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Mechanisms of Therapeutic Activity for Gallium

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I. Introduction

Gallium, a semi-metallic element in group 13 (IIIa) of the periodic table, has shown efficacy in the treatment of several apparently diverse disorders. These disorders can be broadly categorized as: (*a*) accelerated bone resorption, with or without elevated plasma calcium; (*b*) autoimmune disease and allograft rejection; (*c*) certain cancers; and (*d*) infectious disease.

Gallium is effective in suppressing bone resorption and, when present, concomitant elevated plasma calcium. This antiresorptive activity has led to its clinical use in treating hypercalcemia of malignancy (Warrell and Bockman, 1989) and Paget's disease of bone (Bockman and Bosco, 1994; Bockman *et al.*, 1989, 1995). Gallium has also shown clinical efficacy in suppressing osteolysis and bone pain associated with multiple myeloma and bone metastases (Warrell *et al.*, 1987a, 1993), and has been suggested as a treatment for osteoporosis (Warrell, 1995). Accumulating evidence indicates that, in addition to antiresorptive activity on bone, gallium also has anabolic activity.

The semimetal also shows specific immunomodulating activities. It is effective in suppressing adjuvant-induced arthritis (Matkovic *et al.*, 1991), experimental encephalomyelitis (Whitacre *et al.*, 1992), experimental autoimmune uveitis (Lobanoff *et al.*, 1997), and allograft rejection (Orosz *et al.*, 1996) in animal models, without being generally immunosuppressive. Other studies have suggested possible efficacy in mouse models for asthma (Apseloff *et al.*, 1996), type I diabetes (Flynn *et al.*, 1992), and endotoxic shock (attenuation of LPS-induced hepatitis; Krecic *et al.*, 1995). Several in vitro experiments have found gallium effective at inhibiting T cell and macrophage activation and in suppressing the secretion of certain cytokines by these cells (Whitacre *et al.*, 1992; Huang *et al.*, 1994; Makkonen *et al.*, 1995; Drobyski *et al.*, 1996).

Through its antiproliferative and antimitotic activity, gallium has shown moderate efficacy against some cancers (Adamson *et al.*, 1975; Foster *et al.*, 1986). Clinical trials have shown particular efficacy against bladder and urothelial carcinomas (Crawford *et al.*, 1991; Seidman *et al.*, 1991; Einhorn *et al.*, 1994) and some lymphomas (Warrell *et al.*, 1983; Weik *et al.*, 1983; Chitambar *et al.*, 1997).

In addition, there is some evidence that gallium is effective against the organisms causing syphilis, trypanosomiasis (Levaditi *et al.*, 1931), and tuberculosis (Olakanmi *et al.*, 1997).

With such a diverse array of reported therapeutic activities, it is useful to look for some common mechanisms. This report examines the currently known biochemical properties of gallium and how these properties bear on the mechanisms of gallium's therapeutic activities.

II. Solution Biochemistry of Gallium

The aqueous chemistry of gallium is summarized by Baes and Mesmer (1976), Taylor and Brothers (1993), and Tuck (1993) and gallium chemistry as it pertains to physiological solutions is reviewed by Green and Welch (1989), Jackson and Byrne (1996), and Weiner (1996). These subjects will be reviewed only briefly here.

A. Aqueous Chemistry of Gallium

Gallium is trivalent in aqueous solution (Ga^{3+}; outer electron configuration $3d^{10}$) (Baes and Mesmer, 1976). The ion Ga^{3+} is a hard acid according to the classification of Pearson (1967): it bonds most readily and strongly in solution to strong Lewis bases, particularly OH^-. Gallium thus has a strong tendency to form chelates through bonds with oxygen and, to a lesser extent, nitrogen atoms on ligands. (In contrast, "soft acids" such as Ag^+ or Au^+ tend to bond with ligands through weaker Lewis bases such as sulfur.)

The free hydrated ion Ga^{3+} hydrolyzes nearly completely at pH values close to neutral, readily forming highly insoluble amorphous $Ga(OH)_3$. Experimental data on the hydrolysis of gallium are scarce and rather inconsistent, so the data presented in the following equations should be regarded as approximate. Equations 1 to 4 present a conventional model of gallium hydrolysis: The "free" Ga^{3+} ion is octahedrally coordinated to six water molecules (the hexa-aqua ion). The waters are replaced stepwise by hydroxyl groups, with concomitant production of hydronium ions, resulting in an acidic solution. At gallium concentrations above approximately 10^{-2} M, at the resulting pH of approximately 2, an amorphous phase close to $Ga(OH)_3$ precipitates (equation 3.1) (Baes and Mesmer, 1976); remaining gallium follows the hydrolysis pathway shown in equation 3.2. The number of water molecules coordinated to aqueous $Ga(OH)_4^-$ is not known; possible coordinating waters are omitted from equation 4.

$$Ga(H_2O)_6^{3+} + H_2O \rightleftharpoons Ga(OH)(H_2O)_5^{2+} + H_3O^+ \quad [1]$$

$$\log K_1 = -2.6 \quad [1A]$$

$$Ga(OH)(H_2O)_5^{2+} + H_2O \rightleftharpoons Ga(OH)_2(H_2O)_4^+ + H_3O^+ \quad [2]$$

$$\log K_2 = -3.3 \quad [2A]$$

$$Ga(OH)_2(H_2O)_4^+ + H_2O \rightleftharpoons Ga(OH)_3(am) + H_3O^+ + 3H_2O \quad [3.1]$$

$$Ga(OH)_2(H_2O)_4^+ + H_2O \rightleftharpoons Ga(OH)_3(H_2O)_3^+ + H_3O^+ \quad [3.2]$$

$$\log K_3 = -4.4 \quad [3.2A]$$

$$Ga(OH)_3(aq) + H_2O \rightleftharpoons Ga(OH)_4^- + H^+ \quad [4]$$

$$\log K_4 = -6.3 \quad [4A]$$

Note: In the above equations, K_n values are stability constants at 25°C and zero ionic strength, where $K_n = [Ga(OH)_n][H^+]^n/[Ga(OH)_{n-1}]$ (omitting coordinating waters: e.g., $Ga(H_2O)_6^{3+}$ is considered as Ga (3+) and H_3O^+ is considered as H^+). Calculated from data of Baes and Mesmer (1976).

If the acidic ionic gallium solution is neutralized, nearly all the dissolved gallium will precipitate as amorphous $Ga(OH)_3$.

$$Ga(OH)_3(am) \rightleftharpoons Ga^{3+} + 3OH^- \quad [5]$$

$$\log K = -37 \quad \text{(Smith and Martell, 1976)} \quad [5A]$$

The amorphous $Ga(OH)_3$ converts on aging to the apparently stable crystalline phase GaO(OH), which is somewhat less soluble in neutral solution than $Ga(OH)_3$, but is soluble in basic solutions with the formation of $Ga(OH)_4^-$(gallate) (Baes and Mesmer, 1976).

$$GaO(OH)(c) + H_2O \rightleftharpoons Ga^{3+} + 3OH^- \quad [6]$$

$$\log K = -39.1 \quad \text{(Smith and Martell, 1976)} \quad [6A]$$

$$GaO(OH)(c) + H_2O + OH^- \rightleftharpoons Ga(OH)_4^- \quad [7]$$

$$\log K = 0.33(5) \quad \text{(Baes and Mesmer, 1976)} \quad [7A]$$

At pH 7.4 and 25°C the total aqueous solubility of gallium (in equilibrium with solid GaO(OH)) is only approximately 1 μM, with the dissolved gallium existing 98.4% as $Ga(OH)_4^-$ and 1.6% as $Ga(OH)_3$ (Baes and Mesmer, 1976; Harris and Pecoraro, 1983). $Ga(OH)_3$ and GaO(OH) display the amphoteric properties of gallium, increasing in solubility at both high and low pH values; the minimum solubility ($10^{-7.2}$ M) is at approximately pH 5.2. Even at pH 2, however, the total solubility of these species is only approximately 10^{-2} M, and at pH 10 it is only approximately $10^{-3.3}$ M (Baes and Mesmer, 1976). The ready formation of $Ga(OH)_3$ and GaO(OH), having low solubilities over a wide pH range, likely accounts for the low bioavailability of gallium when gal-

lium salts are administered orally (Dudley and Levine, 1949; Collery *et al.*, 1989; Ho *et al.*, 1990).

In addition to precipitating as hydroxides and oxyhydroxides, Ga will also form highly insoluble phosphates at pH values close to neutral. The solubility product of $GaPO_4$ is only 10^{-21} (Smith and Martell, 1976).

B. Comparison of Ga^{3+} and Fe^{3+}

The solution and coordination chemistries of Ga^{3+} are somewhat similar to those of Al^{3+} and In^{3+}, but are very similar to those of Fe^{3+}. As discussed throughout this report, the biochemical similarities of these two ions, particularly regarding protein and chelate binding, are likely responsible for many of gallium's physiological activities. The high degree of correspondence in the chemical behaviors of Ga^{3+} and Fe^{3+} can be attributed largely to comparable values for ionic radii and for measures of ionic (electrostatic) versus covalent contributions to bonding. Some of these factors are compared in table 1; the comparison can be further explored through the references cited in table 1 and through data presented by Martell and Hancock (1996).

In addition to the similarities between Ga^{3+} and Fe^{3+}, some significant differences exist. The biochemically most important differences are: (*a*) Ga^{3+} is virtually irreducible under physiological conditions, whereas Fe^{3+} can be readily reduced to Fe^{2+} (and then reoxidized). The irreducibility of Ga^{3+} under physiological conditions appears to prevent it from entering Fe^{2+}-binding molecules such as heme (Logan *et al.*, 1981), and to prevent it from participating in redox reactions. (*b*) Unbound Fe^{3+} is essentially unable to exist in aqueous solutions at pH values close to neutral (Fe^{3+} precipitates as polymerized, hydrated FeO(OH) in aqueous solution at pH 7.4, limiting Fe^{3+} solubility to approximately 10^{-18} M; Weiner, 1996). The ability of significant $Ga(OH)_4^-$ (gallate) concentrations to exist in plasma (as detailed in Section II.C.) may allow for transport and biochemical reactions not possible for plasma Fe^{3+}, which can only exist bound to proteins or as chelates (Weiner, 1996).

C. Gallium Localization and Speciation in Blood

Unlike simple aqueous solutions, blood contains thousands of dissolved components, including many proteins, small molecule ligands, anions, anionic groups, metal ions, and complexes of all these, in addition to colloidal and cellular components. In vivo studies using trace (subnanomolar) ^{67}Ga find that virtually all gallium in blood is present in plasma, with traces in leukocytes (Clausen *et al.*, 1974; Camargo *et al.*, 1979). These studies also show that nearly all plasma gallium is tightly bound to the iron-transport protein transferrin (Clausen *et al.*, 1974; Vallabhajosula *et al.*, 1980). It is thus worth taking a brief look at the nature of transferrin.

Transferrin (TF)[b] is a protein with two homologous lobe-like domains having a total molecular weight of 79,570. Each domain can independently bind one Fe^{3+} (or Ga^{3+}) ion, together with one carbonate or bicarbonate anion per metal ion (Brittenham, 1991). The total amount of TF in humans is approximately 240 mg/kg, divided equally between plasma and extravascular fluids (Huebers and Finch, 1987). The iron binding capacity of this TF (referred to as the total iron binding capacity) is normally approximately 330 μg/dl (or 3.3 μg/ml); typically, only approximately 33% of TF binding sites are occupied by Fe^{3+} at any one time (Brittenham, 1991). Thus, unoccupied TF binding sites in plasma are normally available for a maximum of approximately 2.2 μg/ml of Fe^{3+}, or approximately 2.7 μg/ml of Ga^{3+}. Due to the movement of TF between the blood and extravascular fluids, its volume of distribution is expected to be large. Further information on TF biochemistry is reviewed by Brittenham (1991) and Huebers and Finch (1987).

Transferrin transports its metal load into cells via the TF receptor, a protein that can bind two TF molecules. This receptor binds most strongly to diferric TF, less strongly to monoferric TF, and weakly to apotransferrin (having no metal ions) at neutral pH levels. The complex of metal-bearing TF and TF receptor is taken into the cell by endocytosis; the endosome is then acidified to release the metal (which occurs at less than pH 5.5), and the TF and TF receptor are reused (Brittenham, 1991). It is generally assumed that Fe-TF and Ga-TF follow this same receptor-mediated path, but this assumption has not been specifically addressed experimentally. Studies by Chitambar and Seligman (1986) suggest that cellular incorporation of Ga-TF interferes with intracellular release of Fe from Fe-TF by preventing sufficient acidification of the endosome.

All nucleated cells of the body appear to express TF receptor, but concentrations vary widely. In normal tissue, the greatest amounts of TF receptor are expressed by hepatocytes, Kupffer cells, erythroid precursors (particularly in marrow), and cells of the placenta, basal epidermis, endocrine pancreas, seminiferous tubules, and mucosal epithelium (Gatter *et al.*, 1983; Huebers and Finch, 1987). Tissue macrophages other than Kupffer cells may also express large amounts of TF receptor (Gatter *et al.*, 1983; Byrd and Horwitz, 1993). Malignant cells generally have very high TF receptor expression (Gatter *et al.*, 1983; Huebers and Finch, 1987). All these cell types have high iron requirements:

[b] Abbreviations: ATP, adenosine triphosphate; EAE, experimental autoimmune encephalomyelitis; Ga-TF, transferrin-gallium; GVEC, gonadal vein endothelial cells; IL, interleukin; INF, interferon; LF, lactoferrin; LPS, lipopolysaccharide; MBP, myelin basic protein; MHC, major histocompatibility complex; mRNA, messenger ribonucleic acid; OP, osteopontin; OVA, ovalbumin; PTH, parathyroid hormone; PTHrP, parathyroid hormone related protein; PTPase, protein tyrosine phosphatase; ROS, rat osteogenic sarcoma cells; RR, ribonucleotide reductase; TF, transferrin; TNF, tumor necrosis factor.

TABLE 1
Some chemical parameters for Ga^{3+}, Fe^{3+}, Al^{3+}, and In^{3+}

Parameter	Unit	Ga^{3+}	Fe^{3+} high spin	Al^{3+}	In^{3+}	Ref.
Ionic radius (octahedral)	Å	0.620	0.645	0.535	0.800	1
Ionic radius (tetrahedral)	Å	0.47	0.49	0.39	0.62	1
Ionization potential (4th ionization potential)	eV	64	54.8	119.99	54	2
Electron affinity (3rd ionization potential)	eV	30.71	30.65	28.45	28.03	2
Absolute hardness (Pearson)	eV	17	12.08	45.77	13	2
Electronegativity (Pauling)	Pauling units	1.81	1.83	1.61	1.78	3
Metal-oxygen bond dissociation energy	KJ mol^{-1}	353.5	390.4	511	320.1	4
First metal-hydroxide formation constant: $K_1 = [MOH^{2-}]/[M^{3+}][OH^-]$	log K_1	11.4	11.81	9.01	10.0	5
Tendency to ionic bonding (H_A)	none	7.69	7.22	10.50	6.30	6

References: (1) Shannon, 1976; (2) Pearson, 1988; (3) Huheey et al., 1993; (4) Kerr, 1996; (5) Smith and Martell, 1976; (6) Hancock and Marsicano, 1980.

dividing cells require iron for ribonucleotide reductase production, which is essential for DNA synthesis; erythroid precursors require iron for the synthesis of hemoglobin; and many cells of the monocyte-macrophage line accumulate and store iron.

Gallium ions can bind to the two metal sites of transferrin with binding constants log K_1 = 20.3 and log K_2 = 19.3 at normal plasma bicarbonate concentrations; these values compare to the Fe^{3+}-TF binding constants log K_1 = 22.8 and log K_2 = 21.5 under the same conditions (Harris and Pecoraro, 1983). For comparison, at neutral pH the binding constant for gallium citrate is approximately log K = 10.0 and for gallium EDTA it is approximately log K = 21.7 (Green and Welch, 1989). Although the affinity of transferrin for Fe^{3+} is approximately 400 times higher than for Ga^{3+}, the replacement of Ga^{3+} by Fe^{3+} is found to proceed very slowly, with an exchange half-life of 4.3 h at 310 K (based on ^1H NMR studies by Kubal *et al.*, 1983). This slowness likely reflects the energy required to "open up" the metal sites once they are occupied.

Taking into account the formation constants of Ga^{3+} with transferrin and other potential ligands, the speciation of nanomolar concentrations of gallium in blood plasma has been considered in detail by Jackson and Byrne (1996); higher concentrations have been considered by Graham E. Jackson (personal communication, 1997). A computer model of blood plasma was used that incorporated 41 ligand and nine metal-ion concentrations (other than Ga^{3+}), plus pH, of plasma. Available transferrin binding sites were set at a concentration of 50 μM. The results of simulations using this model are presented in figure 1. It is notable that up to a total Ga concentration of almost 20 μM, the proportion of Ga-TF remains more than 99.9%; at 50 μM Ga it is approximately 95%, and at higher total Ga concentrations (above TF saturation) the percent of Ga-TF drops off rapidly, replaced predominately by gallate, $Ga(OH)_4^-$. The proportion of gallate changes little through 20 μM: gallate represents approximately 0.035% of a 1 nM plasma Ga concentration, approximately 0.08% of a 1 μM plasma Ga concentration, and approximately 0.15% of a 20 μM plasma Ga concentration.

Although all available data indicate that <50 μM plasma gallium under normal physiological conditions exists bound almost entirely to transferrin, several situations can alter the gallium disposition. A deficiency of apotransferrin, caused either by a lack of the protein or by overload of trivalent metal (such as Ga^{3+} or Fe^{3+}), may not permit all available gallium to be bound to transferrin. In this case, most of the unbound gallium will exist as $Ga(OH)_4^-$; less than 2% of the unbound gallium will exist as $Ga(OH)_3$, and traces will be present as citrate, phosphate, and other species (Harris and Pecoraro, 1983; Jackson and Byrne, 1996; Weiner, 1996). Even in the presence of excess apotransferrin, a large sudden influx of gallium cannot bind to transferrin all at once. Thus, when gallium enters the bloodstream rapidly (as from i.v. administration), the largest portion is initially present as $Ga(OH)_4^-$, even when administered with citrate (Jackson and Byrne, 1996). When gallium enters the bloodstream more gradually (as when administered orally) it is likely to bind almost entirely to TF as it is entering. Few data exist on the therapeutic plasma concentrations of Ga, but they are postulated to be approximately 10 to 15 μM in the acute treatment of hypercalcemia of malignancy (Warrell *et al.*, 1986), which is within the range where Ga is normally more than 99.9% bound to transferrin. At concentrations several times this high, however, significant amounts of gallate will be present even at equilibrium. As discussed in Section II.B., it is possible that large amounts of plasma gallate may be associated with the nephrotoxicity in rats (Newman *et al.*, 1979) and humans (Krakoff *et al.*, 1979) that has sometimes been observed after large i.v. bolus doses of gallium; maximum plasma Ga in these cases was reported to exceed 200 μM.

In addition to binding to transferrin, gallium binds even more avidly to the related protein lactoferrin, which can remove Ga from transferrin (Harris, 1986). Lactoferrin (LF), like TF, is a two-lobed protein with a molecular weight of approximately 80,000, which can bind two Fe^{3+} (or Ga^{3+}) ions (Levay and Viljoen, 1995). The binding constants of Ga-LF in plasma are log K_1 = 21.43 and log K_2 = 20.57, or approximately 90 times those of Ga-TF (Harris, 1986). Apolactoferrin (which possesses antibacterial activity) is concentrated in many epithelial secretions such as milk, seminal fluid, tears, and nasal secretions, typically in amounts of 0.5 to 1 mg/ml (Masson *et al.*, 1966; Larson and Schall, 1971). It

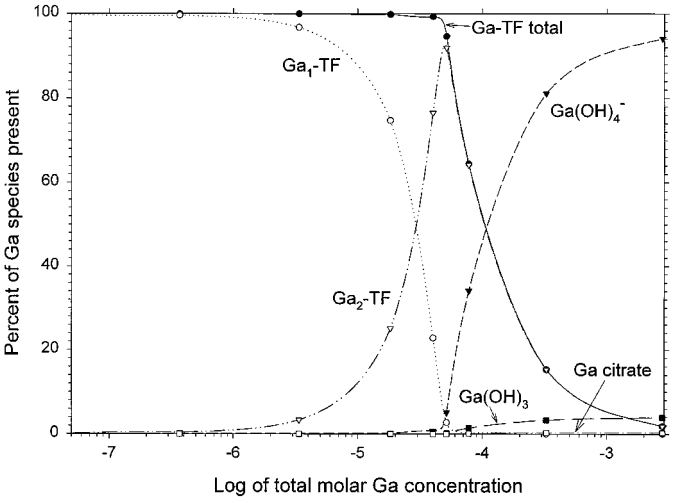

FIG. 1. Gallium species present in normal human blood plasma at pH 7.4, based on calculations of Graham E. Jackson (personal communication, 1997), using the model of Jackson and Byrne (1996). Species shown (which all attain relative concentrations of at least 0.1% over the Ga concentration range examined) are: $Ga(OH)_4^-$; $Ga(OH)_3$; Ga(citrate)(OH^-); TF with one Ga (Ga_1-TF) or two Ga (Ga_2-TF) ions bound in the Fe^{3+}-binding sites, together with a carbonate and two hydroxyl groups per bound Ga ion; and the sum of Ga_1-TF and Ga_2-TF (Ga-TF total).

is also observed to concentrate at sites of inflammation and infection, particularly in granulomateous neutrophils and polymorphonuclear leukocytes (Masson *et al.*, 1969; Bennett and Kokocinski, 1978). Extracellular LF at such sites is derived primarily from activated neutrophils (Birgens, 1991).

A third iron-binding protein to which gallium can bind is ferritin, a very large (MW = 440,000) nearly spherical protein that can hold as many as 4500 Fe^{3+} ions (Brittenham, 1991), situated mainly in a hydrated ferric oxide-hydroxide core (Crichton and Charloteaux-Wauters, 1987). Ferritin is used for iron storage, is present in most cells to varying degrees, and is particularly concentrated in the Kupffer cells of the liver; it is also concentrated in some other tissue macrophages. The transfer of gallium from TF or LF to ferritin appears to be mediated by adenosine triphosphate (ATP), pyrophosphate, and other phosphate-containing compounds (Weiner *et al.*, 1985; Weiner, 1989). In this regard, ATP is more effective than adenosine diphosphate, which is more effective than adenosine monophosphate (Weiner *et al.* 1983). Phosphate-containing compounds appear to promote the translocation of Ga by binding directly to TF (and possibly LF), inducing comformational changes that destabilize the protein-Ga bonds; the Ga then reacts to form dissolved gallate, which can readily donate Ga to ferritin (Weiner, 1989). Bicarbonate (which coordinates to metal in the TF complex) prevents ATP-mediated Ga translocation at serum concentration (30 mM), but not at intracellular concentrations (0.4 and 5 mM) (Weiner, 1989). The presence of ATP, which is abundant in metabolically active sites, thus can serve to enhance the storage of gallium within cells. At sites of inflammation, iron-bearing LF secreted by neutrophils is observed to be taken up by macrophages (particularly activated macrophages), which extract the contained iron and store it in ferritin (Birgens, 1991); the same process may occur for gallium-bearing LF.

It is likely that gallium can substitute for Fe^{3+} in at least some of the many other Fe^{3+}-containing proteins in the body; such a substitution is known to occur in ribonucleotide reductase (Narasimhan *et al.*, 1992).

Gallium is not, however, able to enter Fe^{2+}-bearing proteins, such as hemoglobin and cytochromes (Logan *et al.*, 1981; Chitambar and Seligman, 1986). This behavior is important, because gallium does not enter red blood cells and interfere with oxygen transport.

III. Physiological Transport and Distribution of Gallium

The mechanisms whereby gallium is transported and distributed in the body are of fundamental importance to elucidating gallium's biological activities. A great deal of knowledge has accumulated in this area through the extensive use of gallium radioisotopes to diagnose and study a variety of neoplastic, inflammatory, and bone diseases. This widespread use of gallium radioisotopes has in itself led to considerable research designed to better understand gallium transport and tissue localization.

It has been known since the late 1940s that gallium (mainly as radioactive ^{67}Ga or ^{72}Ga) has a strong affinity for certain tissues, particularly growing or remodeling bone and many tumors (e.g., Dudley and Maddox, 1949; Nelson *et al.*, 1972). Observations beginning in the 1970s have shown that gallium accumulates avidly at sites of inflammation and infection, including sites of granulomatous inflammation (Hoffer, 1980; Tsan, 1985) and synovitis associated with rheumatoid arthritis (McCall *et al.*, 1983). In addition, gallium concentrations are commonly observed in the liver, spleen, kidneys, and in lactating breasts (Larson and Schall, 1971; Larson and Hoffer, 1978; Vistelle *et al.*, 1989).

A. Dose Effect on Tissue Distribution

As most in vivo studies of gallium tissue distribution have used trace quantities of radioactive gallium, the question must be addressed of how results of such studies can be applied to cases where larger, therapeutic doses of nonradioactive gallium are administered. It is noted that early experiments with radiogallium (in the 1940s and early 1950s) used mainly ^{72}Ga, which was administered together with large amounts of stable gallium carrier (at least 5 to 10 mg/kg; Dudley and Maddox, 1949; Bruner *et al.*, 1953b); these doses were in the physiologically active range. (Large amounts of carrier were used because ^{72}Ga was produced by neutron irradiation of stable ^{71}Ga, which cannot be chemically separated from the radioisotope.) More recent studies have used mainly ^{67}Ga, which is a product of proton irradia-

tion of ^{68}Zn. Because ^{67}Ga is readily separated from the parent Zn by chemical means, highly pure carrier-free radioisotope is routinely prepared and used, generally at subnanomolar in vivo concentrations (doses on the order of 10^{-4} mg Ga/kg).

Several studies have specifically addressed the relations between administered Ga dose and resulting Ga retention, excretion, and tissue distribution. Dudley and Marrer (1952) studied retention of Ga in bones of rats and rabbits 24 h after i.v. or s.c. administration of Ga citrate, in doses ranging from 1 to 45 or 50 mg Ga/kg. They found that Ga uptake was directly proportional to dose up to 15 mg/kg, above which the Ga uptake as a proportion of administered dose decreased; the route of administration had no significant effect. Bruner *et al.* (1953b) found that as the dose of Ga citrate (labeled with a trace of ^{72}Ga) in rats increased from 0.5 to 26 mg Ga/kg, a proportionately higher amount was excreted (35% at 0.5 mg/kg to 72% at 26 mg/kg), with a higher percentage excreted in the urine (amount in urine/amount in feces was 6.3 at 0.5 mg/kg and 60.7 at 26 mg/kg). They found, however, that the percentage distribution and differential absorption ratios in body tissues, as measured 0.5 to 4 days after Ga administration, were unaffected by dose over the studied range. In another series of experiments, Bruner *et al.* (1953a) found that the carrier-free ^{67}Ga citrate (dose $<10^{-4}$ mg Ga/kg) tissue distribution pattern in rats was retained up to stable Ga carrier concentrations of at least 0.25 mg/kg, as measured 5 days after dosing. At 2.5 and especially at 25 mg Ga/kg, the proportional amount of Ga retained by soft tissues decreased although the proportional amount retained by bone decreased very slightly, resulting in a higher proportion retained by bone relative to soft tissue. Schomäcker *et al.* (1986) administered to mice trace ^{67}Ga citrate together with carrier Ga in doses of 0 to 150 μg (approximately 5 mg/kg). High carrier Ga doses resulted in increased urinary excretion, blood clearance, and uptake in femur and bone marrow, as well as decreased uptake in tumor, spleen, liver, and muscle. Nelson *et al.* (1972), in human autopsy studies after administration of ^{67}Ga citrate to cancer patients, concluded that there was no significant difference in Ga tissue distribution between subjects who received carrier-free solution and those who received 0.2 mg/kg carrier (although there may have been a slight increase in bone Ga retention in subjects who received carrier, possibly due to Ga uptake by neoplastic bone tumors); in addition, no significant differences were observed between those groups of subjects and single subjects who received 1.0 and 2.0 mg/kg carrier. No effect of carrier and dose-to-death time (which ranged from 3 h to 22.7 days) was observed.

Although the results of these studies are not entirely consistent, all Ga tissue distribution studies find that Ga concentrates at the same tissue sites (e.g., bone, liver, many tumors) regardless of dose, with the relative proportion excreted in urine and retained by bone generally increasing at high doses. As discussed previously, at carrier-free and other low Ga doses, nearly all Ga is expected to bind to transferrin, whereas at high Ga doses (above transferrin saturation, $\approx$50 μM), relative gallate concentrations increase substantially. Even at Ga plasma concentrations well less than 50 μM, the absolute gallate concentration at equilibrium can exceed nanomolar amounts. Rapid renal elimination of the small, charged gallate group is expected. The observed differential dose-dependent tissue distributions may result from preferential uptake of either Ga-TF or gallate by different tissues, as will be discussed in subsequent sections.

B. Distribution of Gallium to Soft Tissues and Tumors from Blood

In light of the discussion on gallium localization and speciation in blood, it appears reasonable to expect gallium to concentrate in tissues having a high concentration of TF, TF receptor, lactoferrin, or ferritin. In fact, to a large extent this is what is observed. Gallium is found to concentrate in proliferating tissue (including most tumors), where large amounts of TF receptor are expressed; in milk, tears, and areas of inflammation that contain large amounts of lactoferrin or of ferritin-rich macrophages; and in the spleen, liver, and other sites that contain an abundance of both TF receptor and ferritin. The situation is evidently more complex, however, as gallium does not concentrate equally in all proliferating tissue, and it does concentrate at sites of bone formation and remodeling, which do not normally contain high concentrations of Fe^{3+}-binding proteins.

The mechanisms for the concentration of gallium in tumors (which are the basis for the widespread use of ^{67}Ga imaging as a diagnostic technique for many malignancies) are still not fully understood and remain somewhat controversial. In almost all cases, the concentration of gallium in malignant tissue correlates with TF receptor expression (Larson *et al.*, 1980; Chitambar and Zivkovic, 1987; Weiner, 1996). For example, one study found that tumor cells from a variety of lymphomas having different affinities for ^{67}Ga showed a high correlation between transferrin receptor (CD71) expression and ^{67}Ga binding avidity (Feremans *et al.*, 1991). Another study (Tsuchiya *et al.*, 1992) found a high correlation between TF receptor expression and ^{67}Ga uptake in lung tumors (squamous cell and adenocarcinomas).

Although it is clear that in the majority of cases gallium enters tumor cells through TF and the TF receptor, several studies make it apparent that gallium can enter tumor and other cells by a TF-independent mechanism, which is probably also used by iron (Chitambar and Zivkovic, 1987; Chitambar and Sax, 1992; Weiner *et al.*, 1996). Some gallium-avid tumors may, in fact, preferentially use TF-independent gallium absorption (Sohn *et al.*, 1993). The TF-independent mechanism, which is

very inefficient in most tumors but nearly as efficient as typical TF-dependent absorption in a few tumors, becomes apparent when TF is in short supply or when it is saturated with iron or other metals (Sohn *et al.*, 1993). No experimental data exist on the details of this proposed mechanism; gallate, as a very small molecule that may easily penetrate some cellular membranes, may play an important role.

The distribution of gallium once incorporated into tumor cells appears variable. Some studies show gallium concentration mainly in nuclei and in ferritin (Clausen *et al.*, 1974), although others find high concentrations in lysosomes or lysosome-like bodies (Swartzendruber *et al.*, 1971; Berry, 1996) or in cytoplasm (Deckner *et al.*, 1971). The reasons for the observed variability in distribution within tumor cells have not been resolved, and probably relate to the diverse methods used in the different studies, and perhaps to differences in the studied cell types and their stages of development.

In addition to concentrating in tumor cells themselves, gallium also concentrates in the lysosomes of associated macrophages (Collery *et al.*, 1984). Gallium concentrated in the lysosomes of macrophages and other cell types appears to be in the form of phosphate granules (Berry, 1996). These gallium phosphates appear to result from the action of endogenous acid phosphatases, which liberate phosphate ions from phosphate ester substrates. The free phosphate can then react with gallium, forming a gallium phosphate precipitate (Berry, 1996).

As well as concentrating in lysosomes, it is likely that gallium within all macrophages concentrates in ferritin, a commonly abundant component of these cells. Human macrophages in vitro acquire iron by both TF-dependent and TF-independent (small-molecule iron chelate) pathways. The TF-independent pathway appears to become important under conditions of iron overload; gallium nitrate can apparently stimulate this pathway although Ga-nitriloacetate can suppress it (Olakanmi *et al.*, 1994). The ferritin content and TF receptor expression of human macrophages are up-regulated by Fe-TF itself, but down-regulated by interferon-gamma (Byrd and Horwitz; 1993). Interestingly, as discussed in Section V., gallium has been found to inhibit interferon-γ secretion by activated T lymphocytes; this inhibitory activity could maintain TF receptor expression and thus TF receptor mediated entry of gallium into macrophages at sites of inflammation.

At sites of inflammation and infection, much gallium may be concentrated by lactoferrin, as mentioned. In addition, gallium may be concentrated by leukocytes, particularly neutrophils and lymphocytes (Camargo *et al.*, 1979; Tsan, 1985). Experimental information on gallium uptake by normal leukocytes is scant, but limited data suggest that uptake by T lymphocytes rises in the presence of TF (Drobyski *et al.*, 1996). Gallium can also accumulate in bacteria, probably concentrating in ferric iron-binding proteins, through siderophore- (ferric iron binding chelator) dependent and -independent mechanisms (Menon *et al.*, 1978; Emery and Hoffer, 1980).

Gallium has, in a few cases, been associated with nephrotoxicity in rats (Newman *et al.*, 1979) and humans (Krakoff *et al.*, 1979). Significant nephrotoxicity has only been observed after large i.v. bolus doses of gallium: maximum plasma Ga in these cases was reported to exceed 200 μM. It is likely that this nephrotoxicity stems from high plasma concentrations of gallate, which, as previously discussed, arise when transferrin saturation is exceeded (approximately 50 μM Ga). Gallate, as a small charged molecule, will be rapidly excreted by the kidneys, where it may transiently reach high concentrations. The mechanism of the renal toxicity in humans is not known, but Newman *et al.* (1979) found that in rats it appears due to mechanical occlusion of the renal tubular lumina by Ga-Ca-phosphate precipitates. It is likely that gallium phosphate solubility is exceeded in regions of high gallate concentration in the renal tubules, leading to precipitation of gallium phosphates, which act as nucleation sites for supersaturated calcium phosphates. This hypothesis is substantiated by the observations of Newman *et al.* (1979) that diuresis of Ga-treated rats significantly reduced urinary Ga concentrations and the severity of renal damage. It is important to note that ever since the earliest clinical studies, gallium has been administered to thousands of individuals via slow intravenous infusion or subcutaneous injection with no significant resulting renal toxicity (e.g., Leyland-Jones *et al.*, 1983). Under such conditions, transferrin saturation will not generally be exceeded, and gallate levels will not rise to toxic levels in the kidneys. If this proposed mechanism for nephrotoxicity is correct, then only patients who are substantially hypotransferrinanemic, whose TF is saturated with iron due to transfusions or other reasons, or whose TF is saturated with other metals, may be at risk for nephrotoxicity when Ga is administered other than by high-dose rapid intravenous bolus. For such patients, or for patients with existing renal damage, proper hydration and use of diuretics should substantially lower the risk of nephrotoxicity.

C. Distribution of Gallium to Bone from Blood

Gallium has long been known to concentrate in skeletal tissue, particularly regions of bone deposition and remodeling (e.g., Dudley and Maddox, 1949; Nelson *et al.*, 1972). In growing bone, gallium is concentrated in the metaphysis, particularly in the hypertrophic cartilage zone (growth plate); it is also concentrated in regions of fracture healing (Dudley and Maddox, 1949; Dudley *et al.*, 1950; Bockman *et al.*, 1986, 1990). To a lesser extent, gallium accumulates on the endosteal and periosteal surfaces of diaphyseal bone (Bockman *et al.*, 1990).

Unlike the gallium uptake of most tumors and soft tissues, most of the gallium uptake by bone does not

appear to involve the TF receptor. Animals or humans lacking TF, or that have saturated TF due to iron or other metal overload, absorb gallium into skeletal tissue at the same or a greater rate than those with normal TF (Lentle *et al.*, 1984; Sohn *et al.*, 1993). People who receive repeated blood transfusions, resulting in iron-saturated TF, show high ^{67}Ga bone activity after injection with ^{67}Ga-citrate, plus increased ^{67}Ga excretion in the urine (and increased kidney activity) (Englestad *et al.*, 1982). Congenitally hypotransferrinanemic mice that received i.v. infusions of ^{67}Ga citrate in isotonic NaCl solution showed moderate increases in ^{67}Ga uptake in all tissues compared with control animals, but marked increases in the renal cortex (9-fold) and bone (20-fold) (Radunović *et al.*, 1997). The same study found that control mice pretreated with the anti-TF receptor antibody RI7 208 were similarly affected. As mentioned, in all such cases of reduced apoTF availability, much gallium will be present in plasma as gallate, $Ga(OH)_4^-$.

Very little information exists on mechanisms of gallium uptake by bone cells. Guidon *et al*. (1991) found that the in vitro gallium nitrate inhibition of bone Gla-protein (osteocalcin) synthesis was TF independent. Kasai *et al*. (1990), however, found that the inhibition of proliferation by gallium of UMR-106–01 osteoblast-like cells is TF dependent, and that TF receptor expression in these cells is regulated in a way similar to other cell types.

The mechanisms of skeletal gallium accumulation remain largely unknown. Gallium is known to adsorb in vitro to synthetic hydroxyapatite (the predominant calcium phosphate phase of bone) and to slow its crystallization and probably its dissolution (Donnelly and Boskey, 1989; Blumenthal and Cosma, 1989); this effect is similar to that observed for the chemically related trivalent metal ions Al^{3+}, Cr^{3+}, and Fe^{3+} (Christoffersen *et al.*, 1987). Adsorbed gallium could come directly from the small amount of gallate present in plasma; it could also come from Ga-TF, after separation of the gallium by high concentrations of phosphate (forming gallium phosphates), by locally acidic conditions (as occur from osteoclastic secretions or from acid phosphatases), or by chelating compounds. Weiner (1989) reported that some phosphates (including ATP and pyrophosphate) can bind to Ga-TF, altering the protein conformation so that Ga is released to form soluble gallate. Once put locally into solution, it is known that gallium can be extracted nearly quantitatively by coprecipitation with calcium phosphate at pH < 7.5 (Dymov and Savostin, 1970). Limited experimental evidence indicates that approximately half the 300 μg/g gallium present in metaphyses of femurs from rats treated with gallium is in tetrahedral coordination, probably in gallium phosphates (Bernstein and Bockman, 1988). These bits of evidence support the concept of some gallium precipitating as inorganic phosphates in bone tissue, particularly in newly mineralizing tissue.

IV. Effects on Bone

Many studies have shown that gallium inhibits bone resorption and lowers plasma calcium through its antiresorptive activity (e.g., Warrell *et al.*, 1984, 1985; Warrell and Bockman, 1989). Several studies provide convincing evidence that gallium also has anabolic (bone-forming) activity (e.g., Bockman *et al.*, 1993; Guidon *et al.*, 1993; Stern *et al.*, 1994). Some attention has been directed to the mechanisms by which gallium acts on the various cellular and noncellular components of bone.

A. Effects on Bone Resorption and Osteoclasts

Several studies have examined the antiresorptive effects of gallium on bone and bone fragments; these studies have led to a close examination of how osteoclasts (bone resorbing cells) are affected. A few of these studies are summarized here.

Devitalized bone powder from rats treated for 2 weeks with gallium nitrate (total dose 45 mg/kg body weight) was less soluble in acetate buffer and less readily resorbed by monocytes (Repo *et al.*, 1988). Bones from gallium-treated growing rats were found to have increased calcium and phosphate content and an increase in the denser fraction of bone (Repo *et al.*, 1988; Bockman, 1991). These effects were dose-dependent in rats treated for 2 weeks at total doses of 4.5, 35, and 45 mg gallium nitrate per kg body weight. Mechanical bone strength was the same in gallium-treated and control animals (Repo *et al.*, 1988; Adelman *et al.*, 1989).

Bone particles from gallium-treated rats (containing 0.39 μg Ga/mg bone), as well as from control rats (containing <0.01 μg Ga/mg bone), were implanted s.c. in growing rats (Donnelly *et al.*, 1991). The gallium-bearing bone particles were found to be significantly more resistant to in vivo resorption than the bone particles from the control rats. This study found that there was no difference in the ability of gallium-treated and untreated bone to recruit osteoclast-like cells (which stained for tartrate-resistant acid phosphatase activity), just in the apparent resorptive activity of these cells.

Gallium nitrate at 0.1 to 100 μg/ml produced a concentration-dependent inhibition of bone resorption by osteoclasts isolated from neonatal rat long bones and cultured on slices of rat cortical bone (Hall and Chambers, 1990). This inhibition was independent of the presence of parathyroid hormone (PTH), and no change in osteoclast morphology or viability was observed, even at the highest dose. Adsorption of gallium to the bone surface was demonstrated by washing the gallium nitrate-treated bone slices and finding that they still inhibited osteoclastic bone resorption. In another study (Warrell *et al.*, 1984), explants of fetal rat bone containing ^{45}Ca incorporated during gestation were pretreated with gallium nitrate at 1, 5, and 10 μg/ml. When the explants were exposed to the resorption stimulators PTH or lymphokine preparations [containing interleukin- (IL)1 plus tumor necrosis factor (TNF) or TNF

alone], dose-dependent inhibition of ^{45}Ca release from the explants was observed; again, no change in osteoclast morphology or number was observed, even after exposure to the highest dose for 72h.

An in vitro study (Blair *et al.*, 1992) found reversible, dose-dependent inhibition of osteoclast activity (bone resorption) from bone-bound gallium at solution [Ga^{3+}] <15 μM, with irreversible cytotoxicity at solution [Ga^{3+}]> 50 μM. This study found that for gallium to be effective at inhibiting osteoclast bone resorption without causing cytotoxicity, it had to be first adsorbed to the bone surface; it was also found that osteoclasts on bone inhibited by approximately 40% the binding capacity of gallium to bone. A related study found that gallium had no effect on anion transport by osteoclast ruffled membrane vesicles, but directly inhibited the vacuolar-class ATPase (an electrogenic proton pump) (Schlesinger *et al.*, 1991).

In patients with Paget's disease who were given ^{67}Ga citrate, Mills *et al.* (1988), using electron microscopic autoradiography, found that ^{67}Ga localized almost exclusively in the nuclei of osteoclasts. The significance of gallium localization in osteoclast nuclei is not clear, but suggests the potential for gallium to affect gene expression and protein synthesis in these cells.

Available studies thus indicate that gallium is adsorbed onto the surface of bone, where it is effective in blocking osteoclastic resorption. At antiresorptive concentrations, gallium does not appear cytotoxic to osteoclasts (as is the antiresorptive agent plicamycin; Bockman, 1991), or to act as a cellular metabolic inhibitor (as do bisphosphonates; Schlesinger *et al.*, 1991).

An area that has received very little attention is the possible action of gallium on zinc-bearing enzymes associated with bone formation and resorption, including collagenases (and related matrix metalloproteinases), ATPases, and carbonic anhydrases. Due to the chemical similarities of gallium and zinc, it is possible that gallium could substitute for zinc in one or more of these enzymes, disrupting their activity. Experiments comparing trace Ga, Fe, Zn, and Cu content of bone in control and Ga-treated rats (2.5 mg/kg Ga nitrate every other day for 14 days) found a slight decrease in Fe/Ca and Zn/Ca levels (but not Cu/Ca levels) in metaphyseal Ga-containing bone from Ga-treated rats (Bockman *et al.*, 1990). These results suggest the possibility that Ga substituted for some Fe and Zn.

B. Effects on Bone Formation (Anabolic Activity) and Osteoblasts

In addition to acting directly on osteoclasts, gallium can, based on considerable experimental evidence, inhibit bone resorption and stimulate bone formation through action on osteoblasts (bone-forming cells). Evidence for in vivo enhancement of bone formation (anabolic activity) is provided by the observed marked increase of bone formation (Stern *et al.*, 1994) and increased bone calcium content (Bockman *et al.*, 1986) in gallium nitrate-treated rats, and the elevated levels of plasma alkaline phosphatase (a marker for bone formation) in postmenopausal women treated with gallium nitrate (Matkovic *et al.*, 1991).

Several in vitro studies have looked at the effects of gallium on rat osteogenic sarcoma (ROS) osteoblast-like cells and on normal rat osteoblasts. Gallium nitrate (buffered with citrate) dose dependently (in concentrations from 5 to 100 μM) decreased constitutive and vitamin D_3-stimulated osteocalcin (OC) and OC mRNA levels in ROS 17/2.8 cells (Guidon *et al.*, 1993). Gallium did not affect the stability of OC mRNA, but rather appeared to block reporter gene expression stimulated by the OC promoter gene. The role of osteocalcin in bone formation is not clear, but a lack of OC is associated with increased bone formation (Ducy *et al.*, 1996), and the protein has been linked to recruitment of osteoclasts (Malone *et al.*, 1982; Mundy and Poser, 1983). Guidon *et al.* (1993) reported that gallium also slightly increased osteopontin (OP) and glyceraldehyde-3-phosphate dehydrogenase mRNA levels in ROS 17/2.8 cells, and suppressed vitamin D_3-stimulated OP mRNA levels, but did not appear to affect other mRNA levels. A more than three-fold increase in c-fos mRNA levels was also observed (Guidon and Bockman, 1990) (c-fos is associated with bone formation).

In agreement with these studies, Jenis *et al.* (1993) found gallium (at 50 μM) to markedly decrease OC mRNA levels in both ROS 17/2.8 and normal rat osteoblast cells. They also showed that mRNA levels for type I collagen (a marker for bone matrix formation) were markedly increased by gallium, although levels for histone H_4 (a marker for cell proliferation) were unchanged. When mice were treated with 0.5 mg/kg/day of gallium nitrate, plasma calcium was reduced and OC steady-state levels in the femur decreased 58%, but type I collagen levels and bone density were not affected. Bockman *et al.* (1993) noted that the dose administered by Jenis *et al.* (1993) was approximately 10% of that used in previous studies where an increase in bone mineral content was observed.

Rat osteoblast cultures (derived from calvaria) were also found to be stimulated by gallium to produce type I collagen and fibronectin by Bockman *et al.* (1993), but only during a particular stage of development. Differentiated osteoblasts at approximately 10 days after confluence responded to gallium, but older and younger cells did not.

Studies on another ROS line, UMR-106, evaluated the effects of gallium on osteoblast stimulation by PTH and some other calcium-regulating hormones (Lakatos *et al.*, 1992). Gallium nitrate, at concentrations as low as 3 μg/ml, inhibited PTH-stimulated calcium transients in these cells. The gallium did not have a general cytotoxic effect, and did not inhibit calcium transients caused by α-thrombin or prostaglandin $F_{1\alpha}$.

It is important to note that PTH has not been observed to stimulate bone resorption by direct action on osteoclasts because they do not appear to express PTH receptor. Instead, osteoblasts, which do carry PTH receptor, respond to PTH and then signal osteoclasts by an unknown mechanism (McSheehy and Chambers, 1986), possibly IL-6 secretion (Grey *et al.*, 1997). An inhibition of osteoblastic response to PTH is thus likely to result in a decrease in PTH-stimulated bone resorption.

C. Effects on PTH Secretion

The parathyroid glands help regulate plasma calcium levels through their secretion of PTH, which stimulates bone resorption and thus raises plasma calcium concentrations. These glands adjust PTH secretion in response to the plasma calcium level, which is monitored by an abundance of extracellular Ca^{2+}-sensing receptor on parathyroid cells (Brown *et al.*, 1993; Segre and Brown, 1996). (Secretion of PTH is also affected to lesser extents by other circulating substances, including 1, 25-dihydroxyvitamin D_3, catecholamines, hormones such as calcitonin, and ions such as Mg^{2+}, Li^{+}, and Al^{3+}; Segre and Brown, 1996.) High plasma calcium inhibits PTH secretion, resulting in diminished bone resorption, whereas low plasma calcium promotes PTH secretion and accelerated bone resorption. The extracellular Ca^{2+}-sensing receptor responds to an increase in plasma calcium by inducing a rise in cytoplasmic Ca^{2+} as well as a decrease in cytoplasmic cAMP in parathyroid cells (Brown, 1991).

Gallium nitrate at 200 μM inhibits PTH release from bovine parathyroid cells, as well as from normal and pathological human parathyroid cells (Ridefelt *et al.*, 1992, 1995); at 100 μM, however, no such effect was observed (Warrell *et al.*, 1987b). According to Ridefelt *et al.* (1992), Ga^{3+} does not act simply as a Ca^{2+} analog, because it does not increase cytoplasmic Ca^{2+} or decrease cytoplasmic cAMP in parathyroid cells. Gallium would thus appear to inhibit PTH secretion by a mechanism not involving the extracellular Ca^{2+}-sensing receptor. Ridefelt *et al.* (1992) proposed that Ga^{3+} may stabilize the plasma membrane in a manner that inhibits exocytosis; this hypothesis has not been tested. Interestingly, the bisphosphonate pamidronate was found to have no effect on PTH secretion (Ridefelt *et al.*, 1995). Some clinical studies in patients with cancer-related hypercalcemia have found that gallium treatment increases plasma PTH levels (Warrell *et al.*, 1987a,b). Ridefelt *et al.* (1992, 1995) point out that this increase is likely due to the lowering of plasma Ca^{2+} by the inhibition of bone resorption; they further point out that in patients with parathyroid carcinoma, gallium treatment decreased plasma Ca^{2+} and slightly decreased plasma PTH (Warrell *et al.*, 1987c). As the effective Ga concentration (200 μM) reported by Ridefelt *et al.* (1992, 1995) is much higher than known safe therapeutic serum levels (approximately 5–25 μM Ga), further studies will be required to explore whether gallium has direct in vivo activity to inhibit parathyroid secretion of PTH.

No published experiments have examined the effect of gallium on secretion of parathyroid hormone-related protein (PTHrP) by some malignant cells. As such secretion is thought to be the major factor in causing cancer-related hypercalcemia, the results of such experiments would shed further light on the mechanisms whereby gallium is effective in treating this condition.

D. Effects on Secretion of IL-6 and Other Osteoclast-Activating Cytokines

Osteoclast formation from progenitor cells, as well as osteoclastic bone resorption, are stimulated by IL-6 together with other cytokines such as IL-1, IL-3, and IL-11 (reviewed by Manolagas *et al.*, 1995). In addition, Paget's disease (Roodman *et al.*, 1992; Roodman, 1996) and multiple myeloma (Klein *et al.*, 1991) are characterized by greatly elevated IL-6 levels in bone lesions. The IL-6 may be secreted by osteoblasts, osteoclasts, and their progenitor cells in bone marrow (Hoyland *et al.*, 1994). The secretion of IL-6 is inhibited by estrogen (Girasole *et al.*, 1992) and androgen hormones (Bellido *et al.*, 1993): the high incidence of senile osteoporosis, particularly postmenopausal osteoporosis, may be due in part to an increase in IL-6 levels as the levels of sex hormones decrease.

As discussed in the following section, gallium inhibits the secretion of IL-6 and some other cytokines from macrophage-like cells. Because osteoclasts are derived from the same line as macrophages, with which they share many characteristics (Manolagas *et al.*, 1995), it is likely that gallium has a similar effect on osteoclasts. The efficacy of gallium in treating Paget's disease (Bockman *et al.*, 1995) and in suppressing osteolysis associated with multiple myeloma (Warrell *et al.*, 1993) are also suggestive of this activity. Substantiation of an anti-IL-6-secreting effect in bone cells by gallium awaits further experimentation.

V. Immunomodulating Activity

Effects of gallium on immune responses were first noted by Maurel (1973) and Bouissou *et al.* (1973). They found that a low dose of gallium sulfate (2.5 mg/kg/day for 5 weeks) aggravated the progression of tuberculosis in guinea pigs and suppressed the allergic response in infected animals to tuberculin injections. The authors hypothesized that gallium was suppressing macrophage and/or T cell response. Delbarre and Rabaud (1976) found that gallium (0.74 and 1.48 mg Ga/kg as gallium sulfate) suppressed the cutaneous tuberculin response in rats.

Several subsequent animal and in vitro studies have shown gallium to suppress certain immune reactions, without being generally immunosuppressive or cytotoxic. Gallium appears to target specific inflammatory and proliferative responses, particularly those mediated

by T lymphocytes and macrophages. A few representative studies are briefly discussed here.

Gallium sulfate (daily doses of 0.185–1.48 mg Ga/kg from day 0 of the study, or 1.48 mg Ga/kg on day 3; Delbarre and Rabaud, 1976) or gallium nitrate (30 mg Ga/kg on day −1, then 10 mg Ga/kg weekly; Matkovic *et al.,* 1991) administered s.c. to rats suppressed the development of adjuvant arthritis, a T cell-mediated autoimmune disease with similarities to Reiter's disease and rheumatoid arthritis in humans. Animals that received gallium developed less synovitis, pannus, subchondral resorption, cartilage degeneration, and periosteal new bone formation than control diseased animals. Related in vitro studies on purified-protein-derivative-specific rat T cells found gallium to block both antigen-specific and mitogenic proliferative responses (Matkovic *et al.*, 1991). Interestingly, the same cell line exposed to gallium but not to antigen or mitogen showed normal to slightly enhanced proliferative activity, indicating that gallium was not directly toxic to the cells. Delbarre and Rabaud (1976) found that lymphocytes from gallium-treated rats had normal responses to specific and nonspecific mitogens.

Experimental autoimmune encephalomyelitis (EAE), a T cell-mediated autoimmune disease used as a model for demyelinating human diseases such as multiple sclerosis, was also suppressed in rats by weekly s.c. administration of gallium nitrate (Whitacre *et al.*, 1992). When only a single gallium injection was administered, the timing was important: maximum disease suppression occurred when administration was on day 6 after induction of EAE, with less suppression resulting from administration on days 3 or 9, and none on day 12. Lymphocytes extracted from gallium-treated and untreated rats were tested in vitro for their proliferative responses. The proliferative response to myelin basic protein (MBP; the antigen used to induce EAE) was suppressed in cells from gallium-treated animals (treated on days 3, 6, 9, or 12) compared with those from nontreated animals. The proliferative response to the mitogen concanavalin A, however, was not suppressed in cells from gallium-treated animals, indicating that gallium did not have a general toxic effect. Further experiments, using a separate MBP-specific T lymphocyte line, found that the proliferative response of such cells to MBP was suppressed when gallium was added to the cell cultures within 48h of initiation of culture; no effect was seen at 62h. All these results indicate that gallium acts to suppress T cell proliferation at early stages of activation, and is not simply toxic to T cells.

Similarly, gallium nitrate (30 mg Ga/kg at −1 day; 10 mg Ga/kg at 1, 4, 7, 10, 13, 16, and 19 days) significantly inhibited the development of experimental autoimmune uveitis in rats (a T cell-mediated disease, induced by injecting the rats with a solution containing retinal S-antigen and *Mycobacterium tuberculosis*) (Lobanoff *et al.*, 1997). Gallium was highly effective at preventing clinical and histological signs of retinal and choroidal inflammation. Lymphocyte proliferative responses in Ga-treated rats to S antigen, purified protein derivative, and concavalin A were all decreased by small, although significant, amounts. Gallium also caused a small decrease in the humoral immune response, measured by a reduction in antibody production to S antigen.

Gallium may have moderate efficacy in ameliorating asthma in B6D2F1/J mice (Apseloff *et al.*, 1996). Asthma was induced in mice by s.c. injection of ovalbumin (OVA) on days 0 and 5 of the experiment, followed by exposure to aerosolized OVA on day 12; the mice were euthanized on day 14. Mice that received 45 mg/kg Ga as gallium nitrate on day 11 showed a significant reduction in histological evidence of asthma compared with mice exposed to OVA but only given saline on day 11. A subsequent experiment in which a small group of mice was given 45 mg/kg Ga nitrate on days 6 and 11 failed, however, to show efficacy.

In a model of endotoxic shock, gallium nitrate (45 mg/kg, s.c. injection) administered 24h before lipopolysaccharide (LPS) injection in *Propionibacterium acnes*-sensitized mice attenuated LPS-induced hepatitis but had no effect on production of tumor necrosis factor-α (TNF-α) (Krecic *et al.*, 1995).

In a mouse model of type I diabetes, gallium suppressed the development of diabetes in nonobese diabetic mice (Flynn *et al.*, 1992). In one study, s.c. gallium nitrate at a dose of 45 mg/kg was administered at 6 weeks of age, and then weekly doses of 15 mg/kg were administered until 20 weeks of age. At 30 weeks, no treated animals had developed diabetes, whereas all of the control animals were diabetic. Although some diabetogenic T cells were still present in the treated animals, their activity was greatly curtailed.

Several other studies have confirmed that gallium is a potent inhibitor of T cell activation and proliferation in rodents and in vitro. Drobyski *et al.* (1996) found that transferrin-gallium (Ga-TF) markedly suppressed alloantigen-induced proliferation of mixed lymphocytes; it also significantly reduced the density of IL-2 receptor on activated T cells and slightly reduced the number of $CD3^+/CD25^+$ T cells in phytohemagglutinin-stimulated cultures. Similar to the iron chelator deferoxamine, Ga-TF significantly increased the density of transferrin receptor (CD71) and the level of transferrin receptor mRNA in activated T cells, but did not affect the number of these cells. Importantly, Ga-TF did not inhibit IL-2 secretion or the induction of IL-2-stimulated lymphokine-activated killer activity. Huang *et al.* (1994) found that although gallium suppressed T cell activation and some lymphokine (including IFN–γ) secretion in cell cultures, it did not directly interfere with the normal inflammatory response of gonadal vein endothelial cells (GVEC), including their response to IFN-γ and TNF-α. This GVEC response, which includes the production of intercellular adhesion molecule-1, favors tissue growth

and repair, and actually appeared to be enhanced by gallium.

The immunological effects of gallium on macrophages have also received some attention. Gallium was found to transiently inhibit the expression of major histocompatibility complex (MHC) class II by murine macrophages (Matkovic *et al.*, 1991). In activated murine macrophage-like RAW 264 cells, gallium was found to inhibit dose dependently the secretion of IL-6, TNF-α, and nitric oxide (Makkonen *et al.*, 1995). Mullet *et al.* (1995) found that gallium nitrate inhibited nitric oxide secretion from activated murine ANA-1 macrophages, but did not inhibit secretion of TNF-α.

The selective immunosuppressive properties of gallium have led to its testing as a possible antirejection therapeutic for allograft subjects. In a mouse model of severe graft versus host disease (using irradiated mice that received transplanted bone marrow and spleen cells), citrate-buffered gallium nitrate administered by continuous infusion over 14 days at 2.6, 3.5, and 4.6 mg/kg/day significantly prolonged survival and attenuated effects of the disease (Drobyski *et al.*, 1996). In another study (Orosz *et al.*, 1996), survival was greatly extended and histological evidence of tissue rejection was reduced in mice that received cardiac allografts and were treated with gallium (as s.c. citrate-buffered gallium nitrate, 30 mg/kg on the day of transplantation and on the third day posttransplant, then 10 mg/kg every third day until day 30). Although acute rejection was inhibited, chronic rejection, including persistent inflammation, was observed (Orosz *et al.*, 1997).

Ghio *et al.* (1997) hypothesize that neutrophilic inflammatory responses (which, as previously mentioned, include the secretion of large amounts of lactoferrin) are triggered in part by iron chelates and chelators that are not indigenous to the organism (including bacterial siderophores). Their hypothesis includes the postulate that Fe^{3+} is reduced to Fe^{2+} in an attempt to free the iron from its exogenous chelate, through superoxide generated by phagocyte-associated NADPH oxidoreductase in the neutrophil; the Fe^{2+} is then reoxidized and combined with lactoferrin. It is tempting to speculate that gallium, which can act as a ferric analog but cannot be physiologically reduced to a divalent form, is caught up in the inflammatory response through binding to exogenous chelators and to lactoferrin. The inability of gallium to be reduced, and perhaps differing biochemical behaviors of gallium-lactoferrin and iron-lactoferrin after uptake by activated macrophages (and possibly T cells), may act to suppress the inflammatory response.

In addition to the immunomodulating mechanisms just discussed, gallium may inhibit some immune reactions by antimitotic activity on lymphocytes, as discussed in Section VI.B. It is, in fact, possible that this will turn out to be the dominant mechanism.

VI. Effects on Mitosis and Cellular Proliferation

A. Neoplastic and other hyperproliferative tissue

Antitumor properties of gallium were first described by Hart *et al.* (1971) based on several in vitro and animal studies. Antineoplastic and antiproliferative activities have been subsequently demonstrated in several in vitro cancer cell lines and in clinical studies (Foster *et al.*, 1986; Seidman *et al.* 1991; Einhorn *et al.*, 1994; Crawford *et al.*, 1991). Although gallium has generally shown only low to moderate clinical efficacy, it continues to be used as an experimental anticancer drug, particularly for lymphatic malignancies (including multiple myeloma; Warrell *et al.*, 1993) and urothelial malignancies. The particular efficacy in these cancers is likely related to the high concentrations of gallium reaching these sites: lymphomas due to their high Ga avidity (high concentration of TF receptor, ferritin, and perhaps LF); multiple myeloma due, in addition, to Ga accumulation in remodeling bone; and urothelial sites due in part to the urinary excretion of much Ga. Efficacy may be expected in metastatic bone disease, kidney and prostate cancers, and other Ga-avid neoplastic sites for similar reasons.

The cellular mechanisms for gallium's antiproliferative properties were first investigated by Maurel (1973), who found gallium to be strongly antimitotic in HeLa cells in concentrations of approximately 0.05 mM $Ga_2(SO_4)_3$ or more. At low antimitotic concentrations, no chromosomal or other structural damage was apparent in the cells; at more than approximately 0.16 mM, cells did not recover mitotic ability and developed structural abnormalities in their nuclei (likely the nuclear fragmentation now known to be characteristic of apoptosis). The lack of chromosomal damage at low antimitotic concentrations was also shown by Hedley *et al.* (1988), who found that gallium at 50 μM did not induce DNA strand breaks or interfere with repair of existing breaks.

An antiproliferative effect of gallium on human and murine leukemic and lymphoma cells, which is greatly enhanced by transferrin, has been repeatedly demonstrated (Rasey *et al.*, 1982; Chitambar and Seligman, 1986; Kovar *et al.*, 1990; Chitambar *et al.*, 1988, 1989). These studies indicate that much of the antiproliferative activity of gallium is due to its competition with ferric iron, particularly at sites of cellular Fe^{3+} uptake and storage, resulting in decreased cellular iron use. Although transferrin-dependent uptake by leukemic cells is predominant, a transferrin-independent pathway also exists (Chitambar and Zivkovic, 1987; Chitambar and Sax, 1992).

Cancer cells that incorporate Ga-TF will generally set themselves on a self-destructive path: The cells become iron-deprived, which up-regulates TF receptor on the cell surface (Chitambar and Seligman, 1986). Increased TF-receptor promotes further Ga-TF incorporation and Fe-deprivation, which ultimately prevents cell division

and may lead to apoptosis. As previously mentioned, Ga-TF may have the additional iron-depriving effect of inhibiting intracellular release of Fe from Fe-TF by preventing sufficient acidification of Fe-TF-containing endosomes (Chitambar and Seligman, 1986).

Once gallium is incorporated in cells, a major antiproliferative mechanism is its inhibition of DNA synthesis through deactivation of ribonucleotide reductase (RR) (Hedley *et al.*, 1988). RR activity, which is essential for DNA replication, is blocked due to a conformational change resulting from the substitution of Ga^{3+} for Fe^{3+} in the M_2 subunit (Narasimhan *et al.*, 1992; Chitambar *et al.*, 1991). The deactivating effect of gallium on cellular RR has been observed in vitro on human CCRF-CEM T lymphoblasts (gallium nitrate, 50 μg/ml; Hedley *et al.*, 1988), and on murine leukemic cells (Ga^{3+}-transferrin, 12.5–100 μM; Chitambar *et al.*, 1991). Because RR levels rise as cells enter the S phase of the cell cycle (Engström *et al.*, 1985), gallium is expected to arrest cells at this phase, as has been observed (Hedley *et al.*, 1988).

Gallium (as gallium nitrate) has also been found to be a potent inhibitor of certain detergent-solubilized cellular membrane protein tyrosine phosphatases (PTPases) in Jurkat human T cell leukemia cells and HT-29 human colon cancer cells (Berggren *et al.*, 1993). Neither gallium nitrate or Ga^{3+}-transferrin, however, inhibited the growth of Jurkat cells, although a gallium-hydrogen peroxide complex strongly inhibited growth and DNA synthesis. Berggren *et al.* (1993) postulated that the different activities may be due to low uptake of gallium by these cells, except from the hydrogen peroxide complex. A relation between the inhibition of PTPases and the antitumor properties of gallium has not been established.

A further antiproliferative mechanism of gallium is its ability, under certain conditions, to induce apoptosis. Human leukemic CCRF-CEM cells incubated for 48h with 12.5 to 100 μM gallium nitrate displayed dose-dependent growth inhibition and apoptosis (Ul-Haq *et al.*, 1995). In these experiments, apoptosis was identified by characteristic chromatin condensation, nuclear fragmentation (apoptotic bodies), and DNA fragmentation. These results were similar to those observed when cells from the same cell line were incubated with the iron chelator deferoxamine; in both cases apoptosis could be prevented by simultaneous administration of ferric ammonium citrate. It was therefor hypothesized that apoptosis induced by both gallium and deferoxamine was due to cellular iron deprivation. Apoptosis was also induced in normal human keratinocytes by incubation with a high concentration (500 μM) of gallium maltolate [tris(3-hydroxy-2-methyl-4*H*-pyran-4-onato)gallium] for 24h; gallium nitrate under the same conditions had no more than a slight effect, and maltol itself (which can chelate iron, although less effectively than deferoxamine) showed no effect at 1500 μM (Bernstein LR and Wilkinson DI, unpublished data, 1996). Whether Ga can induce apoptosis in other cell types, and whether such induction occurs in vivo, remain open questions. The low observed toxicity of Ga in vivo, and the low cytotoxicity of Ga on many cell types (including osteoclasts and osteoblasts), suggests that Ga does not generally induce apoptosis at therapeutic concentrations; it may, however, do so when it reaches especially high concentrations, such as at particularly gallium-avid sites (as some tumors, or cells with high TF receptor expression).

B. Lymphocytes

Lymphocytes, as other cells, require iron for cell division, and the availability of iron can be a limiting factor in their proliferation (Seligman *et al.*, 1992). Several studies have shown that activation of T and B lymphocytes by mitogens, antigens, or cytokines, and the subsequent proliferation of these cells, are greatly inhibited in the absence of diferric TF (Brock, 1981; Brock and Mainou-Fowler, 1983; Djeha and Brock, 1992). Iron in some low molecular weight hydrophilic chelates is taken up by such cells, but unlike diferric TF does not appear to allow their proliferation, because much of the iron forms insoluble intracellular material (Djeha and Brock, 1992). Iron is also taken up from the lipophilic complex ferric pyridoxal isonicotinoyl hydrazone, but in this case the intracellular iron remains in a soluble form that allows proliferation (Djeha and Brock, 1992). It is possible that the observed inhibition of T cell activation and proliferation by gallium (discussed in Section V.) is due at least in part to the presence of significant Ga-TF, which lowers diferric TF concentrations and binds to TF receptor. These effects would, by interfering with the T cell binding of diferric TF, reduce the uptake of iron required for T cell proliferation.

C. Antimicrobial Activity

One study found that intramuscular (30–45 mg/kg) or i.v. (15 mg/kg) gallium tartrate was effective in treating experimental syphilis in rabbits (Levaditi *et al.*, 1931). The same study found that i.v. gallium tartrate, at 225 mg/kg (750 mg/kg was tolerated), eliminated *Trypanosoma evansi* parasites from infected mice. Antimicrobial activity of gallium has also been demonstrated in vitro against pathogens responsible for tuberculosis in humans, *Mycobacterium tuberculosis* and *Mycobacterium avium complex* (Olakanmi *et al.*, 1997). Gallium, as gallium nitrate or gallium-transferrin, was effective against even a multiple drug-resistant variety, and was additive in effect with other antibacterials. Activity was shown both in culture medium alone and in infected macrophages. Another study found a gallium organometallic compound to have efficacy against malarial parasites (Yan *et al.*, 1991) (as an organometallic compound, the configuration of the molecule, rather than gallium per se, may be responsible for this activity).

Very little information is available on possible mechanisms for gallium's antimicrobial activity. It is likely that the activity stems from gallium's ability to enter microbes through their iron transport mechanisms, to disrupt their iron metabolism, and to interfere with their DNA and protein synthesis. The ability of transferrin-bound gallium to enter infected cells, such as infected macrophages, through the transferrin receptor may be an advantage in treating some intracellular infections. The suppression by gallium of T cell and macrophage activation could, however, partially counteract gallium's antimicrobial activities in vivo (at least at some dose levels); such an effect is suggested by the reported aggravated progression of tuberculosis in gallium-treated guinea pigs (Maurel, 1973; Bouissou *et al.*, 1973). Dose versus response studies in vivo using several different infectious agents would elucidate the relationship between the antimicrobial and immunomodulating activities of gallium. Results of such studies would help indicate the therapeutic potential of gallium in treating infections.

VII. Summary and Possible Future Research

The known biological activities of gallium that may be of therapeutic importance, together with their proposed mechanisms, may be summarized as follows.

A. Gallium Affects Bone Resorption and Formation

1. Gallium concentrates at sites of bone resorption and formation. To a lesser extent gallium is adsorbed on endosteal and periosteal surfaces.

2. Gallium on bone surfaces inhibits osteoclastic bone resorption.

a. Not cytotoxic to osteoclasts at osteoclast-inhibiting concentrations.

b. No effect on anion transport by osteoclast ruffled membrane vesicles, but directly inhibits the vacuolar-class ATPase, thus suppressing H^+ production.

c. Additional inhibition may be mediated by effects of gallium on osteoblasts and on PTH secretion.

3. Alters osteoblast functions and has anabolic activity.

a. Decreases constitutive and vitamin D_3-stimulated OC production (decreased OC is associated with increased bone formation, and possibly with decreased recruitment of osteoclasts).

b. Type I collagen (a major component of bone matrix) production increased.

c. PTH-stimulated calcium transients inhibited. (It is thought that PTH can stimulate bone resorption by acting on osteoblasts, which then signal osteoclasts by an unknown mechanism, possibly IL-6 secretion.)

d. C-fos production is overexpressed (c-fos appears associated with increased bone formation).

4. Inhibits PTH secretion from parathyroid cells at high concentrations.

a. Gallium does not appear to act through extracellular-Ca^{2+}-sensing receptors, as it does not increase cytoplasmic Ca^{2+} or decrease cAMP in parathyroid cells.

b. Inhibitory activity only observed in vitro at very high concentrations (200 μM) and may not be manifested in vivo at therapeutic concentrations (approximately 5–25 μM).

c. Effect on PTHrP secretion by malignant and other cells unknown.

5. May inhibit secretion of IL-6 and other bone resorption-promoting cytokines by osteoblasts, osteoclasts, and their bone marrow progenitors.

a. No direct data, but mechanism is suggested by gallium's ability to inhibit these cytokines from macrophage-like cells, and its efficacy in treating Paget's disease and osteolysis caused by multiple myeloma.

B. Gallium Modulates Immune Activity

1. Concentrates at sites of inflammation and infection.

a. Binds to lactoferrin.

b. Concentrated by some leukocytes, particularly neutrophils and lymphocytes.

c. May be taken up by macrophages and stored in ferritin.

d. Uptake by bacteria, partly through siderophores.

2. Suppresses T cell activation and proliferation.

a. Suppression is mainly during early stages of activation.

b. Gallium not cytotoxic to T cells at concentrations that suppress activation and not inhibitory to endothelial cells.

c. Density of IL-2 receptor on activated T cells reduced, but IL-2 secretion and IL-2-stimulated lymphokine-activated killer activity not inhibited.

d. Inhibits production of INF-γ by alloactivated T cells, but does not interfere with effects of INF-γ on endothelial cells.

e. Suppresses development of T cell-mediated disease in animal models of rheumatoid arthritis (adjuvant-induced arthritis in mice), multiple sclerosis (experimental autoimmune encephalomyelitis in rats), uveitis (experimental autoimmune uveitis in rats), and type I diabetes (nonobese diabetic mice)

3. Suppresses macrophage activation.

a. Transiently inhibits expression of MHC class II by murine macrophages.

b. Dose-dependently inhibits secretion of IL-6, TNF-α, and nitric oxide by murine macrophage-like RAW 264 cells.

4. Suppresses rejection of allografts.

a. Suppresses severe graft versus host disease in mice (irradiated mice that received transplanted bone marrow and spleen cells).

b. Suppresses acute tissue rejection in mice that received cardiac allografts.

5. Suppresses certain allergic responses.

a. Suppresses allergic reaction to tuberculin injections in guinea pigs and rats infected with tuberculosis.

6. Antimitotic to some leukocytes and macrophages.

C. Gallium Inhibits Some Cellular Proliferation

1. Accumulates at many sites of accelerated cellular proliferation, particularly in malignant tissue.

a. Gallium bound to plasma transferrin concentrates in proliferating cells that express high concentrations of transferrin receptor.

b. Some malignant tissue concentrates gallium through a transferrin-independent mechanism.

2. Antineoplastic in several cancer cell lines and in some in vivo cancers. Gallium is particularly effective against some lymphatic and urothelial cancers, which are sites where administered Ga reaches high concentrations.

3. Can inhibit DNA synthesis through substitution of Ga^{3+} *for* Fe^{3+} *in the* M_2 *subunit of ribonucleotide reductase, thus blocking its action.*

4. Interferes with iron absorption and metabolism of proliferating cells.

a. May inhibit intracellular release of Fe from Fe-TF by preventing sufficient acidification of Fe-TF-containing endosomes.

b. Fe-deprived cells may up-regulate TF receptor on cell surface, leading to further Ga-TF incorporation, ultimately resulting in prevention of cell division and possibly in cell death.

5. Can inhibit some protein tyrosine phosphatases (cell signaling enzymes involved in regulating cell growth and oncogenic transformation) in Jurkat human T cell leukemia cells and HT-29 human colon cancer cells.

6. Potently inhibits proliferation of human keratinocytes in vitro at 100 μM.

7. Can induce apoptosis.

a. Induces dose-dependent apoptosis in human leukemic CCRF-CEM cells incubated for 48h with 12.5 to 100 μM gallium nitrate.

b. Induces apoptosis in human keratinocytes incubated with 500 μM gallium maltolate for 24h.

c. Antimitotic in HeLa cells at 50 μM, and induces probable apoptosis at more than 160 μM.

d. Apoptosis appears linked to cellular Fe deprivation.

8. Inhibits proliferation of some infectious microorganisms, including those causing syphilis, trypanosomiasis, and tuberculosis.

a. A likely mechanism is the ability of gallium to enter microbes through iron transport mechanisms, to disrupt their iron metabolism, and to interfere with DNA and protein synthesis.

b. Transferrin-bound gallium can enter some infected cells, such as many macrophages, through the transferrin receptor.

As we have seen, a large number of studies (including many not mentioned here) have shed much light on the mechanisms of gallium distribution and activity, and have laid the groundwork for further research. Through these studies it has become apparent that the dominant mechanism underlying most of gallium's diverse activities is its ability to act as a chemically irreducible ferric iron analog in a wide variety of systems. A major exception appears to be the mechanism for gallium's accumulation in remodeling bone; the mechanisms for many of gallium's antiresorptive and anabolic effects on bone may also represent exceptions. Such exceptions likely stem from gallium's ability, unlike ferric iron, to exist physiologically in a small, soluble anionic group: gallate, $Ga(OH)_4^-$. Gallate can reach nanomolar plasma concentrations even in the presence of unsaturated TF, and can reach much higher concentrations in the presence of metal-saturated TF or at sites where TF is not present, or where phosphate interacts with TF at low bicarbonate concentrations.

The observed targeting of gallium through endogenous mechanisms to sites of its potential therapeutic activity holds the promise of further clinical efficacy in several indications. Examples include targeting to areas of bone remodeling, with activity to decrease bone resorption; targeting to gallium-avid malignant tissue, with activity to inhibit cellular proliferation; and targeting to areas of synovitis associated with rheumatoid arthritis, with activity to inhibit inflammation and other autoimmune reactions.

An area particularly ripe for further investigation is the skeletal accumulation of gallium. Although bone was the first tissue in which gallium concentration was described, the mechanisms of skeletal gallium localization remain largely unknown. As previously suggested, gallate appears to play an important role in this localization. The possible role of transferrin, however, needs to be elucidated: (*a*) whether Ga-TF is taken into some bone components through the TF receptor and (*b*) whether, how, and where gallium leaves transferrin (perhaps by interaction with ATP or other phosphates to form gallate) and enters bone by mechanisms independent of the TF receptor. Further avenues to explore, both in vitro and in vivo, include details of the interaction of gallium with phosphate-bearing compounds (e.g., phospholipids, kinases, alkaline and acid phosphatases, ATP, hydroxyapatite) at regions of bone remodeling, the adsorption of gallium on bone components, and the precipitation of gallium phosphates.

Other unexplored or little-explored areas of potential investigation include: (*a*) gallium effects on parathyroid-hormone-related protein secretion from malignant and nonmalignant cells; (*b*) the mechanism of gallium activity on parathyroid cells (which is apparently not mediated by the extracellular-Ca^{2+}-sensing-receptor); (*c*) the effects of gallium, as Ga-TF and gallate, on metal-ion channels, membrane stability, and membrane permeability; (*d*) the effects of gallium on the secretion of cytokines, particularly IL-6, by osteoblasts, osteoclasts, and other bone cells; (*e*) the biochemical mechanisms of

gallium activity on T cells, macrophages, osteoclasts, and other cells, including possible mechanisms that arise from gallium acting as an irreducible ferric analog; (*f*) the effects of gallium on cell signaling enzymes, such as protein kinases and phosphatases, and on their targets; (*g*) the mechanisms of transferrin-independent gallium uptake by non-bone and malignant cells; (*h*) the degree to which the immunomodulating effects of gallium are due to its antiproliferative activity on lymphocytes and other cells; (*i*) the ability of Ga, particularly as Ga-TF, to induce apoptosis in a variety of cell types, and the mechanisms of this induction; and (*j*) antimicrobial activity of gallium. In addition, although the biochemical relation between gallium and iron has received considerable attention, little attention has been paid to the biochemical relations of gallium to other essential trace metals, particularly zinc. Further understanding in these diverse areas could lead to increased and more effective therapeutic applications for gallium.

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