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QUARTERLY

Adenosine 5′-triphosphate — a new regulator of annexin function. A hypothesis[o]

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The paradigm of annexins as phospholipid-binding proteins interacting with membranes in a calcium-dependent manner has been recently questioned in light of observations that some annexin isoforms may behave like membrane integral proteins or remain associated with their target membranes at low, resting, concentrations of Ca^{2+} in the cytoplasm. In addition, an evidence has been presented that some annexins (annexins I, VI and VII) bind *in vitro* ATP and GTP, and upon binding the nucleotide the *in vitro* activity of these proteins is modified. However, annexins do not contain Walker A and B consensus sequences for ATP/GTP binding. This review presents the hypothesis that a new ATP-binding motif exists within the annexin molecules and that ATP may play a role of functional ligand for annexins also *in vivo*.

Annexins are ubiquitous intracellular calcium- and membrane-binding proteins [1, 2]. In most cases studied to date they exhibit a lower affinity to Ca^{2+} than the "EF-hand" calcium-binding proteins, although, they seem to be as sensitive to changes in intracellular calcium concentration as "EF-hand" proteins. Some of the annexin isoforms resemble membrane integral proteins since they are resistant to Ca^{2+} chelating agents and are solubilized from membranes only with detergents [3].

Recently, several annexins have been shown to interact *in vitro* with nucleotides [4–8], the ATP-binding property being the best documented for annexin VI ($K_d \approx 5\,\mu M$) [6, 7]. The nucleotide binding data for annexins are still limited and, therefore, must be taken with an adequate measure of caution. Moreover, annexins lack the Walker consensus motifs A and B for ATP/GTP binding in their structure [4], and there is no convincing experimental results that annexin function is modulated *in vivo* by changes in cellular level of ATP. How-

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Abbreviations: PKC, protein kinase C; PLA_2, phospholipase A_2.

ever, the following *in vitro* observations form the basis of a rationale for annexins as a new subfamily of ATP-binding proteins. ATP and cAMP were found to bind *in vitro* to bovine lung annexin I, influencing its ability to aggregate liposomes and chromaffin granules, and to form calcium channels [4]. Furthermore, with the aid of ^{13}C NMR analysis it has been shown that the binding ratio of ATP to N-terminally truncated human annexin I is 1:1 and the ATP binding site of the protein is located in the repeated domain I which determines the overall phospholipid affinity of annexin I [8]. In addition, the calcium-dependent binding of annexin VI to the hepatocyte plasma membrane was enhanced by ATP, although at Ca^{2+} concentrations higher than the physiological ones [9]. Moreover, evidence has been presented that annexin VI from porcine liver exhibits the ability to interact in a calcium-dependent manner with ATP, either immobilized on agarose beads or in solution [6]. On the basis of the limited amount of observations, one may speculate about the binding of ATP by some annexins to be a new regulatory mechanism for these proteins [10]. In the present review we would like to focus on following possibilities. Some annexins are able to react to local changes in intracellular ATP concentrations, resulting in changes of their interaction with membrane phospholipids and/or proteins. The binding of nucleotides may alter the annexin conformation and function; the nucleotide signal is then stopped by a slow-rate nucleotide hydrolysis, as suggested for annexin VII [5], after which annexins return to their resting state.

INTERACTION OF ANNEXINS WITH ATP: EFFECT ON BINDING OF ANNEXINS TO MEMBRANES

ATP was found to be a potent Ca^{2+} mobilizing agent and to act intracellularly on P2X and P2Y purinoreceptors localized in plasma membrane of various cells [11]. Within the cell ATP may bind to various proteins as ATP-sensitive K^+ channels in plasma membranes [12], ryanodine receptor/calcium release channels in sarcoplasmic reticulum membranes [13], or synapsins I and II in synaptic vesicles [14], what is not necessarily accompanied by nucleotide hydrolysis. This points to the possibility of ATP being a functional ligand for various effector proteins within a cell, amongst the many, some annexin isoforms.

On the basis of many *in vitro* observations, annexins have been proposed to play a role in Ca^{2+} signal transduction and calcium homeostasis [1, 2, 15]. Almost all annexins are able to form *in vitro* voltage-dependent ion channels [16,17]. These proteins have been implicated in endo- [18] and exocytosis [19]; in these processes, in addition to various proteins and ligands, ATP plays an important role (for review see [20]). Through a mode of interaction with membranes, annexins represent a second major group of soluble macromolecules binding to phospholipids, an alternative to C2 domain-containing proteins represented by cytosolic PLA_2, copines and PKC isoforms [21, 22]. Interestingly, for PKC it has been already shown that the stabilization of the membrane-PKC complex by phorbol esters can be reversed by ATP ($K_{1/2}$ 65 μM) [23]. Similarly, for porcine liver annexin VI ATP, at physiological concentration range, shifter the Ca^{2+} concentration required for half-maximal association of annexin VI to the membrane to 10 times higher concentration [6].

Annexins, like PKC and cytosolic PLA_2, alternate within a cell between two major states: soluble at relatively low Ca^{2+} concentrations, and membrane-bound, at higher Ca^{2+} concentrations. The calcium-bound form can interact with membranes in at least two distinct mechanisms: by a reversible Ca^{2+}-dependent mechanism and by a mechanism in which Ca^{2+} is required at an initial step but then the binding becomes Ca^{2+}-independent [2]. In the case of annexin II, the unique N-terminal se-

quence within the annexin molecule (residues 15–24), has been identified and proposed to play a role in a Ca^{2+}-independent specific annexin-endosome interaction. Moreover, it has been suggested, although is still unverified hypothesis, that the Ca^{2+}-independent mechanism of the annexin-membrane interaction is mediated by specific membrane receptors interacting with a particular sequence in the annexin molecule [24]. This hypothesis needs to be probed experimentally in the future.

Various annexin isoforms, were found to be associated with the membranes even when intracellular Ca^{2+} concentrations reached low resting levels, as for example in bovine heart, lungs and brain where annexins V and VI remained in the membrane-bound form under such conditions [9, 25]. It has been also observed that the Ca^{2+}-dependent binding of annexin VI to membranes is resistant to treatment with nonionic detergents [9]. These observations are in favor of an interpretation that annexin may interact not only with membrane components but also with cytoskeletal proteins, as it colocalizes in a cell with actin-binding proteins (α-actinin and fimbrin) [1, 2]. Moreover, the relocation of annexin V to platelet membranes upon cell stimulation and an increase of intracellular [Ca^{2+}] was found to be enhanced by ATP, and its analog, adenosine 5'-[γ-thio]-triphosphate. These results have been interpreted as that the phosphorylation of some membrane proteins or, perhaps, annexin V, is responsible for the tight association of annexin V with platelet membranes [26].

Mentioned above observations may indicate that not only Ca^{2+} binding to the annexins but also other ligands, for example nucleotides, may influence the interaction of annexins with membranes. It has been proposed that annexin VI of porcine liver may have a nucleotide-binding domain differing from other ATP-binding proteins characterized to date (see [27] and explanation below). Evidence was also provided that the binding of ATP to annexin VI may be accompanied by a structural rearrangement within the protein molecule [7] leading to modulation of the functional response of annexin VI determined *in vitro* [6, 28]. In fact, we have recently observed, using circular dichroism and Fourier transformed infrared spectroscopies and caged-ATP, that indeed binding of ATP to annexin VI results is small but well reproducible change in α-helix content of the protein (Bandorowicz-Pikuła, J., Wrzosek, A., Pikuła, S. & Buchet, R., submitted). Similar observations were not made for homologous to annexin VI, porcine liver annexin IV, probably due to a 10 times lower affinity of that protein for ATP than annexin VI [29]. The above mentioned results point for the existence of a close relationship between the Ca^{2+}-, phospholipid-, and ATP-binding sites in the annexin VI molecule [6, 7, 29] as confirmed by other investigators in case of human annexin I, where it was found that histidine 52 located within the phospholipid-binding domain of the annexin is probably involved in binding of ATP [8].

In general, within the characteristic Ca^{2+}-binding site of annexins, the calcium coordination sphere forms a pentagonal bipyramid. The calcium is coordinated by three main chain carbonyl oxygen atoms of the loop (with the consensus sequence G-X-G-T). In addition, coordination is provided by a bidentate carboxylate group from an acidic residue (either D or E) 38 residues carboxyterminal to the G-X-G-T sequence, and water molecules [21, 30]. Upon membrane binding, a water molecule from the apical position of the calcium coordination sphere can be replaced by a phosphoryl oxygen from the phosphatidylserine backbone [21, 31]; such behavior may explain the strong preference of annexins for acidic phospholipids. As a hypothetical possibility, which requires experimental confirmation, it can be speculated that a negatively charged molecule of ATP bound to annexin may mimic the polar head-group of phosphatidylserine. Furthermore, the nucleotide-binding domain of annexin VI could be allocated to a molecular

pocket analogous to the actin hydrophobic pocket [32]. This pocket is placed between two symmetric lobes of the annexin VI molecule, each consisting of four Ca^{2+}- and phospholipid-binding domains [27]. Therefore, it is rather distant from the N-terminal portion of the protein molecule recently shown to confer binding of benzodiazepine derivative, BDA-452 [33]. The loop between the symmetric lobes of annexin VI may participate in the creation of an ATP-binding epitope (either continuous or discontinuous) for the protein. Most annexin VI genus-specific isoforms contain a unique tryptophan 347 residue located within the mentioned loop [7]. ATP, GTP and other nucleotides quench the intrinsic fluorescence of annexin VI which, in the case of trinitrophenyl-ATP, was accompanied by a fluorescence energy transfer between this tryptophan residue and trinitrophenyl-ATP [7].

POSSIBLE IMPLICATIONS OF THE BINDING OF NUCLEOTIDES TO ANNEXINS

The expression of annexins is often tissue-restricted and cell-specific and the genes encoding some annexins are specifically regulated, e.g. by prolactin, progesterone, glucocorticoids, retinoids and thyroid hormones [1, 2]. Moreover, the mechanisms of gene regulation are frequently linked to processes of cell differentiation and proliferation. For example, annexin VI is able to bind protein $p120^{GAP}$, an activator of GTPase $p21^{ras}$ [34]. Annexin II binds, through its tail domain, to tissue plasminogen activator [35]. A promoter for the binding of an early response gene product, AP1, has been identified upstream of the annexin V-encoding gene [1]. Some annexins are encoded by *fos*-induced genes [2], have been found to be involved in regulation of PLA_2 and PKC activities [36, 37], and bind signaling molecules and neurotransmitters or their precursors, e.g. choline [1, 2].

An important role in the annexin functioning is played by phosphorylation catalyzed by various tyrosine and serine/threonine protein kinases [38]. On the other hand, annexins were found to be potent inhibitors of various protein kinases; this inhibition resulting in changes of membrane permeability to ions (chloride conductance) [37, 39] or in the modulation of cellular responses to insulin [40].

Annexins have been observed to undergo translocation from cytoplasm to plasma membranes and phagosomes in response to changes in cytosolic [26, 41, 42] or nuclear [43] Ca^{2+} concentrations upon cell stimulation. Under oxidative stress annexin VI was found to translocate from the plasma membrane to cytosol due to a combination of depletion of cytoplasmic ATP level and the oxidative modification of membrane lipids and proteins, matching the elevation of intracellular Ca^{2+} in alveolar macrophages [44] (Fig. 1). Since annexins are able to form calcium channels *in vitro* [16, 17, 45], it has been suggested that they also do so in membranes of various cellular organelles, for example in mitochondria, where they are believed to play a role in regulation of the Ca^{2+} concentration in mitochondrial matrix [46]. Various annexins are also able to modulate *in vitro* the activity of ion transporters, e.g. the ryanodine receptor/calcium-release channel [47] and Ca^{2+}-dependent chloride channel from epithelial cells [48].

Is it possible that annexins may act as effectors in the nucleotide signaling pathways? Changes in the nucleotide concentration within the cell, especially under metabolic or oxidative stress [10, 49], may engage annexins as effectors and mediators of various intracellular reactions involved in information and energy transduction within the cell. Annexin VII has been found to be a GTPase [5], and some plant annexins showed either phosphodiesterase activity, which was inhibited by phospholipid binding [50], or myosin-like hydrolytic activity towards ATP [51]. Since GTP

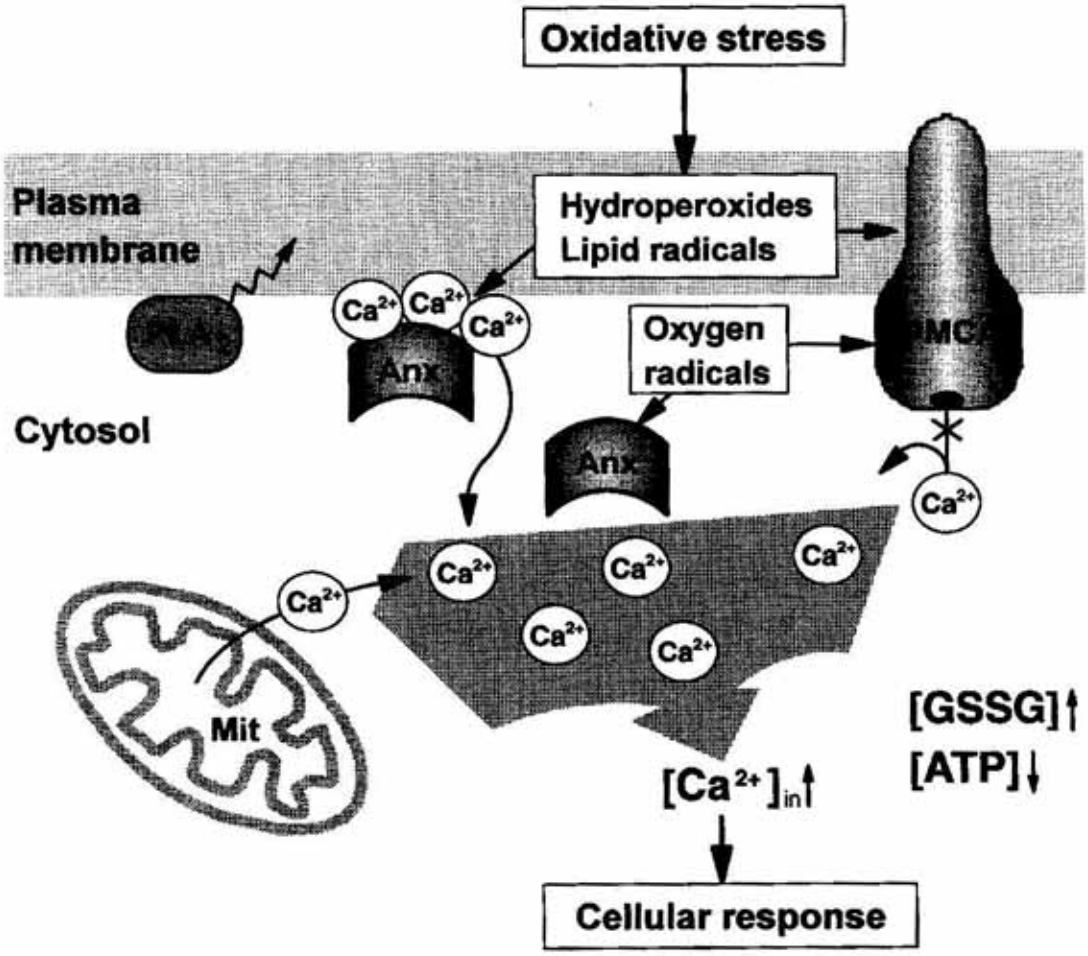

Figure 1. Translocation of annexins from membranes to the cytosol upon oxidative stress in alveolar macrophages.

As a result of lipid peroxidation, hydroperoxides and lipid radicals are formed in membranes influencing both the membrane permeability to calcium ions (as exemplified by inhibition of plasma membrane type Ca^{2+}-ATPase – PMCA) and the translocation of membrane-bound proteins (e.g. annexins – Anx). In addition, oxygen radicals are deleterious to various membrane and cytosolic proteins and nucleic acids. These changes are accompanied by ATP depletion, disturbances in mitochondrial (Mit) Ca^{2+} homeostasis, depletion of the reduced form of glutathione and a concomitant increase of its oxidized form (GSSG), and an increase in the overall cytosolic calcium concentration. Annexins in the cytosol are no longer inhibitory to phospholipase A_2 (PLA_2) which results in further damage to the membrane lipid barrier. These changes lead to various cellular responses mediated by calcium-dependent enzymes and proteins. The dissociation of annexins from damaged membranes, followed by the release of substantial amounts of calcium ions, has been postulated to play a partial but significant role in the cellular responses [44].

and its nonhydrolysable analogue GTPγS are known to promote Ca^{2+}-dependent exocytosis in many cell types by a mechanism thought to involve as yet unknown proteins, Pollard and his co-workers hypothesize that annexin VII may play a role as one of such proteins [5]. The authors used streptolysin *O*-permeabilized cells and found that the initial rate of annexin VII-driven Ca^{2+}-dependent aggregation of chromaffin granules and phosphatidylserine liposomes was increased by GTPγS > GTP [5]. GTP also influenced liposome fusion driven by annexin VII. These effects were accompanied by a specific binding of GTP to annexin VII, followed by hydrolysis of GTP by annexin VII dependent on calcium ($K_{1/2}$ 50 μM), Mg^{2+} ($K_{1/2}$ 400 μM) or other divalent cations (Ba^{2+}, Sr^{2+}). Pollard and his col-

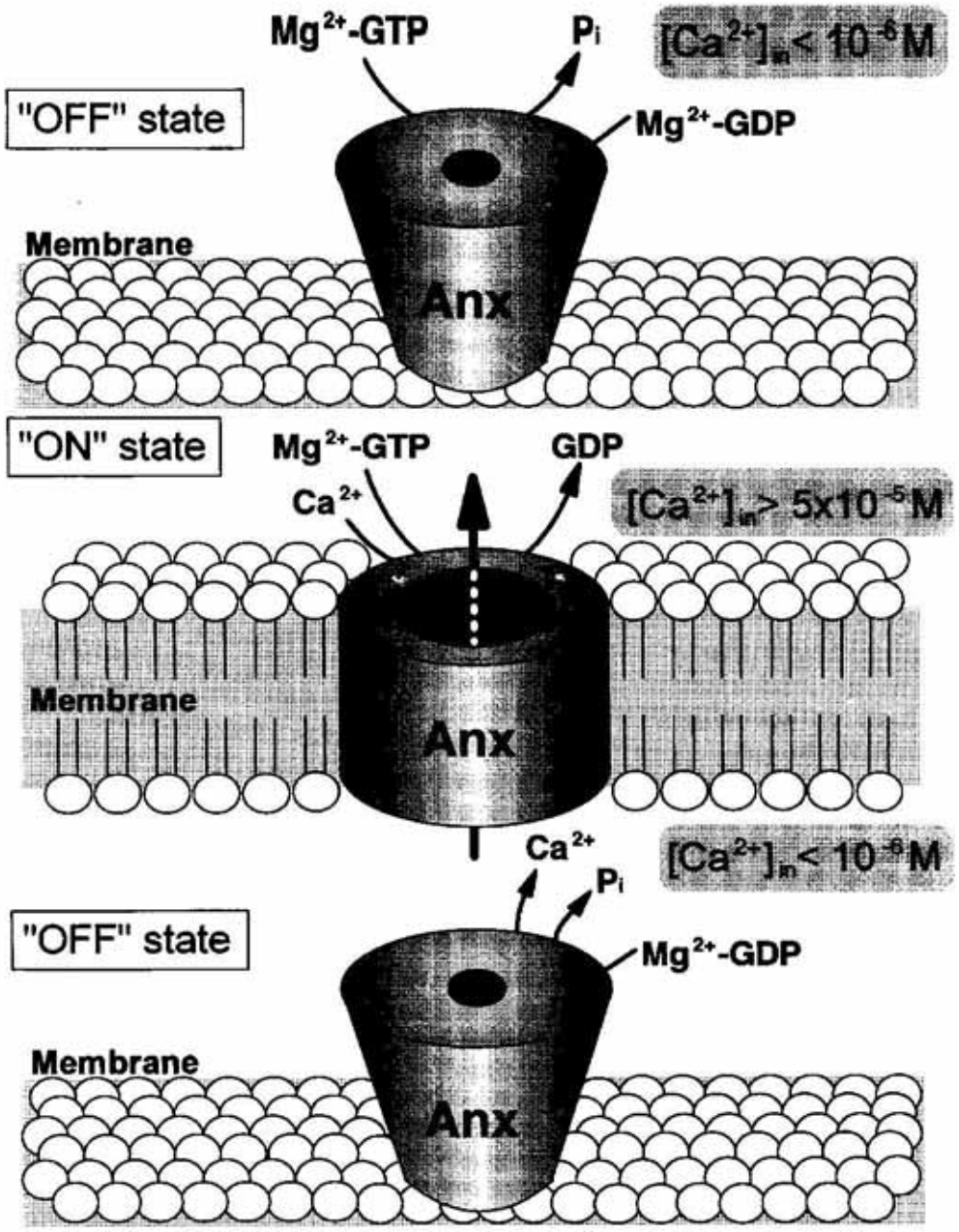

Figure 2. Schematic representation of annexin VII as a GTP-binding protein able to hydrolyze the nucleotide.

At low, submicromolar, resting concentration ranges of Ca^{2+} in the cytosol, the Mg^{2+}-GTP complex binds to the annexin molecule and is eventually hydrolyzed, at a slow rate, to GDP and inorganic phosphate, the latter compound being released. Under these conditions annexin does not bind to the membrane and is not operative, as represented by closed intramolecular channel. Upon elevation of [Ca^{2+}], Ca^{2+} and Mg^{2+}-GTP bind to the annexin molecule, in analogy to the GTP- and calcium-binding protein called calexcitin. Then annexin undergoes a conformational rearrangement, binds to the membrane, releases GDP and annexin is described as in its on state, as depicted by fully open intramembraneous ionic channel. As Mg^{2+}-GTP becomes cleaved in a fast reaction stimulated by Ca^{2+}, and the concomitantly intracellular calcium concentration returns to the resting level, then calcium ions dissociate from the protein and annexin becomes detached from the membrane in its off state, a functionally quiescent form [5, 52].

leagues applied a G protein-like molecular switch model in which the annexin VII-GTP form was active and revealed fusogenic activity. On the other hand, the protein became in-

activated upon Mg^{2+}-GTP hydrolysis, but could be reactivated by elevation of the intracellular Ca^{2+} concentration above 50 μM [5, 52] (Fig. 2).

The interaction of annexin VI with ATP may play an important role in secretion of mineral deposits from chondrocytes. These cells take up calcium ions and accumulate them in a process stimulated by collagens in secretory vesicles, which are primary initiators of extracellular mineral deposition in endochondrial calcification processes [53]. Binding of collagen molecules to secretory vesicles is modulated by annexins II, V, and VI, and the level of expression of these proteins is relatively high in chondrocytes [54]. Chondrocytes also excrete ATP which, in turn, is used to regulate cell maturation and determines the amount and type of mineral compounds produced by these cells [54]. Recently, it has been shown that matrix vesicles, structures that accumulate Ca^{2+} during the initiation of mineral formation in growing bone, are rich in annexin V and evidence was provided that annexin V forms a multiconductance Ca^{2+} channel in the membranes of these vesicles. Moreover, ATP and GTP were found to differentially modulate the activity of this channel: ATP increased the amplitude of the current and the number of conductance states while GTP reduced the number of events and of conductance states [55].

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Annexins have long been known as Ca^{2+}-regulated, phospholipid- and membrane-binding proteins but the key issue remains the determination of their real biological roles. The discovery of some annexin isoforms as intracellular targets for ATP sheds, in our opinion, a new light on determining some important functions of annexins *in vivo*. Therefore, it seems that annexins might be treated as an example of a group of proteins, increasing in number, for which nucleotides would play the role of important ligands and not of a source of metabolic energy. The major goal for future studies should be to design experiments resulting in an understanding of how ATP binding property of some annexins found in *in vitro* experiments is used by these proteins *in vivo*. These studies might result not only in the solution of the structure of a new ATP-binding fold [56] but with establishment of a new function for the annexins. It should be noted that there are good examples of proteins for which the discovery of structural motifs was frequently associated with the identification of discrete mechanisms involved in catalysis of particular chemical reactions.

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