Figure 4B of ref 107). The amino acid residues of the polypeptide chain are described by the usual one-letter code and their position in the sequence. Thus, C331 indicates the active site cysteine residue at position 331. The noncovalent interactions are indicated by dashed lines. The cartoon is not to scale; all distances pertain to non-hydrogen atoms and vary in length from 2.5 to 3.2 Å. The following residues are part of the flap: tyr^{411}, met^{414}, gly^{415}, gln^{441}.

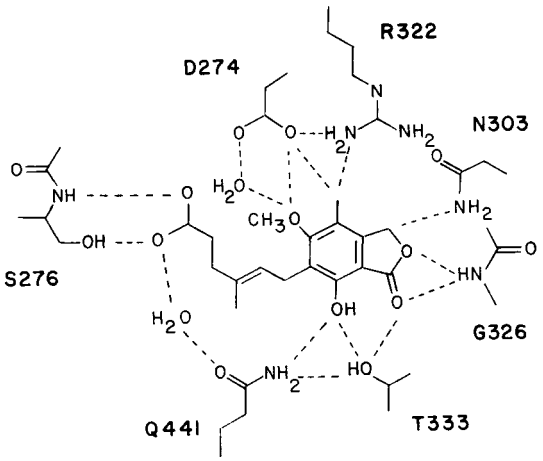


Figure 3. Interactions between hamster IMPDH and MPA (a redrawing of Figure 4C of ref 107). The notations for the amino acid residues and the noncovalent interactions are the same as in Figure 2. The residue gln^{441} is part of the flap sequence.

NAD/MPA site residues are not conserved among the various enzymes from different sources. It has been suggested that the phenolic OH of MPA may be a replacement for the catalytic water molecule involved in the hydration reaction (**36** → **37**).[107] The phenolic hydroxyl of MPA forms hydrogen bonds to thr^{323} and gln^{441} in the hamster enzyme (Figure 3). In the absence of MPA, a water molecule might be stabilized by hydrogen bonds to these amino acid residues and would be positioned to attack at the C2 carbon of the trigonal intermediate, E-XMP* (**36**). In this event, MPA would be "both a nicotinamide ring and a catalytic water mimic".[107] Water molecules that are potential candidates for nucleophilic attack on E-XMP* were also located in the *Streptococcus pyogenes* enzyme as well as amino acid residues that might act as water molecule activators.[109] However, *Tritrichomonas foetus* mutant enzymes (see later) involving lysine/arginine and glutamate/glutamine changes did not support a water mimic role for the MPA phenolic hydroxyl. The conformation of MPA bound to IMPDH is similar to that observed in its crystal structure (**8**); however, the hexenoic acid moiety of bound MPA has a more bent conformation than the fully planar arrangement observed in the crystal structure.

A second domain is defined somewhat differently by two research groups. Colby et al. for IMPDH-hII refer to a "120-residue flanking domain (residues 113−232)",[108] whereas Nimmesgern et al. say that it "includes residues 110−244" with reference to both hamster and IMPDH-hII.[112] The approximate dimensions of this domain are 20 × 20 × 30 Å^3, and it is located between helix α2 and sheet β3. The closest homologue to it is the cystathionine-β-synthase protein (CBS), and in IMPDHs this region is also referred to as the CBS domain; it actually contains two CBS domains.

In the *Borrelia burgdorferi* IMPDH, the CBS domain becomes vestigial since there are only 38 residues. This and other evidence suggesting that the CBS domain is not required for catalytic activity has been strengthened by separate expression of each of the two domains in *E. coli* followed by purification. The core domain construct encompassed residues 1−108 and 244−514 of IMPDH-hII joined together with a 4-amino acid linker containing Ile-Arg-Thr-Gly for a total of 383 residues.[112] It was tetrameric, and its kinetic parameters were very similar to those of recombinant IMPDH-hII. The CBS domain construct included residues 99−244 for a total of 146 amino acids. This construct behaved very differently; it migrated on a gel filtration column as a monomer or a dimer and moreover lacked any IMPDH enzyme activity. It is now abundantly clear that the CBS domain plays no role in the usual catalytic activity of IMPDH. It is hoped that the core domain construct may be amenable to X-ray diffraction analysis so that the active site may be defined more completely.

G. Kinetic Mechanism

There have been extensive investigations of the kinetics of the IMPDH reaction; early work has been summarized[7,113] as have some more recent developments.[10] The tetrameric enzyme shows allosteric behavior, and IMP binds to IMPDH in a negatively cooperative fashion.[114] Initially, the reaction was characterized as a steady-state ordered sequential Bi Bi mechanism with IMP binding before NAD and XMP being released after NADH. A refinement of this mechanism was necessary when recombinant IMPDH-hII was found to be strongly activated (~100-fold) by monovalent cations.[113] In absence of ions such as K^+, Na^+, Rb^+, Tl^+, and NH_4^+, the enzyme had less than 1% of its maximum activity. The data indicated that the enzyme first binds a monovalent cation such as K^+; a potassium ion produces the highest activity.

A complete account of the kinetic mechanism of IMPDHs is beyond the scope of this review. It can

be noted that the most complete descriptions of kinetic details are those for IMPDH-hII[115] and for the *Tritrichomonas foetus* IMPDH.[116] However, the following summary of the kinetics of both human and parasite enzymes may be useful.[117] In particular, note that substrate addition is no longer considered to be ordered: (1) Substrates (IMP, NAD) bind in a random fashion; (2) Hydride transfer is fast and reversible with an equilibrium of 1; (3) Product release is ordered; NADH is released before hydrolysis of the E-XMP* intermediate; (4) NADH release and E-XMP* hydrolysis are both rate limiting.

H. Mycophenolic Acid and Inosine Monophosphate Dehydrogenase in Parasitic Protozoa

In view of significant differences from mammalian systems, the roles of IMPDH and MPA in parasitic protozoa will be discussed separately. Extensive research has been carried out with *Tritrichomonas foetus* (hereafter, *T. foetus*), a flagellated, anaerobic parasite infecting the urogenital tract of cows, thus resulting in bovine trichomoniasis. Early work indicated that the biochemistry of this protozoan was very different from that of mammalian lymphocytes since it does not have the capability for de novo synthesis of purine and pyrimidine nucleotides. The absence of purine biosynthesis is apparently general in parasitic protozoa, and hence, these organisms fall back on salvage pathways. For purine salvage, a major pathway is the utilization of exogenous hypoxanthine derived from the host organism. The host hypoxanthine is converted to IMP by the action of hypoxanthine phosphoribosyltransferase (EC 2.4.2.8, also termed hypoxanthine-guanine-xanthine phosphoribosyltransferase)

Host Hypoxanthine + 5-phospho-α-D-ribofuranosyl 1-diphosphate → IMP + PP

IMP can then be converted in the usual way to AMP and to XMP by action of IMPDH.

Progress was facilitated by the development of a partly defined culture medium for *T. foetus* for use in an anaerobic incubator; it contained hypoxanthine, uracil, thymidine, and 10% dialyzed, heat-inactivated horse serum (described as the HUT medium). Hypoxanthine in HUT could be replaced by adenine, adenosine, guanine, guanosine, or inosine. *T. foetus* can salvage and grow on xanthine; however, with a hypoxanthine−xanthine mixture, the former is utilized preferentially since the phosphoribosyltransferase enzyme (EC 2.4.2.8) prefers hypoxanthine. The human parasite, *Tritrichomonas vaginalis*, is somewhat different since it cannot incorporate hypoxanthine into purine nucleotides; however, adenosine and guanosine are converted to AMP and GMP by the action of kinases.

In 1984, MPA was observed to inhibit growth of *T. foetus* but not *T. vaginalis*;[118] the IC_{50} value was 8 μM. That this action was probably exerted on IMPDH (or less likely, GMP synthase) was shown by the fact that the MPA inhibition was overcome by guanine or guanosine. The incorporation of labeled hypoxanthine into XMP, GMP, GDP, and GTP was completely inhibited by 100 μM MPA, again suggesting a role for IMPDH. The *T. foetus* IMPDH was purified 1000-fold to homogeneity by conventional methods.[119] Unlike most other IMPDHs, the *T. foetus* enzyme did not require K^+. MPA was an uncompetitive inhibitor against IMP ($K_{ii} = 14\ \mu M$) but noncompetitive against NAD ($K_{is} = 20\mu M$, $K_{ii} = 14\ \mu M$; K_{ii} = intercept inhibition constant, K_{is} = slope inhibition constant).[120,121] These values are significantly higher than those for mammalian IMPDHs.

In 1994, the *T. foetus* IMPDH was cloned and sequenced.[122] The ORF in the cloned gene indicated a deduced amino acid sequence of 503 residues with a molecular mass for the protein of 55.5 kDa. The native enzyme subunit had an estimated molecular mass of 58 kDa, and there was a tendency to aggregate in high salt concentrations. There was a relatively low level (25−30%) of sequence identity with other IMPDHs (e.g., human, *E. coli, Leishmania donovani*). Errors in the original sequence for residues 269−276 and 289 have been corrected.[106]

To identify an active site in the *T. foetus* protein, the enzyme was expressed in transformed *E. coli* in high yield and purified by ammonium sulfate precipitation and HPLC on Mono Q.[121] About 3 mg of pure IMPDH was obtained from 1 L of *E. coli* culture. The IMPDH was inhibited irreversibly by 6-Cl−IMP (see earlier), and this reagent protected cys^{319} in the enzyme from reaction with iodoacetamide. Sequence alignment indicated that cys^{319} of *T. foetus* IMPDH was equivalent to cys^{331} of human IMPDH. On incubation with [8-^{14}C]IMP, the enzyme became labeled but only if NAD was present. Analysis of tryptic digests of the labeled protein by HPLC indicated only a single labeled peptide peak. Edman degradation of the peptide gave an unidentified product, probably the adduct between the labeled IMP and cys^{319}. These results were consistent with a nucleophilic attack of IMPDH cys^{319} at the 2 position of IMP to form a covalent intermediate. The process facilitates hydride transfer to NAD^+. The active site geometry of *T. foetus* IMPDH (see earlier) is, overall, similar to that of other IMPDHs; a significant difference is that arg^{382} in the *T. foetus* enzyme forms three hydrogen bonds to the substrate phosphate through the main chain amide and guanidinium group.

The information with respect to other protozoan parasites is less comprehensive. Early studies with *Eimeria tenella*, a parasitic protozoan causing avian coccidiosis, indicated a lack of de novo purine biosynthesis and a high level of salvage enzymes.[123] MPA was shown to be an effective inhibitor for growth of this organism in tissue cultures;[124] this inhibition was reversed by guanine. MPA inhibited IMPDH preparations from the organism, but detailed study was hindered by activity loss on attempts to purify the enzyme. However, some evidence was obtained for an ordered Bi Bi kinetic mechanism (IMP on first, then NAD, NADH off first, then XMP). The MPA inhibition was uncompetitive versus IMP,

NAD, and K^+. This was interpreted as evidence for an E·XMP·MPA complex.

In 1982 it was shown that mizoribine (bredinin, see later) was an antimalarial agent in vitro against drug-susceptible (chloroquine, quinine, pyrimethamine) and drug-resistant strains of *Plasmodium falciparum*, probably by inhibition of IMPDH.[125] It was also stated that in view of its known action on IMPDH, the possible antimalarial activity of MPA and other materials was under investigation; further reports have not been located. At the same time, MPA was found to be partially effective against *Leishmania tropica* amastigotes within macrophages and to inhibit guanosine nucleotide synthesis in *L. tropica* promastigotes (insect forms).[126] From wild-type *Leishmania donovani*, a mutant strain (MPA 100) was obtained by growth in a medium to which increasing concentrations of MPA were added. The mutant strain had a markedly diminished sensitivity to MPA toxicity, and the cells expressed 20−30 times as much IMPDH activity as did parental cells. The gene encoding IMPDH was isolated, and a 2−3 kb *Eco*RV-*Pst*I fragment had an ORF of 514 amino acids encompassing the entire leishmanial IMPDH coding sequence. There was about 50% identity to IMPDH-hII and hamster enzymes. The augmented expression of IMPDH in the mutant was associated with the presence of amplified DNA segments encompassing the IMPDH gene.[127]

Similarly, an MPA-resistant strain of *Trypanosoma brucei gambiense* was obtained. This pathogenic hemoflagellate causes African trypanosomiasis (humans) and nagana (cattle) and becomes resistant to chemotherapy. The MPA resistance was traced to an approximately 10-fold amplification of the gene, *impdh*, encoding IMPDH. There was an increase in chromosome copy number.[128]

V. Mycophenolic Acid and Other Inhibitors of Inosine 5′-Monophosphate Dehydrogenase

A. Species-Specific Inhibition of Inosine Monophosphate Dehydrogenase by Mycophenolic Acid

One interesting and important feature of the MPA inhibition of IMPDHs is that it is species-specific. This is not the case for other IMPDH inhibitors. Mammalian IMPDHs are very sensitive to MPA inhibition, e.g., $K_i = 20$ nM for human IMPDH and 9 nM for hamster IMPDH.[117,129] With microbial enzymes, however, there is resistance to MPA inhibition, e.g., $K_i = 20$ μM for *E. coli* and for parasitic protozoa, $K_i = 9$ or 0.2 μM, respectively, for *T. foetus* and *Leishmania donovani*. The microbial enzymes also tend to bind NAD poorly.[9] Only a small fraction of the MPA resistance of *T. foetus* derives from the kinetic properties of this enzyme.[117] Roughly one-half of the sensitivity to differences (mammalian versus microbial) arises from specific residues in the MPA binding site, which as already noted is the nicotinamide half of the dinucleotide (NAD) binding site. The adenosine region of the NAD/MPA binding site may be responsible for the other portion of the differences. In particular, this subsite is not conserved among IMPDHs from various sources. In multiple inhibitor experiments with *T. foetus* IMPDH and IMPDH-hII, tiazofurin (see later), which binds in the nicotinamide portion, and ADP, which binds in the adenosine portion, were used. While these two inhibitors were independent for IMPDH-hII, they were strongly synergistic for *T. foetus* IMPDH. It appears that the nicotinamide and adenosine subsites are tightly coupled in the parasitic enzyme but not in IMPDH-hII.

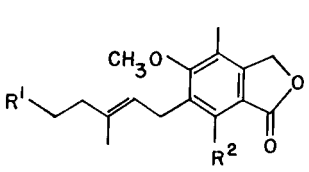
Site-specific mutants provided further definition of the active site. In *Streptococcus pyogenes*, mutation of arg^{406} to ala^{406} gave inactive IMPDH.[109] Since arg^{406} is a component of the flap region, it is clear that the flap has an important role in catalysis. In *E. coli* IMPDH, 11 conserved asp and glu residues (i.e., those providing carboxylate residues) were mutated to ala. Kinetic observations of the mutant enzymes identified five functionally important asp/glu residues. Asp^{13}, asp^{50}, and glu^{469} were involved in the binding of K^+; asp^{248} was involved in NAD binding, and asp^{338} was probably a general acid−base catalyst. Mutations involving glu^{54}, asp^{138}, asp^{200}, asp^{243}, glu^{369}, and glu^{373} had only minor effects on IMPDH function.[130] The general role of acid−base catalysis in the recombinant IMPDH-hII reaction has been discussed.[131]

In other work, three mutants of IMPDH-hII were constructed as follows: $arg^{322} \rightarrow lys^{322}$; $gln^{441} \rightarrow glu^{441}$; arg^{322}, $gln^{441} \rightarrow lys^{322}$, glu^{441}. These mutants had very low IMPDH activity but were somewhat more sensitive to MPA.[117] The following *T. foetus* mutants were constructed: $lys^{310} \rightarrow arg^{310}$; $glu^{431} \rightarrow gln^{431}$; lys^{310}, $glu^{431} \rightarrow arg^{310}$, gln^{431}. These enzymes did have IMPDH activity and were 10−20-fold more sensitive to MPA, suggesting that these residues are important structural determinants for MPA sensitivity.[117] The differences between mammalian and microbial IMPDHs might be useful in anti-infective chemotherapy.[116,119,124] Perhaps a specific inhibitor of microbial IMPDHs could be developed based on the structural information now available.[10]

B. Mycophenolic Acid Analogues

Much research has been carried out in attempts to obtain an analogue of MPA with improved properties. With a few exceptions, the results have been disappointing. With the knowledge of the detailed crystal structures of IMPDH, it is possible that future work will be more productive. Two studies from the 1970s are typical. In one, 65 derivatives and analogues were obtained but none of them had a greater antitumor effect than that of MPA.[132] Three compounds (**40**, $R^1 = COOH$, $R^2 = O-COCH_3$; **40**, $R^1 = CH_2OH$, $R^2 = OH$; **40**, $R^1 = CHO$, $R^2 = OH$) were equiactive with MPA (**40**, $R^1 = COOH$, $R^2 = OH$) against transplanted tumors in rodents, presumably as a result of in vivo conversion to MPA. In the second study, 108 MPA derivatives were prepared, without finding any compound more effective than MPA in suppressing the growth of L-5178Y cells in vitro.[133] One material (**40**, $R^1 = COOCH_3$, $R^2 = O-CONH_2$)

was slightly more active than MPA against some tumors in mice (the Ehrlich tumor, X-5563, and L-1210). Again, this structure could have been converted to MPA in vivo. A more complete listing of early work with other analogues, in many cases without biological data, is available.[134]



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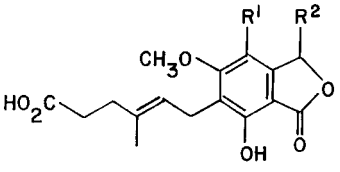

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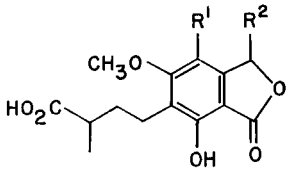

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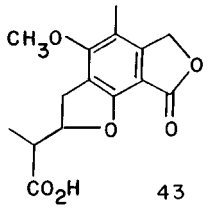

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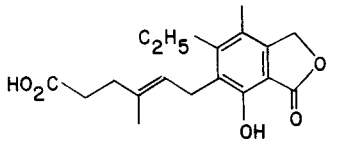
At about the same time, the microbial transformation of MPA was investigated. More than 500 microorganisms (algae, bacteria, fungi, protozoa, streptomycetes, yeasts) were tested, with 21 actually modifying MPA.[135] Compounds formed by four or more organisms were as follows: **41**, $R^1 = CH_2OH$, $R^2 = H$; **41**, $R^1 = CH_3$, $R^2 = OH$; **42**, $R^1 = CH_3$, $R^2 = H$; **42**, $R^1 = CH_3$, $R^2 = OH$; **42**, $R^1 = CH_2OH$, $R^2 = H$; **43**. Thirteen other compounds (not shown) were found less frequently, in five cases by a single microorganism. No tests for biological activity were reported.

In 1990, Nelson et al. reexamined the possibility of producing a more active analogue of MPA as part of the Syntex research program on immunosuppressive materials.[136] In the expectation of finding a more active material, 12 side chain variants were tested for inhibition of mitogen-induced human lymphocyte proliferation and 11 of them for inhibition of IMPDH. Variations were the replacement of the (*E*) double bond of MPA by the (*Z*) isomer or by acetylenic or allenic moieties or by using a sulfur atom or cyclopropane ring to simulate the side chain (it was already known that hydrogenation of the double bond caused a marked loss of antimitotic potency). As the authors wrote, "our expectations were not fulfilled".

Nelson and colleagues returned to the problem in 1996 by preparing many nuclear variants of MPA and testing them against recombinant IMPDH-hII and in many cases against mitogen-induced human peripheral lymphocyte proliferation.[134] Some structure−activity conclusions were reached: the lactone ring and the aromatic methyl were essential; for high potency, a phenolic OH group with adjacent hydrogen bond acceptor (e.g., the lactonic carbonyl) was required. One small success was that replacement of the OCH_3 group of MPA by C_2H_5 (**44**) gave a material that was 2−4 times as potent as MPA both in vivo and in vitro. A more extensive discussion of structure−activity relationships was given in 1996.[137]



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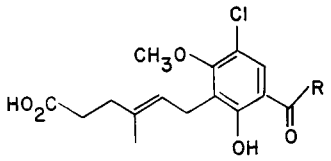

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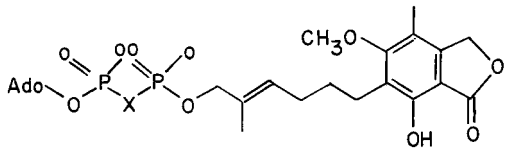
Monocyclic analogues of MPA have been investigated; they are essentially benzenoid structures lacking the lactone ring. Of nine structures, **45** with $R = OCH_3$ and **45** with $R = CH_3$ showed some activity against L-1210 leukemia cells in tissue culture. While the activity was 2 orders of magnitude less than that of MPA, these results represented the first MPA monocyclic analogue with any antitumor activity.[137]

Since MPA is rapidly converted in vivo to its inactive glucuronide (see later), one objective in the analogue search was to obtain a material that would resist glucuronidation. Since evidence indicated that MPA functioned by mimicking the binding of NAD at the active site of IMPDH (see above), an analogue containing some structural features of NAD was synthesized.[138] It was derived from the alcohol form of MPA, MPAlc (i.e., conversion of COOH to CH_2OH), by the addition of an adenosine diphosphate moiety except that in the diphosphate group the O atom of P−O−P was replaced by CH_2 (i.e., P−CH_2−P). The diphosphate derivative itself, mycophenolic adenine dinucleotide, MAD (**46**, Ado = adenosine, X = O), was not expected to penetrate cell membranes and was also likely to be susceptible to attack by esterases. The β-methylene compound, β-methylene-MAD (**46**, Ado = adenosine, X = CH_2), was an inhibitor of IMPDH-hII and was resistant to glucuronidation. The compound may have potential for therapeutic use.



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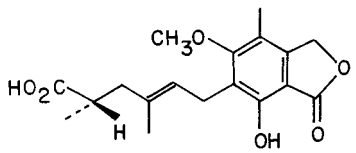

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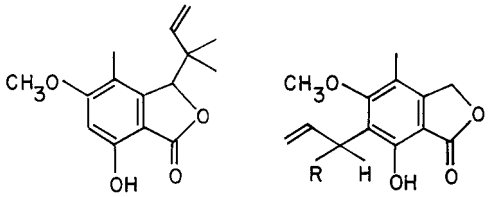
One compound showing a 5-fold increase in activity compared with MPA involved substitution of a meth-

yl group at the position α to the COOH group in the side chain.[139] The (*S*) enantiomer (**47**) had the



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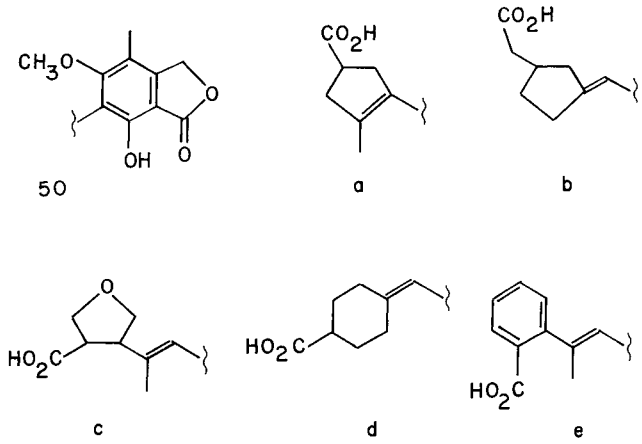
increased activity in in vitro testing. The methyl derivative was prepared from the mycophenolate ester enolate. The syntheses of compounds with substituents on the lactone or benzene ring (**48; 49,** R = H; **49**, R = CH_3) and of side chain derivatives containing cyclic structures (**50a**−**e**) have been briefly reported without biological data.[140,141]

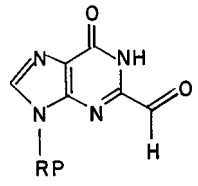


50 a b c d e

C. Inhibition of Inosine Monophosphate Dehydrogenase by Other Compounds

In addition to a few MPA analogues, many other materials, usually with no structural resemblance to MPA, are also IMPDH inhibitors. In early work using MPA, ribavirin (Virazole, 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), and 1-amino guanosine, it was noted that IMPDH preparations were inhibited by ribonucleotides of 6-mercaptopurine (which was also a substrate), 6-thioguanine, 8-azaguanine, and 2,6-diaminopurine, as well as GMP and GTP.[73] In fact, the use of nucleosides and nucleotides became a common theme. Several 8-substituted purine nucleotides and 2-substituted IMP and inosine derivatives inhibited IMPDH from *E. coli*.[142] In further work, functionalized 2-alkyl derivatives of IMP have shown some promise as both reversible and irreversible inhibitors of IMPDH-hII.[143] These materials were designed to react with the active site cysteine residue of the IMPDH enzyme. The most potent (IC_{50} = 0.44 μM) was 2-formyl-IMP (**51**) obtained as its hydrate.

With the passage of time, more exotic structures have been investigated and some have found clinical applications. Only a brief description will be given here, and for more detail, recent reviews should be consulted.[8,10,11,13] These materials can be classified as



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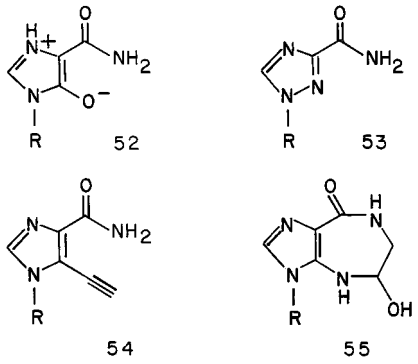
either IMP site-binding or as NAD site-binding inhibitors.

1. IMP Site-Binding Inhibitors

Most of these IMP site-binding inhibitors are ribonucleotides with the general structure of base−ribose−phosphate; in IMP itself, the base is, of course, hypoxanthine. One example is the previously mentioned 6-Cl−IMP. Perhaps the most interesting of these materials is the nucleoside mizoribine, also known as bredinin, and having the structure, 5-hydroxy-1-β-D-ribofuranosyl-1*H*-imidazole-4-carboxamide (**52**).[12] Mizoribine, a secondary metabolite



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of *Eupenicillium brefeldianum*, was discovered in the early 1970s by screening programs in Japan. It had cytotoxic and immunosuppressive activities, being a potent competitive inhibitor of IMPDH. Mizoribine is a prodrug, being a ribonucleoside. The nucleoside undergoes phosphorylation in vivo to form the active, ribonucleotide structure. Mizoribine has had extensive clinical use in Japan in human renal transplantation (where it was approved for use in 1984) and has been effective against some autoimmune disorders.

Other structures are synthetic materials. The previously mentioned ribavirin (**53**) is said to be "the first synthetic, non-interferon-inducing, broad spectrum antiviral nucleoside".[144] Descriptions of this material first appeared in 1972, and it has proved to be a potent antiviral agent in experimental clinical use. It is U.S.-approved for treatment of respiratory syncytial virus and in combination with interferon-α for treatment of hepatitis C.[8,13] Like mizoribine, it is a prodrug, undergoing phosphorylation at the 5′ position to form the active ribonucleotide.[145] While a strong competitive inhibitor of IMPDH, its antiviral action may involve other mechanisms.

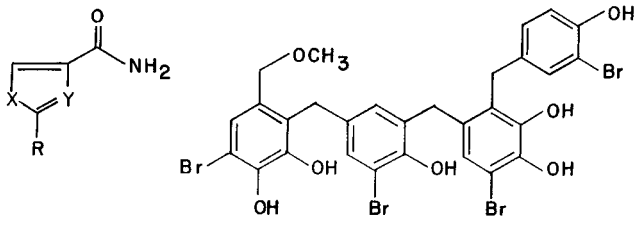
Another synthetic inhibitor undergoing in vivo phosphorylation to form the active metabolite contains an unusual acetylenic group. The nucleoside, EICAR, 5-ethynyl-1-β-D-ribofuranosyl-imidazole-4-carboxamide (**54**), was developed as a potent antiviral

and antileukemic agent in the early 1990s.[14,146] The 5′-phosphate, EICARMP, inhibited IMPDH from L1210 cells and was a potent irreversible inactivator of *E. coli* IMPDH and IMPDH-hII. Covalent alkylation of cys[305] of the bacterial enzyme and cys[331] in the human enzyme was demonstrated; the process involved addition of the cysteine SH group to the triple bond. There were, however, significant kinetic differences between these two enzymes. For the bacterial IMPDH, there was a single-step reaction mechanism, $E + I \rightarrow E{\cdot}I^*$, but for the human enzyme, the reaction had a two-step mechanism: $E + I \leftrightarrow E{\cdot}I \leftrightarrow E{\cdot}I^*$. In view of these human−bacterial differences, it was suggested that IMPDH could be a target for antibiotic drugs.

The final material to be noted here is a synthetic purine nucleoside analogue that contains a seven-membered diazapine ring (hence, termed a "fat base"), **55**. The nucleotide form, obtained by enzymatic phosphorylation, was a slow-binding, reversible inhibitor for human and *E. coli* IMPDH, probably by formation of a covalent adduct with the active site cysteine units.[147]

2. NAD Site-Binding Inhibitors

While the IMP site-binding inhibitors have a common structural theme—they are all ribonucleotides—inhibitors binding at the NAD site are structurally more heterogeneous. The MPA structure has little in common with a group of compounds, the furins. These structures contain a five-membered heterocyclic ring linked to ribose at the 1′ position to form a novel nucleoside. In vivo, they are converted to adenosine dinucleotide analogues of NAD—the nicotinamide ring of the latter dinucleotide is replaced by the five-membered heterocyclic ring. Much attention has focused on tiazofurin (T), 2-β-D-ribofuranosylthiazole-4-carboxamide, **56**, X = S, Y = N, and its adenine dinucleotide, TAD.[11,145] Tiazofurin is converted to TAD in vivo. Tiazofurin was originally prepared as an analogue of ribavirin and has been used clinically as an antitumor agent. One example of its use is that complete hematological remission was obtained in patients with certain leukemias.[8] It should be noted that while the furins have many desirable pharmaceutical features, use of some of them is restricted by significant problems in clinical practice. Thus, tiazofurin can cause severe dose-limiting side effects requiring aggressive treatment in hospital, and some patients are tiazofurin-resistant.



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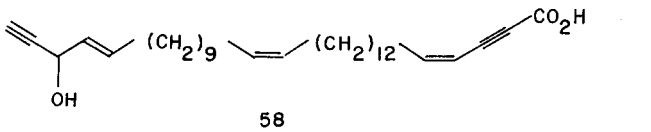

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Despite the structural relationship between NAD and TAD, TAD is a noncompetitive inhibitor of IMPDH with respect to NAD. K_i values range from 0.1 (rat) to 8.5 μM in *E. coli*. Furthermore, a β-methylene variant in which the diphosphate linkage, P−O−P, is replaced by P−CH_2−P has been prepared (compare β-methylene MAD). It would not be expected to be subject to the action of phosphodiesterases. In yet another variation, the group replacing O in P−O−P is CF_2. The β-CF_2-TAD compound was a less effective inhibitor than β-CH_2-TAD but did show an increased differentiation-inducing activity for K562 erythroid leukemia cells.[148] A compound with a modified adenosine ring (2′-F-arabinose instead of ribose) and termed 2′-F-β-CF_2-TAD behaved similarly.

Another variation on the T/TAD theme is to replace the S atom of the thiazole moiety of tiazofurin with Se, forming selenazofurin, **56**, X = Se, Y = N, and its adenine dinucleotide, SAD, and again a β-methylene form, β-SAD. Selenazofurin is more cytotoxic than tiazofurin and has been shown to be effective against viral infections and animal tumors. SAD binds to IMPDH about 10-fold more strongly than TAD. Further variations of these structures are still being investigated.[8]

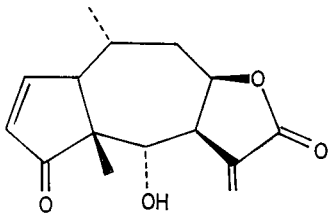
Still other variants of TAD were created by changes in the heterocyclic five-membered ring. These structures are listed below with the abbreviations for the corresponding dinucleotides. The X, Y substituents are those of structure **56**.

	X	Y	dinucleotide
thiophenfurin	S	CH	TFAD
furanfurin	O	CH	FFAD
selenophenfurin	Se	CH	SFAD

These materials showed uncompetitive inhibition against IMP and NAD with IMPDH-hI and IMPDH-hII. SFAD had potencies comparable to those of SAD. TFAD was slightly less potent than SFAD (but more so than TAD), while FFAD was only a weak inhibitor.[13,149] Another approach was to replace the nicotinamide moiety of NAD with the benzene analogue, benzamide. Benzamide riboside was converted in vivo to the benzamide adenine dinucleotide, BAD. BAD was a potent IMPDH inhibitor with the same or slightly higher potency than TAD.[8]

3. Other Inhibitors

Several materials that are neither nucleosides nor ribotides have been described as IMPDH inhibitors. They include the brominated diphenylmethane derivatives, rawsonol (**57**) and isorawsonol from the tropical green alga, *Avrainvillea rawsonii*,[150] pellynic acid, a long-chain ($C_{33}H_{52}O_3$) acetylenic compound (**58**) from the marine sponge, *Pellina triangulata* with IC_{50} = 1.03 μM for IMPDH,[151] and sesquiterpene lactones with the α-methylene-γ-lactone arrangement such as helenalin (**59**) from *Helenium autumnale*.[152] The IMPDH inhibition by helenalin was blocked by IMP, and it was believed that these sesquiterpene



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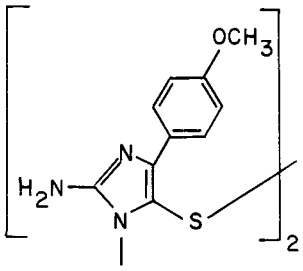

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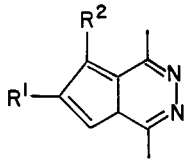

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lactones were bound at the active site of IMPDH. It is possible that E−S$^-$ could attack the double bond of the methylene to form a covalent adduct in a manner similar to the reaction with the triple bond of EICARMP. A similar possibility exists for pellynic acid. Another surprising observation was that an alkaloid, polycarpine (**60**), from the tunicates *Polycarpa aurata* and *P. clavata* was an inhibitor for recombinant IMPDH-hII with IC = 0.03 μM.[153,154] The inhibition was time-dependent and removed by the presence of dithiothreitol. It appeared, therefore, that polycarpine was a reversible sulfhydryl reagent.[153] Polycarpine had significant activity against a variety of mouse tumors and strongly inhibited other enzymes (reverse transcriptases from Raus [*sic*] sarcoma and avian myeloblastosis viruses and rat brain Na^+,K^+-ATPase).[154]

A screening of 80 000 compounds for activity against IMPDH yielded a further unusual structural type—the pyridazine, **61**, $R^1 = NO_2$, $R^2 = H$.[155] Although this compound had insufficient potency to warrant further development, synthesis of variants gave three further pyridazines with better potency against IMPDH and the proliferation of mouse mammary carcinoma cells (EMT6): **61**, $R^1 = R^2 =$Cl; **61**, $R^1 = NO_2$, $R^2 =$ Cl; **61**, $R^1 =$ CN, $R^2 =$ Cl. Compound **61**, $R^1 = NO_2$, $R^2 = H$, inhibited IMPDH by an uncompetitive mechanism with respect to both IMP and NAD. These pyridiazines apparently bind to the enzyme after association of both IMP and NAD. It was believed that the pyridiazines trapped the same enzyme intermediate as did MPA. Remarkably, the first author of the paper describing the pyridiazines was the same T. J. Franklin who had described for the first time the inhibition of IMPDH by MPA in 1968.

VI. Use of Mycophenolic Acid in Medicine

A. Introduction

MPA has become a useful weapon in the physician's armamentarium. There is an enormous literature on its clinical use, and an attempt to review in detail all of this material would not be appropriate in this article. The importance of MPA in medicine is shown by the fact that a search of Medline Express for 1999 with the term "immunosuppressant" gave 206 records. When "immunosuppressant" was coupled with "AND (mycophenolic acid OR mycophenolate)" there were 22 records. Thus, about 11% of work dealing with immunosuppressants concerned MPA in the 12 month period of 1999. Some of the highlights of this work will be described and citations given to recent work and review papers for those with more interest.

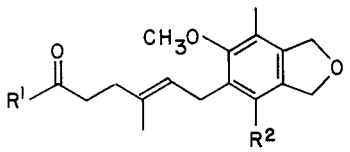
B. Use of Mycophenolic Acid

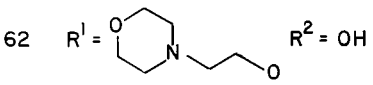
As noted, clinical trials of MPA as an antitumor drug gave only minimal results. However, beginning in 1973, oral MPA was used in treatment of psoriasis. The rationale was that other drugs slowing cellular replication had a beneficial effect on psoriasis when administered systemically.[156] MPA appeared to be an effective and safe treatment with positive results.[156−158] However, in 1997 it was stated that "the question of MPA's role in immunosuppression, the increased incidence of latent viral infections, and the question of carcinogenicity led to the discontinuation of the majority of investigations of MPA as a treatment for psoriasis".[159,160] Psoriasis has also been treated with a prodrug form of MPA (see below).

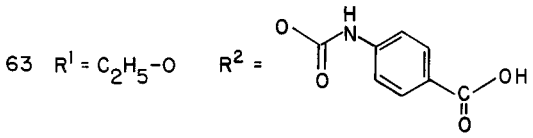
C. Mycophenolate Mofetil, a Prodrug

In 1982, Allison and Eugui established a research program on immunosuppressants at Syntex Research.[74,82,83] At that time, drugs already in use as immunosuppressants (e.g., cyclophosphamide, methotrexate, azathioprine, cyclosporin) had serious problems such as toxicity, lack of reversibility, and increased susceptibility to viral and other infections. Allison and Eugui hoped to develop an improved agent by inhibiting a metabolic pathway more susceptible in human T and B lymphocytes than in other cell types. Their literature search led them to the antitumor effects of MPA and its use in treatment of psoriasis; they found that MPA strongly inhibited responses of human lymphocytes to mitogenic stimulation,[161] and they observed immunosuppressive activities of MPA in mice.[162] A search for a derivative of MPA with improved oral availability led to the discovery of the 2-morpholinoethyl ester of MPA, 2-morpholinoethyl (*E*)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoate, as a valuable prodrug. Mycophenolate mofetil (**62**), known initially by the Syntex number RS-61443, is now known by the brand name of

CellCept (Hoffmann LaRoche) and is abbreviated as MM or MMF (used here). Gratifyingly, this ester had an improved bioavailability in primates as compared to MPA itself. Allison and Eugui justifiably claim that







they have produced "a rationally designed immunosuppressive drug".[83] The interest at Syntex was apparently directed at first to the use of MMF for treatment of rheumatoid arthritis, but clinical development in this direction was initially discontinued.[84,163] CellCept is available in 250 mg capsules with a recommended dosage of 1 g twice a day. Of the two moieties in MMF, the morpholinoethyl compound is synthetic while the MPA component is derived by fermentation. MMF hydrolyzes rapidly in the body by the action of plasma and tissue esterases to MPA itself; MPA is converted to the glucuronide for excretion (see later).

Early work had shown that MPA only weakly suppressed skin graft rejection in mice. However, further investigation in rat heart allograft transplant models showed that it prevented acute and chronic rejection.[164] Moreover, ongoing rejection was reversed by MPA. The use of MMF in renal allograft recipients quickly followed, and an extensive literature developed describing several large-scale clinical trials.[164,165] MMF was generally used as a co-immunosuppressant in connection with other materials. The U.S. Food and Drug Administration approved MMF for prevention of rejection in renal allograft recipients in 1995. From 1995 to 1998, CellCept was used to treat more than 50 000 patients undergoing kidney transplantation.

Interest in the use of MMF in heart transplants grew out of the work of Morris and colleagues,[166] and clinical trials of MMF in heart transplantation were generally encouraging.[167] CellCept was approved in the United States by the Federal Food and Drug Administration for use in heart transplants early in 1998 and by the European Commission in the fall of that year. As in the work on kidneys, it is used in combination with other materials such as cyclosporin and corticosteroids.

There have also been recent reports on the use of MMF in connection with liver transplantation. Long-term complications of other immunosuppressive drugs used by liver transplant recipients include diabetes, hypercholesterolemia, and hypertension. In one study, prednisone, a potential troublemaker, was withdrawn 14 days after liver transplantation; therapy continued with MMF in combination with either cyclosporin or tacrolimus.[168] Post-transplant diabetes, hypercholesterolemia, and hypertension were decreased by this treatment. In a four-year study, MMF appeared to be an effective alternative immunosuppressant for patients failing cyclosporin-based therapy.[169] It was proposed to be of benefit in patients tolerating neither cyclosporin nor tacrolimus. MMF also appeared to improve cyclosporin-induced renal impairment in stable liver transplant patients.[170] It appears likely that MMF will be used increasingly in connection with liver transplantation. MMF also showed improved survival rates for simultaneous pancreas–kidney transplants.[171] In the remarkable transplantation of a human right distal forearm and hand, the immunosuppressive maintenance therapy included tacrolimus, prednisone, and 2 g day^{-1} of MPA.[172] Although as indicated, MPA was specified, it seems likely that MMF was actually used.

MMF has been used for the treatment of rheumatoid arthritis, and by 1993, over 600 patients had used MMF, some for as long as 3.5 years.[173] In treatment of rheumatoid arthritis, MMF is both effective and well tolerated.[174] Recent publications have concerned the experimental use of MMF in the following situations with promising results being recorded in each case: Systemic lupus erythematosus,[163] lupus nephritis refractory to cyclophosphamide,[175] myasthenia gravis,[176] inflammatory eye disease,[177] autoimmune and inflammatory skin disorders[178,179] (including psoriasis—see earlier material on use of MPA itself for this condition), and glomerular disease.[180] MMF blocks replication of HIV in lymphocytes,[181] has a profound and synergistic anti-HIV activity in combination with abacavir,[182] and is active against multidrug-resistant HIV-1.[183]

As for many medications, there are limitations to the use of MMF and certain side effects; the most common are diarrhea, leukopenia, sepsis, and vomiting. Adverse effects on fetal development occurred in experimental animals, and MMF is not recommended for use in pregnant women unless any possible benefit outweighs the potential fetal risk. As with other immunosuppressants, the decreased immune response may lead to an increased susceptibility to infection by opportunistic pathogens. In one situation, a recently transplanted patient with immunotherapy based on tacrolimus, prednisone, and MMF developed a nocardial infection.[184]

There have been several reports indicating that in microorganisms (e.g., *Leishmania donovani*, *Trypanosoma brucei gambiense*, *Candida albicans*) amplification of an IMPDH gene confers resistance to MPA by overexpression of the enzyme. Many citations are given by Farazi et al.[185] Similarly, mutant IMPDH enzymes with altered sensitivity to MPA have been studied in mouse and hamster cells selected for increased resistance to MPA;[186–189] MPA-resistant mutants of IMPDH-hII have also been studied.[185] This work will not be detailed here, but altered sensitivity of IMPDH to MPA is relevant in connection with the question—are there any long-term

effects in patients treated with MMF/MPA? In the previously mentioned work on the treatment of psoriasis, it was noted that use of MPA had actually been discontinued because of uncertainty of the long-term role of MPA in immunosuppression and because there were concerns regarding the incidence of latent viral infections and potential carcinogenicity.[160] The extensive use of MMF in other situations (and also for psoriasis) has apparently indicated that these concerns are minimal.

One recent report concerns patients receiving MMF for periods from two months to three years. As the length of MMF therapy was increased, there was a reduction in the inhibitory action on IMPDH and as well an induction of IMPDH activity. The latter induction of IMPDH activity would be deleterious if followed by restoration or stimulation of the proliferative capacity of lymphocytes. Although this study used a small number of patients ($n = 17$), the results suggested that long-term use of MMF in renal transplant patients still needs careful assessment.[190]

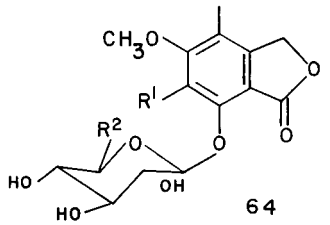
D. Another Prodrug Form of Mycophenolic Acid

Another prodrug form of MPA was developed in Japan, initially as an antitumor agent. In this case, the OH group of MPA was derivatized and the COOH was converted to the ethyl ester to produce ethyl [*N*-(*p*-carboxyphenyl)carbamoyl]mycophenolate (**63**), abbreviated as CAM.[191] In the rat heart allograft model, CAM was more effective than MMF in prolonging rat heart graft survival. Oral administration of CAM suppressed acute experimental allergic encephalomyelitis in Lewis rats.[192] It was suggested that CAM might be a useful adjunct for long-term immunosuppressive therapy for inflammatory diseases of the central nervous system. CAM appeared to have some promise for small bowel transplantation in preliminary studies.[193]

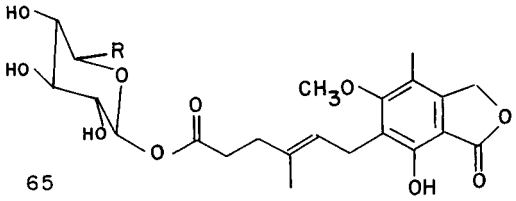
E. Metabolic Products Formed from Mycophenolic Acid in Vivo

The main urinary excretion product of MPA in humans, either from MPA itself or MMF, is the 7-*O*-glucuronide, MPAG (**64**, $R^1 = C_7H_{11}O_2$, $R^2 = COOH$), probably formed by glucuronosyl transfer from UDPglucuronate. MPAG may undergo deglucuronidation and reabsorption as MPA during the enterohepatic circulation in humans.[194] MPAG is also the major metabolite of MPA in plasma.[195] There have been contradictory statements concerning the possible inhibition of IMPDH by MPAG;[196] the inhibitory effects observed may have been caused by trace amounts of MPA in the MPAG preparation.[197,198] During storage of MPAG, hydrolysis to MPA can occur;[199] this deterioration may account for some of the observed discrepancies.

Other metabolites, termed M1, M2 and M3, have been observed in plasma of kidney, liver, and heart transplant patients when MMF was used in immunosuppressive therapy.[195] M1 was identified as the 7-*O*-glucoside of MPA (**64**, $R^1 = C_7H_{11}O_2$, $R^2 = CH_2OH$), i.e., with a glucosyl unit in place of the glucuronosyl unit in MPAG. This metabolite did not cross-



64



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react in an immunoassay for MPA. M2 was identified as the acyl glucuronide of MPA (**65**, R = COOH), i.e., MPA with a glucuronosyl unit at the COOH position. M2, unlike M1, did cross-react in the MPA immunoassay. Moreover, M2 inhibited recombinant IMPDH-hII in a concentration-dependent fashion, just as did MPA; purified MPAG did not inhibit this enzyme.[199] The presence of metabolite M2 in the plasma of transplant patients suggests a possible role in the immunosuppressive activity of MPA/MMF. The third metabolite, M3, present in plasma only in trace amounts, was not identified. It may have been a metabolic product of MPA following partial metabolism by the microsomal cytochrome P_{450} system. The morpholine moiety of MMF is extensively metabolized to CMM, *N*-(2-carboxymethyl)morpholine, and to a lesser extent to HEMO, an *N*-oxide of *N*-(2-hydroxyethyl)morpholine.[194]

VII. Concluding Remarks

The transformation of Gosio's obscure phenolic acid to the valuable pharmaceutical agent, MMF, is a remarkable achievement. It seems appropriate to quote from the conclusions of an extensive pharmacokinetic study of MMF.[194] "As an oral prodrug of mycophenolic acid, mycophenolate mofetil meets its intended role well. It undergoes rapid and essentially complete absorption, complete presystemic de-esterification to mycophenolic acid and delivers mycophenolic acid with close to 100% systemic availability. Mycophenolic acid is itself well-behaved kinetically. It is almost completely metabolised to the pharmacologically inactive and stable phenolic glucuronide, MPAG, which is excreted in urine and in amounts which eventually account for almost all of the administered drug".

It may also be appropriate to point out that various authors have suggested the possibility that IMPDH inhibitors might be useful in antimicrobial or antiinfective chemotherapy. If this can indeed be achieved, the wheel will have come full circle—MPA itself has been described variously as having antibacterial, antifungal, antitumor, and antiviral properties. An interesting situation concerns the fungus *Pneumocystis carinii*. This opportunistic pathogen causes a pneumonia to which immunodeficient patients are very susceptible. MPA is a potent inhibitor of IMPDH

in *P. carinii*;[200] in rats, MMF had an anti-*P. carinii* effect.[201] Thus, there is a surprising situation since as an immunosuppressant, MMF would have been expected to increase the susceptibility of the animals to fungal infections. Clearly there is much yet to learn about the biological properties of MPA and MMF.

VIII. Acknowledgments

I should like to acknowledge 40 years of support and help from my wife, Marian (unfortunately deceased in 1989), who had a role in the IMPDH story. Together with our Pittsburgh colleague, Richard Abrams, and at the same time as Lagerkvist, she described the first isolation of a mammalian IMPDH[64,65,69] and the role of glutamine in mammalian GMP synthase.[61−63,67] Two other Pittsburgh colleagues, James Franzen and John Rosenberg, kindly provided assistance in dealing with some aspects of this review. I thank Lizbeth Hedstrom for valuable comments after reading a draft of this review and the referees who also provided useful suggestions for revisions. I have to thank Drynda Johnston and Ann Rogers of Langley Library, University of Pittsburgh, for cheerful help in locating many of the references.

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