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Original Article

Glyphosate, pathways to modern diseases III: Manganese, neurological diseases, and associated pathologies

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Abstract

Manganese (Mn) is an often overlooked but important nutrient, required in small amounts for multiple essential functions in the body. A recent study on cows fed genetically modified Roundup®-Ready feed revealed a severe depletion of serum Mn. Glyphosate, the active ingredient in Roundup®, has also been shown to severely deplete Mn levels in plants. Here, we investigate the impact of Mn on physiology, and its association with gut dysbiosis as well as neuropathologies such as autism, Alzheimer's disease (AD), depression, anxiety syndrome, Parkinson's disease (PD), and prion diseases. Glutamate overexpression in the brain in association with autism, AD, and other neurological diseases can be explained by Mn deficiency. Mn superoxide dismutase protects mitochondria from oxidative damage, and mitochondrial dysfunction is a key feature of autism and Alzheimer's. Chondroitin sulfate synthesis depends on Mn, and its deficiency leads to osteoporosis and osteomalacia. Lactobacillus, depleted in autism, depend critically on Mn for antioxidant protection. Lactobacillus probiotics can treat anxiety, which is a comorbidity of autism and chronic fatigue syndrome. Reduced gut Lactobacillus leads to overgrowth of the pathogen, Salmonella, which is resistant to glyphosate toxicity, and Mn plays a role here as well. Sperm motility depends on Mn, and this may partially explain increased rates of infertility and birth defects. We further reason that, under conditions of adequate Mn in the diet, glyphosate, through its disruption of bile acid homeostasis, ironically promotes toxic accumulation of Mn in the brainstem, leading to conditions such as PD and prion diseases.

Key Words: Autism, cholestasis, glyphosate, manganese, Parkinson's disease

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INTRODUCTION

Glyphosate is the active ingredient in Roundup®, the most widely used herbicide on the planet.[3,14] Glyphosate enjoys widespread usage on core food crops, in large part because of its perceived nontoxicity to humans. The adoption of genetically engineered "Roundup®-Ready" corn, soy, canola, cotton, alfalfa, and sugar beets has made it relatively easy to control weeds without killing the crop plant, but this means that glyphosate will be present as a residue in derived foods. Unfortunately, weeds among GM Roundup®-Ready crops are developing ever-increasing resistance to Roundup®,[107,221] which requires an increased rate of herbicide application.[26]

In 1987, glyphosate was the 17th most commonly used herbicide in the United States, but, in large part due to the introduction of glyphosate-resistant core crops, it became the number one herbicide by 2001.[146] Its usage has increased steadily since then, in step with the rise in autism rates. Glyphosate's perceived nontoxicity is predicated on the assumption that our cells do not possess the shikimate pathway, the biological pathway in plants, which is disrupted by glyphosate, and whose disruption is believed to be the most important factor in its toxicity.

It may seem implausible that glyphosate could be toxic to humans, given the fact that government regulators appear nonchalant about steadily increasing residue limits, and that the levels in food and water are rarely monitored by government agencies, presumably due to lack of concern. However, a paper by Antoniou *et al*.[12] provided a scathing indictment of the European regulatory process regarding glyphosate's toxicity, focusing on potential teratogenic effects. They identified several key factors leading to a tendency to overlook potential toxic effects. These include using animal studies that are too short or have too few animals to achieve statistical significance, disregarding *in vitro* studies or studies with exposures that are higher than what is expected to be realistically present in food, and discarding studies that examine the effects of glyphosate formulations rather than pure glyphosate, even though formulations are a more realistic model of the natural setting and are often orders of magnitude more toxic than the active ingredient in pesticides.[189] Regulators also seemed unaware that chemicals that act as endocrine disruptors (such as glyphosate[108]) often have an inverted dose–response relationship, wherein very low doses can have more acute effects than higher doses. Teratogenic effects have been demonstrated in human cell lines.[212] An *in vitro* study showed that glyphosate in parts per trillion can induce human breast cancer cell proliferation.[289]

Adjuvants in pesticides are synergistically toxic with the active ingredient. Mesnage *et al*.[189] showed that Roundup® was 125 times more toxic than glyphosate by itself. These authors wrote: "Despite its relatively benign reputation, Roundup® was among the most toxic herbicides and insecticides tested."[189]

The industry dictates that 3 months is a sufficiently long time to test for toxicity in rodent studies, and as a consequence none of the industry studies have run for longer than 3 months. The only study we are aware of that was a realistic assessment of the long-term effects of GM Roundup®-Ready corn and soy feed on mammals was the study by Séralini *et al*. that examined the effects on rats fed these foods for their entire life span.[261] This study showed increased risk to mammary tumors in females, as well as kidney and liver damage in the males, and a shortened lifespan in both females and males. These effects occurred both in response to Roundup and to the GM food alone. These effects only began to be apparent after 4 months.

There are multiple pathways by which glyphosate could lead to pathology.[248] A major consideration is that our gut bacteria *do* have the shikimate pathway, and that we depend upon this pathway in our gut bacteria as well as in plants to supply us with the essential aromatic amino acids, tryptophan, tyrosine, and phenylalanine. Methionine, an essential sulfur-containing amino acid, and glycine, are also negatively impacted by glyphosate. Furthermore, many other biologically active molecules, including serotonin, melatonin, melanin, epinephrine, dopamine, thyroid hormone, folate, coenzyme Q10, vitamin K, and vitamin E, depend on the shikimate pathway metabolites as precursors. Gut bacteria and plants use exclusively the shikimate pathway to produce these amino acids. In part because of shikimate pathway disruption, our gut bacteria are harmed by glyphosate, as evidenced by the fact that it has been patented as an antimicrobial agent.[298]

Metal chelation and inactivation of cytochrome P450 (CYP) enzymes (which contain heme) play important roles in the adverse effects of glyphosate on humans. A recent study on rats showed that both males and females exposed to Roundup® had 50% reduction in hepatic CYP enzyme levels compared with controls.[156] CYP enzyme dysfunction impairs the liver's ability to detoxify xenobiotics. A large number of chemicals have been identified as being porphyrinogenic.[77] Rossignol *et al*.[242] have reviewed the evidence for environmental toxicant exposure as a causative factor in autism, and they referenced several studies showing that urinary excretion of porphyrin precursors to heme is found in association with autism, suggesting impaired heme synthesis. Impaired biliary excretion leads to increased excretion of heme precursors in the urine, a biomarker of multiple chemical sensitivity syndrome.[77] We later discuss the ability of glyphosate to disrupt bile homeostasis, which we believe is a major source of its toxic effects on humans.

Glyphosate is a likely cause of the recent epidemic in celiac disease.[249] Glyphosate residues are found in wheat due to the increasingly widespread practice of staging and desiccation of wheat right before harvest. Many of the pathologies associated with celiac disease can be explained by disruption of CYP enzymes.[156] Celiac patients have a shortened life span, mainly due to an increased risk to cancer, most especially non-Hodgkin's lymphoma, which has also been linked to glyphosate.[85,253] Celiac disease trends over time match well with the increase in glyphosate usage on wheat crops.

Glyphosate is also neurotoxic.[59] Its mammalian metabolism yields two products: Aminomethylphosphonic acid (AMPA) and glyoxylate, with AMPA being at least as

toxic as glyphosate. Glyoxylate is a highly reactive glycating agent, which will disrupt the function of multiple proteins in cells that are exposed.[90] Glycation has been directly implicated in Parkinson's disease (PD).[57] Glyphosate has been detected in the brains of malformed piglets.[155] In a report produced by the Environmental Protection Agency (EPA), over 36% of 271 incidences involving acute glyphosate poisoning involved neurological symptoms, indicative of glyphosate toxicity in the brain and nervous system.[122]

In the remainder of this paper, we first introduce the link between glyphosate and manganese (Mn) dysbiosis, and briefly describe the main biological roles of Mn. We then describe how glyphosate's disruption of gut bacteria may be a major player in the recent epidemic in antibiotic resistance. We then explain how glyphosate can influence the uptake of arsenic and aluminum, and propose similar mechanisms at work with Mn. In the next section, we describe how Mn deficiency can lead to a reduction in Lactobacillus in the gut, and we link this to anxiety disorder. We follow with a discussion on mitochondrial dysfunction associated with suppressed Mn superoxide dismutase (Mn-SOD), and then a section on implications of Mn deficiency for oxalate metabolism. The following section explains how Mn deficiency can lead to the overexpression of ammonia and glutamate in many neurological diseases. The next two sections show how Mn accumulation in the liver is linked to cholestasis and high serum low density lipoprotein (LDL), and how this can also induce increased susceptibility to Salmonella poisoning. We then identify a role for Mn in chondroitin sulfate synthesis, and the implications for osteomalacia. The next two sections explain how glyphosate exposure can lead to Mn toxicity in the brain, and discuss two neurological diseases that are associated with excess Mn, PD and prion diseases. After a section on the link between male infertility and Mn deficiency in the testes, we discuss evidence of exposure to glyphosate and end with a short summary of our findings.

SUPPORTIVE EVIDENCE OF MANGANESE DYSBIOSIS DUE TO GLYPHOSATE

Glyphosate's disruption of the shikimate pathway is due in part to its chelation of Mn, which is a catalyst for enolpyruvylshikimate phosphate synthase (EPSPS), a critical early enzyme in the pathway.[63] A recent study on Danish dairy cattle investigated mineral composition in serum of cattle fed Roundup®-Ready feed.[154] The study identified a marked deficiency in two minerals: Serum cobalt and serum Mn. All of the cattle on eight different farms had severe Mn deficiency, along with measurable amounts of glyphosate in their urine. In Australia, following two seasons of high levels of stillbirths in cattle, it was found that all dead calves were Mn deficient.[184] Furthermore, 63% of newborns with birth defects were found to be deficient in Mn.

Mn, named after the Greek word for "magic," is one of 14 essential trace elements. Mn plays essential roles in antioxidant protection, glutamine synthesis, bone development, and sperm motility, among other things. Although Mn is essential, it is only required in trace amounts. And an excess of Mn can be neurotoxic.

Remarkably, Mn deficiency can explain many of the pathologies associated with autism and Alzheimer's disease (AD). The incidence of both of these conditions has been increasing at an alarming rate in the past two decades, in step with the increased usage of glyphosate on corn and soy crops in the United States, as shown in [Figures 1 and 2]. Although correlation does not necessarily mean causation, from 1995 to 2010, the autism rates in first grade in the public school correlates almost perfectly ($P = 0.997$) with total glyphosate application on corn and soy crops over the previous 4 years (from age 2 to 6 for each child) [Figure 1]. Such remarkable correlation necessitates further experimental investigation. These neurological disorders are associated with mitochondrial impairment[197,241,243,281,316] and with excess glutamate and ammonia in the brain,[2,109,265] leading to a chronic low-grade encephalopathy.[256,260] As we will show later, Mn deficiency is critically associated with these pathologies.

Thyroid dysfunction can be predicted as well, and low maternal thyroid function predicts autism in the fetus.[238] Furthermore, increases in bone fractures in both children and the elderly can also be explained by Mn deficiency, due to its critical role in bone development.[276] Osteoporosis, which is a serious problem

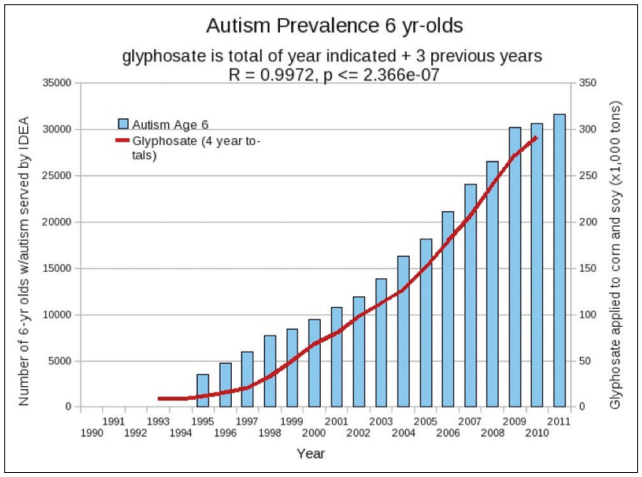

Figure 1: Plots of amount of glyphosate applied to corn and soy crops in the US over the previous 4 years (red), provided by the US Department of Agriculture, compared with number of children enrolled in the first grade in the public school system under the autism category according to the Individuals with Disabilities Education Act (IDEA) (blue bars). (Figure courtesy of Dr. Nancy Swanson)

among the elderly today, is also likely promoted by Mn deficiency,[247] and osteoporosis leads to increased risk to fractures.[98,139,140]

Sprague-Dawley rats fed a Mn-deficient diet had significantly reduced concentrations of Mn in liver, kidney, heart, and pancreas, compared with controls.[18] Furthermore, pancreatic insulin content was only 63% of control levels, and insulin release following glucose administration was also reduced. Mn deficiency not only impairs insulin secretion in Sprague-Dawley rats, but it also causes reduced glucose uptake in adipose tissue,[19] so Mn deficiency could contribute to impaired glucose metabolism in both type 1 and type 2 diabetes, which are a growing problem worldwide.[199] Type 1 diabetes in children is associated with a decrease in Lactobacillus and Bifidobacterium, and an increase in Clostridium, in the gut.[195] These same pathologies are also found in gut bacteria from poultry fed Roundup®-Ready feed.[263] The increased incidence of diabetes in the US is strongly correlated with glyphosate usage on corn and soy, as shown in [Figure 3].

Much remains elusive about Mn's roles in cellular metabolism, but it is clear that it is very important. For instance, Target of Rapamycin Complex 1 (TORC1) accelerates the aging process in cells from yeast to mammals,[231] and Mn inhibits TORC1, but only if it is present in the Golgi.[86] Zinc (Zn) is essential for DNA and RNA replication and cell division. Zn deficiency leads to greatly enhanced Mn uptake by cells, and this induces modifications to messenger RNA such that the ratio of guanine and cytosine nucleotides (C + G) to adenine and thymine (A + T) is sharply increased.[100] Clearly, more research is needed to explain the significance of these phenomena.

We infer, paradoxically, that both Mn deficiency and Mn toxicity, attributable to glyphosate, can occur simultaneously. Because of glyphosate's disruption of CYP enzymes, the liver becomes impaired in its ability to dispose of Mn via the bile acids, and instead it transports the Mn via the vagus nerve to brainstem nuclei, where excess Mn leads to PD. Recently, PD has also increased dramatically, in step with glyphosate usage on corn and soy [Figure 4].

Ironically, while the brainstem suffers from excess Mn, the rest of the brain incurs Mn deficiency due to the depressed serum levels of Mn. Mn is particularly important in the hippocampus, and deficiency there can lead to seizures. A high incidence of seizures is found in children with autism.[302] Seizures are also associated with reduced serum Mn,[54,88,269] and this is consistent with the liver's inability to distribute Mn to the body via the bile acids. Antibiotics have been found to induce seizures.[132]

Mn uptake in the brain is normally enhanced during the neonatal period in rats, and proper development

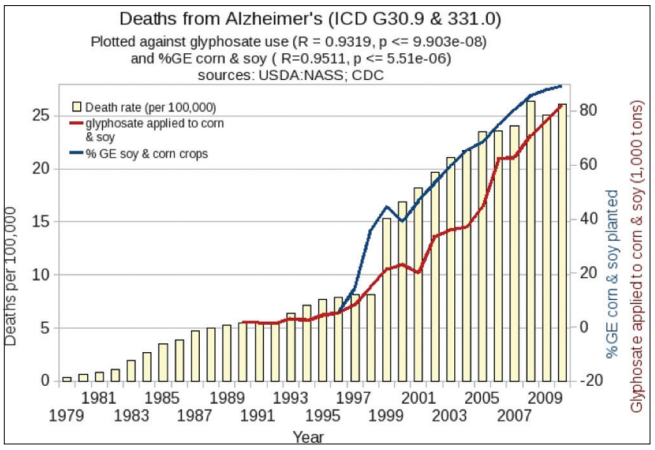

Figure 2: Plots of amount of glyphosate applied to corn and soy crops in the US over time, compared to the rate of death from AD. (Figure courtesy of Dr. Nancy Swanson)

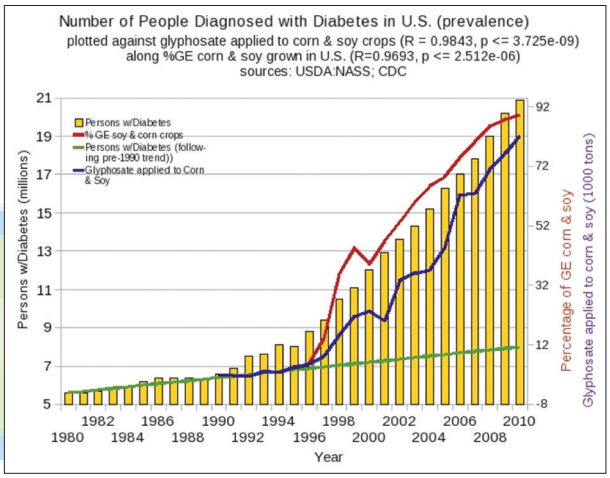

Figure 3: Plots of glyphosate usage on corn and soy crops (blue), percent of corn and soy that is genetically engineered to be "Roundup Ready" (red), and prevalence of diabetes (yellow bars) in the US. (Figure courtesy of Dr. Nancy Swanson)

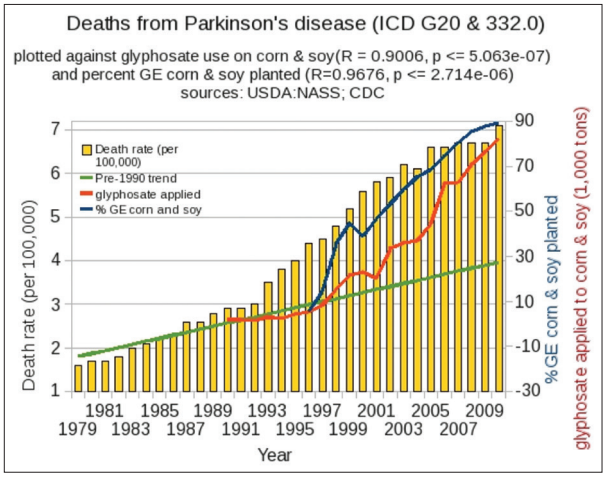

Figure 4: Plots of glyphosate usage on corn and soy crops (blue), percent of corn and soy that is genetically engineered to be "Roundup Ready," (red), and deaths from PD (yellow bars) in the US. (Figure courtesy of Dr. Nancy Swanson)

of the hippocampus depends on Mn.[284] Soy formula increases the risk of seizures in autism,[310] hardly surprising when one considers that soybean crops are now 90% Roundup®-Ready. A recent paper has confirmed that alarmingly high glyphosate residues appear in Roundup®-Ready soy.[35] The US Department of Agriculture analyzed glyphosate residues in soy in 2011, and reported that 91% of the 300 samples tested were positive for glyphosate, with 96% being positive for AMPA, an equally toxic by-product of glyphosate breakdown.[297] Our own analysis confirms that glyphosate is present in infant formula. Out of several soy-based baby formulas we tested, only one contained glyphosate residues. We found levels of 170 ppb in Enfamil ProSobee liquid concentrate. Further testing is underway. Soybean product sourcing and residue testing should be required prior to product manufacturing and is necessary to prevent inadvertent infant exposure.

Another mechanism by which glyphosate in soy formula could cause seizures is through bilirubin production. Serum concentrations of bilirubin were elevated in catfish exposed to sublethal doses of Roundup®, in a dose-dependent relationship.[208] Neonates, due to an immature digestive system, are unable to metabolize bilirubin in the gut, and it can therefore build up in the blood and even penetrate their immature blood–brain barrier to cause seizures.[308]

GLYPHOSATE AND MICROBIAL ANTIBIOTIC INTOLERANCE

Microbial antibiotic tolerance and resistance are a growing problem worldwide, likely fueled by horizontal gene transfer among different bacterial species.[106,121] Multiple-drug resistant commensal bacteria in the guts of both animals and humans form a reservoir of resistance genes that can spread to pathogenic species. Methicillin-resistant *Staphylococcus aureus* (MRSA),[119] *Clostridium difficile*,[183] and *Pseudomonas aeruginosa*[169] are all becoming major threats, especially in the hospital environment. A generic mechanism of upregulated efflux through membrane pores offers broad-domain resistance to multiple antibiotics.[169] Exposure to antibiotics early in life can even lead to obesity as a direct consequence of the resulting imbalance in gut bacteria.[73]

Studies have shown that increased mutation rates due to chronic low level exposure to one antibiotic can induce an accelerated rate of development of resistance to diverse other antibiotics.[151] Glyphosate, patented as an antimicrobial agent,[298] is present in steadily increasing amounts in the GM Roundup-Ready corn and soy feed of cows, pigs, chickens, farmed shrimp, and fish, and it is ubiquitous in the Western diet of humans. *Pseudomonas aeruginosa* can use glyphosate as a sole source of phosphorus,[192] and it is one of a small number of resistant bacterial species with the ability to metabolize glyphosate, a feature that might be exploited for soil remediation.[1] However, DNA mutations due to exposure would enhance tolerance to glyphosate and other antibiotics, perhaps explaining the current epidemic in multiple antibiotic resistant *P. aeruginosa* infections, which have a 20% mortality rate.[190] Antibiotic resistance sequences engineered into GM crops may also play a role in the current crisis concerning antibiotic resistant pathogens.

Glyphosate has also been demonstrated as a remarkable antimicrobial synergist. It greatly increases the cidal effects of other antimicrobials, particularly when combined as salts of glyphosate. A concentration dependent synergy index (SI) ranging from 0.34 to 5.13 has been recorded for the Zn salt of glyphosate.[299] This has serious implications for glyphosate ingested with pharmaceuticals or residues of other widely used agricultural chemicals, such as the herbicides Diquat, Paraquat, 2,4 D and Glufosinate, the fungicide Chlorothalonil and the systemic neonicotinoid insecticides Acetamiprid, Imidacloprid, Thiacloprid, Thiamethoxam, and Clothianidin.

Glyphosate acts as a catalyst for the development of antibiotic resistance genes in pathogens. Since both poultry and cow manure are used as natural fertilizers in crops, it can be expected that a vector for microbial resistance to multiple drugs is through contamination of fruits and vegetables. Indeed, multiple resistance genes have been identified from diverse phyla found in cow manure, including Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria, that is, in phylogenetically diverse organisms.[311]

One of the ways in which glyphosate is toxic to plants is through disruption of chlorophyll synthesis, due to suppression of the activity of the first enzyme in pyrrole synthesis.[61,69,143,319] Pyrrole is the core building block of both chlorophyll and the porphyrin rings, including corrin in cobalamin and heme in hemoglobin and cytochrome enzymes. Several cofactors containing a structurally complex tetrapyrrole-derived framework chelating a metal ion (cobalt (Co), magnesium (Mg), iron (Fe), or nickel (Ni)) are synthesized by gut bacteria and supplied to the host organism, including heme and corrin.[236]

Thus, glyphosate can be expected to disrupt synthesis of these biologically essential molecules. Pseudomonas normally thrives in the small bowel and produces abundant cobalamin that may be a significant source for the human host.[6] *P. aeruginosa*'s successful colonization may be due in part to its ability to produce cobalamin despite the presence of glyphosate. Only recently has it also been recognized that a Mn–porphyrin complex can protect from mitochondrial overproduction of hydrogen peroxide (H_2O_2) in response to ionizing radiation.[274] It can be predicted that homeostasis of all of these minerals

in the gut (Co, Mn, Fe, Ni, and Mg) is impaired in the presence of glyphosate, and this will have serious consequences not only to the gut bacteria but also to the impaired regulation of these minerals. The implications of impaired heme and cobalamin synthesis will be further addressed in a future paper.

A key component of glyphosate's action is its ability to chelate minerals, particularly transition metals such as Mn. Glyphosate forms strong complexes with the transition metals via the amino, the carboxylic, and the phosphonic moieties in the molecule. Each of these can coordinate separately to metal ions or in combination as bidentate or tridentate ligands.[194,296]

ANALOGY WITH ARSENIC AND ALUMINUM

Chronic kidney disease is clearly associated with multiple environmental toxicants.[268] There has been an epidemic in recent years in kidney failure among young agricultural workers in Central America, India, and Sri Lanka, particularly those working in the sugar cane fields.[249] A recent paper reached the unmistakable conclusion that glyphosate plays a critical role in this epidemic.[133] A growing practice of spraying sugar cane with glyphosate as a ripener and desiccant right before the harvest has led to much greater exposure to the workers in the fields. The authors, who focused their studies on affected workers in rice paddies in Sri Lanka, identified a synergistic effect of arsenic, which contaminated the soil in the affected regions. This paper is highly significant, because it proposes a mechanism whereby glyphosate greatly increases the toxicity of arsenic through chelation, which promotes uptake by the gut. Glyphosate also depletes glutathione (GSH)[60,128] and glutathione S transferase (GST) is a critical enzyme for liver detoxification of arsenic.[295] As a consequence, excess arsenic in the kidney causes acute kidney failure, without evidence of other symptoms such as diabetes usually preceding kidney failure.

Arsenic is normally disposed of by the liver through biliary excretion. In rats exposed to arsenic, large amounts of GSH appeared in the bile simultaneously with biliary excretion of arsenic.[113] It was first hypothesized, and later confirmed, that arsenic is transported in bile acids in the form of unstable GSH complexes (monomethylarsonous acid), which release GSH upon decomposing. Since glyphosate disrupts CYP enzymes necessary for bile acid formation,[248,249] as well as depleting GSH,[60,128] it can be expected that glyphosate would disrupt the process of biliary excretion of arsenic, thus forcing arsenic to be redirected toward urinary excretion, leading ultimately to kidney failure.

Glyphosate also chelates aluminum,[230] and it has been reasoned that this enables aluminum to get past the gut barrier more readily through direct analogy with the situation with arsenic, which is also a 3+ cation.[193]

However, it has been demonstrated through experimentation that glyphosate prefers divalent cations. Thus, aluminum would enter the bloodstream via the digestive tract's portal vein to the organs traveling with albumin, which is known to attach and transport many xenobiotics. It is well established that citrate also binds aluminum and promotes its uptake past the gut barrier through a mechanism that parallels glyphosate's binding to aluminum.[68,148] Both are small molecules that easily pass through a leaky gut barrier.

Considering these observations regarding aluminum and arsenic, it is reasonable to expect that something similar might happen with Mn. Unlike these other two, however, Mn plays many essential roles in the body, and so its chelation by glyphosate would interfere with its bioavailability in the general circulation. Just as for arsenic, bile acids play a critical role in Mn homeostasis. Bile is the major excretory route of injected Mn.[17] Malecki *et al*. wrote: "Biliary excretion may be a major homeostatic mechanism for preventing both deficiency and toxicity of Mn."[179, p. 489]

Glyphosate, a dipolar zwitterion, is toxic in part due to its bio-transformative properties as pH varies. We postulate that Mn, which is transported in the blood stream bound to glyphosate, is oxidized to Mn^{3+} following its release in the localized acidic environment of sulfated glycosaminoglycans (GAGs) in the glycocalyx lining the capillary wall.[234] As we will later explain, Mn uniquely is able to travel along axons and across synapses, and this results in a novel path via the vagus nerve for brain toxicity following excess Mn accumulation in the liver.

Mn is a transition metal, and therefore it can catalyze oxidative reactions in neurons via the Fenton reaction.[305] While Mn^{2+} is the form of Mn that catalyzes enzyme reactions, Mn^{3+}, similar to Al^{3+}, is directly toxic to neuronal membranes.[13] *In vitro* studies have shown that Mn^{3+} complexes auto-oxidize catecholamines, and therefore exposure to excess Mn leads to a decrease in the bioavailability of dopamine, serotonin, and noradrenaline in the striatum.[227]

It has been shown that glyphosate enhances the oxidation of Mn from a 2^+ oxidation state to 3^+, both in solution and on an inert surface.[21] It can be inferred, therefore, that Mn^{2+} oxidation to the toxic form, Mn^{3+}, might occur in the artery wall following exposure to superoxide in the presence of glyphosate. Mn^{3+} also enhances glyphosate degradation to AMPA,[21] which would produce the highly glycating by-product, glyoxylate. Glyoxylate and Mn^{3+} would both cause significant arterial damage in association with the inflammatory response.

We suggest that another route for Mn transport is the vagus nerve, which delivers Mn from the liver to the brainstem nuclei. When bile acid synthesis is impaired,

the brainstem nuclei can acquire neurotoxic levels of Mn, while serum levels are simultaneously depressed.

GUT BACTERIA DYSBIOSIS AND ANXIETY

Anxiety disorder is a comorbidity of autism,[110] AD,[288] and PD,[235] and, in this section, we argue that disruption of Lactobacillus due to impaired Mn bioavailability is a likely cause. Anxiety disorder is also correlated with glyphosate usage on corn and soy, as illustrated in [Figure 5].

Glyphosate has been shown to severely deplete Mn uptake by plants, both by the roots and by the shoots.[127] Experiments on plants demonstrated that Mn applied as fertilizer antagonizes glyphosate's effectiveness in weed control,[29] and this implied that Mn chelation was an important part of glyphosate's toxicity to plants. Electron paramagnetic resonance (EPR) spectroscopy analyses conducted by these authors demonstrated that glyphosate's binding to Mn increased with pH as pH rose from 2.8 to 7.5. The pH of plant symplast is typically 7.5, a level at which glyphosate would be an effective chelator of Mn.

Certain species of gut bacteria, such as members of the Lactobacillus family, utilize Mn in novel ways for protection from oxidation damage, and, as a consequence, their requirements for Mn are much higher than those of other species.[13,14] Thus, Mn chelation by glyphosate would lead to reduced numbers of these essential bacteria in the gut. This leads directly to neurological symptoms such as anxiety, due to the influence of the gut–brain axis.[70,75,106] In the small intestine, the pH increases gradually from pH 6 to pH 7.4 in the terminal ileum.[101] At pH 7.4, Mn bioavailability can be expected to be reduced by 50% due to glyphosate chelation.[173]

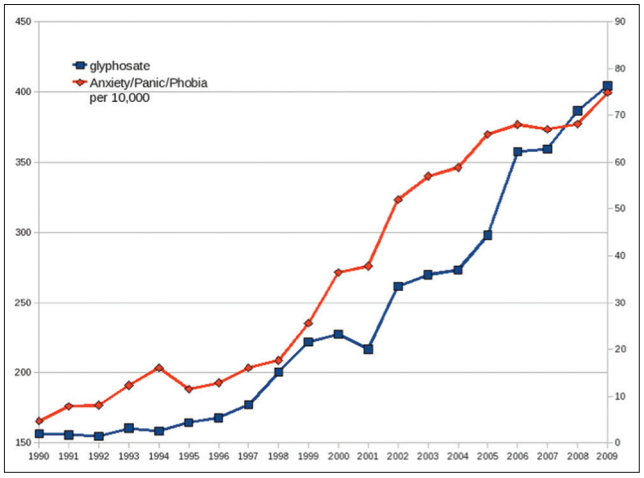

Figure 5: Plots of glyphosate usage on corn and soy crops (blue), provided by the US Department of Agriculture, and rates per 10,000 of phobia, anxiety disorder, and panic disorder (red) in the US, provided by the Centers for Disease Control. (Figure courtesy of Dr. Nancy Swanson)

The liver regulates the amount of Mn in the general vascular circulation, by incorporating any excess into bile acids, which gives the gut bacteria repeated chances to take it up. However, production of bile acids depends upon CYP enzymes, which are disrupted by glyphosate.[248,249] Hence, glyphosate can be expected to lead to severe impairment of Mn bioavailability to the gut bacteria, while at the same time allowing too much Mn to accumulate in the liver.

Lactobacillus tends to reside in the foregut.[286] The pH of the foregut is higher than that of the cecum,[100] so Mn chelation by glyphosate is a bigger issue there, since glyphosate's chelation effects increase with increasing pH. Mn-SOD is an important enzyme in mitochondria for protection from oxidative damage. Most Lactobacillus species lack Mn-SOD, but they have devised a way to protect themselves from oxidation damage due to the superoxide radical by using active transport of Mn in the +2 oxidation state. Many Lactobacilli normally have high intracellular concentrations of Mn.[14] For example, *Lactobacillus plantarum* accumulates over 30 mM of intracellular Mn (II).[13]

A recent study demonstrated that Roundup® in concentrations lower than those recommended in agriculture inhibited microbial growth of three microorganisms that are widely used as starters in fermentation of milk products,[66] including a species of Lactobacillus. Research into genetically engineering an Mn-SOD-encoding gene derived from *Streptococcus thermophilus* into various Lactobacillus species has shown that they can produce Mn-SOD from these heterologous genes and use it to improve their resistance to oxidative stress.[48]

Bruno-Bárcena *et al.*[48] proposed that such genetically engineered Lactobacilli might provide benefit as probiotics to people suffering from colitis or peptic ulcers. Colitis is associated with increased inflammation in the gut, which may be due to impaired function of Mn-SOD. An experiment on a mouse model of colitis demonstrated that *Lactobacilus gasseri* treatment alleviated inflammation in the colon of Il-10 deficient mice.[55] Genetically modified forms of *L. gasseri* as described above, which overproduce Mn-SOD, showed enhanced therapeutic effects.

Several members of the Lactobacillus family are capable of producing the inhibitory neurotransmitter γ-aminobutyric acid (GABA) via the enzyme glutamate decarboxylase, and this may be a reason for their ability to improve symptoms of anxiety. Experiments with Lactobacillus probiotics in mice demonstrated neurochemical and behavioral effects related to changes in GABAergic expression in regions of the brain that control mood.[165] These effects were absent in vagotomized mice, pointing to the vagus nerve as the bacterial communication pathway between gut and brain.

Lactobacillus have been successfully cultivated to produce fermented food containing high levels of GABA, proposed to be a health benefit in probiotics.[40]

Patients suffering from chronic fatigue syndrome (CFS) often have imbalances in microbial flora,[177] along with anxiety as a frequent comorbidity.[104] A pilot placebo-controlled study involved daily administration of *Lactobacillus casei* probiotics over a 2-month period to CFS patients.[232] The outcome was a significant rise in both Lactobacillus and Bifidobacteria in the gut, along with a significant decrease in anxiety symptoms ($P = 0.01$). This study reinforces the gut–brain connection, specifically implicating Lactobacillus in the etiology of anxiety disorder.

MN-SUPEROXIDE DISMUTASE AND MITOCHONDRIAL DYSFUNCTION

Mitochondrial dysfunction, particularly for the neutrophils, which perform important immune system functions, is implicated in CFS,[196] and this could be due to impaired Mn supply to Mn-SOD. Mn-SOD is dramatically upregulated in association with the inflammatory markers tumor necrosis factor α (TNF-α), lipopolysaccharide (LPS), and interleukin-1,[301] presumably to protect mitochondria from oxidative damage. SOD plays an important role in antioxidant defenses, by converting superoxide into H_2O_2, which can then be further detoxified by other enzymes such as catalase.[182] There are three major classes of SOD, which are distinguished by the metal catalyst, which can be copper (Cu), Zn, Mn, iron, or nickel. Eukaryotes rely on a distinct form in the mitochondria, which depends on Mn, whereas Cu/Zn SOD is present in the cytoplasm and extracellularly. Many bacterial species including *Escherichia coli* use Fe-SOD as well as Mn-SOD. The adjuvants in Roundup® may play an important role in enabling glyphosate to penetrate the mitochondrial membrane,[215] where it can interfere with the activities of Mn-SOD via chelation of Mn.

Recent experiments on goldfish involved exposing them for 96 h to Roundup® at concentrations ranging from 2.5 to 20 mg/L.[174] Several metabolites were measured from the liver, kidney, and brain. Remarkably, Roundup® inhibited SOD activity in all three organs examined, by 51–68% in the brain, 58–67% in the liver, and 33–53% in the kidney, and this was the most striking effect that was observed. Unfortunately, they did not specify whether the cytoplasmic Cu/Zn SOD or the mitochondrial Mn-SOD was most affected. Regardless, a plausible explanation of this effect is the chelation of Mn, Cu, and Zn, which are essential cofactors for the two SODs.

A recent study on the fish species *Anguilla anguilla* exposed to environmentally realistic levels of glyphosate over a short time period revealed DNA strand breaks in both liver and gills, along with a suppression of SOD activity in the liver.[116] A plausible explanation is that the breakdown product of glyphosate, glyoxylate, which is a potent glycating agent, would cause DNA damage by attacking Cu, Zn-SOD. Experiments have shown that released Cu in combination with H_2O_2 produced by glycated Cu, Zn-SOD triggers a Fenton reaction, resulting in nuclear DNA cleavage.[138]

Aconitase, an enzyme that converts citrate to isocitrate, is a crucial participant in Complex I of the citric acid cycle in the mitochondria. Many neurodegenerative diseases have been linked to decreased aconitase activity due to oxidative stress, including Friedreich ataxia,[245,249] Huntington's disease,[282] progressive supranuclear palsy,[213] autism,[239] and epilepsy.[300] The presence of a single unligated iron atom in the iron–sulfur cluster of aconitase makes it uniquely sensitive to oxidative inactivation by superoxide.[105] Aconitase inactivation has a cascade effect, because the released iron results in ferrous iron toxicity, further promoting cell death.[52] The pervasive herbicide Paraquat has been implicated in oxidative inactivation of aconitase, and this likely explains its known role in PD.[52]

A postmortem study of brains of autistic individuals showed a striking decrease in aconitase activity in the cerebellum associated with a similar decrease in glutathione redox antioxidant capacity (GSH/GSSG), with the plot producing a near 100% separation between cases and controls.[239] Inadequate clearance of superoxide due to Mn-SOD inactivation can easily account for this observation. Aconitase is a crucial participant in the citric acid cycle in the mitochondria, so this effect has catastrophic consequences on the renewal of adenosine triphosphate (ATP) as an energy source for the neurons.

In another study, cells from children with autism exhibited higher oxidative stress than control cells, including a 1.6-fold increase in reactive oxygen species (ROS) production, 1.5-fold increase in mitochondrial DNA copy number per cell, and more deletions.[198] Furthermore, oxidative phosphorylation capacity of granulocytes from children with autism was 3-fold lower than in controls. These are all indicators of oxidative stress, which could be due to SOD inhibition by glyphosate, mediated by Mn deficiency.

OXALATE

Monsanto's Roundup herbicide, WeatherMax® with Transorb II Technology, is now a preferred formulation and uses a potassium salt of glyphosate (48.8%). The Transorb Technology, which utilizes a dual surfactant system and adjuvants, was first introduced in 1996 as Roundup ULTRA. In 1998, the formula was altered and released as WeatherMAX, then further improved and released in 2005 as the current product.

The inert ingredients have undergone several changes over the years to include formula variations, which included the use of dual surfactants of siloxane copolymers and Polyoxyethylated tallow amine (POEA). As noted by Monsanto, "Promotion of stomatal infiltration of glyphosate by an organosilicone surfactant reduces the critical rainfall period," hence the rain-fastness of Roundup WeatherMax® with Transorb® 2 Technology. In 1995, US Patent #5,464,806 was issued which reflected another product formulation improvement and move from the use of Silwet-77 siloxane surfactant due to phase separation problems, to the use of an acetylenic diol.[142] The new formula provides protection from herbicide loss due to rain within 30 min of application. Additional adjuvants, well known in the paper-making industry, were used to quickly break down cell walls and collapse the plant. These chemicals originally included sodium sulfite with a later change to oxalic acid (oxalate) as patented in 2006.[315] In this patent, it was noted that "it has been discovered that the addition of oxalic acid or salts thereof to glyphosate compositions increases the cell membrane permeability of plant cells or suppresses oxidative burst to increase cellular uptake of glyphosate."

This modification may be related to the recent increase in health issues concerning excess serum oxalate in the United States and elsewhere, linked to both autism and kidney stones. A study comparing children with autism with controls found a 3-fold increase in serum oxalate levels in the children with autism,[152] and it was suggested that this might be due to excess absorption through the gut barrier, and that oxalate crystals in the brain could potentially disrupt brain function. Calcium oxalate crystals are responsible for up to 80% of kidney stones, and there has been an increased incidence of kidney stones recently in the US.[250] In a study on prisoners in Illinois who complained of gastrointestinal distress following a change to a high-soy diet, it was proposed that the unusually high levels of oxalate in the processed soy protein might be responsible for the observed symptoms.[175]

Both the high oxalate content of the soy and the high serum oxalate in humans could be due to impaired oxalate metabolism. Oxalate metabolism by oxalate oxidase in plants and by oxalate decarboxylases in fungi and a few bacteria, such as *Bacillus subtilis*, are both dependent on Mn as a cofactor.[280] Bifidobacteria, which are highly sensitive to glyphosate,[263] possess an oxalate-metabolizing enzyme that depends on magnesium rather than Mn as a cofactor.[102] However, glyphosate decreases the content of both magnesium and Mn in plants.[50] Furthermore, gamma-glutamyl carboxylase, a liver enzyme that metabolizes oxalate, is catalyzed by vitamin K, which depends on the shikimate pathway.[51] It has been shown that patients with calcium oxalate urolithiasis have significantly reduced activity of this enzyme in the liver.[65]

The sulfate ion transporter, Sat-1, plays an important role not only in sulfate transport but also in oxalate transport,[273] as evidenced by the fact that mice with a disrupted Sat-1 gene develop urolithiasis.[252] Glyoxylate is not only a substrate of Sat-1 but it is also a key regulator.[252] The upregulation of SAT-1 by glyoxylate in hepatic cells likely serves to flush oxalate and glyoxylate from the liver, to avoid hepatotoxicity. However, this can lead to nephrotoxicity due to glyoxylate glycation damage and the formation of kidney stones. Due to competition between oxalate and sulfate for transport via Sat-1, glyoxylate, and oxalate, likely, also disrupt sulfate homeostasis in the liver. Sulfate is critical for bile acid formation and for detoxification of xenobiotics such as acetaminophen.

The conversion of glyoxylate to oxalate by the enzyme lactate dehydrogenase is inhibited by oxalate.[87] Hence gloxylate, derived from glyphosate breakdown, would accumulate in the presence of excess oxalate.

Aside from the obvious damaging effects of oxalate crystals on tissues, the oxalate, whose metabolism is impaired due to Mn deficiency, will also interfere with the metabolism of glyphosate, likely greatly increasing both its effectiveness as an herbicide and its toxicity to mammals. Under oxalate stress conditions, both superoxide and the hydroxyl radical are produced in excess amounts.[258] Obviously, the ineffectiveness of Mn-SOD due to Mn deficiency would further enhance the damage due to excess oxalate. SOD activity has been shown to be reduced in association with the urolithic kidney, and methionine supplementation can alleviate this problem.[257] As mentioned previously, methionine is depleted by glyphosate.

AMMONIA, GLUTAMATE, AND NEUROTOXICITY

In this section, we will show that both glutamate and ammonia are implicated as neurotoxins in connection with autism and other neurological diseases, and we will offer the simple explanation that Mn deficiency leads to impaired activity of glutamine synthase and arginase, both of which utilize Mn as a cofactor. Mn deficiency can also explain the increased risk to epilepsy found in autism, due to the fact that Mn decreases T2 relaxation time. Mn-deprived rats are more susceptible to convulsions.[129]

Blaylock and Strunecká[32] have proposed that immune-glutamatergic dysfunction may be the central mechanism of autism spectrum disorders. Ghanizadeh[109] reported that glutamate and homocysteine are elevated in the serum in association with autism, and that glutamine and tryptophan are depleted. Tryptophan, which depends

upon the shikimate pathway in plants and microbes, is the precursor to serotonin and melatonin. An increase in glutamate and a corresponding decrease in glutamine can be entirely explained by an inactive glutamine synthase enzyme. Another extensive study on children with autism compared with controls found low serum tryptophan, high serum glutamate and homocysteine, and significantly reduced free sulfate, as well as high levels of oxidative stress markers,[1] all of which are consistent with these assertions. High serum homocysteine is one associated consequence of folate deficiency:[76] Folate is produced by Lactobacillus and Bifidobacteria from products of the shikimate pathway.[240]

The neurotransmitter glutamate has been implicated as an excitatory neurotoxin in the brain not only in autism but also in association with multiple neurological diseases, including AD, PD, amyotrophic lateral sclerosis (ALS), and multiple sclerosis.[93] Ordinarily, following glutamate release into the synaptic cleft, microglia in the brain take up excess glutamate and convert it to glutamine, using the enzyme glutamine synthase.[204] Glutamine is then released into the extracellular space, taken up by neurons, and converted in the cytoplasm to glutamate to be held within internal vesicles in anticipation of future activity. Conversion to glutamine for the transport stage from microglia to neurons renders the molecule inactive as a neurotransmitter, and therefore as a neurotoxin, when it is out of service.

TNF-α, secreted by activated microglia in the brain, is a major cytokine leading to neurotoxicity in association with multiple neurological diseases. A major component of the damaging effect of TNF-α is the autocrine induction of the release of glutamate from microglia.[285] Experiments exposing immature rats to Roundup®, whether via exposure to the dam during pregnancy and lactation or via acute exposure to the pup for 30 min, demonstrated lipid peroxidation and NMDA receptor activation in the hippocampus, indicative of oxidative stress and glutamate excitotoxicity.[59] Acute exposure increased the release of glutamate into the synaptic cleft, and depleted GSH.

Glutamine synthase depends upon Mn as a cofactor, so depleted Mn supplies would lead to a build-up of glutamate that cannot be returned to the neurons using normal channels. Multiple sclerosis is associated with both depleted Mn in the cerebrospinal fluid[185] and depleted GSH synthase in the white matter lesions.[309] Cerebellar brain samples taken postmortem from 10 individuals with autism demonstrated an anomalous increase in mRNA expression of excitatory amino acid transporter 1 and glutamate receptor AMPA 1, both involved in the glutamate system.[226] Glutamate receptor binding proteins were also abnormally expressed, and AMPA-type glutamate receptor density was low. These effects could be explained as a response to the excess bioavailability of glutamate due to an inability to convert it to the inactive form, glutamine.

Further confirmation of glutamate dysbiosis in autism comes from a study on levels of 25 amino acids in the platelet-poor plasma of high-functioning autistic children compared with normal controls, which revealed that only glutamate and glutamine were abnormally expressed in the children with autism, with a highly significant ($P < 0.002$) excess of glutamate and a highly significant ($P < 0.004$) decrease in glutamine.[264] They linked these findings to glutamatergic abnormalities reported by others.

It is intriguing to us that Mn deficiency leads to a pair of complementary pathologies – excess glutamate along with aconitase deficiency, which together allow for the cells to generate ATP by metabolizing glutamate instead of glucose. Glutamate enters the citric acid cycle beyond Complex I, thus bypassing the step that is impaired by aconitase deficiency. This is a rather elegant regulatory system that provides energy even in the face of Complex I impairment.

The prevalence of epilepsy in the US is similar to that of diabetes, making it a common disorder affecting 2 million Americans.[47] Epilepsy is associated with increased T2 relaxation time in nagnetic resonance imaging (MRI) signaling analysis of the hippocampus, both in the ipsilateral sclerotic hippocampus as well as the contralateral hippocampus and anterior temporal lobe.[42] Following intracerebral injection of Mn, a large amount of Mn accumulates in the hippocampal fissure, which results in a reduction in T2 relaxation time.[78] Epilepsy may therefore be a consequence of insufficient Mn in the hippocampus, which could easily account for the associated increase in T2. Autism is associated with a high risk of epilepsy,[293,162] and the hippocampus has been the focus of many studies on the neurological pathology of autism.[84,95]

Ammonia is a well-established neurotoxin, which accumulates when the urea cycle is unable to keep up with ammonia released from protein breakdown.[290] Ammonia can induce tremor, ataxia, seizures, coma, and death.[49] Ammonia is a highly diffusible gas that readily crosses the blood–brain barrier, and its detoxification depends upon the conversion of glutamate to glutamine, which is catalyzed by glutamine synthase, the enzyme in microglia that relies upon Mn as a cofactor.[71] Thus, impaired function of glutamine synthase leads to the accumulation of both glutamate and ammonia in the brain, both of which are established neurotoxins.

Excess ammonia due to impaired ability to detoxify excess nitrogen via the urea cycle can lead to impaired memory, shortened attention span, sleep–wake cycle disruption, brain edema, intracranial hypertension, seizures, ataxia,

and coma.[37] Arginase, the final enzyme of the urea cycle, is ubiquitous in living systems, and depends upon Mn as a cofactor. Therefore, Mn deficiency due to glyphosate's chelation of the metal would be expected to lead directly to impaired arginase function. The excess accumulation of ammonia due to inactive glutamine synthase combined with the decreased ability to metabolize ammonia to urea constitute a double-hit leading to ammonia toxicity in the brain.

A case study of a 4-year-old girl showed that a genetic defect in arginase could present as pervasive developmental delay not otherwise specified (PDD-NOS) with many similarities to autism.[112] Its manifestations include brain edema and signs of epilepsy, as would be expected with ammonia toxicity in the brain. A Mn-deficient diet administered to rats induced a reduction in hepatic arginase activity ($P < 0.01$), along with a significant rise in plasma ammonia ($P < 0.01$).[43]

Seneff *et al.*[260] put forward the idea that excess ammonia due to disrupted gut bacteria could lead to a chronic low-grade encephalopathy that could explain much of the pathology associated with autism. Furthermore, glyphosate is known to induce excess ammonia production in plants, due to overactivity of the enzyme phenylalanine ammonia lyase (PAL).[86,117,125] This enzyme may also be overactive in gut bacteria exposed to glyphosate, further compounding the problem.

CHOLESTASIS AND BILIRUBINEMIA

Mn stimulates cholesterol synthesis in the liver, presumably because bile acids, derived from cholesterol, are needed to bind to Mn and carry it back to the gut for recycling.[5] However, CYP enzyme inhibition will impair the liver's ability to oxidize cholesterol, since the formation of oxysterols in the liver for incorporation into bile acids depends on several CYP enzymes (members of the CYP3, CYP7, CYP8, CYP27, CYP39, and CYP46 families).[200,217] As a result, it can be anticipated that, when Mn supplies are plentiful, both the Mn and the cholesterol will accumulate to toxic levels in the liver, unless another method can be found for their redistribution. In the case of cholesterol, this may lead to a necessary increase in the synthesis and release of LDL particles. People with a defective CYP7A1 gene have significantly elevated total and LDL cholesterol levels, as well as substantial accumulation of cholesterol in the liver and a markedly decreased rate of bile acid excretion.[225] Neonatal cholestasis and hypercholesterolemia (elevated LDL) were produced in mice with a defective CYP7A1 gene fed a normal chow diet.[96] The increased serum levels of LDL associated with heart disease risk may therefore be a consequence of the production of cholesterol that cannot be exported through the biliary ducts.

Studies with rats have shown that *Lactobacillus plantarum* probiotic supplements lower serum LDL levels.[166] Lactobacillus levels were reduced in chickens exposed to antimicrobial drugs, which resulted in reduced bile salt deconjugation in the gut.[114] Impaired bile salt deconjugation by gut bacteria results in significant weight gain along with elevated plasma cholesterol and liver triglycerides in mice.[136] Thus, glyphosate acting as an antibiotic that preferentially kills Lactobacillus can be expected to lead to elevated serum cholesterol and triglycerides through a similar mechanism.

Cholestasis is a blockage of bile acid flow, which often occurs as a side effect of various pharmaceutical drugs.[211] Glyphosate administration to rats over a period of 13 weeks induced increases in serum bile acids, indicative of cholestasis.[64] Severe cholestasis can induce bilirubinemia,[28] and glyphosate also independently induces bilirubinemia in catfish.[208] At least two other studies have shown bile stagnation in fish exposed to glyphosate.[80,135]

Inflammatory bowel disease (colitis and Crohn's disease) has been increasing in frequency in the US over the past 20 years, in step with glyphosate usage on corn and soy crops, as shown in [Figure 6]. According to analyses by Cappello *et al.* in a hospital-based survey,[53] cholestasis is a common feature of inflammatory bowel disease. They observed a cholestatic pattern in 60% of patients studied, mainly related to older age, longer duration of disease, and hypertension. Gallstones were commonly found, often in association with abnormal bile salt absorption, especially in Crohn's disease patients.

Ironically, while Mn-SOD depends upon Mn as a cofactor, *excess* exposure to Mn can inhibit SOD expression. Experiments exposing rats to excess Mn revealed several

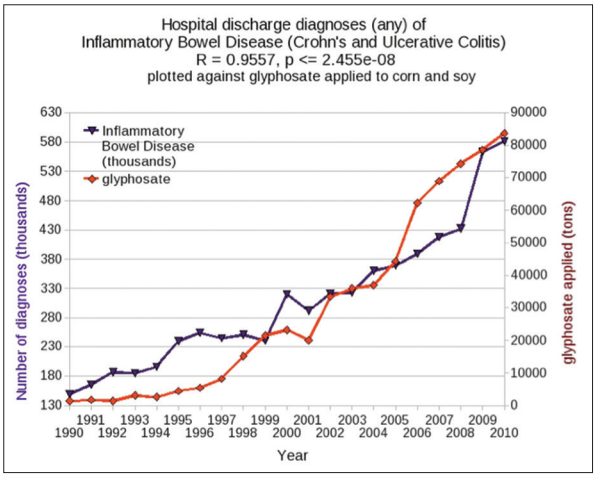

Figure 6: Plots of glyphosate usage on corn and soy crops (blue), and hospital discharge diagnoses of inflammatory bowel disease (Crohn's andulcerative colitis) in the US, over time. (Figure courtesy of Dr. Nancy Swanson)

pathological effects on the liver, including inhibition of SOD and GSH peroxidase, as well as decreased levels of GSH and reduced levels of sodium-potassium ATPase activity.[126] It is striking that glyphosate has also been shown to have these very same effects in animal experiments,[60,174,89] and it is conceivable that these effects may be in part mediated by excess Mn accumulation in the liver.

Bilirubinemia in neonates is a risk factor for autism, particularly when it is unbound and unconjugated.[10] Glucose 6-phosphate dehydrogenase (G6PD) deficiency can induce bilirubin toxicity in neonates,[176] due to the fact that G6PD enables conjugation of bilirubin.[141] Glyphosate was shown to induce a 2.67-fold reduction in G6PD expression in *E. coli*.[171] Studies on goldfish demonstrated that glyphosate significantly decreased G6PD activity in liver, kidney, and brain.[174]

G6PD is the main enzyme responsible for regeneration of NADPH, an essential requirement for GSH reductase activity.[174] Cholestasis is associated with a reduction in the supply of reduced GSH.[317] Furthermore, glyphosate has been shown to reduce the activity of GSH reductase in the liver.[174] Preeclampsia, affecting 4% of pregnancies, is associated with G6PD deficiency in red blood cells along with a reduction in reduced GSH.[3] The ratio of oxidized to reduced GSH is consistently high in association with autism, in plasma, immune cells, and the brain.[239]

A mouse model of cholestasis can be induced by exposing mice to Mn, followed shortly by exposure to bilirubin.[82] Mn induces cholesterol synthesis in the liver, and bilirubin disrupts 7-α oxidation of cholesterol, a crucial step in bile acid formation.[5] Cholesterol 7-α hydroxylase is the CYP enzyme CYP7A1 and is likely disrupted independently by glyphosate. Therefore, excess Mn in the liver works synergistically with glyphosate to induce cholestasis.

The incorporation of cholesterol products into exported bile acids is crucial for regulating cholesterol homeostasis.[205] Bile acid transport depends on ATP,[203] so mitochondrial disruption due to oxidation damage consequential to excess Mn could contribute to disturbed bile acid export, leading again to cholestasis. *In vitro* experiments exposing rat H9c2 cells to glyphosate plus the surfactant TN-20, which is a polyoxyethylene tallow amine commonly used in glyphosate herbicides, demonstrated that the surfactant in conjunction with glyphosate causes collapse of the mitochondrial membrane potential, leading to necrosis and apoptosis.[147] Even in the absence of a catastrophic death cascade, a drop in mitochondrial membrane potential would obviously negatively impact ATP production. The effect could be due in part to polyoxyethylenealkylamine (POEA), which includes polyethoxylated tallow amine surfactants that enable glyphosate to penetrate the mitochondrial membrane.[188] But, in addition, excess Mn, which would accumulate due to the lack of bile flow, itself induces a collapse in mitochondrial membrane potential.[137]

Intracellular accumulation of bile acids, associated with cholestasis, is known to be toxic to hepatocytes. The accumulation of bile acids in the cholestatic liver induces oxidative stress and apoptosis, resulting in damage to the liver parenchyma, and, ultimately, extrahepatic tissues as well.[180] The lipophilic bile acids are much more damaging than the hydrophilic ones,[15] and they can induce proton leakage and the permeability transition pore (PTP), resulting in programmed cellular death.[237] Their toxic effect is directly due to their role as surfactants.[180] Therefore, they enhance the effects of the surfactants in Roundup®, acting in a cascade reaction.

On the other hand, the hydrophilic bile acid, ursodeoxycholic acid (UDCA), is protective, and its protective effects have been proposed to be due to its ability to induce the expression of CYP3A4, a bile-metabolizing enzyme, in hepatocytes.[11] Hydroxylation, which depends on CYP enzymes, converts lipophilic compounds into hydrophilic products. So one can expect that, in the context of the CYP-enzyme suppressing effects of glyphosate, [248,249] lipophilic bile acids would accumulate in hepatocytes, and their export would be impeded by the loss of ATP subsequent to mitochondrial damage, in a positive feedback loop.

Another enzyme class that was discovered to be downregulated in the liver by glyphosate in the goldfish study is GST, an important class of enzymes. The main function of the GST enzymes is the detoxification of electrophilic xenobiotics by GSH conjugation.[275] Beyond their important role in the detoxification of xenobiotics, gene variants where the enzyme is inactive are associated with increased risk to basal cell carcinoma, and a gain-of-function mutation leads to decreased risk to asthma.[275] Arsenic, whose toxicity is synergistically enhanced by glyphosate[132] is a risk factor for basal cell carcinoma.[62] Asthma is reaching epidemic proportions today[91] and is associated with autism.[24,25] GST is increasingly recognized as an important enzyme in gut bacteria, which allows them to assist in the detoxification of xenobiotics.[7]

One final factor in cholestasis is vitamin K deficiency, which is associated with cholestatic liver disease.[278] Chorismate, the intermediary in the shikimate pathway whose synthesis is blocked by glyphosate, is a precursor not only for the three aromatic amino acids but also for tetrahydrofolate and phylloquinone (vitamin K1) in plants.[294] Similarly, menaquinone (vitamin K2) is synthesized by bacteria from chorismate.[27] Thus, disruption of the shikimate pathway contributes to vitamin K deficiency, which can lead to cholestasis.

SALMONELLA INFECTION

Multiple drug-resistant strains of Salmonella are becoming an increasing problem both in the industrialized[191] and in the developing world.[246] This may be due in part to the fact that Salmonella is more resistant to glyphosate than other species. *Salmonella typhimurium* is among a small set of microbes that possess phosphonatase genes that allow it to use the commonly occurring natural phosphonate, 2-aminoethyl-phosphonate (AEPn), as a sole source of phosphorus,[134] which likely contributes to its growth advantage in the presence of the phosphonate, glyphosate. Furthermore, Lactobacillus, which are especially vulnerable to glyphosate, produce antimicrobial compounds that can defend against Salmonella.[81] Salmonella infections often originate from contamination of plant-based foods exposed to manure of chickens and pigs. A study on poultry showed that *Salmonella entritidis*, *Salmonella galliarum*, and *Salmonella typhimurium* were all highly resistant to glyphosate compared with other more benign species.[263]

Our research into the pathology of Salmonella has uncovered a complex interplay of many factors that may be responsible for the epidemic, which includes an important role for Mn, as well as a role for industrial processing of lecithin from soy, and bile acid disruption by glyphosate. Commercial lecithin is a mixture of phospholipids and various metabolites, often derived from soy. In food processing, phospholipase A (PLA) is applied enzymatically to hydrolyze phospholipids in lecithin into lysophospholipids and fatty acids, in order to improve its emulsification, surfactant, and lubricant properties.[97] This may be a factor in inducing both increased virulence and an inflammatory response to Salmonella in the gut, contributing to inflammatory bowel disease.

Salmonella depend on cyclic adenosine monophosphate (cAMP) for flagella formation, and therefore for motility.[318] Salmonella possess a lysophospholipid sensing mechanism that triggers the synthesis of flagellin, mediated by cAMP, and this activates toll-like receptor 5 (TLR5) in macrophages, inducing an inflammatory response.[279] Experiments have shown, as might be expected, that flagella, produced from flagellin, not only enhance mobility but also protect *S. typhimurium* from internal death in macrophages and enhance their ability to multiply within an infected cell.[306]

Salmonella are remarkably resilient in an inflammatory environment, and, in a novel strategy for survival in a hostile environment, they take advantage of tetrathionate produced from oxidation of thiosulfate by ROS as a terminal electron acceptor in processing ethanolamine derived from host lysophospholipids as a source of energy not available to other microbes.[8,313] They can also uniquely use a glycated L-asparagine (fructose-asparagine, F-asn) as a source of both carbon and nitrogen.[8] Concentrations of F-asn, an Amadori product, are surprisingly high in heated fruits and vegetables.[92]

It has only recently been appreciated that Mn plays an important role in the virulence of Salmonella.[144] Salmonella depend on Mn to resist the oxidative attack of macrophages via Mn-SOD.[292] Since glyphosate's chelation of Mn makes it unavailable to gut bacteria, a mystery arises as to how Salmonella might acquire adequate Mn for defense against oxidative damage. Salmonella possess a Mn uptake system based on a protein that is homologous to eukaryotic Nramp transporters. This protein, MntH, is a proton-dependent metal transporter that is highly selective for Mn^{2+} over Fe^{2+},[144] or any other cation. Intracellular Mn^{2+} can accumulate in millimolar amounts even when environmental Mn^{2+} is scarce.

A feature unique to Salmonella is that they are especially adept at binding to cholesterol in the gall bladder, particularly in association with gallstones, and they also possess adaptive responses that allow them to survive the harsh environment of the bile acid milieu, as well as the highly acidic environment of the stomach.[9] In fact, studies have shown that they can survive a lower pH environment than either Shigella flexneri or *E. coli*.[167]

Several different species of Salmonella can form biofilms on human gallstones, which is dependent upon the presence of bile.[222,74] Since bile is an excellent source of Mn, and glyphosate's chelation of Mn is dependent on a basic environment, they could accumulate Mn while resident in the bile acids present in the gallstones. Bile acids offer antibacterial defenses, but Salmonella have developed resistance genes to protect them from bile acids.[123] In association with gallstones, *S. typhi* colonize the human gallbladder and persist in an asymptomatic carrier state.[9,187] Thus, impaired bile acid flow due to glyphosate would promote both gallstones and microbial growth.

Fibrates are hypolipidemic agents that are known to suppress bile acid synthesis via suppression of peroxisome proliferator-activated receptor γ (PPAR-γ).[220] Studies on humans exposed to fibrates have shown reduced activity of cholesterol 7α-hydroxylase (CYP7A1), leading to reduced bile acid production, and concurrent increased risk of gallstones.[271,31] Thus, it is plausible that Salmonella are able as well to gain a foothold on the gallstones caused by suppression of bile-acid production by glyphosate due to CYP enzyme inactivation, and they are able to take up Mn in the immobilized bile acids and utilize it for Mn-SOD production, protecting them from oxidative attack by macrophages. The pathogen responsible for Lyme disease, *Borrelia burgdorferi*, is also uniquely dependent on Mn,[4] and the disruption of Mn homeostasis by glyphosate may therefore play a role in its emergence.

CHONDROITIN SULFATE, OSTEOMALACIA AND ARTHRITIS

Vitamin D deficiency has reached epidemic proportions in the US and increasingly around the world in recent years.[124] In a large population study in the US, Bodnar *et al*.[34] found deficient levels of vitamin D in 83% of Black women and 92% of their newborns, as well as in 47% of White women and 66% of their newborns, despite the fact that over 90% of the women were on prenatal vitamins. This deficiency is associated with an increased risk to bone fractures, likely due to impaired calcium homeostasis.[145] It is even likely that care-takers are being falsely accused ("Shaken Baby Syndrome") of abusing children in their care who suffer from bone fractures.[255] These children are highly vulnerable to bone fractures due to impaired bone development. Bone fractures in the elderly due to osteoporosis have also risen sharply recently in the industrialized world.[139] The cause of a surging incidence of hip fractures across multiple age groups remains a mystery to medical personnel.[140]

Samsel and Seneff[248,249] proposed that the current vitamin D deficiency epidemic is caused by glyphosate, due to glyphosate's interference with CYP enzymes. The metabolite that is usually measured, 25-hydroxy vitamin D, is the product of activation in the liver by a CYP enzyme that is also critical in bile acid formation. However, there is a larger problem with bone development due to impaired Mn homeostasis. Bone mineralization depends critically on Mn, due to its essential role in chondroitin sulfate synthesis.[36,158] Several enzymes in the osteoblasts needed for this crucial step in bone development require Mn as a cofactor, including the polymerase, which polymerizes uridine diphosphate N-acetyle-galactosamine (UDP-N-acetyl-galactosamine) and UDP-glucuronic acid to form the polysaccharide, and galactotransferase, which incorporates galactose from UDP-galactose into the trisaccharide that links the polysaccharide to the glycosylated protein.[157,158] Chondroitin sulfate, together with osteocalcin, forms the ground substance to which collagen adheres to maintain healthy bone, ligaments and cartilage. Sulfate uptake by GAGs in chicks fed a Mn deficient diet was only 50% of that in control chicks, and the deficient chicks suffered from growth retardation and skeletal abnormalities.[36]

Osteoarthritis is another pathology likely related to Mn deficiency, impaired chondroitin sulfate synthesis and impaired vitamin D activation. Vitamin D deficiency is associated with rheumatoid arthritis.[207] Mn is necessary for the synthesis of GAGs or mucopolysaccharides,[156,254] which provide lubrication and protection for the joints. Rheumatoid arthritis is associated with Mn accumulation in the liver,[72] which is consistent with impaired bile flow. Glucosamine sulfate has been demonstrated to be effective in treating osteoarthritis, and it may even delay disease progression.[259] A combination therapy of glucosamine, chondroitin sulfate, and Mn ascorbate was shown to be effective in treating knee osteoarthritis in a placebo-controlled study conducted on US Navy specialists.[160]

A mysterious epidemic of a new disease, called "sea star wasting syndrome," is currently sweeping across the Pacific coast of North America, affecting at least 12 different species of sea stars.[307] We highly suspect that glyphosate plays an important role in this disease, and that it does so by chelating Mn, and therefore disrupting chondroitin sulfate synthesis. A likely source is Roundup® applied to oyster beds to kill the invading sea grass, since oysters are a common food for sea stars.[118] The state of Massachusetts was forced to close oyster beds in Edgartown on Cape Cod recently, due to infection with a pathogen, *Vibrio parahaemolyticus*.[214] This species synthesizes an N-acetyl transferase (NAT) protein, which is capable of detoxifying glyphosate by acetylating the nitrogen moiety,[58] and this could explain its overgrowth as being linked to glyphosate contamination. An analysis of the GAGs isolated from brittle stars showed exceptionally high proportions of di- and trisulfated chondroitin sulfate/dermatan sulfate disaccharides,[233] whose synthesis would be severely impaired by Mn deficiency due to chelation by glyphosate.

A protective layer of mucopolysaccharides called mucus is secreted by corals, and it has been characterized as containing sulfated glycoproteins similar to chondroitin sulfate,[44] which play an important role in controlling pH and the transepithelial movement of electrolytes and water, just as is the case in vertebrate mucosa. Mucus pathology is implicated in coral disease leading to mortality, particularly in the Caribbean.[219] Thus, an interesting hypothesis that should be considered is that glyphosate chelation of Mn is a crucial factor in the worldwide coral die-off.

PARKINSON'S DISEASE

It might be anticipated that a simple Mn mineral supplement could correct for the problem of glyphosate's chelation of Mn, but this assumption is almost certainly false. We suggest that glyphosate's disruption of Mn homeostasis leads to extreme sensitivity to Mn bioavailability, making it easy to err in the direction of either too little or too much.[103] Our investigations into the body's mechanisms for transporting Mn has revealed a likely pathway from the liver to the brain that would induce Mn toxicity in the brainstem nuclei whenever Mn is plentiful but glyphosate is also present. A strong clue comes from the condition, "manganism," closely resembling PD, which develops following

chronic occupational exposure to airborne nanoparticles containing Mn.[163,172,244] In addition to evidence from direct occupational exposure, geographical studies in the US have shown a higher incidence of PD in urban areas with higher industrial release of Mn.[312]

The fact that death from PD has increased in the past two decades in step with glyphosate usage on corn and soy suggests that there may be a role for glyphosate to play in Parkinson's pathology [Figure 4]. A case of accidental acute exposure to glyphosate through skin contact showed a remarkable development of Parkinsonian symptoms beginning just 1 month following exposure.[20] T2-weighted images revealed a hyperintense signal in the globus pallidus and substantia nigra. Another case study involved a 44-year-old female who developed PD after a 3-year period of job-related exposure at a chemical plant.[304] Glyphosate has also been shown to induce Parkinsonian-like effects in the worm, *Caenorhabditis elegans*, characterized by damage to both GABAergic and dopaminergic neurons.[201] The mechanism behind this effect remains obscure. However, a Mn-containing fungicide, MANCOZEB, ethylene bisdithio-carbamate, also induced similar damage.

An experiment where rats were exposed to Mn via intranasal instillation demonstrated that the Mn could enter the brain through the olfactory bulbs by following major neuronal pathways.[291] Mn migrated via both secondary and tertiary olfactory pathways and beyond to ultimately reach most parts of the brain and the spinal cord. By contrast, cadmium was unable to pass through synaptic junctions and was therefore limited in its penetration. These authors concluded that the olfactory nerve is the likely pathway by which Mn gains access to the brain in manganism, thus circumventing the blood–brain barrier. Autoradiographic studies tracking the distribution of radiolabeled Mn injected into rat brain verified that Mn is subject to widespread axonal transport in neuronal circuits.[283]

Elder *et al*.[94] reinforced this idea, in a study involving exposure of rats to ultrafine particles (UFPs) of inhaled Mn oxide. Their conclusion sums up the situation quite well: "We conclude from our studies that the olfactory neuronal pathway represents a significant exposure route of central nervous system (CNS) tissue to inhaled solid Mn oxide UFPs. In rats, which are obligatory nose breathers, translocation of inhaled nanosized particles along neurons seems to be a more efficient pathway to the CNS than via the blood circulation across the blood–brain barrier. Given that this neuronal translocation pathway was also demonstrated in nonhuman primates, it is likely to be operative in humans as well." [94, p. 1178]

Manganism is distinct from PD mainly in that it is the locus coeruleus that is preferentially damaged rather than the substantia nigra.[216] In the rat, at least 40% of all locus coeruleus neurons project to the olfactory bulb.[266] Since glyphosate likely interferes with the normal recycling of Mn via the bile acids, the liver will need to find an alternative route to dispose of excess Mn. Following the example of nerve-fiber migration from the olfactory bulb,[291] and the study on injected Mn,[283] a likely path is the vagus nerve, which has extensive innervation in the liver, with 10 times as many afferent nerves as efferent nerves, and particularly concentrated on the outer surface of the bile ducts.[30] MRI abnormalities indicative of Mn toxicity in the globi pallidi and substantia nigra were noted in three cases of patients with liver disease.[120] PD is associated with nonmotor symptoms that often precede the movement impairment aspects.[320] These include depression and gastrointestinal disturbances.

Berthoud *et al*.[30] proposed that the onset of PD may be associated with impairment of the vagus nerve, and subsequent functional inhibition of the dopamine system. Clinical trials have revealed pathological alteration of the vagus nerve in PD patients.[218] The dorsal motor nucleus of the vagus is an early site of pathology.[38] Liver failure can lead to excessive build-up of Mn in the brainstem, particularly the globus pallidus in the basal ganglia, which regulates voluntary movement. This is due primarily to decreased billiary excretion from the liver,[99,131] as bile acids recycle Mn back to the gut, where it is taken up by gut bacteria or disposed of. Liver cirrhosis is associated with excess Mn in the brain and associated Parkinsonian symptoms.[99] Mn neurotoxicity in the basal ganglia causing Parkinsonian symptoms has also been identified in association with chronic liver failure.[150] Despite the fact that Mn is an essential cofactor for glutamine synthase, excess Mn actually downregulates expression and activity of the enzyme, causing neuropathology. Its toxicity is linked to disruption of the cycling between astrocytes and neurons of glutamine, glutamate, and GABA.[267] Mn inhibits ATP-dependent calcium signaling in astrocytes, which likely contributes to the toxic effects of excess Mn on neurons.[277] Decreased bile flow associated with the birth defect, biliary atresia, leads to Mn accumulation in the liver[23] and in the globus pallidus.[130] As discussed previously, bile acid synthesis and export are disrupted by glyphosate and by surfactants, which is also a common effect of many toxic chemicals whose detoxification would be impaired by glyphosate's disruption of CYP enzymes.[248]

A study using MRI technology to detect Mn distribution in the brain in marmosets and rats following Mn injections revealed an accumulation in the basal ganglia as well as other parts of the brain that were situated near the ventricles, suggesting redistribution via the cerebrospinal fluid.[33] There was also considerable liver damage, especially in the marmosets, including hemosiderosis, congestion, and hepatic necrosis. Marmosets were far more susceptible than rats to Mn toxicity.

Both the substantia nigra and the locus coeruleus are characterized by high concentrations of neuromelanin. While it is unclear what role neuromelanin plays, one hypothesis is that it carries a protective action through its unique ability to accumulate and retain various amines and metallic cations, especially Mn.[168] The neuromelanin produced by the substantia nigra is closely related to dopamine, and therefore derived from tyrosine. The locus coeruleus' melanin is related to noradrenaline, and therefore derived from tryptophan. Both tyrosine and tryptophan are products of the shikimate pathway, and are therefore likely to be deficient in the context of glyphosate exposure to plants and microbes. This would result in a reduced ability to temporarily house excess Mn in the brainstem nuclei until it can be disposed of.

Glyphosate itself likely also contributes directly to PD. PD is caused by degeneration of dopaminergic neurons in the substantia nigra, attributed to mitochondrial dysfunction, oxidative stress, and protein aggregation.[79,251] PC12 cells are a popular model cell line for investigating neurological disease, and they produce dopamine in vesicles, as is appropriate for cells from the substantia nigra. A study on PC12 cells exposed to glyphosate demonstrated that glyphosate induced cell death via autophagy pathways as well as apoptotic pathways.[115]

A study on serum Mn levels in infants and their association with neurodevelopment revealed a U-shaped curve, with both too little and too much Mn leading to impaired development.[67] This study was conducted on children born in Mexico City between 1997 and 2000. We hypothesize that extreme sensitivity to Mn levels can be expected in the context of glyphosate, because it would prevent the liver from disposing of Mn via the bile acids, and therefore cause a flooding of the brainstem nuclei with excess Mn delivered via the vagus nerve. At the same time, Mn uptake into the blood stream from the gut is suppressed, both because of glyphosate's chelation of Mn at higher pH and the impaired recycling to the gut from the liver. This can lead to a paradoxical situation in which the brainstem nuclei are overwhelmed with Mn while the precortex and cortex are deprived because of the low bioavailability from the blood stream. One might postulate that seizures play a role in enhancing Mn redistribution from the brainstem to the cortex by mobilizing Mn transport along axons, and this effect might therefore explain the benefit of electroconvulsive therapy (ECT) to depression.[16]

Dopamine suppresses thyroid stimulating hormone, and therefore dopamine insufficiency can lead to overactive thyroid and potential burnout of the thyroid gland.[270] This problem is compounded by the fact that thyroid hormone itself is derived from tyrosine, one of the three aromatic amino acids that are negatively impacted by glyphosate through disruption of the shikimate pathway. The thyroid gland also depends critically on selenoproteins as antioxidants.[249] Glyphosate's depletion of both selenium and methionine will lead to reduced bioavailability of selenoproteins. It is conceivable that all of these factors working together can explain the strong correlation of glyphosate application to corn and soy with thyroid cancer [Figure 7], as well as the association between maternal thyroid disease and autism.[238]

PRION DISEASES AND MANGANESE

Prions are a special class of proteins that acquire alternative conformations that can become self-propagating. There is a class of neurological diseases called transmissible spongiform encephalopathies that are believed to be caused by misfolding of prion proteins, predominantly accumulating in the gray matter in the brain.[223] These include Creutzfeldt–Jakob disease (CJD) in humans, scrapie in sheep, chronic wasting disease of deer, and bovine spongiform encephalopathy or Madcow disease.

Prion proteins have been shown to bind Cu *in vivo*,[46] and this is probably an important factor in their normal functioning. Cu protects against conversion of prions to the pathogenic form, and studies have shown that gene variants with extra octarepeat inserts exhibit decreased Cu binding in association with markedly increased disease risk.[272] A theory first proposed by Purdey[228,229] is that the prion protein misfolds following binding to Mn instead of Cu. The pathology is then explained by a high Mn to Cu ratio in the diet. Brown *et al.*[45] investigated metal binding properties of prion proteins, and demonstrated that prions only bind to Cu and Mn. Furthermore, Mn binding induces a resistance to protein degradation by

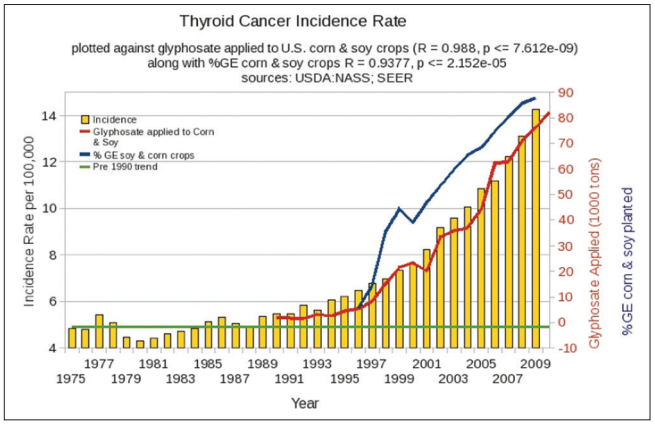

Figure 7: Plots of glyphosate usage on corn and soy crops (blue), percent of corn and soy that is genetically engineered to be "Roundup Ready" (red), and incidence of thyroid cancer (yellow bars) in the US. (Figure courtesy of Dr. Nancy Swanson)

protease, a characteristic feature of prion diseases. Aging of the Mn-bound version of prion proteins leads to loss of function. A later experiment demonstrated that Mn promotes prion protein aggregation.[161] Thus, Mn is causal in the formation of fibrils characteristic of the scrapie isoform of the protein in prion diseases.

The possible link between excess Mn and prion diseases was also supported in a later study by Masánová *et al*.,[181] who compared different regions of the Slovak republic and found a correlation between an elevated Mn/Cu ratio in core food items such as potato and bread, as well as in the soil, and a higher incidence of CJD.

Mn-SOD-/- mice die between days 3 and 13 following birth. They exhibit a marked dilated cardiomyopathy, neurodegeneration and fatty liver disease.[159,164] Mn 5, 10, 15, 20-tetrakis (4-benzoic acid) porphyrin (MnTBAP) rescues mice from this pathology and dramatically improves their lifespan. It operates at 10% of the efficiency of Mn-SOD to oxidize superoxide to H_2O_2. However, these supplemented mice develop a spongiform encephalopathy very similar to CJD.[186] This is most likely caused by excess delivery of Mn to the brain by the MnTBAP.

Purdey[227] has noted that madcow disease epidemiology aligned with the regulatory requirement to apply the organo phthalimido-phosphorus insecticide, phosmet, on the backs of cattle, for the control of warble fly during the 1980s. He maintained that phosmet chelated Cu in the CNS, but also caused oxidation of Mn to Mn^{3+}, leading to its toxicity. Like glyphosate, phosmet also disrupts CYP enzyme function,[262] which would lead to a similar disabling of bile acids.

New experimental data support the idea that the class of prion diseases should be expanded to include AD, PD, and related tauopathies.[224] Indeed, it has been proposed that Cu deficiency may be a factor in AD.[149] Glyphosate chelates Cu down to much lower pH values than those at which it chelates Mn [173,296] [Figure 8], and it has also been shown to oxidize Mn to the +3 oxidation state.[21] Thus, one can surmise that glyphosate might behave similarly to both phosmet and MnTBAP to be causal in prion diseases. In the pH 4 environment adjacent to the sulfates in the glycocalyx, Mn, but not Cu, would be released by glyphosate. Abundant bioavailability of Mn^{3+} alongside Cu deficiency further aggravated by glyphosate could set up a situation whereby excess Mn supplied to the neurons in the cortex, bound to glyphosate, over-competes with Cu in binding to prion protein. One can predict that glyphosate's tenacious binding to Cu will render Cu systemically unavailable, which argues for a role for glyphosate in prion diseases through Cu binding.[45,228,229]

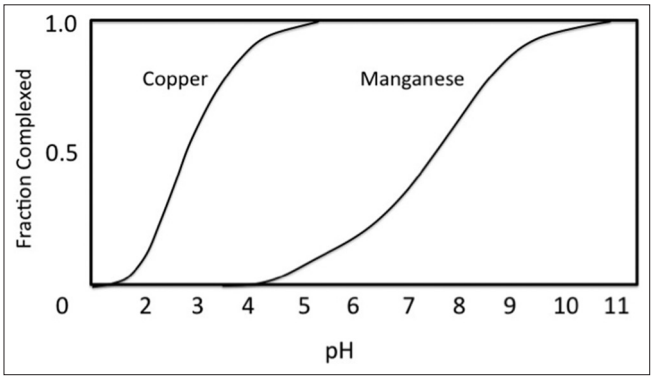

Figure 8: Fractions of metal complexed with glyphosate as a function of pH, for copper and manganese. Figure adapted from Lundager Madsen *et al*[170]

MN AND INFERTILITY

Multiple papers on rodent studies have indicated disruption of the male reproductive system by glyphosate. Acute treatment of 60-day-old male Sprague-Dawley rats to Roundup® caused a marked increase in aromatase mRNA in testicular tissue,[56] likely reflecting an increase in production due to suppression of the activity of aromatase (CYP 19), accompanied by abnormal sperm morphology. Aromatase participates in both hormone synthesis and metabolism. It is an important enzyme in testes that converts testosterone to estrogen, thus regulating the balance of sex hormones.

In vitro studies on Sertoli cells from mouse testis demonstrated that glyphosate opens L-type voltage-dependent calcium channels, leading to calcium-overload cell death.[83] However, glyphosate's disruption of the supply of Mn to sperm may be a more important factor leading to infertility, due to immobilization of the sperm. Sperm are critically dependent on Mn for their motility. Mammalian sperm contain a distinctive form of Mn-dependent adenylate cyclase, which first appears during development in seminiferous tubules simultaneously with the appearance of spermatid cells.[39] It is expressed at the highest levels following sexual maturity. Adenylate cyclase catalyzes the synthesis of cAMP. Bacteria such as *E. coli* and *Salmonella typhimurium* depend on cAMP for flagella formation, and therefore for motility.[318] cAMP-dependent phosphorylation has been linked to activation of motility in sperm flagella from sea squirts[209] and from dogs.[287] Human sperm also depend on cAMP for increased flagellar motility.[210,303] A study on zebrafish demonstrated that glyphosate exposure at concentrations of 5 and 10 mg/L over a 24-h time period reduced sperm motility.[170] Mn stimulated the progressive motility of human sperm in a time- and dose-dependent manner, and this was linked to adenyl cyclase activity.[178] The decrease in male fertility levels today in the industrialized world[111] may therefore be explained by Mn deficiency.

EVIDENCE OF EXPOSURE

Glyphosate-containing herbicides are applied to crops several times each season both for killing weeds and for desiccation just prior to the harvest in non-Roundup®-Ready crops, such as wheat and sugar cane. It accumulates in the leaves, grains, and fruit, and thus cannot be removed by washing and, furthermore, is not broken down by cooking.[41] Nowell *et al*.[206] identified pesticides as a leading cause for declines and deformities among amphibians and pollinators in the United States. A total of 100% of all water surfaces and 96% of all examined fish contained detectable levels of pesticides. In a recent US-based study by Battaglin *et al*.,[22] glyphosate and AMPA were detected frequently in soils and sediment, ditches and drains, rainwater, rivers, and streams.

A recently published study by Shehata *et al*.[264] showed that glyphosate residues can be found in the organs and muscles of chickens that consume glyphosate in their feed, including the liver, spleen, lung, intestine, heart, and kidney. All animals fed a diet contaminated by glyphosate would be subject to such bioaccumulation due to the molecule's ability to act as an attaching ligand. Feed supplementation with humic acid was able to significantly reduce the glyphosate burden in tissues.

A recent paper by Nevison[202] investigated temporal trends in autism since 1988 to assess to what degree the recent observed rate increases are due to increased diagnosis versus increased incidence. She concluded that increased incidence accounts for 75–80% of the tracked increase. She also investigated trend lines for a variety of environmental toxicants potentially implicated in autism. She wrote in the abstract: "Among the suspected toxins surveyed, polybrominated diphenyl ethers, aluminum adjuvants, and the herbicide glyphosate have increasing trends that correlate positively to the rise in autism." As we have stated earlier, glyphosate and aluminum are synergistically toxic.

There has been very little testing of glyphosate levels in either humans or other mammals. Glyphosate is passed in both the urine and the feces. Recent research from Europe has shown that glyphosate is consistently present in significant amounts in the urine of cows consuming Roundup®-Ready feed, as well as in the organs and meat of cattle.[153] Furthermore, they also detected glyphosate in the urine of humans, and the generally healthy population had significantly lower levels than the sick population. Those consuming a predominantly organic diet also had a significantly lower glyphosate burden. Another study found detectable levels of glyphosate in lungs, liver, kidney, *brain*, gut wall, and heart of malformed euthanized 1-day-old Danish piglets, and the authors proposed that glyphosate could be the cause of the deformities.[155]

CONCLUSION

Glyphosate is the most widely used herbicide on the planet, in part because of its perceived low toxicity to humans. In this paper, we propose that glyphosate's chelation of Mn, working together with other known effects of glyphosate such as CYP enzyme suppression and depletion of derivatives of the shikimate pathway in microorganisms, may explain the recent increase in incidence of multiple neurological diseases and other pathologies. We have shown that glyphosate's disruption of Mn homeostasis can lead to extreme sensitivity to variations in Mn bioavailability: While Mn deficiency in the blood leads to impairment of several Mn dependent enzymes, in contrast, excess Mn readily accumulates in the liver and in the brainstem due to the liver's impaired ability to export it in the bile acids. This pathology can lead to liver damage and PD. Mn depletion in the gut due to chelation by glyphosate selectively affects Lactobacillus, leading to increased anxiety via the gut–brain access. Both low Lactobacillus levels in the gut and anxiety syndrome are known features of autism, and Lactobacillus probiotic treatments have been shown to alleviate anxiety. Increased incidence of Salmonella poisoning can also be attributed to glyphosate, through its impairment of bile acid synthesis. Low Mn bioavailability from the blood supply to the brain leads to impaired function of glutamine synthase and a build-up of glutamate and ammonia in the brain, both of which are neurotoxic. Excess brain glutamate and ammonia are associated with many neurological diseases. At the same time, impaired function of Mn-SOD in the mitochondria results in mitochondrial damage, also a hallmark of many neurological diseases. Mn deficiency can account for poor sperm motility and therefore low fertilization rates, as well as poor bone development leading to osteoporosis and osteomalacia. Sea star wasting syndrome and the collapse of coral reefs may in fact be an ecological consequence of the environmental pervasiveness of the herbicide. Many diseases and conditions are currently on the rise in step with glyphosate usage in agriculture, particularly on GM crops of corn and soy. These include autism, AD, PD, anxiety disorder, osteoporosis, inflammatory bowel disease, renal lithiasis, osteomalacia, cholestasis, thyroid dysfunction, and infertility. All of these conditions can be substantially explained by the dysregulation of Mn utilization in the body due to glyphosate.

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Commentary

Glyphosate-based herbicides (GBH) are the major pesticides used worldwide. Initially patented as a metal ion chelator, glyphosate rapidly jumped to a leading position as an active ingredient of commercial pesticides from the 1970s when Monsanto discovered its herbicidal activities. The herbicidal mode of action of glyphosate is primarily to inhibit the shikimic acid pathway,[2] causing a shortage of aromatic amino acids. Since this biochemical pathway does not exist in vertebrates, it is generally assumed that glyphosate is safe for mammals, including humans.[8] As a consequence, GBH are used in private gardens, city parks and along roads and railway lines, as well as within an agricultural context on food and feed crops. All these diverse applications of GBH have resulted in escalating levels of human exposure and thus body burden.

Glyphosate is an aminophosphonic analog of glycine. The fact that glycine and other amino acids like glutamate function as neurotransmitters and play a crucial role in brain function, makes the potential neurotoxic effects of glyphosate a matter of concern.[5] The potential of glyphosate to act as a neurotoxin is further supported by its structural similarity to the glutamate receptor agonist 2-amino-3-phosphonopropionic acid. Indeed, GBH exposure induces glutamate excitotoxicity through L-VDCC and NMDA receptor activation in immature rat hippocampus, by reducing glutamate uptake and metabolism within glial cells, and by increasing glutamate release in the synaptic cleft.[3] However, the lack of esterase inhibition by glyphosate (the neurotoxic mechanism of most organophosphate compounds), was considered by regulatory authorities as sufficient grounds to avoid having to undertake a complete assessment of glyphosate's neurological effects.[6]

The most recent reevaluation of the acceptable daily intake (ADI) for glyphosate within the European Union (EU) conducted by the German regulatory agency (BfR), states that this has been determined by scrutiny of approximately 450 regulatory toxicological studies and 900 publications from the scientific literature.[4] Based on the review of all these investigations, the BfR concluded that the "no observed

adverse effect level" of glyphosate was in the region of 30–50 mg/kg body weight (bw) per day in rats and the ADI was thus calculated at 0.5 mg/kg bw/day, which constitutes a recommended increase from the current 0.3 mg/kg bw/day. However, these glyphosate ADI values have previously been challenged as review of the same studies and especially extending to feeding studies in animals other than rats, suggested that the current ADI of 0.3 mg/kg bw/day was at least three times higher than what the data suggest should be the case.[1] Nevertheless, with a review of such a relatively large number of studies by the official regulatory authorities, the assessment of the toxicological effects of glyphosate is being considered as complete. The notional strength of glyphosate's safety profile has also resulted in it being neglected in some national wide-scale toxicity testing schemes such as the U.S. Environmental Protection Agency (EPA) ToxCast program.

However, a debate continues as to the soundness of this BfR-led assessment as the studies taken into account were performed with an experimental design adapted to the study of poisoning effects based on the principle of "the dose makes the poison"; that is, the higher the dose the greater the poisoning effect. Of major concern is that none of the studies referred to incorporated testing principles derived from a contemporary understanding of (neuro) endocrinology or developmental epigenetics.

In the classic theory of toxicology, as applied for the study of glyphosate toxicity at a regulatory level, a nontoxic threshold is evidenced by decreasing the level of exposure and assuming that toxic effects observed are a linear response to the dose. Lower doses corresponding to environmental exposures are assumed to be safe and are not tested. However, in contrast to a classical poison, an endocrine disruptive chemical (EDC) will alter the functioning of hormonal systems and induce adverse effects at various dosage levels. Such EDC effects will, in some cases, occur in a nonlinear (non-monotonic) manner at environmentally relevant levels of exposure and will not be observed at higher doses. In addition and not surprisingly, EDC effects can be sex-specific in nature. Such nonmonotonic and sex-specific EDC effects have been extensively described for common pollutants.[7] Although nonmonotonic and sex-specific effects have been reported in many cases with GBH, the regulatory authorities considered these as false positive outcomes rather than a suggestion of potential EDC effects.[4]

Major endpoints of toxicity such as neurodevelopmental, reproductive, and transgenerational effects in humans still needs to be investigated at the glyphosate ADI and other concentrations reflective of human levels of exposure. Given its increasing wide-scale use and consequent rise in exposure, we urgently call for greater research efforts on the toxicology of glyphosate and its commercial herbicide formulations, as well as pesticide neurodevelopmental effects in general. Furthermore, pesticide combinatorial (additive or synergistic) effects remain a poorly investigated subject and area of concern that needs to be addressed. Such studies are particularly relevant to the brain since it is physiologically dependent on neurosteroids, making it potentially very sensitive to endocrine disruptive compounds.

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