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IRENEUSZ P. GRUDZIŃSKI

BIOCHEMICAL TARGETS OF NITRIC OXIDE-INDUCED TOXICITY

BIOCHEMICZNE „PUNKTY UCHWYTU" DLA TOKSYCZNOŚCI INDUKOWANEJ TLENKIEM AZOTU

Department of Applied Physiology
Military Institute of Hygiene and Epidemiology
Kozielska St., 01–163 Warsaw, Poland
Head: Prof. dr hab. med. *J. Faff*

Nitric oxide (NO) has become one of the most intensively studied molecules in recent years. Although its beneficial role has been well established, a large body of adverse effects was also attributed to NO and/or its red-ox derivatives, implicating a dual (friend and foe) role of this agent in biological systems. Peroxynitrite ($ONOO^-$), a product from the reaction of NO with superoxide anion ($O_2^{\cdot-}$) was recognized as a potent toxic endogenous agent. This review will discus some of the discrete and/or silent chemical aspects of both NO produced from L-arginine and peroxynitrite in an attempt to elucidate the potential biochemical target(s) of NO- and/or peroxynitrite-induced toxicity.

INTRODUCTION

Over the last two decades, nitrite oxide (NO, nitrogen monoxide) has been well established as a principal and unique type messenger in the cardiovascular, nervous and immune systems [3–5, 12, 21, 32, 37, 41, 42, 45, 47, 58, 66]. The $NO^{\cdot}$ radical has been found to be produced through the net 5-electron oxidation of L-arginine by oxygen in a reaction involving enzyme, nitric oxide synthase (NOS), reduced nicotinamide-adenine dinucleotide phosphate (NADPH), flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN), protoporphyrin IX, tetrahydrobiopterin, calcium ion (NOS1 and NOS3), and calmodulin as cofactors [24–27, 34, 52, 57, 63]. Although, the biochemical characterization of nitric oxide synthase, formally [L-arginine, NADPH: oxygen oxydoreductase (nitric oxide forming); EC 1.14.13.39] closely resembled a NADPH-cytochrome P450 reductase [23, 35, 63], the presence of FAD and FMN and the striking sequence homology to NADPH-cytochrome P450 reductase indicated that NOS is the first catalytically self-sufficient and soluble P450 enzyme (dioxygenase), containing both a reductase and a cytochrome-P450 domain [11, 23]. The NOS synthase has been found to be up regulated at several molecular levels, including transcription, translation, and postranslation modification such as tyrosine phosphorylation, binding of prosthetic groups, palmitoylation, and finally dimerization [23]. In recent years, three isoforms of NOS, termed nNOSμ (NOS1 – neuronal), iNOS (NOS2 – inducible) and eNOS (NOS3 – endothelial) have been successfully cloned in

mammals, and nNOS, iNOS and eNOS genes were identified and localized on chromosomes 12q24.2, 17cen-q11.2 and 7q35–36, respectively [11].

NITRIC OXIDE AND PEROXYNITRITE CHEMISTRY

Nitric oxide (NO) is an uncharged molecule with seven electrons from nitrogen and eight electrons from oxygen, of which eleven are valence shell electrons, leaving one unpaired electron in the highest orbital. This chemical structure means that NO is a free radical and reacts rapidly with other molecules that contain unpaired electrons (Table I). Since the highest occupied molecular orbital of NO is anti-bonding in nature, it may be expected that this electron will be even lost to generate the nitrosonium ion (NO^+), or it will be also reduced by addition of twelfth electrons to form the nitroxyl ion (NO^-) [62]. The steady-state concentration of NO in biological systems is determined by its rate of formation as well as its rate of decomposition to stable forms such as nitrite (NO_2^-) and nitrate (NO_3^-) [33]. Interestingly, orally ingested nitrites to humans can be acidified in the stomach with subsequent dimerization and dehydratation to yield dinitrogen trioxide (N_2O_3) and dinitrogen tetraoxide (N_2O_4), and the agent(s) can be further converted to NO and/or NO_2 [67]. Subsequent reactions of these nitrogen species with secondary and/or tertiary amines will potentially yield carcinogenic N-nitroso compounds [67]. It should be noted that NO was also generated in post-ischemic tissues by either direct disproportionation or reduction of inorganic nitrite (NO_2^-) under acid and hypoxia condition [74]. Since the calculated half-life of circulating nitrate amounts up to 8 hours in humans, and about half of circulating (blood) nitrate depends directly on oral nitrate and nitrite intakes, a great deal of caution should be especially paid when estimating daily NO formation *via* its breakdown metabolites such as nitrate, nitrite and/or peroxynitrite [24, 30, 31, 46, 56], a product of reaction between NO and superoxide anion (O_2^-) [53].

The cellular formation of peroxynitrite ($ONOO^-$, oxoperoxonitrate(1-)), which is recognized as a principle inorganic toxin of biological importance, is extremely fast-speed reaction due to the fact that both species ($NO^\cdot$ and $O_2^{-\cdot}$) are free radicals. The kinetic rate of reaction between $NO^\cdot$ and $O_2^{-\cdot}$ ($1.9 \pm 0.2 \times 10^{-10}$ M^{-1} s^{-1}) is approximately 3–4 times larger then that for the superoxide dismutase (SOD)-catalyzed decomposition of $O_2^{-\cdot}$ ($2.3 \pm 0.1 \times 10^{-9}$ M^{-1} s^{-1}), therefore the cellular formation of peroxynitrite from NO and superoxide may plausibly predominate over the superoxide decomposition by SOD to yield hydrogen peroxide (H_2O_2), which is finally decomposed by catalase (CAT) (Table I). The peroxynitrite anion is relatively stable and long-lived molecule, however its acid form (ONOOH) rapidly decays to inorganic nitrate (NO_3^-) with a half-life of 1–2 second at physiological pH and temperature [36]. The pKa value of peroxynitrous acid has been determined around 6.5 at 25°C, however it was also reported that the pKa value of this acid depends on buffer composition and concentration; and it can be very from 6 to up to 8 (or above) in Hepes, formate or ammonia buffers. Interestingly, below its pKa peroxynitrous acid (the *trans* isomer) isomerizes mainly to nitrate, while above its pKa decomposition to nitrite and dioxygen is generally predominated [36, 68]. In principle, the reactions of heterolysis followed by reaction of the nitryl cation with water, the reaction(s) of homolysis followed by reattachment of the hydroxyl radical to form nitrate, and finally the internal rearrangement(s) have

Table I. Chemistry of nitric oxide and its red-ox derivatives in biological environments.

NITRIC OXIDE (NO) FATE IN CELLS	TYPE REACTION
L-arginine → NO + L-cytruline (NOS-catalyzed)	NO formation from L-arginine (eNOS, nNOS, iNOS)
$2NO^{\cdot} + O_2 \rightarrow 2NO_2$ $2NO^{\cdot} + 2NO_2 \rightarrow 2N_2O_3$ $2N_2O_3 + 2H_2O \rightarrow 4NO_2^- + 4H^+$	NO oxidation to nitrite
$NO_2^- + H^+ \rightarrow HNO_2$ $2HNO_2 \rightarrow N_2O_3 + H_2O$ $N_2O_3 \rightarrow NO + NO_2$	Nitrite reduction to NO(acid pH)
$NO + O_2^{\cdot -} \rightarrow ONOO^- \rightarrow ONOOH \rightarrow {}^{\cdot}NO_2 + OH^{\cdot}$	Peroxynitrite formation from NO and superoxide anion($K = 1.9 \times 10^{-10}$ $M^{-1}s^{-1}$)
$2O_2 + NADPH \rightarrow 2O_2^{\cdot -} + NADP^+ + H^+$ (oxidase) $O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$ (SOD-catalyzed) $H_2O_2 \rightarrow H_2O + {}^1/_2\, O_2$ (CAT-catalyzed) $H_2O_2 + NO_2^- \rightarrow {}^{\cdot}NO_2$ (MPO-catalyzed) $H_2O_2 + Fe^{+2} \rightarrow HO^{\cdot} + OH^- + Fe^{+3}$ $H_2O_2 + O_2^{\cdot -} \rightarrow HO^{\cdot} + OH^- + O_2^-$	Superoxide decay to hydrogen peroxide ($K = 2.3 \times 10^{-9}$ $M^{-1}s^{-1}$) (Enzyme involved) *Fenton* reaction *Haber-Weiss* reaction
$2ONOOH \rightarrow H_2O + ONOO^- + {}^{\cdot}NO_2$ $2ONOO^- \rightarrow O_2 + 2NO_2^- \rightarrow 2NO_3$	Peroxynitrite decay Izomerization to nitrite/nitrate
$HbO_2 + NO \rightarrow$ metHb $+ NO_3^-$ Hb(deoxyHb) $+ NO \rightarrow$ HbNO $HbO_2 + 4NO_2^- + 4H+ \rightarrow$ 4metHb $+ 4NO_2^- + O_2 + 2H_2O$	NO reaction with hemoglobin to form nitrite/nitrate
$SOD\text{-}Cu^{+2} + H_2O_2 \rightarrow SOD\text{-}Cu^{+1} + O_2^{\cdot -} + 2H+$ $SOD\text{-}Cu^{+1} + H_2O_2 \rightarrow SOD\text{-}Cu^{+2}\text{-}OH^{\cdot} + OH^-$ $SOD\text{-}Cu^{+2}\text{-}OH^{\cdot} + DH_2 \rightarrow SOD\text{-}Cu^{+2} + DH^- + H_2O$ $SOD\text{-}Cu^{+2}\text{-}OH. + H^+ + NO^{\cdot} \rightarrow SOD\text{-}Cu^{+2} + H_2O + NO^+$ $H_2O_2 + NO^+ \rightarrow ONOO^- + 2H^+$	NO reaction with SOD Cu+2/+1 (red-ox states) to peroxynitrite *via* hydrogen peroxide
$ONOOH \rightarrow ONOO^- + H^+$ $ONOOH \rightarrow NO_3^- + H^+$	Peroxynitrite izomerizationto nitrate (week acid)
$ONOOH \rightarrow NO_2^+ + OH^-$ $NO_2^+ + OH^{\cdot} \rightarrow NO_3^- + H^+$	Heterolysis followed by reaction of the nitril cation with water
$ONOOH \rightarrow NO_2^{\cdot} + OH^{\cdot} \rightarrow NO_3^- + H^+$	Homolysis followed by reattachment of the hydroxyl radical to form nitrate
$ONOO^- + 2H^+ + e \rightarrow {}^{\cdot}NO_2 + H_2O$ $ONOO^- + 2H^+ + 2e \rightarrow NO_2^- + H_2O$	NO formation from peroxynitrite
$2NH_4^+ + 2OH^- + 3O_2 \rightarrow 2NO_2^- + 2H^+ + 4H_2O$ $2NO_2^- + O_2 \rightarrow 2NO_3^-$	Nitrification processes Nitrite oxidation to nitrate

been recognized as the major pathway(s) by which peroxynitrous acid could isomerize in aqueous solutions [68] (Table I).

BIOCHEMICAL TARGETS OF NITRIC OXIDE-INDUCED TOXICITY

The broader chemistry of nitric oxide (NO) involves a number of interrelated red-ox derivatives, especially those linked to dioxygen (O_2), superoxide ($O_2^{\cdot-}$), transition metal complexes, iron-sulphur sites, and numerous nucleophilic centers of biological importance, whose potential susceptibility to nitrosative attack has been extensively studied [21, 32, 45, 47]. Nitric oxide is highly diffusible molecule (diffusion constant of NO in aqueous solution amounts to 3300 mm x s^{-1} at 37°C, which is 1.4-fold larger than the diffusion coefficient of oxygen), and its biological action(s) is mainly achieved by direct cyclic guanosine monophosphate (cGMP)-targets, including cGMP-dependent protein (G) kinase (NO-G-kinase), cyclic nucleotyde-gated channels, and/or phosphodiesterases (PDE II/III) [46]. Nitric oxide binds to the haeme moiety of soluble guanylate cyclase (GC), forming a metal-nitrosyl adduct that is activated to catalyze the conversion of guanosine triphosphate (GTP) to cGMP, which further activates the protein G family, leading to a cascade of responses at the levels of transcription and translation [7, 46]. Interestingly, the outcome of recent studies also indicates that NO stimulates adenosine diphosphate (ADP)-ribosylation processes [59]. It should be noted that ADP-ribosylation processe(s) is the cGMP-independent reaction(s) through which NO influences signal transduction pathways (Fig. 1). Of these, the small GTP-binding protein $p21^{ras}$ seems to be one key signaling target of NO in cells, leading to downstream apoptotic events including modulation of the mitogen-activated protein kinase (MAPK) and/or cysteine protease (caspase)-induced cascades [38]. Since NO can either induce apoptosis or protect cells from programmed cell death, this dualism is largely unexplained, and plausibly depends on several still poorly characterized molecular factors [6, 39]. Nitric oxide is unique as a signal transduction mediator because its action principally depends on direct binding to the metal and/or thiol (SH) active sites in numerous of cytoplasmic components. For example, the active (SH) site of glyceraldehyde-3-phosphate dehydrogenase is frequently subjected to NO-dependent reaction, which often results in ADP-ribosylation and finally enzyme inhibition [44]. Similarly, the NO-dependent inactivation of the low molecular weight phosphotyrosine protein phosphatase family and sulfhydryl-containing enzymes is also postulated to mediate NO-triggered signal transduction pathways [9]. Although proteins such as guanylate cyclase is activated by low NO concentration in cells, a large body of evidences showed that microsomal CYP enzymes and non-haeme iron proteins such as aconitase, lipooxygenase, and xantine oxidase (XO), and other iron-sulfur complex or thiol (SH)-containing proteins are strongly inhibited by NO, albeit minimally or from much grater concentrations of NO [72]. Interestingly, NO has been shown to bind reversibly to the heme-a3 of cytochrome c oxidase, and extremely low (nM) concentrations of NO have been found to inhibit electron transport at physiological oxygen levels [13]. Since mitochondrial processes are one of the major sources of superoxide anion ($O_2^{\cdot-}$), which forms continually by electron leakage from the respiratory chain, the NO agent diffusing into mitochondria may also react with superoxide ($O_2^{\cdot-}$) to yield peroxynitrite ($ONOO^-$) [51]. Interestingly, the cellular expression of

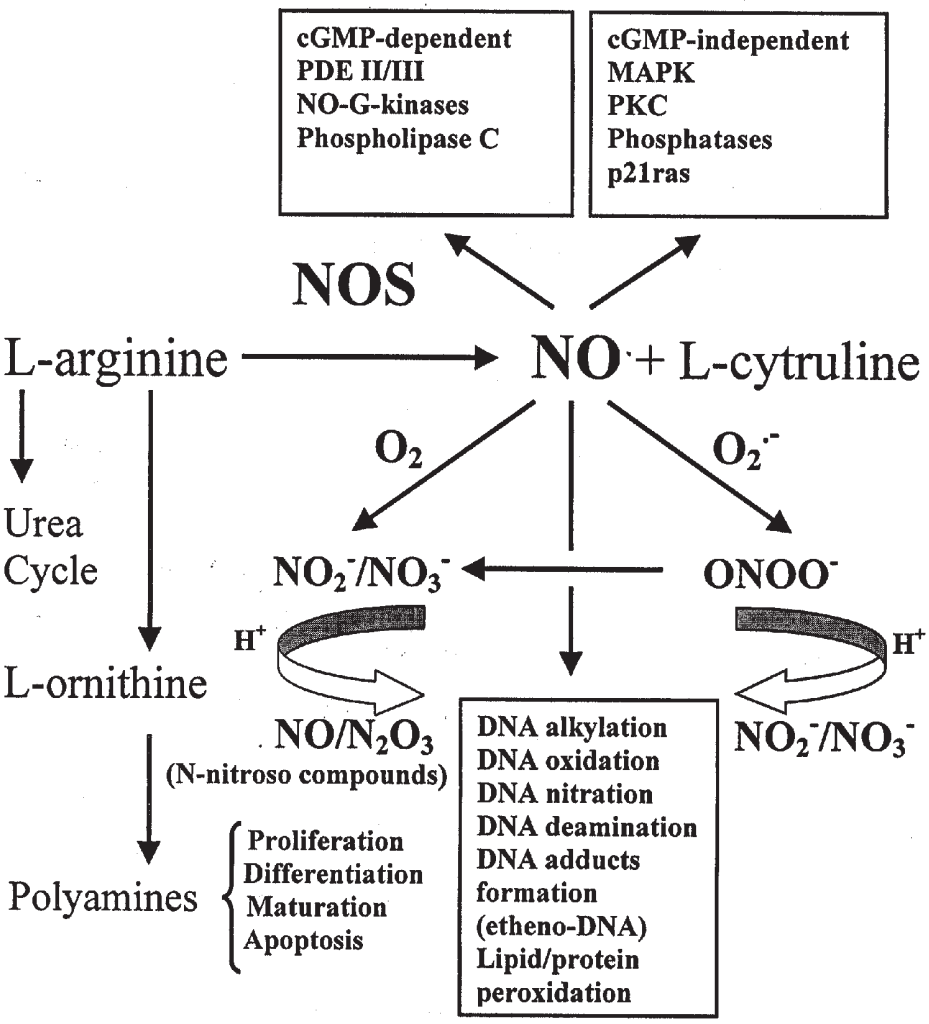

Fig. 1. Biochemical targets of nitric oxide and/or nitrite/peroxynitirte-induced toxicity. Detailed biochemical pathway for nitric oxide (NO) synthesis and NO-induced effect(s) was adopted and modified based on previously published papers by *Grudziński* [26, 27]. See text for abbreviations and comments.

iNOS, a first-step enzyme in NO over-production has been found to be closely associated with inflammatory reactions that followed infections and/or carcinogenic processes, and it was also accompanied to peroxynitrite toxicity in target organs [49, 50].

BIOCHEMICAL TARGETS OF PEROXYNITRITE-INDUCED TOXICITY

Nitric oxide (NO) exerts a dual role in cells showing both pro-and/or anti-oxidative properties as well as pro-and/or anti-apoptotic actions [6, 39, 48]. On the other hand,

the peroxynitrite anion ($ONOO^-$) was mainly recognized as powerful oxidant molecule of biological importance that exhibits complex chemistry, leading to severe damages in a wide range of biological targets, including nucleic acids, proteins, lipids, and non-protein sulfhydryls [8, 19]. For example, the $ONOO^-$ agent was found to nitrate tyrosine and protein-associated tyrosine residue to yield 3-nitrotyrosine, and it also reacted with carbon dioxide (CO_2) to yield nitro-oxocarboxylate anion ($O_2N\text{-}O\text{-}CO_2^-$) via the intermediate biological product, nitroso-dioxycarboxylate ($O{=}NOOCO_2^-$) anion [8, 19]. It seems plausible that the nitroso-dioxycarboxylate anion can also lead to other reactive metabolites including NO_2^+, $^{\cdot}NO_2$, and $^{\cdot}CO_3$, which are recognized as powerful oxidizing and nitrating species [19] (Table I). Interestingly, activated human polymorphonuclear neutrophils have recently been shown to convert inorganic nitrite (NO_2^-) into nitryl chloride (NO_2Cl) and the $^{\cdot}NO_2$ radical through myeloperoxidase (MPO)-mediated systems, which also utilized both nitrite and hydrogen peroxide (H_2O_2) to nitrate tyrosine-residues in proteins [20]. It should be noted that nitryl chloride is the major product of reaction between nitrite and MPO-derived hypochlorous acid and the direct oxidation of nitrite by MPO and H_2O_2 yields plausibly $^{\cdot}NO_2$ and then it oxidizes lipids in the absence of some transition metal catalysts [48]. Peroxynitrite is known to hydroxylate phenylalanine at three position of the aromatic ring, plausibly via $OH^{\cdot}$-linkage mechanisms(s) due to homolysis of peroxynitrous acid [69]. Furthermore, the $ONOO^-$ agent reacts with catecholamines to form semiquinones and quinines, and it affects some seleno-organic compounds and cellular-important anti-oxidant molecules including haemoproteins, porphyrin derivatives, ascorbate, α-, β-, and γ-tocopherols, β-carotene, uric acid, glutathione, N-acetylcysteine, bilirubin, and/or finally melatonin [2, 17, 19, 22, 61, 70]. Peroxynitrite readily inactivates aconitase by oxidizing the 4Fe4S cluster to an unstable oxidative state and it also inhibits α1-antiprotease by oxidation of an active site methionine [10]. It should be noted that $ONOO^-$ also inactivates mitochondrial electron transports, and converts xantine dehydrogenase (XDH) to the oxidase form (XO), possibly via sulfhydryl oxidation mechanism(s) [29, 55]. More importantly, the agent strongly oxidizes and nitrates nucleic acids to yield 8-oxo-7,8-dihydro-2'-deoxyguanosine, 8-oxoguanine and 8-nitroguanine, and some other unidentified moieties, and it depletes energy production and ATP storage in cells [8, 19, 43, 64, 65]. It should be emphasized that damaged DNA by $ONOO^-$ and/or NO is enable to activate poly(ADP-ribose) polymerase (PARP), probably to assist in DNA repair processes, and it up-regulates p53 gene but also mitigates cellular energy (ATP) due to re-synthesize of NAD^+ from nicotinamide [18]. Although, peroxynitrite could only react with guanine moieties (unlike N_2O_3 which could react with adenine, guanine, and cytosine), the $ONOO^-$ agent was found to cause DNA strand breaks much more effectively then that induced by NO and/or N_2O_3, the predominant nitrosating agent(s) arising from inorganic nitrite (NO_2^-) at the acid condition in the pyloric stomach of rodents and humans [15].

CONCLUDING REMARKS

It was proposed from a broad range of biochemical studies that NO produced from the amino acid L-arginine can mediate a numerous of biochemical and/or molecular targets in cells. One predominant factor that has been identified which influences NO

being beneficial versus detrimental is plausibly the relative rates of production and/or concentrations of NO and/or its red-ox derivatives especially peroxynitrite ($ONOO^-$). Once issue is certain, a final product of reaction between NO and superoxide ($O_2^{\cdot-}$) will remain to be precisely revealed in order to understand the molecular scenario of its activity. Evidence from experimental studies suggests that superoxide production and subsequent inactivation of NO may be important in peroxynitrite formation and the elevation of its cellular toxicity. Since peroxynitrite predominate in several common pathologies in humans including *Parkinson's*, *Alzheimer's*, and *Huntington's* diseases [54, 60, 73], coronary heart failure [16], arteriosclerosis [1], asthma [14], diabetes [28], and finally cancer [71], a detailed effort should be paid to examine NOS genes in further pre-clinical experiments.

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I.P. Grudziński

BIOCHEMICAL TARGETS OF NITRIC OXIDE-INDUCED TOXICITY

Summary

Nitric oxide (NO) has become one of the most intensively studied molecules in recent years. Although its beneficial role has been well established, a large body of adverse effects was also attributed to NO and/or its red-ox derivatives in biological systems. Peroxynitrite ($ONOO^-$), a product of reaction between NO and superoxide anion ($O_2^{\cdot-}$) was recognized as a potent pro-oxidant endogenous toxicant. The agent was found to induce DNA and protein oxidative damages leading to increased risk(s) of severe human pathologies including cancer. In this review, the discrete chemical aspects of both nitric oxide and peroxynitrite have been discussed in an attempt to elucidate the major biochemical target(s) of NO-and/or peroxynitrite-induced toxicity.

I.P. Grudziński

BIOCHEMICZNE „PUNKTY UCHWYTU" TOKSYCZNOŚCI INDUKOWANEJ TLENKIEM AZOTU

Streszczenie

Tlenek azotu (NO) jest jedną z najintensywniej badanych cząsteczek w ostatnich latach. Pomimo iż wykazano szereg korzystnych efektów związanych z jego działaniem, istnieje znaczna liczba dowodów eksperymentalnych wskazujących na toksyczny wpływ tlenku azotu i/lub jego pochodnych red-ox w układach biologicznych. Peroksyazotyn ($ONOO^-$) będący produktem reakcji pomiędzy NO i rodnikiem ponadtlenkowym ($O_2^{\cdot-}$) został uznany za potencjalny toksyczny i pro-oksydacyjny endogenny związek, który indukował oksydacyjne uszkodzenia DNA i białek, zwiększając tym samym ryzyko powstawania szeregu patologii u ludzi w tym procesów nowotworowych. W obecnej pracy przedstawiono wybrane chemiczne aspekty działania NO i $ONOO^-$ wskazując na główne biochemiczne i/lub molekularne „punkty uchwytu" dla toksyczności indukowanej tlenkiem azotu i/lub peroksyazotynem.

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