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METAMORPHOSIS OF RESEARCH ON ION-COUPLED METABOLITE TRANSPORT

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A defining moment in the history of active transport research came in August 1960, in a symposium at the Czeckoslovak Academy of Sciences, which became known among membrane biologists as the Prague Symposium (Kleinzeller and Kotyk, 1961). By that date, the enzymatic nature of sodium transport in animal cells had been demonstrated (Skou, 1957) and a generalized concept of transport-related, vectorial metabolism was being formulated (Mitchell, 1961). Specifically concerning uptake of organic metabolites, a pivotal observation had been made by Riklis and Quastel (1958): that active transport of sugars by intestinal villus membrane is dependent upon sodium ions in the luminal bathing solution. This finding was extended by Crane *et al.* (1961), who suggested in Prague that sugar and sodium ions might be simultaneously transported, and was further discussed by Mitchell. Subsequently, both Crane and Mitchell elaborated hypotheses of ion-coupled sugar transport: Na^+–glucose in mammalian intestine (Crane, 1962) and H^+–galactoside in *Escherichia coli* (Mitchell, 1962), which became incorporated into a comprehensive picture of proton-linked 'chemiosmotic' processes in bioenergetics.

Despite an initial hard resistance to these hypotheses, particularly among established bioenergeticists, the two decades following the Prague Symposium saw major research efforts in active transport focus upon thermodynamic 'proofs' of coupling between the movements of ions and sundry organic metabolites, upon detailed kinetic modelling of 'cotransport' mechanisms and upon elementary chemical characterization of selected transporters.

Like ion pumps, most ion–metabolite symports and some antiports were shown to transport electric charges, therefore being termed 'rheogenic' to denote their generation of transmembrane currents (Schultz, 1980), or 'electrophoretic' to denote the normal action of membrane voltage in driving metabolite transport. [The term 'electrogenic' has also been applied, but this is a misnomer, since the normal physiological action of these ion-coupled porters is membrane *de*polarization.] The ability of ion-coupled porters to concentrate (or exclude) specific metabolites depends, therefore, upon the transmembrane *electro*chemical potential difference for the coupled ion(s), $\Delta\bar{\mu}_k$, where k is usually Na^+ or H^+, or – as recently shown in certain insect tissues – K^+ (Wieczorek *et al.* 1991). In animal cells, the total ion-motive force, $\Delta\bar{\mu}_k/z_kF=\Delta\Psi-E_k$, is normally small, often positive to -100 mV. Because 59 mV corresponds to a transmembrane

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concentration ratio of 10 for a single-charge process, observed ratios for neutral organic substrates exceeding a few hundred-fold demand ion–substrate coupling ratios of 2:1 (or greater). This, indeed, appears to be the most common ratio for animal-cell Na^+ symporters (Turner, 1985; Kimmich, 1990).

Thermodynamic constraints upon symporters and antiporters in H^+-coupled systems – particularly those in plant, fungal and bacterial plasma membranes – are quite different. There, resting $\Delta\Psi$ alone commonly ranges from -150 to -300 mV, and extracellular acidity (pH 6 to pH 2) can easily add another -100 to -300 mV to the total ion-motive force. The theoretical steady-state concentration ratios for ion-coupled porters operating in such environments would be very large, often in excess of 10^6 with coupling ratios of only 1:1. It is mysterious, then, why even in such situations ion:substrate transport ratios are also preponderantly 2:1. One possible answer is that excess ionic charge accommodates non-specificity in porter function, i.e. permits a single species of protein molecule to build large gradients for metabolites of different *intrinsic* charge. An interesting case in point is the general amino-acid (AA) permease in *Neurospora crassa*, which couples two H^+ to a single AA molecule and transports neutral and basic AAs at the same rate, but conducts two charges for each neutral AA and three charges for each basic AA (Sanders *et al.* 1983). Another possibility is the physical circumstance that *external* control of reaction rates, a conspicuous feature of all integrated metabolic systems, is most easily imposed on reactions occurring far from their *intrinsic* equilibrium, as is generally understood in biochemistry (see, for example, Newsholme and Start, 1973).

Prior to the widespread adoption of molecular biological methods (that is, in the period that might now be termed technological *pre*history), the standard biochemical approaches to study of ion symports and antiports were freeze-fracture electron microscopy, physical characterization of detergent-stabilized proteins, reconstitution of extracted proteins into liposomes followed by functional characterization, and reactivity measurements employing various specific protein reagents. Such studies determined expectations for the structural entities mediating coupled ion–metabolite transport, and they took the first steps towards structure–function analysis by identifying protein functional groups and chemical asymmetries.

Particularly revealing in this respect was work on the ATP/ADP antiporter of the mitochondrial inner membrane, which is not otherwise represented in this volume. This system regulates exchange of ADP and ATP into and out of the mitochondrial matrix in response to changes of cellular metabolic status (Klingenberg *et al.* 1969) and is the most abundant single protein of the inner membrane (Riccio *et al.* 1975). Its function is sensitive to $\Delta\Psi$, with matrix-negative voltages (thus, energized mitochondria) driving ATP towards the cytoplasm and ADP towards the mitochondrial matrix (Krämer and Klingenberg, 1980). The asymmetric structure of the antiporter could be demonstrated by its sensitivity to single-sided inhibitors: bongkrekate (approximately $10\,\mu mol\,l^{-1}$) at the matrix side, where it traps the antiporter in a high-affinity state for ADP and ATP (but *not* AMP or other nucleotides); and atractylate (also approximately $10\,\mu mol\,l^{-1}$) at the cytosolic side, where it displaces ADP and exposes fresh tyrosines to iodination (Klingenberg *et al.* 1980). It was the first exchanger protein to be isolated and purified (by

means of atractylate binding; Riccio *et al.* 1975), and the first to be functionally reconstituted into artificial membranes.

Its primary structure was determined as early as 1982 by protein-chemical methods (Aquila *et al.* 1982) and found to contain six hydrophobic helices (see review by Klingenberg *et al.* 1992). Its electrophoretic mobility in SDS indicated a molecular mass of 29 kDa, compatible with the sequence of approximately 300 amino acids. But nucleotide-binding studies on extracted, lipid-protected protein and on Triton-extracted micelles indicated a single active site per 60 kDa, thereby suggesting functional operation as a dimer (Klingenberg, 1985). The ADP/ATP antiporter belongs to a family of mitochondrial ion-exchanger proteins, including another nucleotide-dependent one, which appears to catalyze H^+/H^+ or OH^-/OH^- exchange. This protein appears to be limited to brown adipose tissue, where it short-circuits (uncouples) the mitochondrial membrane and thereby enhances thermiogenesis (Klingenberg, 1990).

Since the early 1980s, when application of molecular-biological techniques to membrane studies began in earnest, transport research generally – and that on ion-coupled metabolite porters in particular – has been totally transformed. The four papers which follow trace out this transformation in microcosm. Central aspects of the discovery and characterization of the K^+–amino acid symporter in the gut of phytophagous insect larvae are presented by Giordana and Parenti (1994), with a detailed description of the voltage- and K^+-dependence of amino acid transit, and an exploration of the transport specifications for amino acids by means of analogue studies. Functional differentiation of the transporter along the gut is made evident, in differential pH- and voltage-sensitivity, which is likely to reflect differential expression of isoforms from genes in a single family. Transport and storage of Ca^{2+}, the most polyfunctional of all cytosolic ions, is described by Cunningham and Fink (1994) for the yeast *Saccharomyces cerevisiae*, explored almost entirely by techniques of yeast genetics. Calcium is sequestered into vacuoles and Golgi membrane spaces coupled to the hydrolysis of ATP *via* two distinct P-type ATPases, and it appears to be released – at least under certain conditions – by a Ca^{2+}/H^+ antiporter, which is calcineurin-regulated. The possibility must also be considered that calcium *channels* play a role in calcium release, at least from the vacuoles (Bertl and Slayman, 1990). Physiological function, distribution, cloning and heterologous expression of renal and intestinal Na^+–anion symporters are presented by Murer and Biber (1994). Comparison is made between the transport properties of these porters assayed in brush-border membrane preparations and porters expressed in oocytes of the African clawed frog *Xenopus laevis*. The operant stoichiometry appears to be Na^+:anion^{2-}=3:1 for both symporters. The separately cloned intestinal and renal Na^+–SO_4^{2-} symporters are identical (595 amino acids, molecular mass 66 kDa) and appear to represent a new class of porter proteins. Only renal phosphate porters have been cloned, but two distinct proteins have been found, with molecular masses of approximately 51 kDa and approximately 69 kDa. Hydropathy analysis suggests that all four cloned proteins have (at least) eight transmembrane α-helices.

Major emphasis in the work on the lactose permease of *E. coli* (Kaback *et al.* 1994), over the past 5 years, has been on structure–function analysis: i.e. on the production of mutations – usually single amino-acid replacements – whose physiological (transport-

kinetic) consequences are then carefully analyzed. These experiments highlight a fundamental change in the operant scientific method: it is now far easier to determine and modify the primary structures of transport proteins than it is to define function. And yet the ability, however strained, to make minute functional comparisons between precisely altered proteins represents a kind of *differential membrane physiology*, and here – just as in mathematics – differential analysis is a way to enhance sensitivity.

There are several conspicuous limitations of method and data, however, which currently preclude a genuine understanding of the dynamic molecular processes comprising metabolite transport. Most obvious is the lack of even a single good crystal from which to establish unequivocally the tertiary structure (folding) of a porter molecule. The failure of conventional crystallization methods when applied to *bona fide* ion-transport proteins is discussed by Kaback *et al.* (1994), along with the intriguing new approach of trying to crystallize a fusion protein made from the lactose permease plus a small soluble protein which *by itself can* be readily crystallized. A second limitation is that the actual carrying out of transport by a two-substrate enzyme is inherently complicated, involving a minimum of six conformational states in theoretical discrete models (Sanders *et al.* 1984), eight or more kinetically identifiable conformations in practical descriptive models (e.g. Schultz and Curran, 1970; Aronson, 1985), and perhaps many more in certain 'simple' ion exchangers such as the erythrocyte Cl^-/HCO_3^- exchanger or in channel-like processes where continuous energetic transitions appear to be involved (Liebovitch, 1989). The chance of trapping a significant number of these enzymes in defined crystal states is negligible and, indeed, this fact may weigh heavily in the so-far intractable problem of producing useful crystals of porter proteins. A third limitation is that simple functional assays (overall transport rate, apparent substrate affinity, etc.) are inadequate measures of complex reactions, even when the resources to evaluate hundreds or thousands of site-directed mutations are available.

What is needed, then, is a way to look *inside* porter molecules and to determine which conformational states are enhanced and which diminished by replacement of a particular amino acid or cassette. Again, Kaback *et al.* (1994) and his laboratory have undertaken an exhausting but potentially powerful approach: that of stripping the lactose permease of cysteine residues, then inserting cysteines at any desired point (or at regular intervals) and covalently attaching fluorescent labels, some of which – if luck abides – will become conformation-sensitive. A related approach is to use extrinsic differential fluorescent probes, covalently or non-covalently bound, of which a battery has been developed for studying transport ATPases. Fluorescent probes, of course, can respond to a variety of factors related to conformation: changes in local electric field, relative hydrophobicity or anisotropy, for example. And the possibility exists of combining conformation-specific fluorescence measurements with electrical-kinetic measurements in order to label *many* distinct porter conformations, as has recently been initiated by Bühler *et al.* (1991) on the Na^+/K^+-ATPase.

Electrical-kinetic analysis, in itself, is a potentially powerful technique which until recently has been rather neglected for ion-coupled metabolite porters. This was partly because earlier efforts (Hansen and Slayman, 1978; Lapointe *et al.* 1986; Smith-Maxwell *et al.* 1990) yielded data which were intrinsically difficult to interpret. However, more

recent and more sophisticated studies, using patch-electrode techniques under particularly favorable conditions (isolated single secretory cells, giant patches), have begun to yield data which satisfy plausible reaction models for symporters and antiporters (Jauch *et al.* 1986; Matsuoka and Hilgemann, 1992). The technique has also been given a compelling boost by the ability – *via* gene transfection – to express ion-coupled metabolite transporters in *selected* cell membranes which are more favorable for electrophysiological study than most membranes handed out by nature. Wright and his colleagues (Parent *et al.* 1992*a,b*; Wright *et al.* 1994) have aggressively pursued this kind of analysis, using *Xenopus* oocytes as an expression system and using a variety of specified amino-acid mutants of the mammalian intestinal Na^+–glucose symporter for transfection and differential current–voltage measurements. That particular work has begun to yield *major* insights into the conformational localization of certain amino-acid substitutions in the porter molecule; the results are discussed in proper detail in the last article of this chapter (Wright *et al.* 1994).

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