*Thiol Metabolism and Redox Regulation of Cellular Functions*
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# Reduction of the Endoplasmic Reticulum Accompanies the Oxidative Damage of Diabetes Mellitus

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## 1. Thiol Metabolism in the Endoplasmic Reticulum

The average redox potential of the endoplasmic reticulum (ER) is about –160 mV, but theoretical calculations and some experimental results suggest that redox potential gradients and redox potential inhomogenities are typical of the subcompartment. The ER redox potential was thought to be mantained mostly by the glutathione/glutathione-disulfide redox buffer (GSH:GSSG = 1 trough 3:1), and can be described by the thiol/disulfide ratio [1]. But there are many other systems, which are able to influence the thiol metabolism. The *in vitro* mechanism to alter the redox state includes sulfhydryl oxidase, a NADPH-dependent oxigenase and the vitamin-K redox cycle [2]. The direct role of hem, ubiquinone, Fe-S clusters and molecular oxygene was excluded recently in yeast models [3]. The possible involvement of flavin adenine dinucleotide (FAD) in the electron transport was also documented on yeast, where the addition of FAD accelerated the disulfide bridge formation by the ER-resident enzyme, ERO1p [3]. The yeast lumenal ER protein, ERV2 was also described as a flavoenzyme, and is able to accelerate $O_2$-dependent disulfide bridge formation [4]. Besides flavin adenine dinucleotide, increasing number of evidence supports the involvement of an other, well known redox system on ER redox state. Ascorbate/dehydroascorbate concentration is in milimolar range in the ER lumen, and asorbate is a very important cofactor of the enzymes catalyzing prolyl- and lysyl-hydroxylation. The characteristics of their transport was well described by G. Bánhegyi and co-workers [5]. Cytoplasmic ascorbic acid is first oxidized and dehydroascorbic acid is transported to the lumen by facilitated diffusion. Inside, the increasing concentration of oxidized form can help to mantain the transitional redox state, and take part in the GSH and protein thiol oxidation [6]. Moreover, protein disulfide isomerase itself has dehydroascorbate reductase activity [7]. The membrane-bound antioxidant agent, tocopherol was also mentioned as a possible contributor of the electron transport by ascorbic acid [8]. The importance of the glutathione/glutathione-disulfide redox buffer was recently described. The estimated redox potential of the ER (–160 mV) correlates with the current GSH/GSSG ratio, and its total concentration (1 to 2 mM) is high enough to affect redox environment and protein redox states [9]. But the processes setting the balance between GSH and GSSG have not yet been clearly identified. Glutathione synthetase, responsible for the *de novo* GSH generation, is located only in the cytoplasm, so GSH must enter to the ER lumen through transporters. A much faster GSSG transport was hypothesized to sustain the oxidative environment, but recent data are quite controversial

on this topic [5]. Another possible way for the increase of GSSG concentration is described by some publications involving specific enzymatic GSSG generation by intraluminal redox enzymes [10].

The next chapter of glutathione research, its role on the protein folding process, is also under reevaluation today. The increasing importance of GSH is underlined as a counterbalance for the ERO1p-mediated oxidation in yeast. According to this model, the oxidizing equivalents (whose precise nature is still unidentified) coming from the cytosol, and transmitted through the ER membrane by the ERO1p protein, oxidize protein disulfide isomerase and consequently the secreted proteins. To avoid hyperoxidizing conditions a part of these oxidized proteins (and perhaps ERO1p itself) are reduced by GSH and this process takes part in the maintenance of the relatively low ER GSH/GSSG ratio [11]. The final step of ER glutathione metabolism is the secretion of GSH, which occurs by the vesicular transport system. The concentration of the glutathione in the vesicles targeting secreted and membrane surface proteins to the cell membrane is about 1 mM [12].

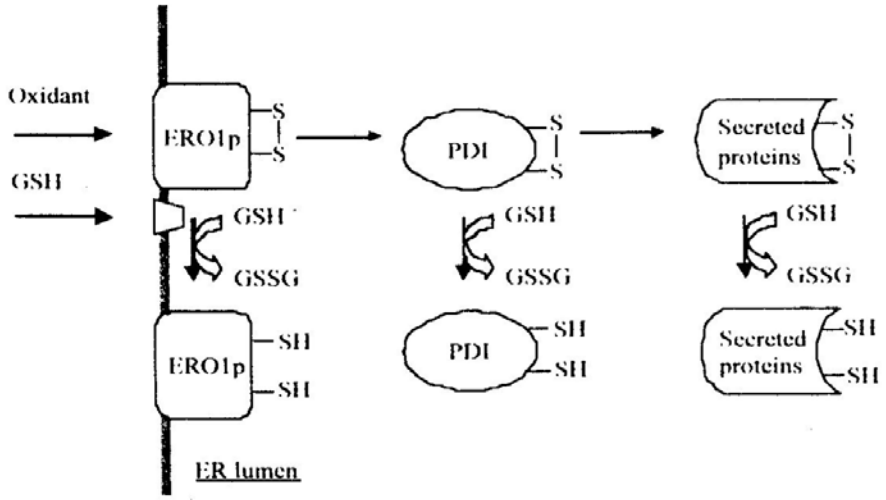


**Fig. 1. Redox balance in the ER [10]**

## 2. Disulfide bond formation in the ER

The cytoplasmic redox potential (-230 mV) and the high GSH/GSSG ratio (30 through 100:1) is not favourable to the formation and existence of disulfide bridges. Therefore, only 0.1% of the total protein thiol groups is bound in intramolecular or mixed disulfides permanently. Transitional disulfide bridges can be observed in case of many enzymes. This function is connected to a thiol-disulfide catalytic cycle [13,14]. Therefore, folding of the secreted and integral membrane proteins, which usually have many disulfide bridges to stabilize their tertiary and quaternary structure, must occur in the more oxidizing environment of the ER. The newly synthetized polypeptide chain labeled by the N-

terminal signal peptide targeting it to the ER, enters to the lumen, where molecular chaperones and folding catalysts help to get its final structure. Molecular chaperones, like BiP and Grp94 trap the unfolded protein and stabilize it against aggregation, incorrect folding, but don't participate in the disulfide bond synthesis directly [15,16]. The formation of native disulfide bridges may occur without enzymatic catalysis under highly diluted and mild oxidative conditions as Christian Anfinsen and co-workers demonstrated almost 40 years ago [17], but the redox folding assistance, such as protein disulfide isomerases can accelerate and make the thiol-disulfide exchange more efficient in the crowded and dinamically changing environment of the ER. These proteins have some common caracteristics. They are intraluminal, mostly soluble enzymes containing the ER retention signal, and at least one thioredoxin-domain and a special amino acid sequence (CXXC) in their active site. The major steps of the enzymatic catalysis are the formation of a transitional enzyme-substrate complex, connected by intermolecular disulfide bridges and then the rearrangement of these bounds. Protein disulfide isomerases can create, cleave and isomerize the disulfide bonds depending on the state of the substrate and the redox eqilibrium [18,19].

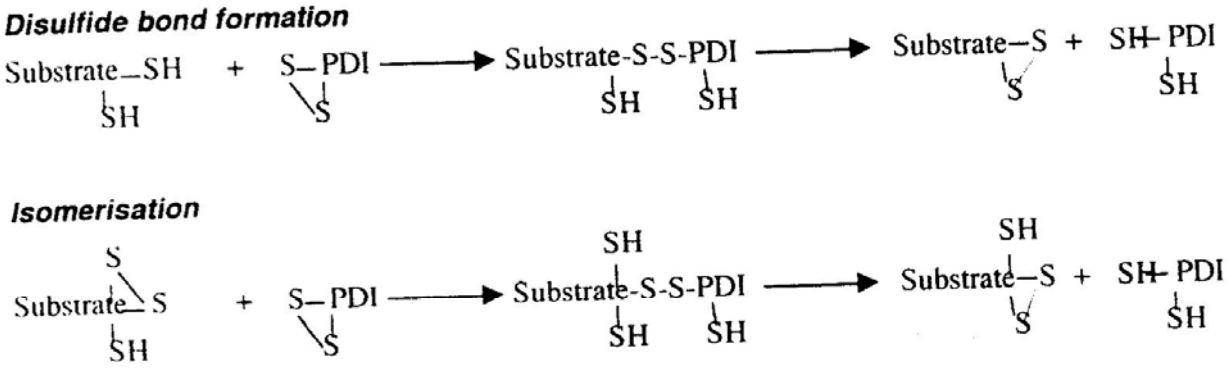


**Fig. 2. The mechanism of thiol-disulfide exchange**

The best known member of the PDI family is the 58 kDa protein disulfide isomerase, which is usually called as *Erp58*, or simply as *protein disulfide isomerase (PDI)*. This PDI contains two thioredoxin domains, and its local concentration in the ER is about 200 to 300 μM [19,20]. It has both "molecular chaperone" and "antichaperone" activities on different substrates. As a chaperone it prevents protein aggregation. Just conversely, as an antichaperone PDI mediates aggregate formation, when the amount of unfolded or aggregation-prone proteins is far greater than that of ER chaperones [21]. PDI seems to be a redox-regulated chaperone: binding of the cholera-toxin substrate to PDI is stronger, when the protein is reduced and cholera toxin dissociates under oxidizing conditions [22]. PDI is also a constant part of various enzyme complexes assisiting in quite different processes (e.g. prolyl-4-hydroxilase, tryacylglycerol transfer protein, etc.) and can bind the hormone, estrogen [23]. But the most important PDI function is to form native disulfide bridges on newly syntethized and reversibly denatured proteins. This is a circular process and the reduced PDI is reoxidized by the ER membrane protein ERO1p [24]. EROp1 was discovered a couple of years ago in yeast, but later mammalian isoforms were also

identified. EROp1 contains a CXXCXXC motif in its active site, and is able to oxidize both GSH and PDI. PDI was found to bind directly to the ERO1p. Neither structural, nor functional interaction between the ERO1p and other members of the protein disulfide isomerase family, Erp72, Erp57 were detectable so far. In mammals, the two human ERO1p isoforms (α and β) were cloned, and only the expression of β form is induced by the unfolded protein response. This is a flavoprotein, which is able to accept electrons coming from the secretory and other substrate proteins through protein disulfide isomerase and donate them to unknown participants [10].

***ERp72*** is a protein disulfide isomerase containing three thioredoxin domains. By its active redox and disulfide isomerase activity can replace PDI in PDI-deficient cells. *In vivo* it binds calcium, and the *in vitro* binding of different peptides could be also demonstrated together with some chaperone-like activities [20,25].

***Erp57*** is a protein disulfide isomerase specialized to glycoproteins. It has two thioredoxin domains, and forms complexes by calnexin and calreticulin the two ER lectin-like chaperones. The isomerase activity of the calreticulin/ or calnexin/Erp57 complex is higher than that of Erp57 alone. Erp57 interacts specifically with N-glycosylated polypeptides and its function is strongly determined by the lectin-like chaperones and the glycosylation-state of the substrate [26,27].

***ERp44*** is the newest member of the thioredoxin domain-containing ER luminal proteins, but the primary structure of its active site (CRFS) is different from that of other PDI-s in the ER. Erp44 helps immunoglobulin folding, binds covalently to ERO1p and can influence its redox state [28].

There are many ER-resident proteins (Erp28, P5, Erp55) associated to these folding catalysts, which lack the thioredoxin domain, and they do not likely play a direct role in redox folding. However, they are suggested as possible co-chaperones of the redox folding process [29,30].

According to our current knowledge two different ways of disulfide bond formation are present in the ER. In the first, the electron transport occurs from the reduced substrate through protein disulfide isomerase to the ERO1p. The ERO1p flavin adenine dinucleotide group gives it to unidentified participants, which can integrate the thiol metabolism to the general cellular redox metabolic pathways. The second way (performed mostly by ERp72, and Erp57) seems to be uncoupled from ERO1p, and requires other, probable small molecular mediators [31].

## 3. Disturbances of Redox Protein Folding

As we discussed above, the redox regulation of the ER is closely connected to redox protein folding. Therefore, it is not surprising that any major disturbances of the ER milieu, especially that of the thiol metabolism can lead to inefficient protein folding, and the consequent accumulation of non-native, aggregation-prone polypeptides. These changes induce ER stress, and provoke the unfolded protein response [32].

*Unfolded protein response (UPR)* was first characterized in yeast, but we have an increasing number of data about the mammalian pathway [33]. In this system the increased concentration of the unfolded proteins indicates the danger, and the cell, by a complex signal transduction mechanism, accelerates the expression of different ER proteins, which are necessary to survive and handle non-native proteins. The sensors of the UPR are ER

transmembrane proteins, which are able to bind unfolded proteins with their luminal domain. In case of ER stress, ER chaperones become overloaded, and free unfolded proteins emerge occupying the sensor binding sites. PERK is a stress-induced protein kinase, it can phosphorylate eiF-2α, and thus it prevents any further protein synthesis under ER stress. However, the synthesis of UPR induced proteins must be proceeded *via* a different mechanism and is not inhibited by PERK. ATF6 has a cytosolic transactivation domain, which is cleaved from the ER upon stress, goes to the nucleus and helps the transcription of genes containing ER stress response element (ERSE) [34]. Ire1 proteins have a cytoplasmic ribonuclease activity. In ER stress they undergo oligomerization and phosphorylation, and are suspected to splice the XBP-1 transcription factor mRNA resulting in a more efficient XBP-1 translation and concomitant induction of ERSE-regulated genes [35]. UPR induces molecular chaperones, members of the redox folding pathway, glycoprotein folding catalysts, enzymes of lipid metabolism, participants of the vesicle transport, protein translocation and ER-associated protein degradation [36].

The *ER overload response (EOR)* is induced, when misfolded or normal proteins (because of the accelerated expression of viral proteins) accumulate in the ER lumen, fulfill the space, and disturb the normal function of the ER [37]. In EOR, besides the induction of chaperones, a special pathway is also activated, and generates an immune response against the infected of disorganized cell. ER overload induces a $Ca^{++}$-efflux to the cytosol, followed by reactive oxigen species generation. The increased concentration of ROS will activate the NFκB transcription factor, which is responsible for the induction of several inflammatory genes, such as interferon, interleukines and other cytokines. Besides these changes the expression of the other proteins participating in antigen presentation is also increased [36].

**Table 1. Agents that activate UPR or EOR**

| | UPR | EOR |
|---|---|---|
| 2-Deoxyglucose | + | + |
| Tunicamycin | + | + |
| Brefeldin A | + | + |
| Buthylhydroperoxide | + | + |
| Dithiothreitol | + | - |
| Heavy metal ions | + | - |
| Calcium ionophores | + | - |
| Castanospermine | + | - |
| Cycloheximide | - | + |
| TNFalpha | - | + |
| Overexpression of proteins | + | + |

## 4. Changes of the ER Redox Status in Diabetes Mellitus and in Other Pathological States

Several *in vitro* studies prove that the changes of the thiol/disulfide ratio, inhibition of the electron transport disrupt the redox folding process. On the contrary, many other factors, which can inhibit the proper protein folding by different mechanisms, will alter the redox balance. Thus, the redox folding process and thiol metabolism are closely connected in the ER. Although the interrelations of the different processes were recognized, the possible folding consequences of the pathological states, which are suspected to affect the redox state of the cellular compartments have been largely unnoticed.

It was previously shown that the presence of reducing agents (DTT, β-mercaptoethanol) or strong oxidants (butylhydroperoxide, metal ions) in the cell culture medium blocks disulfide bridge formation [38].

Oxidative/reductive changes of the cellular environment were observed in many diseases as the cause or the consequence of the pathological state. The redox potential is hardly mantained upon hypoxic conditions. Acute or chronic hypoxia is general in many common diseases (ischemic injury of the tissues - myocardial infarction, stroke; tumor growth; pulmonary diseases; poisoning). Here, not only the low $O_2$ concentration, but the ATP depletion and the reperfusion-induced generation of reactive oxygen species (ROS) all disturb the normal redox balance [39]. The oxidative stress is characteristic to inflammations also, where ROS are produced by the phagocytes. Drugs can also influence thiol metabolism. NO-donor chemicals (nitrogycerol, nitroprusside sodium, etc) require free protein thiols to act on the vascular smooth muscles. Besides their direct effect we should keep in mind that many drugs are metabolised by the microsomal oxigenases, the P450 proteins, and that these redox enzymes might be in contact with other redox systems than the thiol/disulfide pathway [40].

The diabetes mellitus is described as a complex metabolic disease characterized by the absolute or relative shortage of insulin. One of the consequences of the metabolic disorganization is the increased generation of ROS [41,42]. The oxidative stress is initially prevented by the different antioxidative defense systems, but later on these mechanisms become exhausted, and oxidative damage develops. The elevated concentration of the oxidative stressors is the result of the disorganized function of the mitochondria, glucose autooxidation, and the free radicals generated by the non-enzymatically glycated proteins. These changes are typical of the extracellular space, but the signs of the oxidative stress are also detectable in the cytosol [39]. The thiol metabolism might be affected by other factors too. There are several evidences showing that the intracellular level of ascorbic acid, tocopherol and FAD is lower in both diabetic animal models and diabetic patients [43]. These changes are caused partly because of the accelerated cofactor consumption, and partly by the deficient cofactor-transport. Both the plasmamembrane transporter of ascorbic acid and dehydroascorbic acid and their microsomal uptake are blocked by high glucose concentration [5,44] The activity of some FAD-containing enzymes is significantly lower in experimental diabetes. These small molecules are all suggested to take part in the thiol metabolism of the ER, and more experiments required to decide if they are responsible for the suspected disturbances of the redox state and protein folding .

In our recent work examining streptozotocin induced diabetic rats, we found that in spite of the oxidative changes of the extracellular space, the redox environment of liver microsomal vesicles was shifted to more reducing state [45]. Diabetic microsomal redox status was characterized by an increased total disulfide content and by and increased protein-thiol:disulfide ratio. The dehydroascorbate reductase activity was also higher in diabetes [45]. The cytoplasmic parameters remained to be unchanged. A similar redox shift was also observed in some ER proteins involved in the disulfide bond formation. Importantly, a significant portion of the protein disulfide isomerase and ERp72 were found to be in reduced form (data not shown). These changes were detected by the verification of the mobility shift resulted by the covalent modifications of SH-groups on the reduced proteins [46]. In diabetic samples we found more PDI-containing aggregates, which were only partially sensitive to reducing agents (data not shown).

## 5. Discussion and Perspectives

Redox changes in diabetes alter the structure and perhaps the function of the ER redox protein folding-machinery in liver. That might be a possible explanation of the decreased hepatic protein secretion observed in some studies [47], and can also contribute to the accelerated protein turnover detected upon oxidative stress conditions [48]. The exact consequences of the more reducing environment are not clear yet. However, the regulatory role of the redox state is well known in many processes. The chaperoning activity of the PDI was found to be redox sensitive, the accumulation of the reduced form can lead the formation of more noncovalently bound complexes and a slowdown in redox-chaperoning [22]. The reducing conditions can also decrease protein stability and increase ER protein degradation. These redox changes in diabetes may influence the transport, presence and redox function of extracellular PDI on the plasma membrane [49].

We do not know, how typical are the changes of the ER metabolism we observed in diabetes mellitus. Are these changes common consequences of the oxidative stress or of any other aspecific events? More experiments and the use of different models is necessary to answer this question, and to understand the mechanisms involved in the pathology of the ER thiol metabolism, and to their evaluation as possible therapeutic targets in various diseases.

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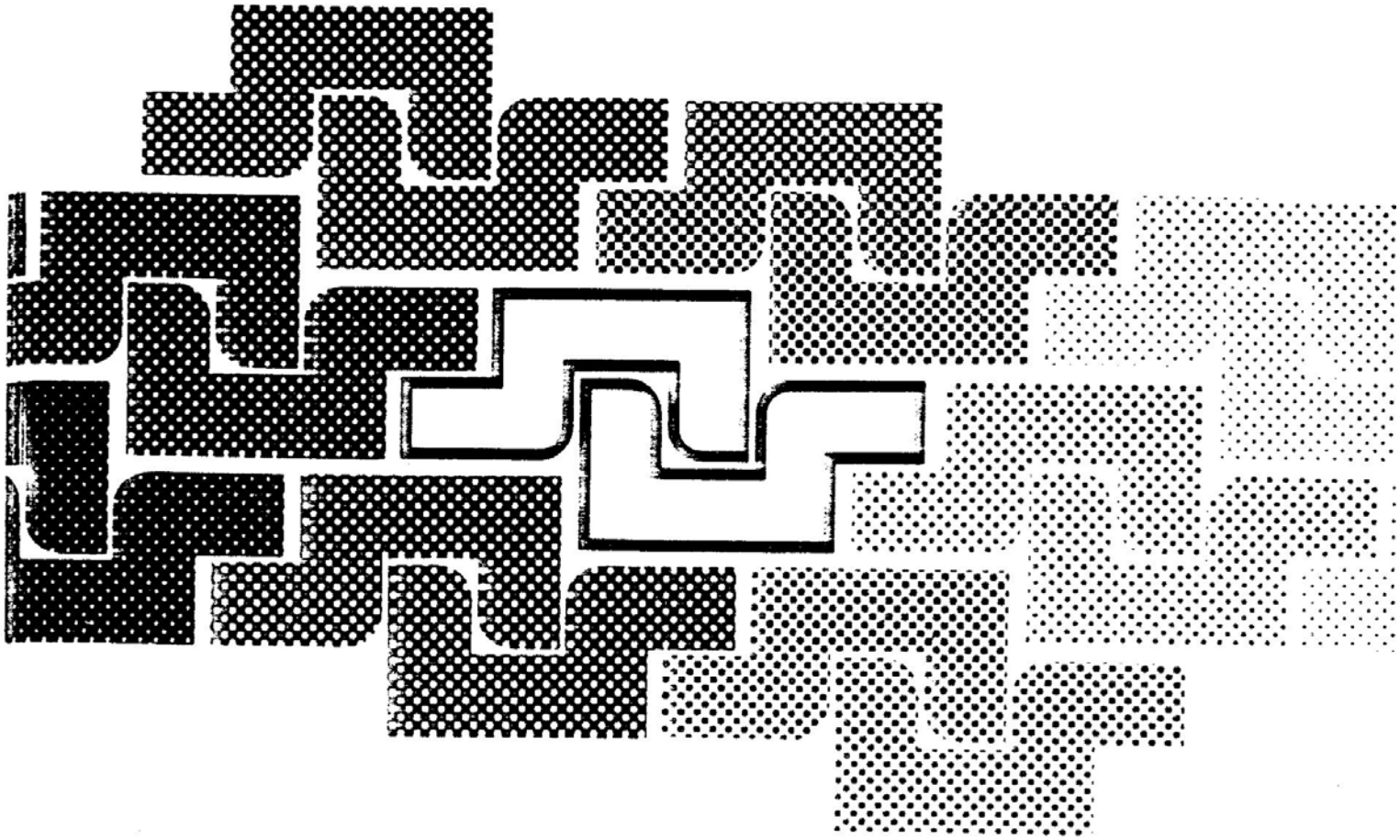

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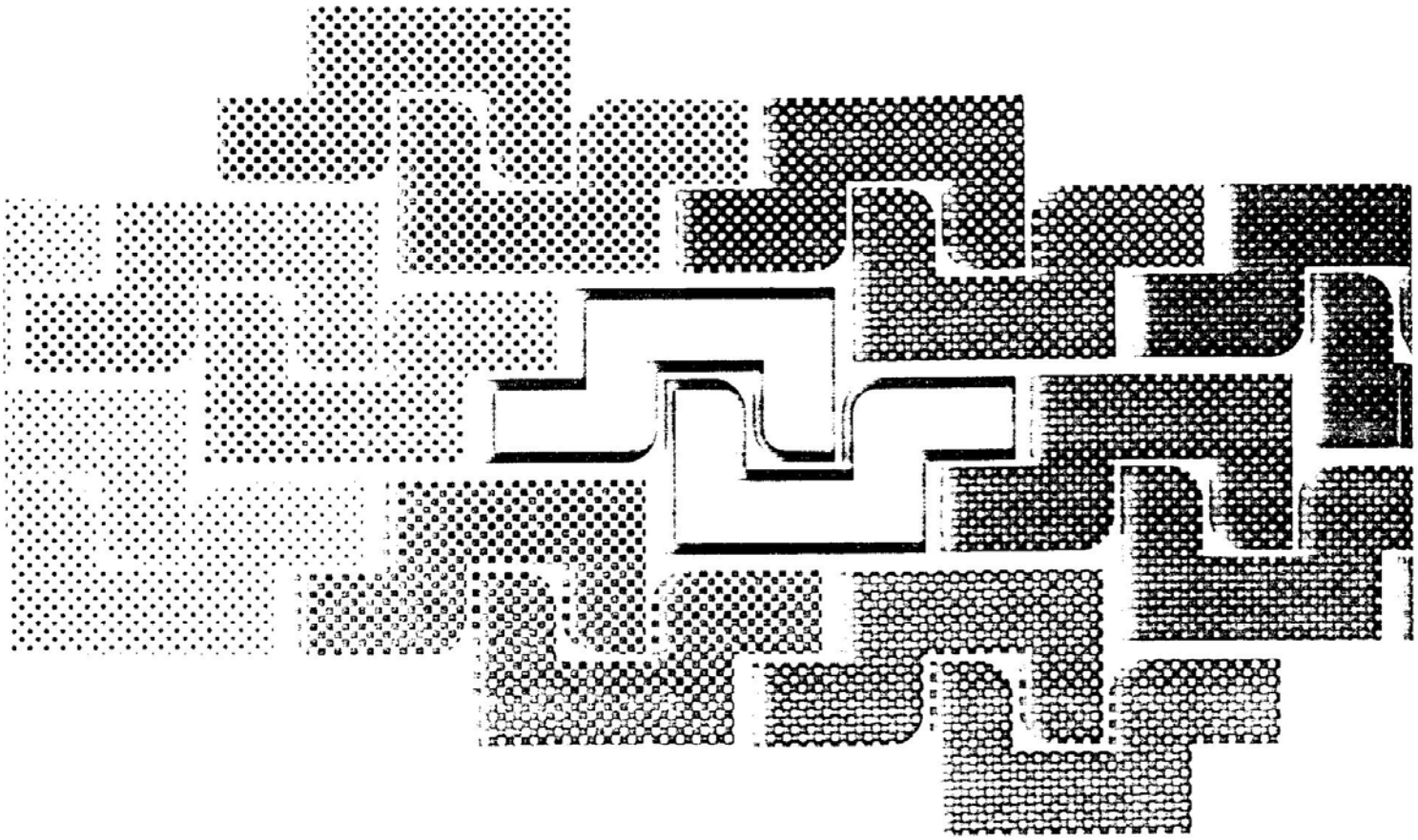

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