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COMMENTARY

FORMATION OF HIGHLY REACTIVE VINYLGLYOXYLATE (2-OXO-3-BUTENOATE) FROM AMINO ACIDS WITH GOOD LEAVING GROUPS IN THE γ-POSITION

TOXICOLOGICAL IMPLICATIONS AND THERAPEUTIC POTENTIAL

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Enzymatic oxidation of α-amino acids to α-keto acids (Fig. 1, **1** → **2**) or to α-imino acids (Fig. 1, **1** → **3**) activates the C—H bond adjacent to the carbonyl or imino group relative to that in the parent amino acid. If the parent amino acid has a good leaving group (X) in the γ position (Fig. 1, **1**), a β,γ-elimination reaction may occur from the α-keto acid (Fig. 1, **2**) giving rise to vinylglyoxylate (2-oxo-3-butenoate, Fig. 1, **4**) or from the α-imino acid (Fig. 1, **3**) to yield 2-imino-3-butenoate (Fig. 1, **5**); the latter compound will hydrolyze to yield vinylglyoxylate (Fig. 1, **5** → **4**). Vinylglyoxylate is expected to serve as an excellent Michael acceptor and to react with, for example, the sulfhydryl groups of cysteine, glutathione, and proteins (Fig. 1, **4** → **6**). The toxicity of several naturally occurring and synthetic, γ-substituted α-amino acids may involve the formation of vinylglyoxylate. In this commentary, we review the evidence that vinylglyoxylate may be a metabolite of some γ-substituted amino acids and that its formation may contribute to the observed toxicity of certain amino acids.

S-*Adenosylmethionine*

S-Adenosylmethionine is notoriously unstable [1, 2]. The powerful leaving group (X = CH_3S-adenosyl) may be lost via a β,γ-elimination reaction without activation of the β C—H bond. Homoserine lactone (α-amino-γ-butyrolactone) and 5′-methylthioadenosine are formed in both the enzymatic [by adenosylmethionine cyclotransferase (EC 2.5.1.4) present in bacteria and yeast; [1] and references cited therein] and the mild, chemical decomposition of *S*-adenosylmethionine [1]. The reaction proceeds via a nucleophilic attack by the carboxyl oxygen on the γ carbon of the amino acid moiety of *S*-adenosylmethionine. During the chemical decomposition, about 4% of the reaction appears to follow a pathway involving vinylglycine as an intermediate [1].

Eisenberg and Stoner [3] observed that *S*-adenosyl-L-methionine is the amino donor in the transamination of 7-oxo-8-aminopelargonic acid, an intermediate in the biotin biosynthetic pathway, to 7,8-diaminopelargonic acid in *Escherichia coli*. The expected α-keto acid could not be detected. Further studies indicated that the α-keto acid analogue of *S*-adenosyl-L-methionine is unstable and is converted to 5′-methylthioadenosine and vinylglyoxylate [4]. *S*-Adenosyl-L-methionine is a substrate for both a bacterial and a snake-venom L-amino acid oxidase [5], and oxygen is consumed and ammonia is formed during the reaction; the α-keto acid product was not characterized. Based on the work of Eisenberg and Stoner [3], it is likely that the oxidation product will decompose to vinylglyoxylate and 5′-methylthioadenosine. Whether or not oxidative fragmentation of *S*-adenosylmethionine occurs *in vivo* in mammals is not known. From the above discussion, it is evident that a major product of fragmentation (whether oxidative or nonoxidative) is 5′-methylthioadenosine, which is also produced enzymatically during polyamine biosynthesis. Interestingly, 5′-methylthioadenosine carbon and sulfur are salvaged in bacteria [6] and mammals [7] (for a discussion see Ref. 8) by enzymatic conversion to α-keto-γ-methiolbutyrate, which is in turn transaminated to methionine.

L-*Methionine*-RS-*sulfoximine*

More than 40 years ago, L-methionine-*RS*-sulfoximine (Fig. 1, X = —S(=O)(=NH)CH_3) was observed as a product of the action of the bleaching agent nitrogen trichloride on flour proteins. The L,*S*-diastereoisomer of L-methionine-*RS*-sulfoximine (but not the L,*R*-diastereoisomer) is a potent inhibitor of glutamine synthetase and induces convulsions in experimental animals (for original references, see Ref. 9). When [^{35}S]methionine sulfoximine was given to mice, label was retained for long periods as methionine sulfoximine phosphate [10]. Several additional labeled metabolites were also detected, one of which was thought to be the corresponding α-keto acid.

Several workers have presumed that the convulsant activity of L-methionine-*RS*-sulfoximine is related to inhibition of brain glutamine synthetase, whereas others have been unable to show a direct, causal link between inhibition of brain glutamine

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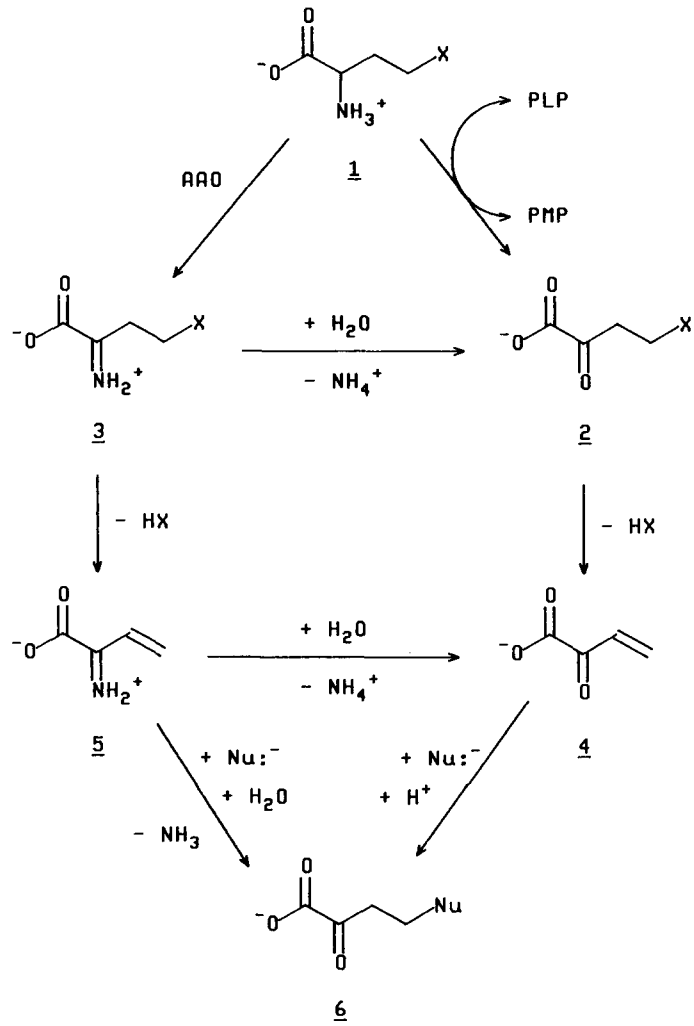

Fig. 1. Metabolism of 4-substituted α-aminobutyrates to vinylglyoxylate. Key: (1) 4-substituted α-aminobutyrate, (2) 4-substituted-2-oxobutyrate, (3) 4-substituted 2-iminobutyrate acid, (4) vinylglyoxylate (2-oxo-3-butenoate), (5) 2-imino-3-butenoate, and (6) Michael addition product of 4 with tissue nucleophiles ($Nu:^-$). Abbreviations: AAO, amino acid oxidase; PLP, pyridoxal 5′-phosphate; and PMP, pyridoxamine 5′-phosphate.

synthetase and the onset of seizures (for original references, see Ref. 11). Moreover, L-methionine-*S*-sulfoximine also inhibits γ-glutamylcysteine synthetase [12]. Administration of L-methionine-*RS*-sulfoximine to rats lowers hepatic and renal, but not brain, glutathione concentrations [13]. Nevertheless, rat brain γ-glutamylcysteine synthetase is inhibited after giving L-methionine-*RS*-sulfoximine [11]. To gain more insight into the toxicity of L-methionine-*RS*-sulfoximine, preparation of the α-keto acid analogue was attempted by incubating the amino acid with L-amino acid oxidase and catalase [9]. The α-keto acid could not be isolated from the reaction mixture; subsequently, it was realized that the α-imino acid formed initially rapidly decomposes to 2-imino-3-butenoate and methane sulfinamide [9]. Methane sulfinamide is unstable and is readily converted to methane sulfonic acid and methane sulfonamide [9]. L-Methionine-*RS*-sulfoximine is a substrate for glutamine transaminase K (EC 4.4.1.13), and the α-keto acid can be generated by transamination of L-methionine-*RS*-sulfoximine with phenylpyruvate in a reaction catalyzed by glutamine transaminase K. The α-keto acid analogue of L-methionine-*RS*-sulfoximine is more stable than the α-imino acid and breaks down relatively slowly to vinylglyoxylate and methane sulfinamide over a period of several hours [9].

Selective inhibitors of glutamine synthetase (α-ethyl-DL-methionine-*RS*-sulfoximine; Ref. 14) and γ-glutamylcysteine synthetase (DL-buthionine-*RS*-sulfoximine; Ref. 15) are now available. α-Ethyl-DL-methionine-*RS*-sulfoximine inhibits glutamine synthetase and causes convulsions. The convulsant activity of L-methionine-*RS*-sulfoximine is therefore probably related to the inhibition of brain glutamine synthetase and not to the inhibition of brain γ-glutamylcysteine synthetase [14, 15]. Moreover, because α-ethyl-DL-methionine-*RS*-sulfoximine cannot be converted readily to an α-keto acid, by anal-

ogy it appears that the convulsant activity of L-methionine-*RS*-sulfoximine is also not related to its conversion to the α-keto acid *in vivo*. Nevertheless, oxidation of L-methionine-*RS*-sulfoximine to its corresponding α-keto acid may have consequences deleterious to other tissues. Methionine sulfoximine has been identified recently as the toxic agent in the tropical plant *Cnestis glabra* (family Connaraceae) [16].

L-Homocyst(e)ine

Oxidation of L-homocysteine by L-amino acid oxidase in the presence of catalase yields a complex mixture of α-keto acids [17]. This complexity is due partly to the ease with which homocysteine is nonenzymatically oxidized to homocystine; homocystine, in turn, may be converted to mono- and diketo analogues. In addition, about 20–30% of the original homocysteine sulfur is converted to hydrogen sulfide [5, 17]. It is probable that hydrogen sulfide arises from the initial enzymatic product of homocysteine oxidation (i.e. α-imino-γ-mercaptobutyrate) by a β,γ-elimination reaction [17]. This conclusion is supported by the tentative identification of the mono- and diketo analogues of homolanthionine in the reaction mixture; these may arise by Michael addition of homocysteine or α-keto-γ-mercaptobutyrate to vinylglyoxylate or to 2-imino-3-butenoate followed by hydrolysis of the imino acid to the keto acid. The monoketo analogue of homolanthionine may also be converted to the diketo analogue by L-amino acid oxidase [17]. It is also possible that a γ-elimination reaction occurs with the mono- or diketo analogues of homocystine. The enzymatic oxidation products of homocystine are unstable [17], consistent with the expectation that RSS^- is a better leaving group than HS^- (see discussion in Ref. 18).

Many transaminases have broad substrate specificity, and it is possible that the α-keto acid analogue of homocysteine is produced continuously *in vivo* at a low level via non-specific transamination reactions. If this α-keto acid were to accumulate, secondary production of vinylglyoxylate may prove deleterious. Cooper and Meister [8, 17, 19] have suggested that one of the roles of the glutamine transaminases is to prevent accumulation (due to non-specific transamination reactions) of potentially toxic α-keto acids (e.g. phenylpyruvate, *p*-hydroxyphenylpyruvate, α-keto-γ-methiolbutyrate, α-keto-γ-mercaptobutyrate). The reaction with glutamine as substrate will be driven toward α-keto acid removal, because α-ketoglutaramate, the product of glutamine oxidation, either cyclizes or is rapidly deamidated to α-ketoglutarate. Rat kidney thiopurine *S*-methyltransferase catalyzes methylation of β-mercaptopyruvate and α-keto-γ-mercaptobutyrate (cysteine and homocysteine are not substrates) [20]. Because both the initial α-keto acid (α-keto-γ-mercaptobutyrate) and the product of the thiopurine *S*-methyltransferase reaction (α-keto-γ-methiolbutyrate; Fig. 1, **2**, $X = SCH_3$) are substrates for glutamine transaminase K, the kidney appears to have a mechanism for ensuring detoxification and recycling of homocysteine carbon and sulfur.

L-Canavanine

L-Canavanine (2-amino-4-guanidinooxy-butanoate) [Fig. 1, where X is often depicted as $—ONHC(=NH)NH_2$ but is more correctly shown as $—ON=C(NH_2)_2$] is an amino acid analogue of arginine in which the 5-methylene group is replaced by oxygen. L-Canavanine is found in many plants and seeds of the *Leguminosae spp.* (subfamily Papilionoideae) and probably functions as a source of nitrogen sequestered in the guanidinooxy portion of the molecule [21] and, due to its toxicity, as a chemical defense against herbivores [22]. L-Canavanine has been implicated as a cause of the systemic lupus erythematosus-like syndrome observed after the ingestion of large amounts of alfalfa seeds or sprouts by cynomologus monkeys [23] or humans [24].

The toxicity of L-canavanine is probably due, in part, to its incorporation into proteins in place of arginine. The pK_a of the oxyguanidinium group of L-canavanine is 7.0, whereas that of the guanidinium group of arginine is 12.5 (see discussion in Ref. 18). Because a considerable fraction of the oxyguanidinium group would be uncharged at neutral pH, drastic changes in the three-dimensional structure of L-canavanine-containing proteins compared to the natural proteins may occur. It is also possible that the toxicity of L-canavanine is due, in part, to the production of secondary, toxic metabolites (see discussion in Ref. 18). β,γ-Elimination of hydroxyguanidine occurs after oxidation of L-canavanine at the α-carbon with L-amino acid oxidase [18]. Direct conversion of L-canavanine to the α-keto acid by the action of a transaminase, a dehydrogenase, or an amino acid oxidase has not yet been demonstrated in mammalian tissues *in vivo*, although it seems plausible that α-oxidation may occur *in vivo*. The α-keto acid analogue of arginine (α-keto-δ-guanidinovalerate) is a known metabolite that accumulates in patients with hyperargininemia [25].

L-Homocysteine S-*conjugates*

The nephrotoxicity, genotoxicity, and, perhaps, nephrocarcinogenicity of several chloro- and fluoroalkenes are attributable to hepatic glutathione *S*-conjugate formation, metabolism to the corresponding cysteine *S*-conjugate, translocation to the kidney, and bioactivation by renal cysteine conjugate β-lyase (β-lyase) to yield thioacylating intermediates [26–28]. Cysteine *S*-conjugates of nephrotoxic haloalkenes cause renal proximal tubular damage [29, 30]. β-Lyase catalyzes β-elimination reactions from cysteine *S*-conjugates, and the initial metabolites are either haloalkyl- or haloalkenylthiols [31–33]. Rat renal cytosolic β-lyase is identical with soluble glutamine transaminase K [34]. In the absence of the usual α-keto acid amino group acceptor, the enzyme catalyzes competing half-transamination and β-elimination reactions with cysteine *S*-conjugates. To maintain maximal rates of β-elimination reactions, an α-keto acid is required to convert the pyridoxamine 5′-phosphate form of the enzyme, produced after half-transamination, back to the pyridoxal 5′-phosphate form of the enzyme [34, 35]. Rat

kidney mitochondria also contain β-lyase activity, which appears to be attributable, at least in part, to mitochondrial glutamine transaminase K [36]. *S*-(1,2-Dichlorovinyl)-L-cysteine is a substrate for both soluble and mitochondrial glutamine transaminase K/β-lyase, which catalyzes the elimination of an unstable thiol that may tautomerize to yield a thionoacyl halide or lose HX to form a thioketene, both of which are thioacylating intermediates [31–33]. Formation of such an intermediate may account for the nephrotoxicity of certain cysteine conjugates or their mercapturic acids [31–33, 37–39]. Much evidence indicates that kidney mitochondria may be the target for *S*-(1,2-dichlorovinyl)-L-cysteine and other halogenated cysteine (or glutathione) conjugates [27, 28, 40–43].

To characterize further the bioactivation of *S*-conjugates, Anders and coworkers [44] synthesized *S*-(1,2-dichlorovinyl)-L-homocysteine, the homocysteine analogue of *S*-(1,2-dichlorovinyl-L-cysteine, and *S*-(1,2-dichlorovinyl)-DL-α-methylhomocysteine. *S*-(1,2-Dichlorovinyl)-L-homocysteine was considerably more nephrotoxic than *S*-(1,2-dichlorovinyl)-L-cysteine. A γ-cystathionase-type γ-elimination reaction accompanied by the formation of α-ketobutyrate and ammonia was ruled out; evidence was presented indicating that enzymatic transamination of *S*-(1,2-dichlorovinyl)-L-homocysteine yielded the corresponding α-keto acid. The α-keto acid spontaneously undergoes β,γ-elimination to yield two reactive metabolites: vinylglyoxylate and 1,2-dichlorovinylthiol. *S*-(1,2-Dichlorovinyl)-DL-α-methylhomocysteine cannot be converted to an α-keto acid, and this compound is not nephrotoxic. Again, the pyridoxal 5′-phosphate-dependent enzyme responsible appears to be glutamine transaminase K [44]. Cooper and Meister [17, 19, 45] have pointed out that amino acids with the structure $X(CH_2)_nCH(NH_2)CO_2H$ and where n = 1 or 2 and X is a non-charged group (e.g. glutamine, phenylalanine, tyrosine, *S*-methylcysteine, methionine) are good substrates for rat kidney glutamine transaminase K. More recently, Ricci *et al.* [46] have shown that the specificity of the bovine kidney glutamine transaminase K, like the rat enzyme, is broad; amino acids where X is charged [e.g. cystine, lanthionine, *S*-(2-aminoethyl)-L-cysteine] are also substrates. Glutamine transaminase K is a remarkable enzyme: not only does it have a pocket at the active site capable of accommodating a variety of amino acid side-chains, but it may act as a β-lyase if the electron-withdrawing properties of X (where n = 1) are sufficiently powerful. It would be even more remarkable, however, if the enzyme were to act as a γ-lyase; rather, γ-elimination occurs nonenzymatically and spontaneously from *S*-(1,2-dichlorovinyl)-L-homocysteine after formation of the corresponding α-keto acid [16, 44].

In addition to *S*-(1,2-dichlorovinyl)-L-homocysteine, *S*-(2-benzothiazolyl)-L-homocysteine, L-canavanine, and L-methionine sulfoximine are cytotoxic in rat renal proximal tubular cells and are metabolized by snake-venom L-amino acid oxidase to vinylglyoxylate, which was trapped by reaction with methanethiol [47] *S*-(2-benzothiazolyl)-L-homocysteine is also a substrate for purified bovine kidney glutamine transaminase K/β-lyase. The formation of vinylglyoxylate may be associated with the observed cytotoxicity of these 4-substituted 2-aminobutanoates. This contention is supported by the observation that 2-hydroxy-3-butenoate, which is also cytotoxic [47], may be metabolized by rat renal L-2-hydroxy (L-amino) acid oxidase.

Possible therapeutic benefits of γ-substituted amino acids

Vinylglyoxylate is so reactive that it has not yet been isolated in pure form, although it can be trapped as an addition product [9, 17, 18]. This reactivity may have clinical relevance: D-amino acid oxidase is present in peroxisomes and microperoxisomes of a variety of animal tissues (reviewed in Ref. 48). In humans, the enzyme activity is particularly abundant in liver [49], kidney [50], and brain [49, 50]. In general, in mammals activity is highest in kidney followed by liver. Glutamine transaminase K activity is present in the cytosolic and mitochondrial fractions of many tissues, but is also most active in kidney, followed by liver [8]; the enzyme is also present in human kidney cytosol and mitochondria, and the cytosolic enzyme has been purified [51]. It is curious that the relative tissue distributions of D-amino acid oxidase and glutamine transaminase K are so similar. If non-physiological D-amino acids (such as D-methionine or D-phenylalanine) are encountered *in vivo*, they may be converted to the corresponding L-amino acid in the kidney by the action of the two enzymes. Suitably designed compounds that are substrates for D-amino acid oxidase or glutamine transaminase K are expected to be "metabolically targeted" to the kidney and may be effective in treating kidney infections and kidney cancer. In humans, about 5% of all cancers occur in the kidney, and metastatic renal cell carcinoma is essentially resistant to all currently used therapeutic agents [52]. Renal carcinoma appears to arise from the proximal nephron [53], and, as noted above, this region of the kidney is most susceptible to damage from cysteine *S*-conjugates. Because chloroalkene-derived glutathione and cysteine *S*-conjugates are mutagenic [54–56], it is tempting to speculate that there may be a link between the environmental exposure to chloroalkenes and human kidney cancer. The identical site of action of cysteine *S*-conjugates and the site of origin of renal cancer indicate that the targeting of prodrugs of cancer chemotherapeutic agents to the kidney may afford a useful therapeutic strategy. Experiments designed to exploit this concept have been reported recently [57, 58]. *S*-(6-Purinyl)-L-cysteine is a substrate for renal β-lyase, and the chemotherapeutic agent 6-mercaptopurine is formed as a metabolite in the kidney. The logical extension of this strategy would be to prepare *S*-(6-purinyl)-L-homocysteine, which may yield both 6-mercaptopurine and vinylglyoxylate, and may be an effective, targeted chemotherapeutic and cytotoxic agent.

Hydroxyguanidine has been investigated as a possible anticancer agent because of its structural similarity to hydroxyurea, a well known chemotherapeutic drug, and to guanidine, which has antiviral

activity [59]. Treatment of three cellular tumors, including leukemia L1210 and a solid tumor in rats [59] and leukemia L1210 in mice [60], with hydroxyguanidine and related compounds resulted in a significant increase in survival time. Suitable substitution of hydroxyguanidine [i.e. RCH=NNHC(=NH)NHOH where R is an aromatic or heterocyclic ring] results in compounds that are strongly cytotoxic in cultured L1210 leukemia cells and that are 10- to 100-times more effective than the parent hydroxyguanidine [61]. The major cytotoxic effects of hydroxyurea, hydroxyguanidine, and analogues appear to be due to the inhibition of ribonucleoside diphosphate reductase, resulting in the depletion of deoxyribonucleotides required for DNA synthesis [62, 63]. The mechanism of the inhibition is not clear but may involve binding of the inhibitor to an iron atom at the active site [64]. Hydroxyguanidine has not been tested clinically, presumably because it appears to offer no advantage over hydroxyurea and other anticancer drugs already in use and also because other compounds with both anticancer and antiviral activities are more potent [65]. It is conceivable that hydroxyguanidine, formed intracellularly from D-canavanine after oxidation by D-amino acid oxidase, may be an important chemotherapeutic agent in the treatment of kidney neoplasms. If renal tumor cells or the surrounding tissue express D-amino acid oxidase activity, hydroxyguanidine may accumulate in the tumor cells after administration of D-canavanine. Furthermore, most of the hydroxyguanidine would be expected to be eliminated in the urine, thereby minimizing possible toxic side-effects in other organs. In addition, the vinylglyoxylate that is formed may also exhibit local cytotoxic effects. Thus, D-canavanine, or suitably designed D-canavanine analogues, may be useful because they can give rise to two potentially toxic metabolites that may act synergistically. Similarly, D-homocysteine conjugates upon oxidation with D-amino acid oxidase or L-homocysteine conjugates after transamination may also yield two toxic metabolites (one of which is vinylglyoxylate) that may act synergistically. Whether or not the administered dose can be controlled to minimize toxicity to healthy renal kidney cells and to retain toxicity to kidney cancer cells or infectious agents remains to be ascertained.

Conclusions

The possibility that γ-substituted amino acids, after oxidation at the α-carbon, may give rise to vinylglyoxylate has been largely overlooked, although at least five types of amino acids may give rise to this reactive α-keto acid. It is probable that vinylglyoxylate is a metabolite of certain amino acids *in vivo*. Because of its reactivity, vinylglyoxylate is likely to react rapidly with sulfhydryl groups (cysteine, glutathione, protein SH groups) *in vivo*. Formation of vinylglyoxylate may partly contribute to the toxicity of L-canavanine, L-methionine-*S*-sulfoximine, L-homocysteine, and L-homocysteine *S*-conjugates. The reactivity of vinylglyoxylate may have clinical utility in the treatment of kidney cancers or infections. Two enzymes (D-amino acid oxidase and glutamine transaminase K) that metabolize many γ-substituted amino acids are especially abundant in kidney; preferential formation of vinylglyoxylate in the kidney may be attainable by administration of suitable γ-substituted amino acids.

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