RECOLLECTIONS

Excursions in biophysics by a classical enzymologist

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My career in biochemistry has been greatly influenced by 14 years spent at Washington University in St. Louis, including service from 1961 to 1968 on the staff of the Biochemistry Department. I worked for 5 years with Carl Cori until he left for Harvard in 1966, principally on the role of 5'-AMP as an activator of muscle glycogen phosphorylase (cf. Helmreich & Cori, 1964). This work led to my deep involvement in another fundamental aspect of metabolic regulation besides reversible phosphorylation, namely allosteric regulation, a concept that was developed at the same time by Jacques Monod (1963): "Allostérisme c'est le deuxième secret de la vie." Phosphorylase belongs to the group of enzymes with allosteric control of catalytic activity (Monod et al., 1965). I have continued research on allosteric regulation of phosphorylase first with Maria Michaelides, Boyd Metzger, and Luis Glaser, and later with Lewis and Jeanine Kastenschmidt (cf. Michaelides et al., 1964; Metzger et al., 1967; Kastenschmidt et al., 1968a, 1968b). In the latter work with the Kastenschmidts we used equilibrium binding of substrates and the cofactor AMP, rather than reaction rates to study the allosteric transitions of phosphorylase activation.

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Ernst J.M. Helmreich, after having obtained an M.D. degree, received his graduate training in organic chemistry at the Technical University in Munich and subsequently served as Assistant Professor of Physiological Chemistry at the University of Munich in 1953–1954. From 1954 to 1956 he was a Visiting Fellow of the National Academy of Sciences, USA, in the laboratory of Professor Carl F. Cori in the Department of Biological Chemistry at Washington University in St. Louis, and from 1956 to 1961 he was Assistant Professor, with a joint appointment in the Department of Medicine, carrying out research with Professor Herman N. Eisen. From 1961 to 1968 Dr. Helmreich was a Professor of Biological Chemistry at Washington University. In 1968 he returned to his native Germany to become Professor and Chairman of the Department of Physiological Chemistry at the University of Würzburg, which he now serves as Emeritus Professor.

Among many honors, Professor Helmreich was President of the German Society of Biological Chemistry, he received the Order of Merit of the Federal Republic of Germany, and he was elected to the prestigious *Leopoldina*, the German Academy of Natural Scientists, and to the Bavarian Academy of Science. More recently, he has been a Fogarty International Scholar at the National Institutes of Health. Dr. Helmreich's major scientific contributions have been on the role of pyridoxal phosphate in glycogen phosphorylase catalysis and the molecular mechanism of signal transmission from β -adrenergic receptor to adenylate cyclase.

My interest in the conformational transitions of enzymes after the binding of allosteric effectors led me to spend a sabbatical in Manfred Eigen's laboratory in Göttingen in 1967. He had achieved dissection of reaction sequences into elementary steps by temporarily disturbing equilibria through a temperature jump and other means. When I arrived in Göttingen, Manfred Eigen and Kasper Kirschner had just finished a paper that elegantly established the value of their method for kinetic studies of allosteric transitions of an enzyme, in this case yeast triosephosphate dehydrogenase (Kirschner et al., 1966). The binding of bromothymol blue to phosphorylase *b* had been shown to be temperature dependent by Agnès Ullman, Roy Vagelos, and Jacques Monod (1964). However, the temperature-jump method was not as successful for phosphorylase, which showed several binding transitions for the dye that were difficult to separate in time.

But the sabbatical in Göttingen had long-lasting consequences for me. During this extended period of time in Germany, my friends and colleagues tried to persuade me to return to Bavaria where I was born and raised and where my mother was still living. They reminded me how much I loved hiking and skiing in the Alps. In Würzburg, the chair of physiological chemistry was vacant. Carl Martius had been chairman after the war but had left for the Eidgenössische Technische Hochschule in Zürich, and his successor, Fritz Turba, a protein chemist, died soon after he came to Würzburg. Würzburg is an old university, first founded in 1402, but closed in 1413 and then permanently established in 1575. Würzburg University is a scientifically respectable address, having at one or the other time men like Rudolf Virchow, R.A. von Koelliker, Emil Fischer, Wilhelm Conrad Röntgen, Wilhelm Wien, Julius von Sachs, Theodor Boveri, and Eduard Buchner on the faculty. Karl Landsteiner studied in Würzburg as a student of Emil Fischer. Aside from the university, Würzburg, with its surrounding hills rising above the Main river valley, is an attractive area where excellent wine is grown. However, it was difficult for me to make up my mind. I vacillated and changed my mind a few times. The day on which I finally committed myself to take the Würzburg position happened to coincide with a lecture given by Manfred Eigen in Munich, just after he had learned in October 1967 that he had received the Nobel prize. After the lecture we met with Manfred to celebrate, and in the good spirits of the moment, I sent a picture postcard from a well-known bar in Munich to my wife in St. Louis informing her that I had finally decided to accept the chairmanship in Würzburg. This was not well received by my family, as I soon discovered upon my return to St. Louis. I guess

I never could convince them that my decision to go to Würzburg was made in a sober state. In any event, my decision to return to Germany after 14 years in America had been made and I started work in Würzburg in the summer of 1968. Later I recalled my experiences as a re-immigrant under the heading of Mark Twain's *European travelogue: An innocent abroad*. Now 25 years after my return, I can say that I do not regret having returned to the country in which I was born. In a way I could not foresee from my impressions of the fifties, German science has been reborn. I respect the younger generation that now occupies chairs and responsible positions in German science for their talent, their commitment, and their achievements.

By this time I had established a close and friendly relationship with Anne and Carl Cori and I visited them occasionally in Boston. On one of these occasions just after I had returned to Germany, Carl asked me what I planned to do in Würzburg. I told him that I wanted to find out how pyridoxal phosphate functions in glycogen phosphorylase and that I also wanted to study the structure and function of the β -adrenergically stimulated adenylate cyclase. Carl was in favor of the second choice, but not of the first choice. He considered it a secondary problem because the primary role of pyridoxal phosphate as an essential cofactor of all enzymes involved in amino acid metabolism was already established by the work of Esmond E. Snell in the US, Alexander Braunstein in Moscow, and their respective schools. Instead, he tried to persuade me, but unsuccessfully, to look at the de novo synthesis of glycogen by phosphorylase, a problem that was much on his mind, and at that time, he seemed to be right. As Esmond Snell liked to say, glycogen phosphorylase was at best only an honorary member of the club of pyridoxal phosphate-dependent enzymes. Also there is more pyridoxal phosphate bound to glycogen phosphorylase in man and mammals than in all other pyridoxal phosphate-dependent enzymes together, but nobody knew for what it was there. In 1957, shortly after pyridoxal phosphate was found to be a stoichiometric constituent of muscle glycogen phosphorylase in Carl Cori's laboratory in St. Louis (cf. Baranowski et al., 1957), Edmond H. Fischer and Edwin G. Krebs in Seattle used NaBH_4 to reduce the cofactor-enzyme bond (a Schiff's base) to a secondary amine, and found to everyone's surprise that reduced phosphorylase with irreversibly attached cofactor retained activity (cf. Fischer et al., 1958). This brilliant and simple experiment showed that if pyridoxal phosphate actually should play a role in phosphorylase catalysis, it would have to do this in a manner different from that of all other known pyridoxal phosphate-dependent enzymes. Other pyridoxal phosphate-dependent enzymes are inactivated on reduction because the 4-formyl group of pyridoxal 5'-phosphate is directly involved in transaldamination reactions in the course of catalysis.

I started to work on the role of pyridoxal phosphate in glycogen phosphorylases right after I came to Würzburg. It was a long haul. The starting point was our work on allosteric regulation of phosphorylase. In the first years we excluded, based on the earlier work, the possibility that pyridoxal phosphate is just a structural ligand, merely keeping the quaternary structure together to allow for regulation and catalytic activity (Feldmann et al., 1976). In addition, Eddy Fischer's laboratory and our group synthesized and tested pyridoxal phosphate analogs for binding and reactivation of apophosphorylase (cf. Pocker & Fischer, 1969; Pfeuffer et al., 1972). But, the first clue to the solution of that puzzle was found by Knut Feldmann, a physi-

cochemist working with me. I had recognized quite early the value of NMR spectroscopy in enzymology. Utilizing ^{31}P -NMR spectroscopy with one of the first 180-MHz wide-bore instruments, Knut and Bill Hull from the Bruker company showed that in all active forms of glycogen phosphorylases the 5'-phosphate of the cofactor was dianionic or became dianionic in the course of conversion of inactive to active phosphorylase (cf. Feldmann & Hull, 1977). This breakthrough immediately suggested a plausible explanation for the essential role of pyridoxal 5'-phosphate in glycogen phosphorylases, namely that of a proton-donor-acceptor shuttle in general acid-base catalysis (cf. Feldmann et al., 1978; Helmreich & Klein, 1980). However, our proposal was questioned by Neal Madsen who, like myself, was once a postdoc in Carl Cori's laboratory (Withers et al., 1981a). Neal offered an alternative interpretation based on his studies of the binding of glucose 1,2-cyclic phosphate to phosphorylase also by ^{31}P -NMR spectroscopy. The cyclic phosphate was thought to be a transition state analog of glucose 1-phosphate. The data allowed two interpretations, either the phosphate of pyridoxal phosphate becomes reprotonated, as we had postulated, or the phosphate of pyridoxal phosphate is constrained and tightly coordinated. Neal preferred the latter possibility and proposed that the cofactor phosphate group as a tightly coordinated dianion is an electrophilic species, which could effect a partial withdrawal of electrons from the substrate P_i , thus labilizing the glycosidic bond. An electrophilic (Lewis acid) mechanism, such as that proposed by Madsen and Withers (1986), would not involve proton transfer to or from the coenzyme phosphate, but rather it would imply that the phosphate stays dianionic throughout the catalytic cycle. Such a mechanism would basically be an operationally irreversible phosphoryl transfer mechanism with formation of a pseudo-pyrophosphate bond. To the contrary, the Würzburg group always considered phosphorylases as glucosyl—rather than phosphoryl—transferases. Fortunately we were able to resolve the issue by studying the reaction of phosphorylase with glycosylic substrates (Klein et al., 1982, 1984; Palm et al., 1990). I would have probably never thought of using glycosylic substrates if Helmut Klein, who was an organic chemist before he joined my group, had not suggested them. Formation of a transferable glucosyl residue from a glycosylic substrate involves a stereoselective protonation and leads to products retaining the activating proton, therefore, these compounds are ideally suited to the study of a reaction mechanism involving substrate protonation. We were lucky that these nonglycosidic compounds were readily accepted by phosphorylase as substrates.

I have recently reviewed these results (Helmreich, 1992, containing citations of the relevant original literature). Among the reactions of phosphorylase with glucals studied by us, the reaction with D-glucio-heptenitol (Hehre et al., 1980) proved to be especially important because it turned out to proceed only in one direction, e.g., glucosyl transfer to P_i yielding heptulose 2-phosphate. The fact that heptulose 2-phosphate formed from heptenitol is a "suicide" inhibitor that binds with high affinity ($K_i = 2\text{--}14\ \mu\text{M}$) and in the absence of a primer made it a welcome tool for the X-ray crystallographers (McLaughlin et al., 1984; Johnson et al., 1990). Thanks to the formation of heptulose 2-phosphate in the crystal, interaction of the phosphate of the cofactor with the substrate P_i could be demonstrated, which was consistent with the proximity of the phosphates already made apparent by Neal Madsen's and Toshio Fukui's work

(Withers et al., 1981b). The X-ray crystallographic structural determination of the rabbit muscle phosphorylase *b*-heptulose 2-phosphate complex together with the earlier discovery of the stereospecific protonation of D-glucal (Klein et al., 1982) provided a proof of a general (Brønsted) acid-type activation mechanism for the phosphorylase reaction.

In the forward direction, phosphorolysis of α -1,4-glycosidic bonds in oligo- or polysaccharides is started by protonation of the glycosidic oxygen by the substrate orthophosphate followed by stabilization of the incipient oxocarbenium ion and subsequent covalent binding to form α -glucose 1-phosphate. In the reverse direction, protonation of the phosphate of glucose 1-phosphate destabilizes the glycosidic bond and promotes formation of a glucosyl oxocarbenium ion-phosphate anion pair. In the subsequent step the phosphate anion facilitates nucleophilic attack of a terminal glucosyl residue on the carbonium ion bringing about α -1,4-glycosidic bond formation and primer elongation. Both in the forward and in the reverse reactions, the phosphate of the cofactor pyridoxal 5'-phosphate acts as a general acid (PL-OPO₃H⁻ or PL-OPO₃²⁻) as predicted by the NMR experiments and functions as a proton shuttle to protonate the substrate phosphate. To accomplish this, the phosphates of the cofactor and the substrate must approach each other within a hydrogen bond distance (2.8–3.0 Å) corresponding to a distance of the phosphorus atoms of 4.7–5.3 Å. The phosphorus atoms in the phosphorylase-heptulose 2-phosphate complex are actually only 4.8 Å apart. In the case of the phosphorylase-glucose 1-phosphate complex, the oligosaccharide bound to the catalytic site is thought to help in turning the phosphate of glucose 1-phosphate toward the cofactor phosphate, as in the case of heptulose 2-phosphate, so that pyridoxal phosphate can function in catalysis. Moreover, as pointed out by Hajdu et al. (1987), in the case of the natural substrates glucose 1-phosphate or P_i, it is the allosterically triggered movement of Arg-569 that brings about the formation of the transition state intermediate. This ensures that the cofactor phosphate is able to act as a good base to extract a hydrogen from the oligosaccharide, whereas when the reaction proceeds in the direction of glycogen degradation, the glycosidic bond is weakened by direct protonation from the phosphate (Hajdu et al., 1987).

Dieter Palm, who had worked with me on phosphorylase since the beginning, continues with Reinhard Schinzel in Würzburg to work on the phosphorylase mechanism using site-directed mutagenesis. They have recently identified maltodextrin phosphorylase Glu-637 (in conjunction with Lys-533) as the amino acid side chains most likely involved in the control of the protonation state of the phosphate of pyridoxal phosphate in *Escherichia coli* (Schinzel & Palm, 1990; Schinzel, 1991).

It is now apparent why nature uses pyridoxal 5'-phosphate in phosphorylase catalysis, because no amino acid side chain could accomplish an equally well-balanced proton transfer. Moreover, pyridoxal 5'-phosphate has a different positional mobility than amino acid side chains constrained by protein structure. This, together with the versatile role of the phosphate group are cogent reasons why in the case of glycogen phosphorylases protonatable groups of a vitamin are more advantageous than those of amino acid side chains to facilitate catalysis.

It gave me great satisfaction that the catalytic mechanism proposed by us for glycogen phosphorylase, including the unique participation of a vitamin B₆ derivative, pyridoxal phosphate, was shown to be compatible with the X-ray structural evidence

obtained in Louise Johnson's laboratory in Oxford (Johnson et al., 1990). But quite apart from the catalytic mechanism, the beautiful work of the X-ray crystallographers has unraveled many new and interesting aspects, especially on the control of activity by allosteric activation (Johnson & Barford, 1990). It may be anticipated that in the not too distant future, phosphorylase may become one of the best studied allosteric enzymes deserving a place alongside hemoglobin, which thanks to the work of Max Perutz is the allosterically regulated protein "par excellence" (cf. Perutz, 1990).

While still working on glycogen phosphorylase, I remained a member of the "metabolic interconversion of enzymes" club. The meetings of this group were the best I ever attended. One of the meetings was held in Avila, Spain, and organized by the late Alberto Sols. I never will forget Carl Cori's response as I told him that I would have to miss the Avila meeting because of an important conference in Munich where the possible funding of the Biocenter in Würzburg, which I had planned, would be decided. He showed little sympathy with me that I should trade an interesting scientific meeting in one of the oldest and most picturesque Spanish cities for a boring meeting with bureaucrats. Moreover, he could not quite see why I wanted new laboratory space because our laboratories, which he had visited in the "Kollegienhaus," were built for Rudolf Virchow in 1848. At this occasion, he said to me: "Es ist nicht wichtig, daß der Käfig aus Gold ist, die Hauptsache ist, daß der Vogel singt." It is not important that the cage is golden, all that counts is that the bird sings.

My interest in studying the role of pyridoxal phosphate in glycogen phosphorylase catalysis identifies me as a classical enzymologist rooted in an era of biochemistry, where the clarification of the role of small molecular weight cofactors and vitamins was hailed as a fine achievement. It was therefore predictable that I would again use the techniques of classical enzymology to study β -adrenergic signal transduction. What originally attracted me to that problem was not only the challenge of finding out how hormones act, it was also the challenge of purifying membrane-bound enzymes present in minute amounts. I was scientifically well prepared for that task as one of Carl Cori's former students, but most importantly, I had the good fortune of finding a gifted and hardworking collaborator who was up to the formidable task that awaited us. It was due to the skills and tenacity of Thomas Pfeuffer and his wife Elke that we had a good start in this competitive field in the early seventies. We were among the first to make use of metabolically stable GTP analogs, such as GTP γ S, introduced by Fritz Eckstein, and Gpp(NH)p and Gpp(CH₂)p, which were 10–40 times more potent than GTP as activators of isoproterenol-stimulated adenylate cyclase from pigeon erythrocyte membranes. Because treatment of membrane preparations with these stable analogs protected the hormonally activated proteins against adverse detergent effects, we were able to isolate and enrich a protein fraction that contained about 90% of the radioactive metabolically stable GTP-derivative (Gpp(NH)p) originally bound to membranes. The solubilized GTP-binding protein fraction could then be separated from adenylate cyclase by chromatography on Sepharose, resulting in about a 40–80-fold purification from membranes. Removal of the nucleotide-binding protein from adenylate cyclase was also achieved by affinity chromatography with GTP γ S coupled to Sepharose via a spacer and resulted in a 75% loss of Gpp(NH)p activation.

These results provided the first evidence indicating that adenylate cyclase and the protein that binds GTP are separate moieties (Pfeuffer & Helmreich, 1975). Thomas Pfeuffer subsequently identified the GTP-binding moiety as a protein with an M_r of $\sim 42,000$ and showed that activation of adenylate cyclase by hormone (isoproterenol) or fluoride was transmitted by this protein (Pfeuffer, 1977). Moreover, Dan Cassel and Thomas Pfeuffer (1978) showed that the same M_r , 42-kDa protein was also labeled on treating pigeon erythrocyte membranes with cholera toxin and ^{32}P -NAD, and, on reconstitution with adenylate cyclase, conferred a greater GTP stimulation to the separated catalytic component. A greater GTP activation was expected because Cassel and Selinger (1977) had already shown that cholera toxin inhibits the catecholamine-stimulated GTPase activity making GTP in cholera toxin-treated membranes as effective an activator as nonhydrolyzable GTP analogs.

In the following years, Thomas Pfeuffer and my group advanced in different directions: Whereas Thomas went on to purify adenylate cyclase, I tried with Wolfgang Burgermeister and later with Mirko Hekman to purify the turkey erythrocyte β -adrenoceptor. Thomas succeeded first and purified bovine brain adenylate cyclase to homogeneity, cleverly making use of a forskolin affinity column (Pfeuffer et al., 1985), but I could not keep pace with Bob Lefkowitz and his group, although we eventually also managed to purify the β -adrenoceptor to homogeneity. Moreover, we synthesized useful photoaffinity labels (cf. Burgermeister et al., 1982). Because we had managed to purify by now classical methods all the components of the β -adrenoceptor/G-protein-dependent adenylate cyclase system, Thomas Pfeuffer, Alex Levitzki, and I agreed in 1984 at a meeting in St. Paul de Vence to join forces and to collaborate in an effort to start with the separate purified components and reconstitute the whole signal transmission chain in lipid vesicles. This was achieved at about the same time as Alfred Gilman and his laboratory in Dallas reported on their reconstitution experiments (May et al., 1985). In our case success was due in large part to the skillful and hard work of two young ladies, a graduate student from Alex's laboratory in Jerusalem, Debby Feder, and a postdoc from Korea, Mie-Jae Im (cf. Feder et al., 1986).

In the course of this work, Mie-Jae had observed that separate $\beta\gamma$ -subunits from trimeric G-proteins alone could bind to the β -adrenoceptor (Im et al., 1988). Moreover, when $\beta\gamma$ -subunits purified from bovine brain were added to purified β -adrenoceptor in lipid vesicles in the presence of G_s also purified from turkey erythrocytes, the $\beta\gamma$ -subunits promoted the interaction of the β -adrenoceptor with G_s and increased its GTPase activity (Hekman et al., 1987). These observations became the starting point of a new line of research that I have followed ever since. On the basis of more recent experiments, we have proposed that $\beta\gamma$ -subunits are not only anchors for G-proteins in membranes but also can anchor G-proteins to the receptor and perhaps even select by means of specific recognition sites α -subunits for interaction with receptors (Kurstjens et al., 1991).

I find the specificity of protein-protein interactions and the conformational transitions in G-protein-receptor-effector signaling systems and their functional consequences intriguing. Usually, a well-defined chemical change in a low molecular weight intrinsic or extrinsic ligand of a receptor makes the receptor capable of productive coupling to G-proteins. The prime example is the 11-*cis* $\rightarrow$ all-*trans* transition of the intrinsic ligand retinal in rhodopsin on bleaching. My personal view, if not to say

my personal bias, is that heterologous binding interactions, for example of $\beta\gamma$ -subunits of trimeric G-proteins with receptors and effectors are as specific as their binding to their respective α -subunits, and moreover, that protein-protein interactions are as important as fatty acid acylation and isoprenylation reactions for anchoring or targeting proteins to membranes. Posttranslational modifications may even drastically change the affinities of the interacting proteins for each other. In contrast to interactions with membrane lipids, interactions between proteins in the membrane can provide the specificity required to distinguish among different receptors and targets. Such a sorting and editing function of the $\beta\gamma$ -subunits might be important for the multiplicity of coupling reactions of G-proteins with receptors and targets. With that in mind, we are now engaged in collecting quantitative data on the binding of different α - and $\beta\gamma$ -subunits to each other and to receptors using fluorescence resonance energy transfer (cf. Heithier et al., 1992). Moreover, in order to understand the mechanisms underlying signal transmission processes, it is necessary to characterize the rates of and constraints on the motions of the interacting membrane proteins. The experimental access for solving this problem is difficult because the proteins involved in signal transmission, except for rhodopsin, do not have a natural intrinsic chromophore.

One approach was suggested from another period of study at Washington University. In the winter of 1980–1981, I went to the laboratory of Elliot Elson, who had just moved from Cornell to Washington University in St. Louis. Thanks to Elliot, I had an interesting and stimulating sabbatical. We were joined by Yoav Henis, a former postdoc of Elliot who is now a professor at Tel Aviv University. With his skills and his excellent and patient experimentation, we were able to show by means of fluorescence recovery after photobleaching that the majority of antagonist-bound β -adrenergic receptor is immobilized in the membranes of cultured secondary liver cells over a temperature range of 4 to 37 °C (Henis et al., 1982). A possibility suggested by these findings is that the G-protein and the target enzyme, e.g., adenylate cyclase, may be sequestered in the same local regions on the cell surface as the β -receptors. In this case, lateral diffusion of the receptor over micrometer distances as measured by fluorescence recovery after photobleaching would not be required, and lateral or rotational diffusion over submicrodistances may suffice for activation. Two shortcomings of these experiments were that the fluorescent ligand we were using produced a weak signal and possessed hydrophobic properties that might cause it to have unspecific interactions with membranes. Therefore, the work is far from being completed. We are still trying to find an answer to the questions raised above, concerning the mobility and distribution of the components of the β -adrenergic signal transmission chain in the membrane and the forces and structures that cause immobilization of β -receptors in the resting, nonactivated state. Last year, I spent summer and fall at the Max Planck Institute of Biophysical Chemistry in Göttingen with Donna and Thomas Jovin, searching for more suitable and stronger fluorescent probes for monitoring β -adrenergic receptors in intact cells.

Although I have been retired as chairman of the Department of Physiological Chemistry at Würzburg University Medical School since the fall of 1991, I am able to continue my research in the Division of Clinical Biochemistry and Pathobiochemistry of the Medical Clinic of Würzburg University. Ulrich Walter, the head of the division, and his American wife, Suzanne

Lohmann, moved to Würzburg about 10 years ago from Paul Greengard's laboratory at Yale University.

During 1993–1994 I am at the NIH as a Fogarty Scholar. Thus, I keep myself busy. And who would want to quit now, in times that may someday be judged as the most exciting period in the history of the biological sciences. Now, for the first time, successful attacks on the most challenging problems of cell biology and their reduction to the molecular level have become feasible, problems so complex that nobody would have dared to challenge them only 20 years ago.

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