MACROMOLECULAR INTERACTIONS IN THE REGULATION OF GLYCOLYSIS

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To my great discovery,
my wife Asuka

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TABLE OF CONTENTS

LIST OF TABLES

Table Page

Chapter VI

LIST OF FIGURES

NON-STANDARD ABBREVIATIONS

ADH	Alcohol dehydrogenase
ALD	Aldolase
ALT	Alanine aminotransferase
ε-AMP	$1,N^6$-ethenoadenosine 5'-monophosphate
ε-ATP	$1,N^6$-ethenoadenosine 5'-triphosphate
BSA	Bovine serum albumin
F6P	Fructose 6-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
αGDH	α-Glycerophosphate dehydrogenase
αGDH_1	α-Glycerophosphate dehydrogenase monomer
αGDH_2	α-Glycerophosphate dehydrogenase dimer
HK	Hexokinase
LDH	L-Lactate dehydrogenase
LOD	Lactate oxidase
MOPS	3-(N-morpholino)propanesulfonic acid
ε-NAD^+	$1,N^6$-ethenoadenine nicotinamide dinucleotide
PC12	Rat adrenal pheochromocytoma cells
PFK	Phosphofructokinase
PK	Pyruvate kinase
POP	Pyruvate oxidase
RBL 2H3	Subline 2H3 of rat basophilic leukemia cells
WFI	Water For Injection.

CHAPTER I

INTRODUCTION

The view of the cell as watery bag of enzymes and substrates is no longer tenable. Inside the cell, order and structure is the name of the game, and it is *not* a diffusion-dominated game. Numerous so-called soluble enzymes specifically and dynamically interact with other proteins and intracellular components. These interactions often result in an alteration of the kinetic properties of the participating enzymes and in a non-random distribution of enzymes inside cell compartments. The 'organization' of functionally related enzymes in close proximity to one another can promote the creation of distinct pools of metabolites and 'substrate channeling,' a microcompartmentation of metabolites and potentially important mechanism for metabolic regulation. The modulation of interactions involving so-called soluble enzymes can also provide an important mechanism for adjusting the intracellular distribution and kinetic properties of enzymes to suit the changing metabolic needs of the cell.

The general hypothesis for the research presented in this dissertation is that macromolecular interactions involving the so-called soluble enzymes of the glycolytic pathway have important functional consequences, and that the modulation of these interactions by changes in intracellular conditions represents an important mechanism for the regulation of glycolytic flux.

Research from two separate but related projects is presented in this dissertation. The research presented in chapter III was undertaken as part of a larger investigation into associations among dehydrogenases and the correlation of these associations with direct

NADH channeling (see Zeljko Svedruzic's Ph.D. dissertation). The aim of the research presented in that chapter was to extend recently published work (Yong *et al.*, 1993, *Biochemistry 32*, 11124-11131) showing an NADH-modulated association between α-glycerophosphate dehydrogenase (αGDH) and lactate dehydrogenase (LDH). Our plan was to (1) better characterize this unprecedented and potentially important association which provides a mechanism for direct NADH channeling, and (2) test the generality of NADH-modulated associations among dehydrogenase pairs. However, better characterization of the αGDH/NADH/LDH system conclusively established that the reported association was the result of an artifact. This result, which was not entirely unexpected, has important implications. Chapter III was written to be submitted for publication in *Archives of Biochemistry and Biophysics* after incorporation of supporting analytical ultracentrifugation data.

The research presented in chapter IV and V represents the first part of a research project whose ultimate goal is the study of glycolysis *in situ* in permeabilized cells. The [^{14}C]lactate determination method described in chapter IV was developed to greatly facilitate and improve planned measurements of substrate channeling in the glycolytic pathway *in situ* using the isotopic dilution method. The method described in chapter V was developed to address the critical need for a simple and convenient method to assess cell micropermeabilization. Chapter IV was published in *Analytical Biochemistry*. Chapter V was written to be submitted for publication in the same journal.

Chapter VI, 'The Undiscovered Country', offers a brief overview of ongoing research based upon, and extending, the research described in previous chapters. In that chapter, I present our discovery of an interaction between αGDH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and discuss the potential physiological importance of this interaction. I also present evidence that the generally accepted size-exclusion limit of α-toxin pores in the plasma membrane of nucleated cells is overestimated due to the occurrence of cell lysis. Finally, I discuss our future plans regarding the research just described and the study of glycolysis *in situ* in α-toxin-permeabilized cells.

CHAPTER II

REVIEW OF SELECTED LITERATURE

"...Nature has no bottom: its most basic principle is 'organization'. If nature puts two things together she produces something new with new qualities, which cannot be expressed in terms of qualities of the components.
...Whenever we separate two things, we lose something, something which may have been the most essential feature."

Albert Szent-Györgyi (1963)

ENZYMOLOGY,
THE NEW PARADIGM

The purification of enzymes gave birth to modern enzymology by overthrowing the old vitalist concept that enzymatic catalysis is inseparable from living cells, revealing the protein nature of enzymes, and permitting detailed structure/function studies of the protein catalysts (Friedrich, 1986; Lehninger, 1982). Today, almost 75 years after the first enzyme was crystallized (Lehninger, 1982), much of what is known in enzymology has been learned from the study of purified enzymes under very unphysiological conditions as regards ionic strength, enzyme and substrate concentrations, the presence of other enzymes, proteins, and further macromolecules, etc. (Friedrich, 1986). However, the last decade has witnessed an explosion of biochemical research focusing on the various aspects of macromolecular interactions occurring under more physiological conditions (Srere, 2000). The consensus emerging from this research is that the traditional paradigm, that simple 'solution chemistry' rules apply to the cell/tissue as a whole, is untenable (for a recent review see Luby-Phelps, 2000). It is even argued by

some (Wheatley, 1998), that it should have been clear from an early stage, that diffusion could not play more than a very restricted role in metabolic regulation.

Since current understanding of metabolism is primitive and fragmentary, it is not surprising that first descriptions are simplistic. However, in enzymology, simplistic descriptions (and simplifying assumptions) are proving to be the most difficult to revise (Ovàdi and Srere, 2000). Indeed, much as the old vitalist concept attached to enzymes lingered on (for an historical review see Florkin, 1972), so does the idea inculcated into biochemistry students that intracellular processes are essentially diffusion-dominated (Wheatley, 1998; Agutter *et al.*, 1995). Although many researchers now strictly reject the traditional paradigm, many others still strictly adhere to it, and, as elegantly pointed out by Hochachka (1999a), many also appear to suffer from a kind of schizophrenia, working within the traditional paradigm in some studies, but not in others.

Enzymology therefore appears to be undergoing a paradigm shift as envisioned by T. S. Kuhn (1970). Conceptually, the major difference between the new and traditional paradigm is the emphasis placed on intracellular order and cell architecture, i.e., on the microanatomy of the cell (Hochachka, 1999b; for reviews on the architecture of the cytoplasm see Clegg, 1984; Luby-Phelps, 2000; Pagliaro, 2000; Bhargava, 1985). In the new paradigm or framework, most metabolic systems operate within an ordered milieu and this has fundamentally important functional consequences (for reviews on, and e.g. of, supramolecular organization and its consequences see Agius and Sheratt, 1997;

Ovàdi, 1995; Stadtman and Chock, 1992; Srere *et al.*, 1990). The new framework recognizes that the cell is a membrane-bound microcosm housing complex organelles, membranous networks, cables, trabeculae, channels, pumps, and motors, and that circulation and macromolecular interactions dominate intracellular processes (Hochachka, 1999a; for reviews on intracellular circulation see Wheatley, 1985; Clegg and Wheatley, 1991; Wheatley and Malone, 1990). In short, this framework embraces the Aristotelian concept that the whole is greater than the sum of its parts. Therefore, after having walked away from the cell, enzymology, equipped with powerful new tools, is beginning to walk back to the cell.

THE CYTOPLASM, A PLACE WHERE MACROMOLECULES MEET

The concentration of proteins in the cytoplasm and the mitochondria may be as high as 20 to 30% w/w (Luby-Phelps, 1994) and 56% w/w (Srere, 1981), respectively, compared to 10 to 80% w/w (with an average of 40%) in protein crystals (Fulton, 1982). Although it is not known how much of this protein is in solution (Luby-Phelps, 1994, 2000), clearly the cell is a very crowded place (Fulton, 1982; Luby-Phelps, 2000; also see Zimmerman and Trach, 1991). In such a crowded environment, excluded volume effects (i.e., steric repulsion between macromolecules) can drive changes in the tertiary and quaternary structure of enzymes that lead to altered kinetic behavior, specific activity,

and substrate specificity (for review see Minton, 1997). Excluded volume effects can also promote macromolecular interactions (for reviews see Zimmerman and Minton, 1993; Minton, 1997; also see Minton, 1992, 1995, 1999, 2000 and references therein; also see Berg 1990) including the binding of so-called soluble enzymes to complementary surface sites (e.g. Walsh *et al.*, 1989; Walsh and Knull, 1988; Merz *et al.*, 1987; Minton and Wilf, 1981; Reinhart, 1980).

Macromolecular 'crowding' coupled with high protein concentrations and widespread weak interactions (see Pagliaro, 1993; 2000) may help explain the controversial 'microtrabecular lattice' first observed ca. 25 years ago (for reviews see Porter, 1986, 1990). The microtrabecular lattice is an elaborate network of protein strands (surrounded by water-rich space) that connect virtually all cytoplasmic structures (for reviews see Porter, 1986, 1990; Clegg, 1984, 1992; also see Penman, 1995). It has been suggested that this lattice composed of actin fibers, microtubules, and other structural elements, houses a majority of the so-called soluble cytoplasmic proteins, including all the enzymes of the glycolytic pathway (for reviews see Clegg, 1984, 1992). This view is supported by several independent lines of evidence including the observation that several glycolytic enzymes can play a structural role by crosslinking F-actin and/or microtubules (for reviews see Ovàdi, 1995; Knull and Minton, 1996, also see section 5 and references therein).

In recent years, increased research efforts along with new and improved techniques (e.g., Ashmarina *et al.*, 1994; Sherry and Malloy, 1996) have led to the discovery that numerous enzymes heretofore assumed to be in solution in intact cells (because they were recovered in the high-speed supernatant fraction of conventional cell/tissue homogenates; see Clegg, 1983, 1984; Masters, 1984) have a much more complex organization and are partially associated with other enzymes, structural proteins, and cell components (for reviews see Ovàdi and Srere, 2000; Srere, 2000; Pagliaro, 2000; Luby-Phelps, 2000; Srere, 1998; Ovàdi and Orosz, 1997; Ovàdi and Srere, 1996; Knull and Minton, 1996; Ovàdi, 1995; Clegg, 1984, 1992; Masters, 1992; Uyeda, 1992; Knull and Walsh, 1992; Srere, 1987; Kurganov *et al.*, 1985). As a result, there is an emerging consensus that specific associations involving so-called soluble proteins are widespread. Since proteins from highly diverse organisms have highly conserved structural features, it has been suggested that there is evolutionary pressure to conserve the 'quinary structure' of proteins, i.e., the transient complexes formed by the association of proteins with other macromolecules (McConkey, 1982; see also Srivastava and Bernhard, 1986a). It has even been argued (Srere, 1984) that quinary structure may be in part responsible for the relatively large size of enzymes (estimated average M_r ca. 140,000; Sols and Marco, 1970).

Specific associations involving so-called soluble enzymes and insoluble components of the cell such as membranes and cytoskeletal proteins (e.g., actin and

tubulin) permit a non-homogeneous distribution of enzymes inside cell compartments, i.e, a 'microcompartmentation' of enzymes (e.g., Wang *et al.*, 1997; Minaschek, *et al.*, 1991, 1992; Pagliaro and Taylor, 1988, 1992; for reviews see Knull, 1990; Knull and Walsh, 1992). A startling example of the functional importance of the microcompartmentation of glycolytic enzymes was recently provided by genetic studies of the metabolism of *Drosophila* flight muscle. Whereas earlier studies had shown that aldolase (ALD), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and α-glycerophosphate dehydrogenase (αGDH) colocalize mainly at Z-discs and M-lines, Wojtas *et. al.* (1997) using clever genetic manipulations (that influenced binding, but not overall catalytic efficiency) showed that the mislocation of these *active* enzymes in the non-myofibrillar cytoplasmic space renders *Drosophila* flightless. The results of Wojtas *et. al.* (1997) demonstrate the importance of the microcompartmentation of so-called soluble enzymes, and represent compelling evidence that diffusion is not the dominant means for regulating enzyme-substrate encounters.

MICROCOMPARTMENTATION OF METABOLITES AND SUBSTRATE CHANNELING

As mentioned earlier, it is still commonly assumed that metabolism proceeds from an abundant aqueous pool of metabolites that freely diffuse to and from the active sites of enzymes. However, the concentrations of many metabolic intermediates in the cell are

low when compared to the concentration of complementary high affinity binding sites on enzymes (Srere, 1967; for reviews see Sols and Marco, 1970; Ottaway and Mowbray, 1977; Srivastava and Bernhard, 1986a,b; 1987a; Bernhard and Srivastava, 1990; Ushiroyama *et al.*, 1992). Therefore, in many metabolic pathways (e.g., glycolysis), nearly all intermediates are expected to be bound to enzyme sites, i.e., microcompartmentalized, as opposed to being uniformly distributed in the aqueous environment (op. cit.).

An important consequence of this microcompartmentation is the creation of distinct pools of intermediates inside cell compartments. For example, at least two separate mutually non-accessible pools of glucose-6-phosphate exist in the cytoplasm of hepatocytes, one linked to glucogenolysis, the other to gluconeogenesis (Christ and Jungermann, 1987; Kalant *et al.*, 1987, 1988). Two distinct pools of lactate and pyruvate also appear to exist in glioma cells, one connected to oxidative metabolism, the other to aerobic glycolysis (Bouzier *et al.*, 1998). Glycolytic and glycogenolytic intermediates are also separated into two incompletely mixing pools in vascular smooth muscle (Lynch and Paul, 1983, 1987; Hardin and Kushmerick, 1994), as are the glycolytic and gluconeogenic intermediates in vascular smooth muscle (Hardin and Roberts, 1995ab) and liver (for review see Berry *et al.*, 1992). Furthermore, in vascular smooth muscle, the ATP produced by oxidative metabolism is selectively utilized by the ATPases associated with contraction (Paul, 1983; Hardin and Paul, 1995), whereas the ATP produced by

glycolysis is preferentially utilized by the ATPases of ion pumps (Campbell and Paul, 1992; Hardin *et al.*, 1992; Paul *et al.* 1989 and references therein). The preferential utilization by ion pumps of glycolytically generated ATP is explained by the localization of a glycolytic enzyme cascade on the plasmalemma (Paul *et al.* 1989).

Another important but related consequence of the microcompartmentation of metabolites is that it promotes the use of enzyme-bound intermediates as substrates for other enzymes. When an enzyme-bound intermediate is used as a substrate, the intermediate is transferred directly from one enzyme to the next without completely equilibrating with the surrounding medium, in contrast with the more conventional diffusional coupling of enzymes (for reviews see Agius and Sherratt, 1997; Ovàdi, 1995; Ovàdi, 1991; Spivey and Merz, 1989; Srivastava and Bernhard, 1986a,b; 1987a). The metabolic process by which pathway intermediates are transferred (either directly or by proximity effects; see Goldman and Katchalski, 1971) is termed 'substrate channeling' or 'metabolite channeling.' If a reaction proceeds with both channeling and diffusional components, this is often termed 'leaky' channeling in contrast to 'tight' channeling. Tight channeling is thought to occur in metabolic pathways where the intermediates have but one fate (e.g., protein synthesis). Leaky channeling is consistent with pathways where the intermediates are shared among two or more pathways, as in glycolysis.

The microcompartmentation of so-called soluble enzymes, via interactions with the cytoskeleton and other insoluble cell components, can also promote substrate

channeling by 'organizing' sequential enzymes of a pathway in close proximity to one another. For example, there is compelling evidence that all of the so-called soluble enzymes of the Krebs TCA cycle form a 'metabolon' on the inner surface of the mitochondrial matrix (for reviews see Ovàdi and Srere, 2000; Srere *et al.*, 1997). A metabolon is defined as a metabolic supramolecular assembly of sequential enzymes formed through quinary interactions and capable of substrate channeling (Srere, 1985). Do the microcompartmentalized enzymes of the glycolytic pathway also form a metabolon? Perhaps on the plasmalemma of vascular smooth muscle cells, or on the plasma membrane of erythrocytes? Or, perhaps in the sarcomeres of skeletal muscles, or in the microtrabecular lattice of brain cells?

The potential advantages of substrate channeling are far more extensive than is generally recognized (Spivey and Merz, 1989), faster rates being the only factor normally considered. These potential advantages include: 1) isolating intermediates from competing reactions; 2) circumventing unfavorable equilibria and kinetics imposed by bulk-phase metabolite concentrations (Ushiroyama *et al.*, 1992; Ovàdi *et al.*, 1994); 3) protecting unstable intermediates (Rudolph and Stubbe, 1995); 4) conserving the scarce solvation capacity of the cell (Atkinson, 1969); 5) enhancing catalysis by avoiding unfavorable energetics of desolvating substrates (Dewar and Storch, 1985); 6) reducing lag-transients (time to reach steady-state response to a change in substrate upstream in a coupled reaction path; Easterby, 1981; Westerhoff and Welch, 1992); 7) providing new

means of metabolic regulation by modulation of enzyme associations (e.g., Beeckmans *et al.*, 1990), and increased sensitivities to regulatory signals (Kholodenko *et al.*, 1993); and 8) reducing quantities of intermediates needed to maintain locally high concentrations (to ensure high degrees of saturation of adjacent enzymes; Spivey and Merz, 1989) — this last advantage has been the subject of some controversy (see Cornish-Bowden, 1997; Easterby, 1997; Kholodenko *et al.*, 1997). Substrate channeling is therefore a potentially very important metabolic process. It has even been proposed that metabolite channeling may have played an important role in the origin of life (Edwards, 1996).

Substrate channeling appears to be widespread. It has been demonstrated in numerous systems, in all parts of the cell, and in microbes, plants, and animals (for recent reviews see Ovàdi and Srere, 2000; Agius and Sherratt, 1997; Ovàdi, 1995). Compelling data now exist on systems *in vitro, in situ,* and *in vivo* (op. cit.). Systems *in situ* and *in vivo* are important to demonstrate the existence, behavior, and physiological relevance of substrate channeling. Systems *in vitro* are needed to clarify molecular mechanisms.

Evidence of substrate channeling has been presented for several pairs of glycolytic enzymes *in vitro.* However, much of this evidence is questionable and some has been challenged (for review see Ovàdi and Orosz, 1997). Systems involving the direct transfer of NADH between dehydrogenases of opposite chiral specificity have been

the most controversial.[1] In particular, the evidence presented for the direct transfer of NADH between αGDH and LDH (Srivastava and Bernhard, 1987b; Srivastava *et al.*, 1989) has been repeatedly challenged (Chock and Gutfreund, 1988; Wu *et al.*, 1990; Wu *et al.*, 1991; Gutfreund and Chock, 1991; Brooks and Storey, 1991; Wu *et al.*, 1992). These challenges have raised serious questions regarding the validity of the steady-state and fast-kinetic data presented in support of direct NADH channeling and the interpretation of the steady-state data. However, the arguments presented against the validity of the steady-state data and soundness of its interpretation are demonstrably flawed (Svedruzic and Spivey, *in preparation*). Furthermore, the seemingly persuasive evidence against channeling provided by fast-kinetic and association experiments (Chock and Gutfreund, 1988; Wu *et al.*, 1990; Wu *et al.*, 1991; Brooks and Storey, 1991) is based on the *assumption* that the lack of direct NADH transfer and detectable αGDH-LDH complex in the absence of the catalytic reaction necessarily indicates a corresponding lack of NADH transfer and complex in the presence of the catalytic reaction. As we will show, this is an invalid assumption (chapter III of this dissertation; Svedruzic and Spivey, *in preparation*). Notwithstanding, evidence of an equilibrium complex between αGDH and LDH at a moderate sub-saturating concentration of NADH was recently presented (Yong *et al.*, 1993). However... see next chapter.

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[1] See the *J. Theor. Biol.* (1991) Special issue, Physiological Significance of Metabolite Channelling. A review by Judit Ovàdi, with critical commentaries by many authors. Vol. 152, no. 1.

Evidence of substrate channeling *in situ* has been presented for the phosphoglucoisomerase-phosphofructokinase (PFK) enzyme pair in cross-linked and permeabilized erythrocytes (Zähner and Malaisse, 1993), and for 5 pairs of glycolytic enzymes including the hexokinase (HK)-PFK and pyruvate kinase (PK)-LDH pairs in Dextran sulfate-permeabilized cells (Clegg and Jackson, 1989, 1990). Unfortunately, because of a lack of proper controls the evidence, which is based on isotopic dilution experiments (for a methodological review see Spivey and Ovàdi, 1999), is unconvincing. However, weak evidence in support of substrate channeling in the glycolytic pathway was provided by the observation that increased disruption of cell integrity decreased the initial rates of anaerobic glycolysis (Jackson *et al.*, 1990). This suggests that disruption results in a greater buildup of glycolytic intermediates which, in turn, suggests a disruption of substrate channeling. More recently, direct evidence of substrate channeling was also provided by the observation that Dextran sulfate-permeabilized smooth muscle cells produce lactate from glucose without releasing 'detectable' amounts of glycolytic intermediates in the surrounding medium (Hardin and Finder, 1998). Unfortunately, this evidence is unconvincing because of the lack of a negative control (with disrupted cells) confirming the unstated assumption that intermediates could be detected in the *absence* of channeling. More definitive studies are therefore needed to confirm the suspected presence of substrate channeling in the glycolytic pathway *in situ*.

SPECIFIC MACROMOLECULAR INTERACTIONS INVOLVING SO-CALLED SOLUBLE ENZYMES, AND THEIR KINETIC CONSEQUENCES

Specific macromolecular interactions involving so-called soluble enzymes are widespread. For example, all glycolytic enzymes have been shown to associate specifically with other enzymes, proteins, and various insoluble cell components (Knull, 1990; Knull and Minton, 1996; Clarke *et al.*, 1985; Masters, 1992; Ovàdi, 1995; Ovàdi and Srere, 2000; Uyeda, 1992; Lushchak, 1998a). Evidence of the specificity of the association of glycolytic enzymes with cytoskeletal proteins (and microtrabecular lattice?) was recently provided by the mapping of a glycolytic enzyme binding domain on tubulin (Volker and Knull, 1997), the identification of binding domains on actin for ALD, GAPDH, and PFK (Humphreys, *et al.*, 1986a,b; Mejean *et al.*, 1989), and the mapping of actin binding sites on ALD and αGDH (O'Reilly and Clarke, 1993; Wang *et al.*, 1996; Wojtas *et al.*, 1997). Some glycolytic enzymes that appear to lack an actin binding site (e.g., triosephosphate isomerase) have also been shown to 'piggy-back' bind to actin-binding enzymes (e.g., GAPDH and ALD; Bronstein and Knull, 1981; Clarke *et al.*, 1985).

Specific macromolecular interactions involving so-called soluble enzymes can alter the kinetic properties of the participating enzymes. For example, the association of ALD, GAPDH, hexokinase (HK), LDH, PK, and PFK with cytoskeletal proteins and/or other insoluble cell components was shown to generally activate HK, PK, and PFK, and

either inhibit or activate the other enzymes depending on a summation of factors (Knull, 1990; Knull and Minton, 1996; Clarke *et al.*, 1985; Masters, 1992; Ovàdi, 1995; Ovàdi and Srere, 2000; Uyeda, 1992; Lushchak, 1998a).

Studies of PFK *in vitro* at physiological enzyme concentrations (Boscà *et al.*, 1985; Bär *et al.*, 1990), and *in situ* in cross-linked permeabilized erythrocytes (Aràgon *et al.*, 1980), also indicate activation of the enzyme resulting from homo- and hetero-macromolecular interactions. PFK and fructose 1,6-bisphosphatase were also shown to associate at physiological enzyme concentrations (but not under diluted conditions) with mutual effects on their kinetics, PFK was activated whereas fructose 1,6-bisphosphatase was inhibited (Ovàdi *et al.*, 1986). In addition, fructose 2,6-bisphosphate, the most potent activator of both muscle and liver PFK under dilute conditions *in vitro,* was shown to activate the liver isozyme, but not the muscle isozyme, at physiological enzyme concentrations (Boscà *et al.*, 1985; Bär *et al.*, 1990). Particulate PFK was also shown to be activated by serotonin, in contrast to soluble PFK (Assouline-Cohen *et al.*, 1997). Finally, the calcium-dependent association of PFK with calmodulin and a number of other proteins was also shown to induce reversible inactivation (and phosphorylation) of the enzyme (Mayr, 1984ab; Zhao *et al.*, 1991).

THE MODULATION OF AMBIQUITY AS A MECHANISM OF METABOLIC REGULATION

Ions, small molecules, and macromolecules have been shown to modulate 'ambiquity', i.e., the distribution of so-called soluble enzymes between soluble and bound states (see Wilson, 1978, 1980). The modulation of ambiquity has been extensively characterized for PFK, and less so for other glycolytic enzymes such as ALD, GAPDH, and HK (for reviews see Ovàdi, 1995; Knull and Minton, 1996; Masters, 1992; Uyeda, 1992; Knull, 1990; Clarke *et al.*, 1985).

The association of PFK with insoluble cell components was shown to be increased by low pH (Brooks and Storey, 1997), normal and pathological (high) concentrations of Ca^{2+} (Chen-Zion *et al.*, 1992; Ashkenazy-Shahar and Beitner, 1997; Assouline-Cohen and Beitner, 1999), insulin (Chen-Zion *et al.*, 1992; Ashkenazy-Shahar *et al.*, 1998), platelet-derived growth factor (Livnat *et al.*, 1994), and epidermal growth factor (Livnat *et al.*, 1993a).

In contrast, the association of PFK with insoluble cell components was shown to be decreased by ATP, ADP, GTP, and high pH (but not by fructose 1,6-bisphosphate and fructose 2,6-bisphosphate; Roberts *et al.*, 1988; Roberts and Somero, 1989), phospholipase A_2 (Lilling and Beitner, 1990; Ashkenazy-Shakar and Beitner, 1997) pathologically high concentrations of Ca^{2+} (Ashkenazy-Shahar and Beitner, 1997; Assouline-Cohen and Beitner, 1999), serotonin (Ashkenazy-Shahar and Beitner, 1997b),

calmodulin antagonists (promising drugs in the treatment of cancer, skin injury and muscle damage; Beitner, 1998; Glass-Marmor and Beitner, 1997), cytochalasin B and D (F-actin depolymerizing agents; Brooks and Storey, 1997), and Taxol (paclitaxel; a microtubule polymerizing agent; Glass-Marmor, L. and Beitner, R., 1999). The effect of Taxol on the ambiquity of PFK is probably due to competition for binding sites on tubulin rather than increased polymerization of microtubules (Lloyd and Hardin, 1999).

Calmodulin antagonists were also shown to prevent changes in the ambiquity of PFK by Ca^{2+} (Chen-Zion *et al.*, 1993), serotonin (Ashkenazy-Shahar and Beitner, 1997b), insulin (Ashkenazy *et al.*, 1998), platelet-derived growth factor (Livnat *et al.*, 1994), and epidermal growth factor (Livnat *et al.*, 1993a), strongly suggesting that (Ca^{2+})calmodulin is involved in their action. The effect of insulin on the ambiquity of PFK was also shown to be abolished by uncoupling mitochondrial oxidative phosphorylation (Livnat *et al.*, 1993). There is also evidence that the phosphorylation of PFK affects its ambiquity (Luther and Lee, 1986; Kuo *et al.*, 1986). In addition, there is extensive data indicating changes in the ambiquity of most glycolytic enzymes in response to changes in physiological status [e.g. exercise (Clarke *et al.*, 1985; Guderley *et al.*, 1989), hypoxia and anoxia (Duncan and Storey, 1992; Lushchak *et al.*, 1998ab), and starvation (Lowery *et al.*, 1987)], and during ontogeny (Humphries *et al.*, 1986ab). Diabetes was also shown to affect the ambiquity of PFK in muscle (Chen-Zion *et al.*, 1994).

The modulation of the ambiquity of ALD by 2-deoxyglucose and cytochalasin D was also demonstrated in living cells using fluorescence imaging techniques (Pagliaro and Taylor, 1992). In addition, controlling the availability of the glycolytic binding site in the protein band 3 was shown to modulate glycolytic flux in erythrocytes by over 30 fold (Low *et al.*, 1993). Cell cycle-related changes in F-actin distribution were also correlated with glycolytic activity in XTH-2 cells (Bereitner-Hahn *et al.*, 1995) — a loose fibrillar pattern (providing an increased surface for the binding of glycolytic enzymes) was associated with increased glycolytic activity.

Given the functional importance of the microcompartmentation of enzymes and its effect on the enzymes kinetic properties, these and other observations strongly suggest that the modulation of the ambiquity of glycolytic enzymes may represent an important mechanism for regulating the glycolytic flux to suit the changing metabolic needs of cells (see Wilson, 1978, 1980).

PERMEABILIZED CELLS, A USEFUL TOOL FOR THE STUDY OF METABOLISM *IN SITU*

Permeabilized cells have been extensively used to study intracellular processes *in situ*, but not intermediary metabolism (for reviews see Felix, 1982; van Noorden and Junges, 1995). With the exception of the studies mentioned in the preceding sections, only a few studies on the regulation of glycolysis in permeabilized cells have been

published. Furthermore, the main subject of some of these studies was not glycolysis, but rather the characterization of cells permeabilized with various agents (McCoy *et al.*, 1976; Gankema *et al.*, 1981; Mick *et al.*, 1988; see also Scholnick *et al.*, 1973).

The regulation of glycolysis *in situ* was the focus of two studies using saponin-permeabilized rat adipocytes. The first study investigated the stimulation of the glycolytic pathway and Krebs TCA cycle by free Ca^{2+} (Mick *et al.*, 1991). The second study examined the effect of fructose 2,6-bisphosphate, glucose 1,6-diphosphate, and ribulose 1,5-diphosphate on glycolytic flux (McCormick *et al.*, 1992). This second study represents the *only* demonstration that potent activators of glycolytic enzymes *in vitro* can actually modulate glycolytic flux *in situ*.

Given the functional importance of the structural organization of metabolism, the paucity of studies of intermediary metabolism *in situ* in permeabilized cells is surprising. One possible deterrent to such studies may have been the difficulty in preparing permeabilized cells without incurring the loss of so-called soluble enzymes. However, powerful new tools are now available for the controlled permeabilization of the plasma membrane to ions and small molecules, without undesirable permeabilization to macromolecules.

α-TOXIN FROM *STAPHYLOCOCCUS AUREUS*, A MOLECULAR TOOL FOR THE PERMEABILIZATION OF CELLS

α-Toxin from *Staphylococcus aureus,* a pore-forming protein endowed with hemolytic, cytotoxic, dermonecrotic, and lethal properties, is a foe turned ally (Bhakdi and Tranum-Jensen, 1991). Since its introduction as a molecular tool for the study of intracellular processes (Cassidy *et al.*, 1978; Ahnert-Hilger *et al.*, 1985; McEwen and Arion, 1985), α-toxin has been used widely to permeabilize cells and tissues to ions and small molecules (for reviews see Bhakdi *et al.*, 1993; Thelestam and Blomqvist, 1988). α-Toxin, also known as α-hemolysin, is an extracellular protein of M_r 33,400 (293 amino acids; Gray and Kehoe, 1984) that is secreted by most pathogenic strains of *S. aureus* (Bernheimer, 1965).

S. aureus α-toxin is secreted as an active water-soluble molecule that binds as a monomer to susceptible cells via two distinct interactions (Hilderbrand *et al.*, 1991). At low concentrations (< 50 nM for rabbit erythrocytes), the toxin binds exclusively to a high affinity binding site that has been tentatively identified as band 3 in erythrocytes (Kantor and Fackrell, 1998). At higher concentrations (> 200 nM for rabbit erythrocytes), the toxin will absorb nonspecifically to lipid bilayers. This accounts for the permeabilization of (nonsusceptible) cells lacking the high-affinity binding site (e.g., human erythrocytes). The threshold for nonspecific absorption varies with cell type and

appears to be determined by the mosaic architecture of the membrane surface (Bhakdi and Tranum-Jensen, 1988). However, membrane damage of both susceptible and nonsusceptible target cells occurs via a common mechanism involving toxin oligomerization and pore formation (Hilderbrand *et al.*, 1991).

After binding to target membranes, α-toxin monomers undergo lateral diffusion and collisions at the membrane surface leading to oligomerization into a stable pre-pore heptameric ring-structure. A pre-pore to pore transition is required for formation of the water-filled transmembrane channel (for review see Bhakdi *et al.*, 1996). This transition is partially cooperative and proceeds through multiple intermediate stages (Valeva *et al.*, 1997). Ultimately, the transition involves insertion of the glycine-rich central region of each monomer into the membrane (Krasilnikov *et al.*, 1997; Valeva *et al.*, 1995, 1996; Ward *et al.*, 1994) to form a mushroom-shaped heptameric pore-complex whose 3D structure has been resolved by X-ray crystallography (Song *et al.*, 1996). The heptameric pore-complex (Gouaux, *et al.*, 1994) does not appear to possess a rigid structure within the membrane, but adopts rather a flexible conformation (Vécsey-Semjén, *et al.*, 1999).

Until recently, the functional diameter of α-toxin pores was believed to be similar in erythrocytes and nucleated cells (ca. 1.3 nm ; Bhakdi *et al.*, 1993; Sabirov *et al.*, 1993). However, it has become clear that the pore size of α-toxin can exhibit considerable heterogeneity depending on cell type (for review see Bhakdi *et al.*, 1993). Pore-complexes can be completely devoid of permeabilizing properties (e.g., in human

granulocytes treated with up to 20 μg of α-toxin per ml). The channel of the pore-complexes can be small enough to allow efflux of K^+, Na^+, and $^{86}Rb^+$, but not influx of Ca^{2+} or larger molecules such as propidium iodide or efflux of carboxy fluorescein (e.g., in lymphocytes and keratinocytes treated with up to 1 μg of α-toxin per ml; Jonas *et al.*, 1994; Walev *et al.*, 1993; Bhakdi *et al.*, 1993). And, α-toxin pores can be large enough to allow the passage of Ca^{2+} and small molecules such as nucleotides (e.g., in erythrocytes at low, moderate, and high concentrations of α-toxin).

The observation that cells become permeable to larger molecules when exposed to high concentrations of α-toxin, has lead to the suggestion that small pores may be created when the toxin binds to high affinity sites on the plasma membrane, whereas α-toxin molecules absorbed non-specifically to cells might be responsible for creating larger pores (Jonas *et al.*, 1994). Interestingly, biochemical and physicochemical analyses suggest that pore-complexes containing less than seven monomers can form in planar lipid bilayers (devoid of high affinity binding sites) at low temperatures and low α-toxin concentrations (Belmonte *et al.*, 1987; Tobkes *et al.*, 1985). However, an alternative explanation for the apparent increase in the size of α-toxin pores with increasing dosage is that exposure to high concentrations of α-toxin creates membrane lesions *between* pore-complexes as a result of overpermeabilization. This idea is discussed further in Chapter VI.

Divalent cations can suppress α-toxin pore-complex formation (with decreasing efficiency: $Zn^{2+} > Ca^{2+} > Mg^{2+}$), but relatively high concentrations are required (Pasternak *et al.*, 1985). Ca^{2+} but not Zn^{2+}, appears to also induce the closure of pre-formed channels (Zn^{2+} and Mg^{2+} can also induce channel closure in the presence of an applied transmembrane voltage; Bashford *et al.*, 1996; Sunzel *et al.*, 1995; Ward and Leonard, 1992). The effect of pH and temperature on the permeabilization of plasma membranes by α-toxin appears to depend on the presence or absence of high affinity binding sites (Bhakdi *et al.*, 1984; Hilderbrand *et al.*, 1991).

Finally, α-toxin possesses a number of properties that make it an excellent tool for the controlled permeabilization of cell membranes. It is very stable (so storage is not a problem), soluble in aqueous buffers, and has no ionic requirements (so chelating agents can be used). It permeabilizes cells over a wide range of temperatures (4 to 37°C). It selectively permeabilizes the plasma membrane, leaving the intracellular organelles intact (α-toxin pores are too small to allow entry of the toxin itself into the cell). It forms stable pore-complexes that permit the transmembrane flux of ions and small molecules, but not of macromolecules (Bhakdi and Tranum-Jensen, 1988; 1991). It attacks virtually all mammalian cells, albeit with greatly varying efficiency depending on the presence or absence of high-affinity binding sites (Hilderbrand *et al.*, 1991). And, at a given dosage, it permeabilizes membranes in a self-limiting manner that eliminates the need for delicate

control of permeabilization, in contrast to permeabilization with detergents and other agents.

MACROMOLECULAR INTERACTIONS IN THE REGULATION OF THE GLYCOLYTIC PATHWAY

The glycolytic pathway, one of the most centrally situated, most ancient, and best characterized pathway in metabolism (Knull and Minton, 1996), is often assumed to be well understood. However, rates of glycolytic flux in intact cells cannot be described adequately from the kinetic properties of isolated glycolytic enzymes. Indeed, one of the hallmark properties of the glycolytic pathway, namely that large scale changes in flux are reflected in only modest changes in the concentration of pathway intermediates, has not been satisfactorily explained (Hochachka, 1999a). Given the functional importance of the structural organization of metabolism, it appears likely that a better understanding of the role of macromolecular interactions in the regulation of glycolysis is needed to formulate adequate models of glycolytic flux regulation.

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CHAPTER III

EVIDENCE AGAINST THE NADH-MODULATED COMPLEX FORMATION BETWEEN α-GLYCEROL-3-PHOSPHATE DEHYDROGENASE AND L-LACTATE DEHYDROGENASE[1]

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PREFACE

The aim of the study presented in this chapter was to extend recently published research (Yong *et al.*, 1993, *Biochemistry 32*, 11124-11131) showing an NADH-modulated equilibrium association between α-glycerophosphate dehydrogenase (αGDH) and lactate dehydrogenase (LDH). Our study was undertaken to address deficiencies in this research which was (1) limited to one method, one enzyme pair, and three concentrations of NADH, and (2) missing potentially critical data (NADH absorbance was not monitored). Our research hypotheses were that this unprecedented and potentially important NADH-modulated association (1) provides a mechanism for the direct channeling of NADH, (2) explains the failure of past studies to detect association between αGDH and LDH, and (3) allows K_a's for pairs of channeling dehydrogenases to be correlated with the kinetic and structural properties of the enzymes.

Our results prove the null form of our hypotheses by conclusively showing that the reported NADH-modulated association is an artifact. This result, which was not entirely unexpected, has important implications. First, it indicates that if NADH is directly channeled between αGDH and LDH, as evidenced by enzyme buffering tests, it does *not* require detectable association between the dehydrogenases at equilibrium as heretofore assumed. The results of this study along with other results obtained in our lab may therefore have uncovered the true and unsuspected nature of direct NADH

channeling systems. Second, our results indicate that the transient-state kinetic data on the rate of transfer of NADH from a donor dehydrogenase (E_d) to an acceptor dehydrogenase (E_a) cannot be considered evidence against direct NADH channeling, since direct NADH channeling does not require detectable levels of E_a-NADH-E_d complex under the conditions of these experiments (i.e., in the absence of the catalytic reaction). This point is especially important since most enzymologists have heretofore considered the transient-state kinetic data, presented as evidence against channeling, more rigorous and definitive than the steady-state (enzyme buffering) data, presented in support of channeling.

Finally, this and other studies from our lab have led us to develop a kinetic model in complete accord with the enzyme buffering data and lack of association of the channeling dehydrogenases in the *absence* of their catalytic reaction (Spivey and Svedruzic, *in preparation*). This kinetic model also explains the several other well documented cases (see discussion), where a complex between an enzyme and its protein substrate is essential for the reaction, but cannot be detected in the absence of the catalytic reaction.

ABSTRACT

There is considerable evidence that NADH can be transferred ('channeled') directly from the active site of a 'donor' dehydrogenase (E_d) to the active site of an

'acceptor' dehydrogenase (E_a) if the enzymes are of opposite chiral specificity for NADH. This direct transfer requires the formation of a ternary (E_d–NADH–E_a) complex in the steady-state of the reaction. Furthermore, it is generally assumed that the concentration of a ternary complex at equilibrium (i.e., in the absence of the cosubstrate of E_a) and in the steady-state of the reaction must be comparable. Therefore, unsuccessful attempts to detect an equilibrium complex between L-lactate dehydrogenase (LDH; EC 1.1.1.27; 'A' type chirality) and α-glycerol-3-phosphate dehydrogenase (αGDH; EC 1.1.1.8; 'B' type chirality) — a model dehydrogenase pair for which there is strong evidence for the direct transfer of NADH — have been interpreted as evidence against direct channeling of NADH. However, using a modified Hummel-Dreyer chromatographic technique, Yong *et al.* (1993, Biochemistry 32, 11124–11131) recently obtained results indicating significant equilibrium association between LDH and αGDH, and a most intriguing biphasic modulation of this association by NADH. In the present study, we describe experiments reproducing the chromatographic study of Yong *et al.* (op. cit.) with additional controls. We conclusively show that the reported modulation of complex formation between LDH and αGDH is an artifact that we tentatively attribute to the partial dissociation of dimeric αGDH. In addition, we present evidence that the mechanism for the direct transfer of NADH does not necessarily require the presence of a detectable ternary complex in the absence of the catalytic reaction.

INTRODUCTION

There is considerable evidence that NADH can be transferred ('channeled') directly from the active site of a 'donor' dehydrogenase (E_d) to the active site of an 'acceptor' dehydrogenase (E_a) if the enzymes are of opposite chiral specificity for the C_4 hydrogen of NADH (for review see Spivey, 1991). This direct transfer is equivalent to E_a using E_d–bound NADH (E_d–NADH) as its substrate. The K_m of E_a for E_d–NADH is in the micromolar range (often below 10 μM) for all characterized dehydrogenase pairs (Srivastava and Bernhard, 1985; Ushiroyama *et al.*, 1992). Therefore, under steady-state conditions, E_a must be saturated with E_d–NADH at sub-millimolar [E_d–NADH]. It is generally assumed that comparable saturation of E_a must occur at equilibrium in the absence of the reaction (i.e., in the absence of the cosubstrate of E_a). Indeed, it has even been predicted that sufficient quantity of E_d–NADH–E_a complex must exist at equilibrium to be detected by physical methods (Keizer and Smolen, 1992).

Early attempts to detect an equilibrium complex between L-lactate dehydrogenase (LDH; EC 1.1.1.27; 'A' type chirality) and α-glycerol-3-phosphate dehydrogenase (αGDH; EC 1.1.1.8; 'B' type chirality), a model dehydrogenase pair for which there is strong evidence for the direct transfer of NADH, were unsuccessful (Wu *et al.*, 1991; Brooks and Storey, 1991). However, using a modified Hummel-Dreyer chromatographic technique, Yong *et al.* (1993) recently obtained results indicating significant equilibrium

association and a most intriguing biphasic modulation of this association by NADH. Four plausible reasons can be invoked to explain why Yong *et al.* (1993) detected association when others did not: 1) their study included a moderate sub-stoichiometric concentration of NADH, in contrast to one of two earlier studies (Wu *et al.*, 1991); 2) Hummel-Dreyer chromatography is more sensitive to weak protein-protein complexes than the chromatographic techniques used in the earlier study that used a sub-stoichiometric concentration of NADH (Brooks and Storey, 1991); 3) gel matrices can enhance protein association (Fried and Crothers, 1981; Minton, 1995; Ashmarina *et al.*, 1994) — this could explain the failure to detect association in analytical ultracentrifugation experiments (Wu *et al.*, 1991); and 4) the association is an artifact.

Yong *et al.* (1993) reported association constants (K_a's) of 0.04 ± 0.02 and $2.0 \pm 0.6\ \mu M^{-1}$ at [NADH]/[αGDH] = 0 and 0.5, respectively, and no association at [NADH]/[αGDH] = 2.0 or when αGDH was replaced with alcohol dehydrogenase (ADH; EC 1.1.1.1; "A" type chirality) as a negative control for association. The reported K_a of $2.0\ \mu M^{-1}$ is sufficiently high to explain the saturation of E_a with E_d–NADH at the micromolar [E_d–NADH] used in steady-state channeling experiments (Srivastava and Bernhard, 1985). This K_a is also in excellent agreement with theoretical predictions and the suggestion that, for structural reasons, a complex between LDH and αGDH is most likely at moderate sub-saturating concentrations of NADH (Keizer and Smolen, 1992; Weber and Bernhard, 1982). Therefore, the reported biphasic modulation of the

association between an E_d and E_a suggested an unprecedented but reasonable mechanism of dehydrogenase interactions of potentially great importance to both NADH channeling and enzyme modulation.

Because the association study of Yong *et al.* (1993) was limited to one method, one pair of dehydrogenases, and three experimental conditions, we performed extensive measurements with several methods to test for either E_d–E_a or E_d–NADH–E_a complex formation using several dehydrogenase pairs including the LDH/αGDH pair. Non-chromatographic studies found a few pairs of dehydrogenases that associate in polymer media such as agarose and polyethylene glycol (Svedruzic and Spivey, *in preparation*). However, no evidence of association was found for several pairs of dehydrogenases for which there is evidence for the direct transfer of NADH, including the LDH/αGDH pair studied by Yong *et al.* (1993). In the present article, we describe experiments reproducing, with additional controls, the chromatographic study of Yong *et al.* (1993). We conclusively show that the reported modulation by NADH of complex formation between LDH and αGDH is an artifact. In addition, we present evidence that the mechanism for the direct transfer of NADH does not necessarily require the presence of a detectable E_d–NADH–E_a complex at equilibrium.

MATERIALS AND METHODS

Materials

The TSK-GEL G3000SWXL high performance liquid chromatography column (7.8 mm ID x 30 cm length; 32160 theoretical plates as determined by the supplier), and the TSK-GEL SWXL guard columns (6 mm ID x 4 cm length) were purchased from TosoHaas. Efforts were made to use enzymes from the commercial source (Sigma Chemical Co.) used by Yong *et al.* (1993). However, because of supply difficulties, αGDH from rabbit muscle was obtained from three different sources: Sigma Chemical Co. (Catalog No. G-6751), ICN (Catalog No. 104938, discontinued), and Boehringer Mannheim (Catalog No. 127 779). The specific activities and chromatographic elution profiles of αGDH were similar for all sources. Specifically, the unidentified NADH-binding impurity discussed in the following sections was present in all αGDH preparations. LDH from porcine heart was obtained from ICN (Catalog No. 151532). Preliminary experiments performed with LDH from Sigma Chemical Co. (Catalog No. L-9889) indicated that this source was unsuitable for this study because of low specific activity and a broad trailing peak in chromatographic elution profiles (data not shown). Alcohol dehydrogenase from equine liver (ADH; Catalog No. 102 741), and fatty acid-free BSA were from Boehringer Mannheim. ADH from Sigma Chemical Co. (Catalog No. A-6128) was unsuitable for this study because of heavy contamination (data not

shown). Disodium β-NADH (Catalog No. N-8129), glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (Catalog No. G-8404), and dihydroxyacetone phosphate were from Sigma Chemical Co. MOPS (enzyme grade) and NaCl (ACS grade) were from Fisher Scientific. 1,4-Dithio-DL-threitol was from Fluka Chemical Co. Norit SA3 (formerly Norit A) activated charcoal was from Aldrich Chemical Co. This charcoal was used without further treatment. All other chemicals were either from Sigma Chemical Co. or Fisher Scientific. Water purified with a NANOpure ultrapure water system (Barnstead) was used to prepare all buffers and solutions. All enzymes were received from the suppliers on ice (with the exception of the enzymes from Boehringer Mannheim) and used without further purification. Chromatography was performed with a ÄKTAexplorer liquid chromatography system from Pharmacia Biotech with a 10 mm pathlength 8 µl UV flow cell.

Enzyme preparation and assays

Prior to all measurements, the enzymes were dialyzed for less than 8 hours at 4°C against MOPS buffer (50 mM MOPS, pH 6.9 at room temperature, 100 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 0.02% (w/v) NaN_3). The enzyme dialysates were centrifuged (15 min at 16,000 x **g** at 4°C) to remove insoluble material and used without delay. The $(NH_4)_2SO_4$ concentration in the dialysates was determined with the Nessler method (Hawk *et al.*, 1954). All injected samples contained less than 100 µM

$(NH_4)_2SO_4$. Nessler reagent was prepared as described in The CRC Handbook of Chemistry and Physics 78th ed. (1996). Prior to $(NH_4)_2SO_4$ determination, the samples were diluted to decrease interference from the MOPS buffer to a negligible level. Notwithstanding, MOPS buffer was included in the $(NH_4)_2SO_4$ standards to correct for the presence of buffer in the diluted samples. The concentrations of ADH, αGDH, and LDH were determined spectrophotometrically using molar absorptivities at 280 nm of 3.61×10^4 (Warner L. Peticolas, personal communication), 4.78×10^4 and 1.7×10^5 (Yong *et al.*, 1993), respectively. The M_r of these proteins are 79, 78, and 144 kDa, respectively. NADH was determined enzymatically under the assay conditions of αGDH except that much higher [αGDH] and *ca.* 100 μM NADH were used.

Where indicated, enzymes were treated with activated charcoal to remove enzyme-bound nucleotides using a modification of the procedure of Taylor *et al.* (1948). Briefly, activated charcoal powder was suspended in the enzyme dialysates (containing 3.5-10.5 mg of protein per ml) at a final charcoal concentration of 10 mg/ml. The suspensions were incubated 10 min on ice before centrifugation (15 min at 16,000 x **g** at 4°C) to remove the charcoal. The treatment was repeated once to ensure complete removal of the nucleotides.

αGDH was assayed as described in Bergmeyer (1974) except that 10 mM dihydroxyacetone phosphate, 160 μM NADH, and 0.4% (w/v) BSA were used. ADH and LDH were assayed as described in Bergmeyer (1983) with the following

modifications: in the LDH assay the concentrations of sodium pyruvate and NADH were 500 and 160 μM, respectively; in the ADH assay, absolute ethanol was used and the concentration of semicarbazide hydrochloride was 74 mM. Unless otherwise specified, 0.5 mM DTT, 0.1% (w/v) BSA, and *ca.* 0.02% (w/v) NaN_3 were included in the assay buffers. All assays were performed at 25°C.

The specific activities of the dialyzed αGDH, ADH, and LDH were 410 ± 30 (n = 4), 3.30 ± 0.30 (n = 2), and 360 ± 30 units per mg protein (n = 4), respectively. One unit of enzyme activity is defined as the quantity of enzyme that transforms one μmol of substrate per min under the specified assay conditions.

Modified Hummel-Dreyer Chromatography

A guard column was connected between the pump and the injection loop of the chromatography system to prevent loss of resolution while protecting the main column from the protein in the column equilibration buffer. The gel filtration column was equilibrated with MOPS buffer containing αGDH or ADH at the concentrations indicated in the figure legends, and NADH at a concentration corresponding to [NADH]/[αGDH] = 0, 0.5, and 2.0 or [NADH]/[ADH] = 0.5. In addition, 2 μg/ml of glucose-6-phosphate dehydrogenase (*ca.* 650 U/mg) and 2 mM glucose-6-phosphate were included in the equilibration buffer (except when [NADH]/[αGDH] = 0) to regenerate NADH which is slowly oxidized by intrinsic or contaminating NADH oxidase activity

associated with LDH (see Spivey and Ovàdi, 1999). The low constant concentration of glucose-6-phosphate dehydrogenase used precludes its contribution to changes in recorded absorbances. The samples (100 µl) were pumped through the column at a flow rate of 0.4 ml/min. Samples contained a fixed concentration of LDH (0 or 1.65 µM) and varying concentrations of αGDH or ADH in MOPS buffer containing glucose-6-phosphate dehydrogenase, glucose-6-phosphate, and NADH, as above. Absorbance was monitored continuously at 280 and 340 nm. Chromatography was performed in a cold room (*ca.* 4°C) with the column equilibration buffer kept on ice. Temperature of the effluent at the exit of the column ranged from 7.1 to 9.6°C. Within a single experiment, the variation in temperature did not exceed 0.9°C.

Enzyme-bound nucleotides and free NADH

NADH and untreated and charcoal treated dialyzed enzymes were injected separately onto the size exclusion column equilibrated with MOPS buffer. Absorbance was monitored continuously at 260, 280, and 340 nm. The 280 nm/260 nm absorbance ratios of ADH and LDH were similar before and after charcoal treatment: 1.46 ± 0.01 (n = 6) vs. 1.46 ± 0.01 (n = 7), and 2.01 ± 0.01 (n = 7) vs. 2.02 ± 0.01 (n = 7), respectively. The ratio for ADH is characteristic of the apo-enzyme (Hans W. Adolf, personal communication). In contrast, charcoal treatment slightly increased the 280 nm/260 nm absorbance ratio of αGDH from 1.55 ± 0.01 (n = 8) to 1.60 ± 0.01 (n = 6).

The ratio for the treated enzyme is in good agreement with published values for nucleotide-free αGDH (Ankel, 1960; Fondy *et al.*, 1971; Telegdi, 1964). The 280 nm/340 nm absorbance ratio of free NADH was 0.62 ± 0.04 (n = 6).

Data presentation and analysis

Unless otherwise specified all data are reported as means ± SD. Untransformed original data are presented. Enzyme concentrations are expressed in terms of native enzymes. Since native LDH is a homo-tetramer and native αGDH and ADH are homo-dimers, the [NADH]/[enzyme subunits] ratios are 0.25, 0.5, and 0.5 of those tabulated in terms of concentrations of native LDH, αGDH, and ADH, respectively. When injected [αGDH], [ADH], and [NADH] are expressed in terms of a deficit or excess, it is in reference to their respective concentration in the column.

Chromatographic elution profiles were analyzed with UNICORN software, version 2.30 (Pharmacia Biotech). Horizontal baselines were user defined. Uncertainties in baseline definition caused systematic errors in the integrated peak/trough areas that propagated into the K_a's and became the predominant source of error in K_a determination. A lowest and highest probable baseline was thus defined for each elution profile and a K_a corresponding to each baseline was calculated, as described by Yong *et al.* (1993), for each series of elution profiles. K_a's are presented as lower and upper limits of probable K_a values. However, for clarity of presentation, the plots of integrated αGDH or ADH

peak/trough areas vs. [αGDH] or [ADH] were graphed using the average of the peak/trough areas integrated from each elution profile using the lowest and highest probable baseline.

Differences between the nominal [αGDH] or [ADH] in the column and the nominal concentration of injected αGDH or ADH needed to produce a flat baseline in Hummel-Dreyer elution profiles collected at 280 nm in the absence of LDH were determined as described by Yong *et al.* (1993) for each series of elution profiles and used to correct the [αGDH] or [ADH] in the column by arbitrarily assuming that the [αGDH] or [ADH] in the injected samples were accurate. The alternative assumption that the concentrations in the column were accurate would generate similar K_a's since the K_a's are calculated from the differences between column and sample concentrations. The observed small differences in [αGDH] and [ADH] originate from differences in loading processes (Yong *et al.*, 1993) and preparation of the column equilibration buffer and the injected samples. No such correction in [NADH] was made because the differences between the nominal [NADH] in the column and the concentration of injected NADH needed to produce a flat baseline at 340 nm in the absence of LDH were always small relative to the [NADH] corresponding to the NADH troughs observed at 340 nm in the presence of LDH.

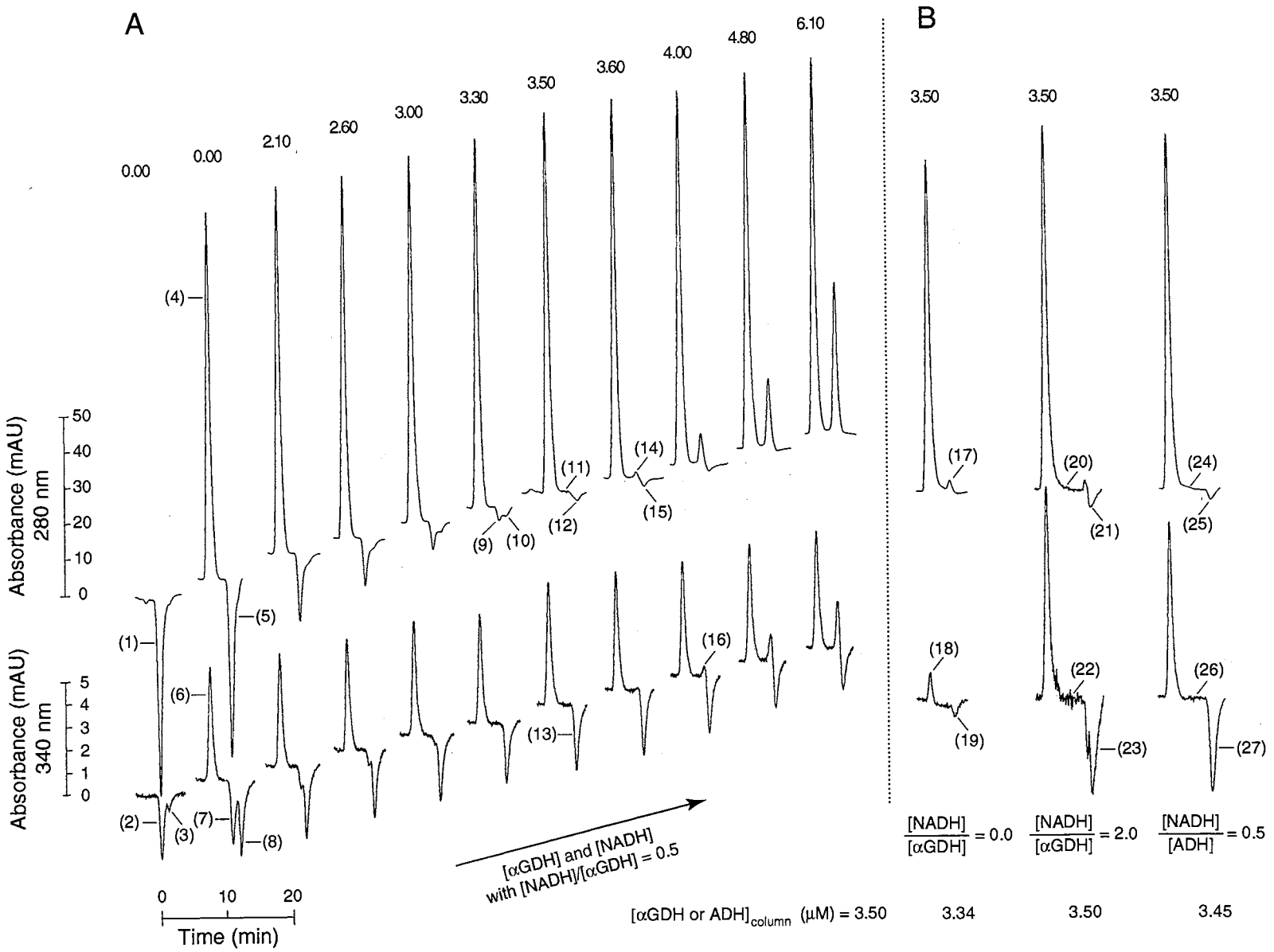
RESULTS

The chromatographic profiles we present were recorded at 280 and 340 nm to monitor [protein] and [NADH], respectively. The elution profiles recorded at 280 nm reproduce the complete data of Yong *et al.* (1993) for LDH and αGDH whereas the profiles recorded at 340 nm provide critical information not found in their study.

In Fig. 1, we show Hummel-Dreyer chromatographic elution profiles of LDH and αGDH collected at three different [NADH]/[αGDH] ratios. In the absence of association between LDH and αGDH, a flat baseline (i.e., the absence of a peak or trough) is expected at the retention time of αGDH when the [αGDH] in the injected samples is equal to the [αGDH] in the column. However, in the presence of detectable association, αGDH is expected to elute more rapidly as part of an aggregate of higher mass than αGDH alone thereby causing a trough at the αGDH retention time. Therefore, in the presence of association, the [αGDH] in the injected sample required to produce a flat baseline at the retention time of αGDH (at 280 nm) will need be higher than the [αGDH] in the column since 'extra' αGDH will be needed to fill the trough.

As shown in Fig. 1A (baseline 11), when the [αGDH] in the injected samples was equal to the [αGDH] in the column, a flat baseline was obtained at the retention time of αGDH. The K_a of 0.00 to 0.01 μM^{-1} calculated from this baseline is incompatible with the K_a of 2.0 ± 0.6 μM^{-1} reported by Yong *et al.* (1993) for the same experimental

FIG. 1. Hummel-Dreyer chromatographic elution profiles of LDH and αGDH or ADH. A: Series of elution profiles of LDH and αGDH collected at [NADH]/[αGDH] = 0.5. The column was equilibrated with 3.50 μM of αGDH. The concentration of LDH in the injected sample was 0.00 or 1.65 μM, while that of αGDH was 0.00, 2.10, 2.60, 3.00, 3.30, 3.50, 3.60, 4.00, 4.80, and 6.10 μM as indicated above each profile. B: Selected elution profiles of LDH and αGDH at [NADH]/[αGDH] = 0 and 2.0, and LDH and ADH at [NADH]/[ADH] = 0.5. The column was equilibrated with 3.34 and 3.50 μM of αGDH, and 3.45 μM of ADH, respectively. The [LDH] and [αGDH] in the injected samples were 1.65 and 3.50 μM, respectively. The time and absorbance scales of A also apply to B. Note the 10-fold difference between the 280 and 340 nm absorbance scales. Peak assignments for A and B (see discussion; expressed in terms of a deficit or an excess of a molecule in the injected sample relative to the column): (1) and (5) deficit of αGDH_2 (the contribution from a deficit of X and a deficit of NADH bound to both αGDH_2 and X is insignificant); (2) and (7) deficit of αGDH_2-bound NADH plus a small contribution from a deficit of αGDH_2; (3) deficit of NADH bound to X (the contribution from a deficit of X is probably insignificant); (4) LDH (the contribution from LDH-bound NADH is insignificant); (6) LDH-bound NADH plus a small contribution from LDH; (8) deficit of NADH bound to X, the area of this trough is larger than the area of trough (3) because of the binding by LDH of NADH (in the injected sample and the column) which would have been bound to X in the absence of LDH (the contribution from a deficit of X is probably insignificant) (9) deficit of αGDH_2 (the contribution from αGDH_2-bound NADH is insignificant); (10), (12), and (15) deficit of NADH bound to X due to the binding by LDH of NADH which would have been bound to X in the absence of LDH plus possibly a significant deficit of X due to the association of the impurity with LDH; (11) and (20) absence of a peak/trough at the retention time of αGDH_2; (13) and (19) deficit of NADH bound to X due to the binding by LDH of NADH which would have been bound to X in the absence of LDH (the contribution from a deficit of X is probably insignificant); (14) and (17) excess of αGDH_2 (the contribution from excess αGDH_2-bound NADH is insignificant); (16) excess of αGDH_2-bound NADH (the contribution from excess αGDH_2 is insignificant); (18) LDH plus LDH-bound NADH; (21), (23), (25), and (27) deficit of free NADH due to binding by LDH; (22) and (26) absence of trough at the retention time of NADH bound to X; (24) absence of a peak/trough at the retention time of ADH. Typical elution profiles are shown.


A
B
0.00
0.00
2.10
2.60
3.00
3.30
3.50
3.60
4.00
4.80
6.10
3.50
3.50
3.50
Absorbance (mAU)
280 nm
50
40
30
20
10
0
Absorbance (mAU)
340 nm
5
4
3
2
1
0
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)
(12)
(13)
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(16)
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(18)
(19)
(20)
(21)
(22)
(23)
(24)
(25)
(26)
(27)
0
10
20
Time (min)
[αGDH] and [NADH]
with [NADH]/[αGDH] = 0.5
[αGDH or ADH]column (μM) = 3.50
3.34
3.50
3.45
[NADH]/[αGDH] = 0.0
[NADH]/[αGDH] = 2.0
[NADH]/[ADH] = 0.5

conditions. However, as explained in the discussion, this discrepancy is *not* a consequence of differences in the data (only small quantitative differences exist between our data and the data reported by Yong *et al.*, 1993), but rather of the mistaken identification by Yong *et al.* (1993) of a small trough in the chromatographic elution profiles (see discussion).

The small trough at issue (see Fig. 1A, e.g., troughs 10, 12, and 15) eluted 1 min 8 s ± 1 s (n = 4) and 4 min 11 s ± 1 s (n = 4) later than αGDH and LDH, respectively, at a retention time of 23 min 19 s ± 2 s (n = 4). At precisely the same retention time, a trough was observed at 340 nm (Fig. 1A, e.g., trough 13; note the 10-fold difference between the 280 and 340 nm scales). For reasons discussed in the next section, we tentatively attribute these small troughs to the presence of monomeric αGDH (αGDH_1) in the column equilibration buffer. The presence of αGDH_1 (or less probably, of an NADH-binding impurity) is clearly indicated by the presence of trough 3 in Fig. 1A.

Key elution profiles collected at [NADH]/[αGDH] = 0 and 2.0, and [NADH]/[ADH] = 0.5, are presented in Fig. 1B. These profiles were selected from series of elution profiles available as supporting information. K_a's of 0.00 to 0.01, 0.00 to 0.02, and 0.00 to 0.01 μM^{-1} were calculated from the series of profiles collected at [NADH]/[αGDH] = 0 and 2.0, and [NADH]/[ADH] = 0, respectively. These K_a's are in excellent agreement with the K_a's reported by Yong *et al.* (1993) with the exception of a

small quantitative difference in the K_a calculated from the profiles collected at [NADH]/[αGDH] = 0 (K_a = 0.00 to 0.01 μM^{-1} in our study vs. 0.04 ± 0.02 μM^{-1} in Yong *et al.*, 1993).

DISCUSSION

Chromatographic data and interpretation

As explained in the RESULTS section, in the absence of association between LDH and αGDH, a flat baseline is expected at the retention time of dimeric αGDH (αGDH_2) when the [αGDH] in the injected samples is equal to the [αGDH] in the column. This was observed at all three [NADH]/[αGDH] ratios. However, in all the elution profiles collected at [NADH]/[αGDH] = 0.5, a small trough (Fig. 1A, e.g., troughs 10, 12, and 15) was observed eluting slightly later than αGDH_2 (23 min 19 s vs. 22 min 11 s). Although the difference in retention time between αGDH_2 and this trough is small, the retention time of this trough corresponds to a M_r of ca. 48 kDa, i.e., roughly half the M_r of αGDH_2 (78 kDa). This clearly indicates that this trough does not correspond to αGDH_2. It also suggests that this trough, which we refer to as trough X in the remainder of this article and in the legend of Fig. 1, corresponds to αGDH_1. This interpretation is strongly supported by our observation that commercially prepared

αGDH and other dehydrogenases undergo partial dissociation as judged by sedimentation velocity experiments (unpublished results).

In their Hummel-Dreyer chromatographic study of LDH and αGDH, Yong *et al.* (1993) misassigned to αGDH_2 the partially resolved trough X, which we tentatively assign to αGDH_1. It should noted that in contrast to our results, trough X is absent from the elution profiles presented by Yong *et al.* (1993) for [NADH]/[αGDH] = 0.5 when the [αGDH] in the injected sample is lower than the [αGDH] in the column, but not when it is higher (Fig. 5A). Several lines of evidence, including the comparison of the elution profiles presented in Fig. 2 (original profile) and 5A (smoothed profiles) of Yong *et al.* (1993), indicate that this conspicuous and misleading absence is an unfortunate consequence of data smoothing.

The misassignment of trough X to αGDH_2 led Yong *et al.* (1993) to interpret this trough as an indication of association between LDH and αGDH_2. As mentioned in the preceding section, association would have caused αGDH_2 to elute more rapidly as part of an aggregate of higher mass thus causing a decrease in absorbance (i.e., a trough) at the αGDH_2 retention time. The discovery that trough X observed at [NADH]/[αGDH] = 0.5 was completely absent at [NADH]/[αGDH] = 2.0 and near the detection limit at [NADH]/[αGDH] = 0, also led Yong *et al.* (1993) to conclude that the association between LDH and αGDH_2 was NADH-modulated in a biphasic manner. However, the

elution profiles we collected at 340 nm (the λ_{max} of NADH) offer new insight into the origin of trough X and its intriguing NADH-dependence. As shown in Fig. 1A, at [NADH]/[αGDH] = 0.5 a small trough is observed at 340 nm (e.g., trough 13) at the precise retention time of trough X (e.g., trough 12). For reasons discussed below, we attribute this small trough to a deficit of NADH bound to molecule 'X', i.e., αGDH_1 or less probably, an NADH-binding impurity. Since NADH absorbs at 280 nm (the 280 nm/340 nm absorbance ratio of free NADH in our instrument was 0.62 ± 0.04, n = 6), the deficit of X-bound NADH observed at 340 nm requires a corresponding trough at 280 nm. The X trough must therefore be attributed, at least in part (see below), to a deficit of X-bound NADH. This offers a simple explanation for the otherwise puzzling NADH-dependence of trough X, which mirrors the dependence of the small trough observed at 340 nm.

The NADH-dependence of the small trough observed at 340 nm is easily explained. The trough corresponding to a deficit of free NADH at [NADH]/[αGDH] = 2.0 (Fig. 1, trough 23) was barely detectable at [NADH]/[αGDH] = 0.5 (trough truncated from the profiles shown Fig. 1A). This indicates that at [NADH]/[αGDH] = 0.5 essentially all the NADH was in the bound state. Since the small trough observed at 340 nm at [NADH]/[αGDH] = 0.5 was absent when LDH was omitted from the injected sample and the concentration of injected αGDH was

equal to the [αGDH] in the column (data not shown), we attribute this small trough to a deficit of X-bound NADH resulting from the binding by LDH of NADH (in the injected sample and the column) which would be bound to X in the absence of LDH (when considering the binding of NADH by LDH, it is important to keep in mind that when LDH was present in the samples the [NADH]/[dehydrogenase] ratio was lower in the injected samples than in the column).

At [NADH]/[αGDH] = 0, a minuscule trough corresponding to a deficit of X-bound NADH (trough 19) was also produced by the mechanism just described (a trough is visible because traces of NADH were present, see MATERIAL AND METHODS). In contrast, at [NADH]/[αGDH] = 2.0, the binding of NADH by LDH produced a trough corresponding to a deficit of free NADH (trough 23) rather than a trough corresponding to a deficit of X-bound NADH (see baseline 22) since a significant fraction of the total NADH was present as free NADH. The NADH-dependence of the trough corresponding to a deficit of X-bound NADH (e.g. trough 13) is thus explained by the absolute requirement that essentially all the NADH be present in the bound state for this trough to be present.

Since the absorbance ratio of the area of the X trough (280 nm) and the trough corresponding to a deficit of X-bound NADH (340 nm) is 0.95 to 1.30 whereas the 280 nm/340 nm absorbance ratio of free NADH is only about 0.62, the trough observed at 340 nm may not, as suggested above, completely account for the area of trough X

(280 nm). On the other hand, the higher absorbance ratio of the trough areas may simply reflect a difference between the 280 nm/340 nm absorbance ratio of free and X-bound NADH. Nevertheless, the possibility that some X associates with LDH cannot be strictly excluded.

The anomalously high concentration of injected [αGDH] required to fill the small trough that Yong *et al.* (1993) attributed to a deficit of αGDH_2 is easily explained when the trough is assigned to X. The high concentration is required to produce a αGDH_2 peak that is broad enough to completely fill trough X by overlap (see Fig. 1A). In light of the elution profiles collected at 340 nm, the observation by Yong *et al.* (1993) of a "strange chromatogram" with an anomalous peak eluting slightly faster than αGDH (see Fig.1A, e.g., peak 14) is also easily explained. The anomalous peak which Yong *et al.* (1993) attributed to αGDH associating with LDH is actually a peak of αGDH (injected at a concentration higher than the [αGDH] in the column) which is truncated by overlap with trough X and thus apparently displaced towards a slightly shorter retention time. It should be noted that in contrast to our results, Yong *et al.* (1993) reported the appearance of the anomalous peak at an injected [αGDH] approximately 1.1 μM lower than the [αGDH] in the column. Superficially, this discrepancy appears to indicate that Yong *et al.* (1993) detected association that we failed to detect. However, the reported value of 1.1 μM is based on an uncertain extrapolation of data (Fig. 5B of Yong *et al.*, 1993), data

in which the anomalous peak is in fact not observed when the injected [αGDH] is less than 0.1 μM below the [αGDH] in the column. Such a small difference in concentration is not significant within experimental error (note the difference between the duplicate points in Fig. 5B of Yong *et al.*, 1993). In fact, while collecting elution profiles at [NADH]/[αGDH] = 0.5, we observed a small αGDH peak in one of 4 replicate elution profiles collected at an injected [αGDH] nominally equal to the [αGDH] in the column (unpublished results).

In their study, Yong *et al.* (1993) replaced αGDH with ADH as a negative control for association. ADH and αGDH have a similar molecular mass (79 vs. 78 kDa) and because ADH has the same chiral specificity as LDH, according to the NADH channeling dogma (Srivastava and Bernhard, 1986) it is not expected to transfer ('channel') NADH directly to LDH and thus associate with LDH. This negative control appeared to support the conclusions of Yong *et al.* (1993). However, our results show that ADH did not bind all the NADH present at [NADH]/[ADH] = 0.5 (Fig. 1B). This is most likely the result of a low binding capacity of the commercial dehydrogenase due to non-native NADH-binding sites (often a significant fraction, typically *ca.* 40%, of the NADH-binding sites of commercially prepared dehydrogenases are not competent for binding; Spivey and Ovàdi, 1999). However, regardless of the reason, free NADH was available for LDH to bind. A trough corresponding to missing free NADH is thus observed (rather than a

trough corresponding to NADH bound to the monomeric ADH which may or may not have been present). Because the retention time of free NADH is much greater than that of ADH, the deficit of free NADH observed at 340 nm does not affect the elution pattern of ADH at 280 nm.

As mentioned earlier, at [NADH]/[αGDH] = 0 Yong *et al.* (1993) reported, in contrast to our results, a small trough at the retention time of αGDH_2 indicating association between LDH and αGDH_2 ($K_a = 0.04 \pm 0.02$). However, it should be noted that the K_a reported by Yong *et al.* (1993) is near the detection limit of the system. In addition, as shown in Fig. 1B, at [NADH]/[αGDH] = 0 a small trough (trough 19) is observed at 340 nm at the retention time of X. Although this trough is too small to have had a significant effect on the elution profile collected at 280 nm, this does not appear to have been the case in the study of Yong *et al.* (1993). Indeed, in Fig. 2 of their study an elution profile collected at [NADH]/[αGDH] = 0 shows a small trough partially overlapping the small αGDH_2 trough at 280 nm. These observations together with our inability to reproduce data showing association between LDH and αGDH make us seriously question the association reported by Yong *et al.* (1993) at [NADH]/[αGDH] = 0.

In summary, based on the overwhelming evidence presented, we conclude with confidence that the reported biphasic modulation by NADH of the association between

LDH and αGDH is an artifact. We also tentatively conclude that this artifact is due to the partial dissociation of αGDH_2. The results of our study thus underscore the importance of monitoring the absorbance of the association modulators (NADH in this case) used in modified Hummel-Dreyer chromatographic studies.

Direct NADH channeling and lack of E_d–E_a association at equilibrium

A lack of detectable association between LDH and αGDH at equilibrium is in general agreement with extensive measurements performed by our group using several different methods with many dehydrogenase pairs of opposite chiral specificity (Svedruzic and Spivey, *in preparation*). As mentioned earlier, association was detected between a few E_d/E_a pairs in polymer media such as agarose and polyethylene glycol, but not between αGDH and LDH (even in the presence of NADH at sub-saturating concentrations) and never in free solutions like those used in the kinetic tests for direct channeling of NADH. In addition, a recent tryptophan fluorescence investigation of dynamic protein associations also failed to detect association between αGDH and LDH (Gabellieri and Strambini, 1994). However, a lack of detectable equilibrium association does not necessarily indicate a lack of association during the steady-state of the reaction. Several examples can be cited where protein-enzyme or enzyme-enzyme complexes occur during the catalytic reaction, but no complex can be detected in the absence of the reactions. Three examples were recently compiled by Rudolph and Stubbe (1995) who

also presented extensive evidence for their substrate channeling system involving the enzyme pair glutamine phosphoribosylpyrophosphate amidotransferase and glycinamide ribonucleotide. Another example is provided by the observation that the medium chain acylCoA dehydrogenase forms a catalytic complex with the electron transferring flavoprotein (the oxidizing cofactor of the enzyme), but no enzyme-electron transferring flavoprotein complex can be detected at equilibrium (D. K. Srivastava, personal communication).

In addition, using simple and rigorous enzyme kinetic theory, we have shown that enormous enhancements of dehydrogenase associations can be achieved during the catalytic cycle, which are consistent with the NADH channeling model (Spivey and Svedruzic, *in preparation*). As mentioned earlier, Keizer and Smolen (1992) came to a different conclusion. However, their study considered only single substrate mechanisms and ignored the obligatory release of the apo–E_d from the E_d–NADH–E_a complex prior to addition of the second substrate. With such simplified and unrealistic mechanisms, a large enhancement of enzyme association during the steady-state reaction is indeed not possible, a fact that we had independently concluded (unpublished study). However, utilizing the correct 'substituted-enzyme' (ping-pong) mechanism makes an enormous quantitative difference. We are therefore currently pursuing experiments to test for the presence of E_d–NADH–E_a complexes during the catalytic reaction.

ACKNOWLEDGEMENT

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SUPPORTING INFORMATION

FIG. 1. Series of Hummel-Dreyer chromatographic elution profiles of LDH and αGDH collected at [NADH]/[αGDH] = 0. The column was equilibrated with 3.34 μM of αGDH. The concentration of LDH in the injected sample was 1.65 μM, while that of αGDH was 0.00, 2.10, 2.60, 3.00, 3.30, 3.50, 3.70, 4.00, 4.80, and 6.10 μM. Typical elution profiles are shown.

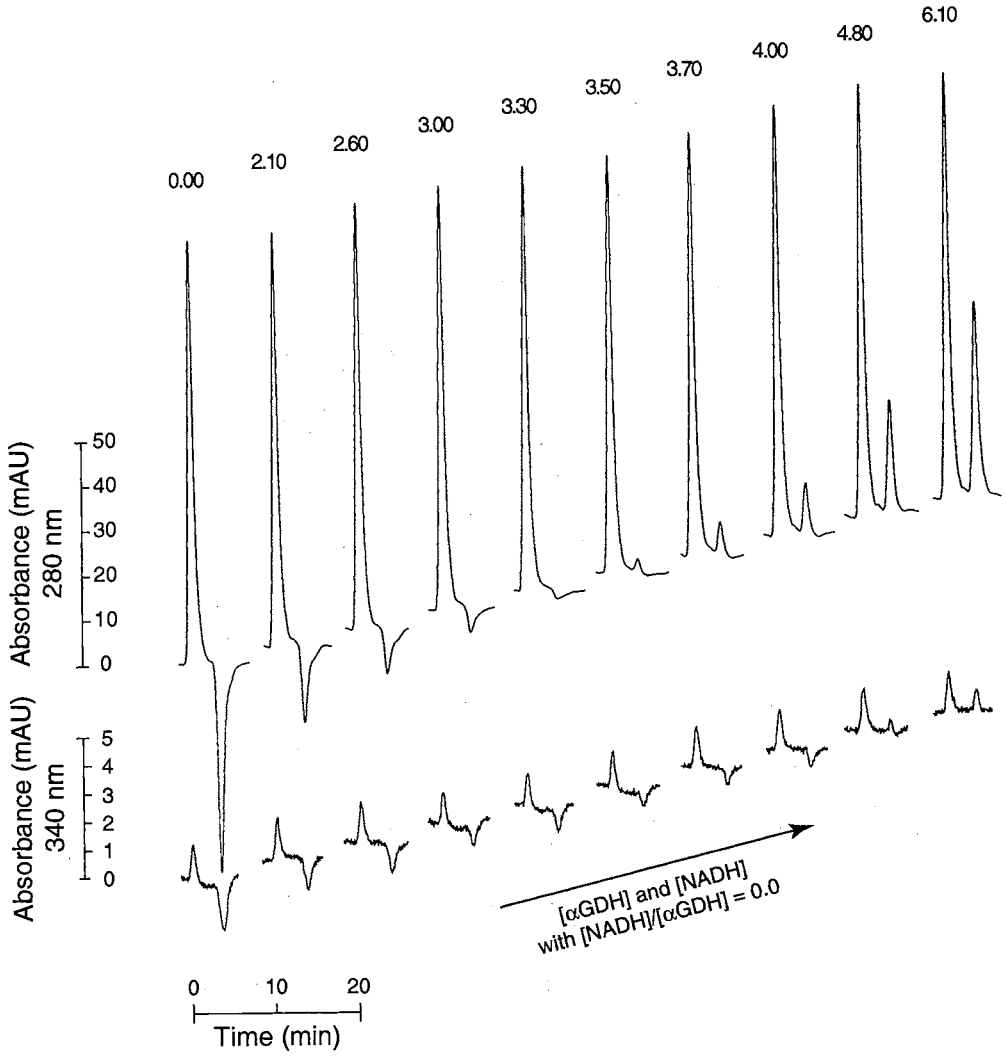

0.00
2.10
2.60
3.00
3.30
3.50
3.70
4.00
4.80
6.10
Absorbance (mAU)
280 nm
50
40
30
20
10
0
Absorbance (mAU)
340 nm
5
4
3
2
1
0
[αGDH] and [NADH]
with [NADH]/[αGDH] = 0.0
0
10
20
Time (min)

FIG. 2. Plot to estimate the [αGDH] needed in the injected sample to produce a flat baseline in the Hummel-Dreyer chromatographic profile of LDH and αGDH at [NADH]/[αGDH] = 0. The [LDH] in the injected sample was 1.65 μM. The [αGDH] in the column is denoted by a square symbol. Symbols and error bars represent means ± SD of two or more measurements. SD ≤ 0.21 mAU min are covered by the symbols.

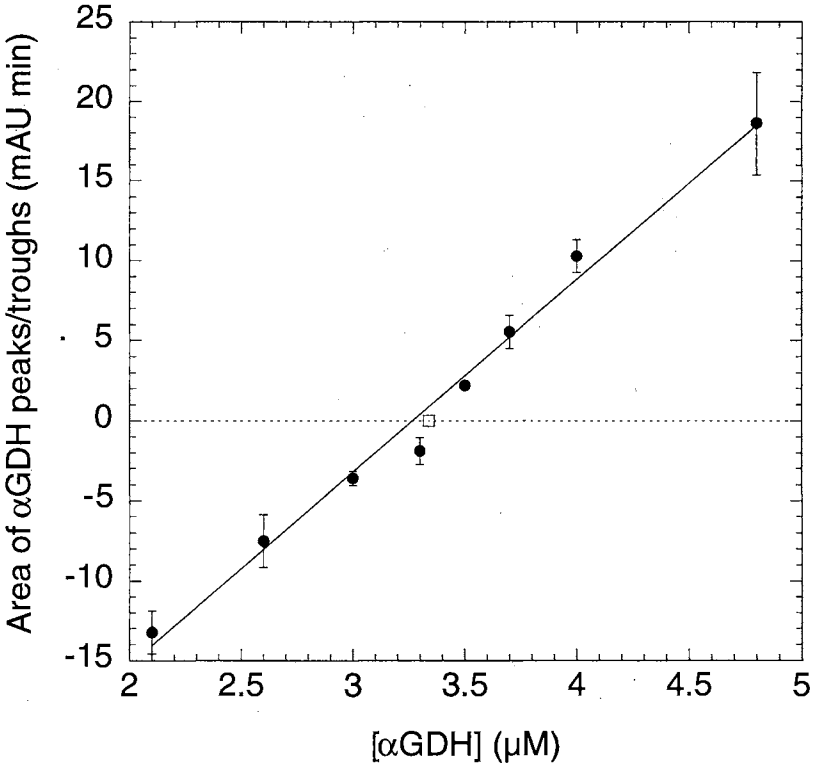

Area of αGDH peaks/troughs (mAU min)
25
20
15
10
5
0
-5
-10
-15
2
2.5
3
3.5
4
4.5
5
[αGDH] (μM)

FIG. 3. Series of Hummel-Dreyer chromatographic elution profiles of LDH and αGDH collected at [NADH]/[αGDH] = 2.0. The column was equilibrated with 3.50 μM of αGDH. The LDH concentration in the injected sample was 1.65 μM, while that of αGDH was 0.00, 2.10, 2.60, 3.00, 3.30, 3.50, 4.00, 4.80, and 6.10 μM. Typical elution profiles are shown.

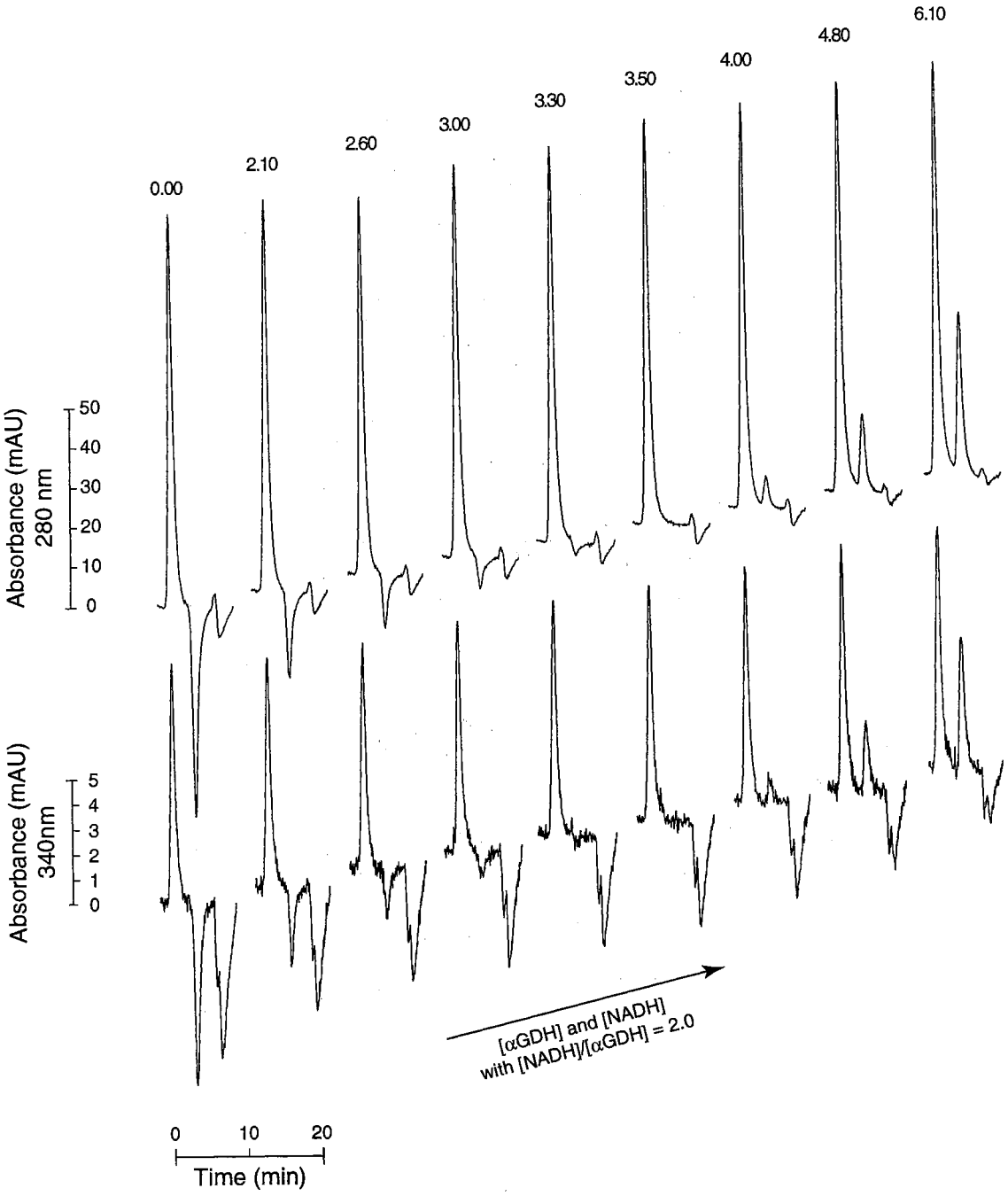

0.00
2.10
2.60
3.00
3.30
3.50
4.00
4.80
6.10
Absorbance (mAU)
280 nm
50
40
30
20
10
0
Absorbance (mAU)
340nm
5
4
3
2
1
0
[αGDH] and [NADH]
with [NADH]/[αGDH] = 2.0
0
10
20
Time (min)

FIG. 4. Plot to estimate the [αGDH] needed in the injected sample to produce a flat baseline in the Hummel-Dreyer chromatographic profile of LDH and αGDH at [NADH]/[αGDH] = 2.0. The [LDH] in the injected sample was 1.65 μM. The [αGDH] in the column is denoted by a square symbol. Symbols and error bars represent means ± SD of two or more measurements. SD ≤ 0.28 mAU min are covered by the symbols.

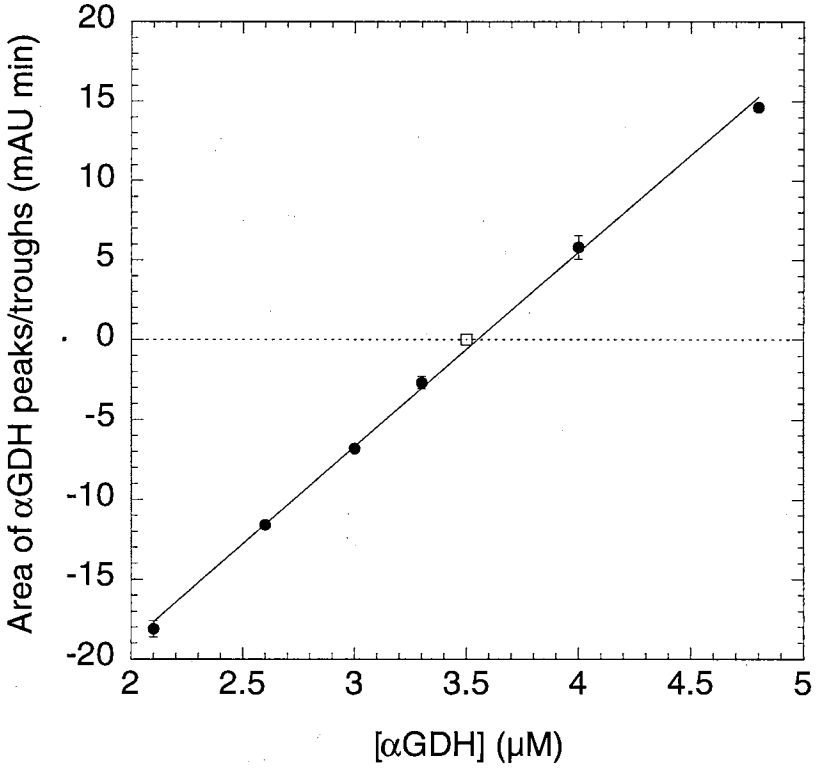

Area of αGDH peaks/troughs (mAU min)
20
15
10
5
0
-5
-10
-15
-20
2
2.5
3
3.5
4
4.5
5
[αGDH] (μM)

FIG. 5. Series of Hummel-Dreyer chromatographic elution profiles of LDH and ADH collected at [NADH]/[ADH] = 0.5. The column was equilibrated with 3.45 μM of ADH. The LDH concentration in the injected sample was 1.65 μM, while that of ADH was 0.00, 2.10, 2.60, 3.00, 3.30, 3.50, 4.00, 4.80, and 6.10 μM. Typical elution profiles are shown.

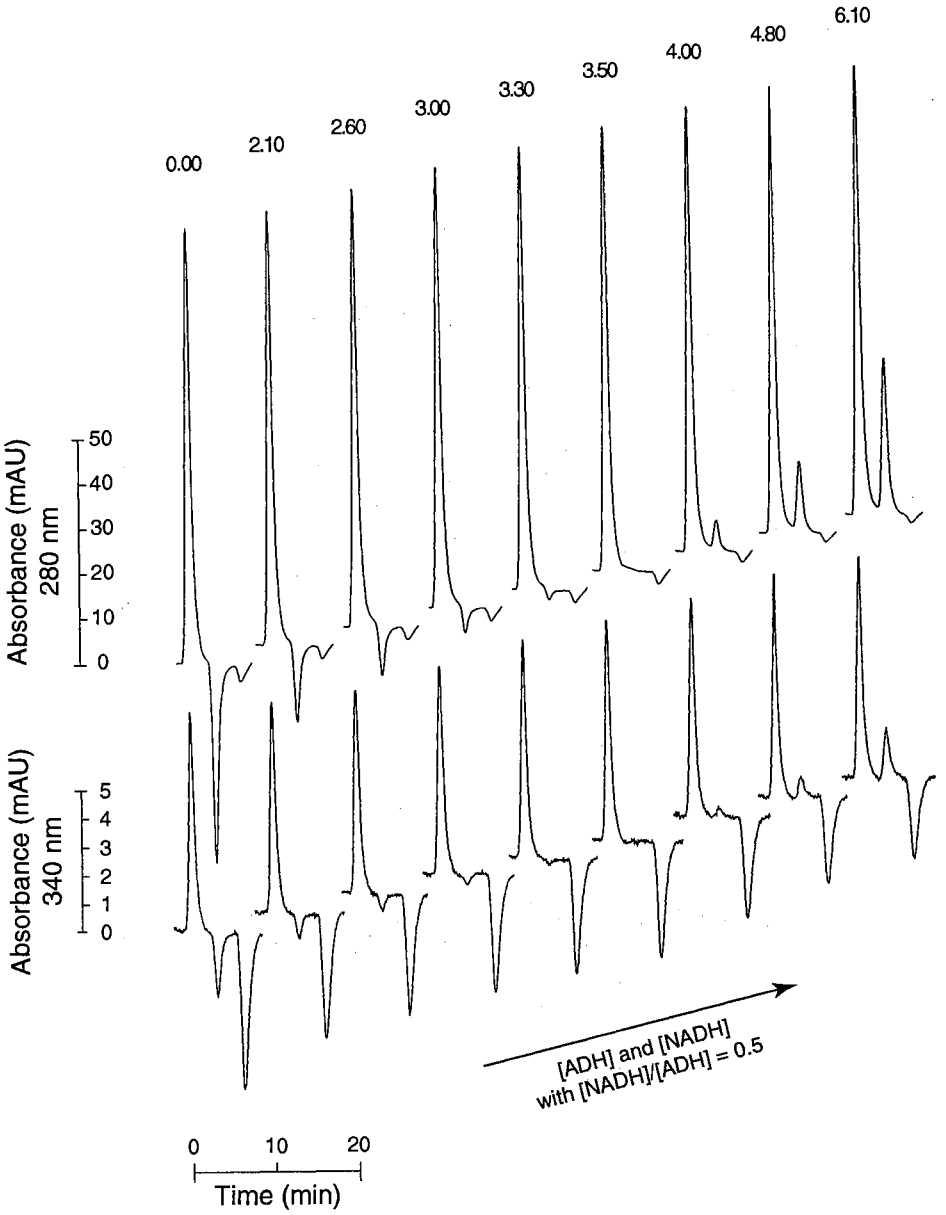
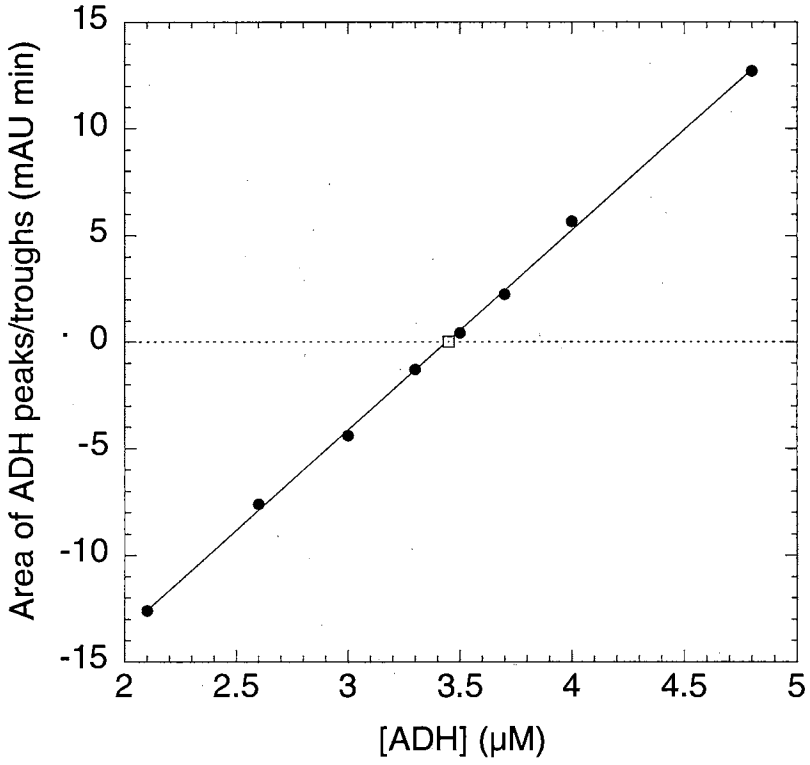

0.00
2.10
2.60
3.00
3.30
3.50
4.00
4.80
6.10
Absorbance (mAU)
280 nm
50
40
30
20
10
0
Absorbance (mAU)
340 nm
5
4
3
2
1
0
[ADH] and [NADH]
with [NADH]/[ADH] = 0.5
0
10
20
Time (min)

FIG. 6. Plot to estimate the [ADH] needed in the injected sample to produce a flat baseline in the Hummel-Dreyer chromatographic profile of LDH and ADH at [NADH]/[ADH] = 0.5. The [LDH] in the injected sample was 1.65 μM. The [ADH] in the column is denoted by a square symbol. Symbols and error bars represent means ± SD of two or more measurements. SD ≤ 0.16 mAU min are covered by the symbols.


15
10
5
0
-5
-10
-15
2
2.5
3
3.5
4
4.5
5
Area of ADH peaks/troughs (mAU min)
[ADH] (μM)

CHAPTER IV

DETERMINATION OF THE SPECIFIC RADIOACTIVITY OF [^{14}C]LACTATE BY ENZYMATIC DECARBOXYLATION AND $^{14}CO_2$ COLLECTION[1]

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PREFACE

In this chapter, we present a method for the determination of the specific radioactivity of L-[^{14}C]lactate that is simple, precise, convenient, specific, and reliable. This method was developed to greatly facilitate and improve our planned measurements of substrate channeling *in situ* in the glycolytic pathway using the isotopic dilution method. Because of the widespread use of L-[^{14}C]lactate in tracer metabolic studies, the method presented is also of considerable general interest. This chapter was published in *Anal. Biochem.* (1997) 253: 190–195.

ABSTRACT

We present an enzymatic method for the determination of L-[^{14}C]lactate specific radioactivity in complex biological samples containing other radiolabeled compounds. The method is based on the conversion of L-lactate to L-pyruvate by lactate oxidase (no EC number assigned) and the decarboxylation of L-pyruvate by pyruvate oxidase (EC 1.2.3.3). The $^{14}CO_2$ produced by the enzymatic decarboxylation of L-pyruvate is quantitatively captured in a CO_2 trap and its radioactivity is measured. The method is simple, specific, and precise (2% relative SD). It can be conveniently used for routine multiple determinations of L-[^{14}C]lactate specific radioactivity in tracer metabolic studies. Under specified conditions, the method can also be used to determine the specific radioactivity of L-[^{14}C]pyruvate.

INTRODUCTION

Numerous methods are available to determine the specific radioactivity of [^{14}C]lactate in samples of biological origin containing other radiolabeled compounds. The most commonly used methods are based on the isolation of lactate by ion-exchange (MacRae, 1992; Owens, 1984; Katz, 1981; Gertz, 1981; Prior, 1977), ion-exclusion (Gavino, 1987; De Bruyn, 1988), and thin-layer chromatography (Hochwald, 1985; Leichtweiß, 1981). Other less popular, although probably more discriminating, methods are based on the isolation of lactate by ion exchange chromatography followed by electrophoresis (Ozand, 1978); the oxidation of L-lactate to L-pyruvate by lactate dehydrogenase (EC 1.1.1.28) followed by the derivatization of pyruvate and the purification of the derivative by crystallization (Reilly, 1975) or hydrophobic interaction chromatography (Roca, 1985); and the trapping of $^{14}CO_2$ released by the direct decarboxylation of L-lactate by lactate oxidase from *Mycobacterium smegmatis* (EC 1.13.12.4; McCormick, 1991). Several of these methods and related ones (Gianotti, 1984; Butler, 1989) are also used to determine the specific radioactivity of [^{14}C]pyruvate.

Determinations of [^{14}C]lactate specific radioactivity that rely on a single-step isolation of lactate from other radiolabeled compounds are often of questionable specificity, and determinations based on more elaborate isolation procedures are time consuming. Low recovery and reproducibility are also problems in some determinations

based the isolation of lactate (McCormick, 1991). The rapid and specific microdetermination method developed by McCormick *et al.* (1991) requires significant quantities of lactate oxidase (EC 1.13.12.4), which is no longer available commercially. Furthermore, low concentrations of some common anions, notably chloride, interfere with that enzymatic method.

To overcome these deficiencies, we have developed a convenient and specific enzymatic method for the determination of L-[^{14}C]lactate specific radioactivity in neutralized $HClO_4$ extracts of animal tissues. Under specified conditions, the method can also be used for the determination of L-[^{14}C]pyruvate specific radioactivity. The method is based on the oxidation of L-lactate to L-pyruvate [reaction I] by lactate oxidase from *Pediococcus* sp. (LOD; no EC number assigned), and the decarboxylation of L-pyruvate [reaction II] by pyruvate oxidase from *Pediococcus pseudomonas* (POP; EC 1.2.3.3).

$$\text{L-lactate} + O_2 \rightarrow \text{L-pyruvate} + H_2O_2 \quad [I]$$

$$\text{L-pyruvate} + P_i + O_2 + H_2O \rightarrow \text{acetyl phosphate} + CO_2 + H_2O_2 \quad [II]$$

The CO_2 released by the enzymatic decarboxylation of L-pyruvate is quantitatively collected in a CO_2 trap and its radioactivity is measured in a liquid scintillation counter.

MATERIALS AND METHODS

Materials

L-[1-^{14}C]Lactate and L-[U-^{14}C]lactate were purchased from American Radiolabeled Chemicals; L-[1-^{14}C]pyruvate was from Amersham Life Science; $NaH^{14}CO_3$, benzethonium hydroxide, catalase (catalog no. C-100), lactate standard, and LOD were from Sigma Chemical Co.; Hydrofluor liquid scintillation cocktail was from National Diagnostics; POP from Calbiochem; fatty acid-free bovine serum albumin, and alanine aminotransferase (ALT) were from Boehringer-Mannheim, and ethanolamine, and Microselect grade glutamic acid were from Fluka Chemical Co. All other chemicals and biochemicals were purchased from either Sigma Chemical Co. or Fisher Scientific.

Preparation of $NaH^{14}CO_3$ and [1-^{14}C]pyruvate solutions

A stock solution of $NaH^{14}CO_3$ (10 μM; 150 μCi/ml) was prepared by dissolving solid $NaH^{14}CO_3$ in freshly prepared 50 mM KOH containing 0.02% sodium azide. A stock solution of [1-^{14}C]pyruvate (3 mM; 75 μCi/ml) was prepared by dissolving solid [1-^{14}C]pyruvate in deionized water degassed with argon. Aliquots of the [1-^{14}C]pyruvate solution were frozen and stored under argon at -80°C.

Enzyme assays

The enzymatic activities of POP, LOD, and ALT were routinely assayed using the quality control assay procedures provided by the suppliers. One unit of enzyme activity is defined as the quantity of enzyme that transforms one μmol of substrate per min under the specified assay conditions.

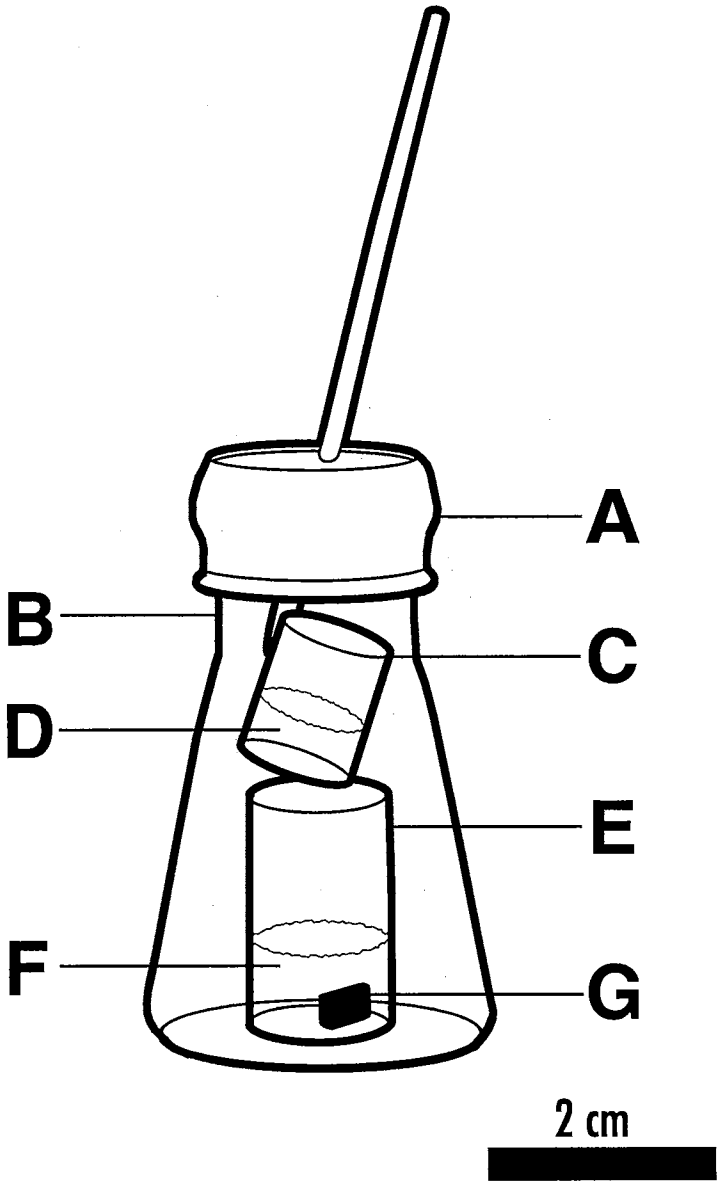
Sample collection and extraction

Blood was collected from the ear (medial artery) of a New Zealand White rabbit in a 7-ml Vacutainer using a 20-G heparinized hypodermic needle. The blood was extracted with 1 vol of 7.5% (v/v) $HClO_4$ as detailed elsewhere (manuscript in preparation). Aliquots of the extracts (pH adjusted to ca. 7.0 with 5 M K_2CO_3) were frozen and stored in liquid N_2. The aliquots were thawed at room temperature just before use.

Center well flasks

Incubation flasks (10 ml capacity; Kontes Catalog No. 882300-0010) were modified to conveniently accommodate small volumes of up to 1.25 ml of reaction mixture by fusing a glass tube (9-mm internal diameter x 18-mm length) to the bottom of each flask (see Fig. 1). The tubes were fused at the center of the flasks so that, if desired,

FIG. 1. CO_2-collection flask. (A) Double-seal rubber stopper; (B) incubation flask; (C) polypropylene center well; (D) CO_2-trapping solution; (E) glass reaction chamber (disposable reaction chamber not shown); (F) reaction mixture; (G) stirring bar.


A
B
C
D
E
F
G
2 cm

a 2-ml capacity microcentrifuge tube cut at the 1-ml mark could be inserted into the glass tube to act as a disposable reaction chamber.

Solutions for the determination of [^{14}C]lactate specific radioactivity

(1) Glutamate—NaOH buffer, 520 mM, pH 8.0. (2) Potassium phosphate buffer, 20 mM, pH 7.0. (3) KH_2PO_4—NaOH buffer, 1 M, pH 6.7. (4) Cocarboxylase (thiamin pyrophosphate chloride), 10 mM in solution 2, pH adjusted to 7.0 with a KOH solution. (5) Disodium flavin adenine dinucleotide, 1 mM in solution 2. (6) BSA 1% (w/v). (7) $MgCl_2$, 100 mM. (8) LOD suspension (250 U/ml at 37°C) prepared by resuspending lyophilized LOD from *Pediococcus sp.* in ice-cold 3.2 M ammonium sulfate in solution 2, pH 6.8. (9) POP suspension (420 U/ml at 37°C) prepared by dissolving lyophilized POP from *P. pseudomonas* in ice-cold solution 2 made 30 µM FAD with solution 5, and slowly adding, with constant stirring, finely ground ammonium sulfate to a final concentration of 3.2 M. (10) CO_2-trapping solution prepared by mixing 1 vol of fresh ethanolamine with 2 vol of ethylene glycol (Cheung, 1989). All pH adjustments were performed at room temperature.

Stability of the solutions

Solutions 1, 4 and 5 were kept for a month and solutions 2, 3, and 7 for several months at 4°C without noticeable effects on the determination of [1-^{14}C]lactate specific

radioactivity. Solution 6 was prepared fresh on the day of use. Suspensions 8 and 9 were kept at 4°C in dark bottles. No decrease in specific activity was detected after 2 months of storage. Solution 10 was kept for a month at room temperature, under argon, in a dark bottle. Solutions 2, 3 and 7 were made 0.02% (w/v) sodium azide to help prevent microbial growth.

Removal of pyruvate from blood extracts

This procedure is based on the use of ALT to remove pyruvate, as described in the enzymatic determination of lactate with lactate dehydrogenase (Noll, 1984). Aliquots (50 µl) of blood extracts spiked with either [1-^{14}C]pyruvate or [U-^{14}C]lactate were individually mixed with 100 µl of deionized water and 50 µl of glutamate—NaOH buffer. The aliquots were warmed to 37°C and ALT (*ca.* 800 U/ml of ammonium sulfate suspension) was added to a final concentration of 3.5 U/ml. Following a 30-min incubation at 37°C, ALT was completely and permanently inactivated by adding 100 µl of ice-cold 15% (v/v) $HClO_4$ to each aliquot. ALT was inactivated to avoid competition with POP for [^{14}C]pyruvate during the determination of [^{14}C]lactate specific radioactivity. The aliquots were incubated on ice for 10 min and the pH of each aliquot was adjusted to ca. 7.0 by adding a predetermined volume of 5 M K_2CO_3. The suspensions were centrifuged 5 min at 15,000g at 4°C and the supernatants were used as samples in determinations of [U-^{14}C]lactate specific radioactivity.

Determination of [^{14}C]lactate specific radioactivity

The reaction mixture is based on Calbiochem's assay mixture for determining POP activity. Unless otherwise specified, assay flasks were prepared by introducing into each reaction chamber a magnetic stirring bar (5 x 2 mm) and the following reaction components: 125 µl of deionized water, 40 µl of KH_2PO_4—NaOH buffer, 25 µl of BSA solution, 20 µl of $MgCl_2$ solution, 5 µl of cocarboxylase solution, 2 µl of FAD solution, 2 µl of catalase solution (EC 1.11.1.6; *ca.* 5.5 x 10^6 units per ml), 25 µl of blood extract (pH 6.0 to 8.0; the pH of the strongly buffered reaction mixture is not affected by the extract in this pH range), 0.5 µl of LOD suspension, and 2.4 µl of POP suspension. Immediately after addition of the POP suspension, each center well flask was sealed with a double seal rubber stopper (Kontes, Catalog No. 882310-0000) in which was inserted a polypropylene center-well (Kontes, Catalog No. 882320-0000) containing 150 µl of the CO_2 trapping solution (see Fig. 1). The reaction mixtures were quickly stirred on a magnetic stirrer and the flasks were incubated for 90 min (unless otherwise specified) at 37°C. At the end of the incubation 125 µl of 30% (v/v) $HClO_4$ was injected into the reaction chamber of each flask by means of a hypodermic needle inserted through the stopper. Care was taken to avoid spilling acid into the polypropylene center wells. The acidified reaction mixtures were stirred with a magnetic stirrer and incubated for 1 to 2 h at room temperature to allow quantitative trapping of the CO_2.

At the end of the CO_2-collection period, the flasks were opened and the polypropylene center wells were cut from their support rods and dropped into 6-ml capacity scintillation vials containing 4 ml of Hydrofluor scintillation cocktail. The vials were vigorously shaken and 1.2 or 1.5 ml of water was added to the content of each vial to form a stable gel. Radioactivity was measured to the desired precision in a liquid scintillation counter (Packard, model 1900CA). Despite the relatively high capacity of Hydrofluor gel to retain dissolved CO_2, we found it necessary to almost completely fill the scintillation vials to obtain counts that were stable for a few hours (for an alternative strategy see Gaines, 1988).

Radioactivity of the acidified reaction mixtures was measured by transferring the entire reaction mixture from each assay flask into a separate 6-ml scintillation vial containing 4 ml of scintillation cocktail. Following this transfer, each reaction chamber was washed twice with 450 or 600 µl of deionized water and these washes were added to the corresponding vial of acidified mixture. The vials were vigorously shaken and radioactivity was measured as above. The extent of decarboxylation was expressed as the percentage of total radioactivity ($^{14}CO_2$ + reaction mixture) recovered as $^{14}CO_2$ radioactivity.

Kinetics of CO_2 collection

CO_2-collection flasks were prepared as described above with the regular assay mixture except that 75 µl of water and 50 µl of diluted $NaH^{14}CO_3$ solution were used in place of the usual 125 µl of water, and the POP and LOD suspensions were substituted by a 3.2 M ammonium sulfate solution. In some flasks, 150 µl of benzethonium hydroxide (1.0 M in methanol) was used in place of the usual CO_2-trapping solution. $HClO_4$ (30%, v/v) was injected in the reaction chambers as above, and the flasks were incubated at room temperature for 30 min, 1 and 24 h. Radioactivity of the collected $^{14}CO_2$ was measured as above. The extent of $^{14}CO_2$ collection is expressed as the percentage of $NaH^{14}CO_3$ radioactivity recovered as $^{14}CO_2$ radioactivity in the center wells.

Lactate determination

Total L-lactate was determined enzymatically as described by Noll (1984) using a lactate standard for calibration. The endogenous lactate concentration in the blood extract was 1.58 ± 0.01 mM (n = 3).

Data presentation

All data are presented as mean ± SD.

RESULTS AND DISCUSSION

Enzymes

LOD from *Pediococcus* sp. is commonly used in colorimetric[3] (Bozimowski, 1985; Shimojo, 1989) and amperometric (e.g., Bardeletti, 1986; Wandrup, 1989; Shimojo, 1991; Wang, 1993; Spohn, 1996) determinations of L-lactate. LOD was selected for our study because of its low K_m for L-lactate (0.7 mM),[4] its high substrate specificity,[3,4] and its high specific activity under POP assay conditions (data not shown). POP from *Pediococcus* sp. was used by Bozimowski *et al.* (1985) for the colorimetric determination of L-pyruvate. It was selected for our study because of its relatively low K_m for L-pyruvate (1.7 mM at 37°C) and its high substrate specificity[5] (Bergmeyer, 1983).

Kinetics of CO_2 collection

The extent of $^{14}CO_2$ collection in our assay system was 96.2 ± 1.0% after 30 min, 98.8 ± 0.8% after 1 h, and 99.4 ± 0.8% after 24 h of incubation (n = 5 in each case). For

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[3] Lactate determination kit from Sigma Chemical Co., Catalog No. 735-10

[4] Product data sheet for LOD from Sigma Chemical Co., Catalog No. L 0638

[5] Product data sheet for POP from Calbiochem, Catalog No. 550700

our routine assay conditions we used a CO_2-collection period of 1 to 2 h. Long (e.g., overnight) collection periods are not recommended since non specific hydrolytic release of $^{14}CO_2$ from ^{14}C-labeled compounds can cause a significant overestimation of the measured specific radioactivity (data not shown). Interestingly, rates of $^{14}CO_2$ collection with the ethanolamine:ethylene glycol mixture were much faster than with the more commonly used benzethonium hydroxide (better known under the trade name Hyamine hydroxide). Only 68.9 ± 2.1% (n = 5) of the total $^{14}CO_2$ was collected with benzethonium hydroxide after 1 h of incubation.

Lactate decarboxylation by pyruvate oxidase

Control experiments performed with the regular assay mixture without LOD revealed that 88.8 ± 0.2% (n = 4) and 95.4 ± 0.2% (n = 4) of [1-^{14}C]lactate were decarboxylated in the presence of 1.0 and 2.0 U/ml of POP, respectively. In contrast, no significant decarboxylation of [1-^{14}C]lactate was observed (0.20 ± 0.01%, n = 5) when both POP and LOD were omitted from the assay mixture. Direct assays of POP revealed that contamination by LOD was always less than 3 x 10^{-5} units of LOD per unit of POP. This level of contamination cannot account for more than 0.5% of the [1-^{14}C]lactate decarboxylation observed in the presence of POP (1 U/ml) and absence of LOD. We conclude that POP readily decarboxylates lactate. Therefore, POP cannot be used to selectively decarboxylate [^{14}C]pyruvate in the presence of [^{14}C]lactate. To circumvent

this problem, tissue extracts can be pretreated with ALT to selectively remove [^{14}C]pyruvate (see below). It should be noted that since the decarboxylation of lactate by POP does not produce H_2O_2, it does not interfere with the colorimetric determination of pyruvate with POP and horseradish peroxidase (Bozimowski, 1985).

Removal of [1-^{14}C]pyruvate from tissue extracts

[1-^{14}C]Pyruvate was conveniently removed from spiked blood extracts by treating the extracts with ALT. Determinations of [1-^{14}C]pyruvate specific radioactivity performed before and after ALT treatment indicate a nearly complete transformation of pyruvate (96.6 ± 0.2%, n = 4). In contrast, control experiments performed with [U-^{14}C]lactate-spiked blood extracts indicate that ALT treatment does not affect lactate (98.8 ± 0.4% of [U-^{14}C]lactate recovered after ALT treatment, n = 4). This result is consistent with the widespread use of ALT in lactate determinations (Noll, 1984). [U-^{14}C]Lactate was used in control experiments because an unidentified contaminant in the commercial [1-^{14}C]lactate was found to react with ALT and the glutamate solution, producing a potent unidentified inhibitor of POP and possibly LOD. As a cautionary note, it should be mentioned that radioactivity was not evenly distributed among the carbons of [U-^{14}C]lactate. We determined, using the method present, that 49.9 ± 0.2% of the total radioactivity of [U-^{14}C]lactate was on carbon-1 (n = 4).

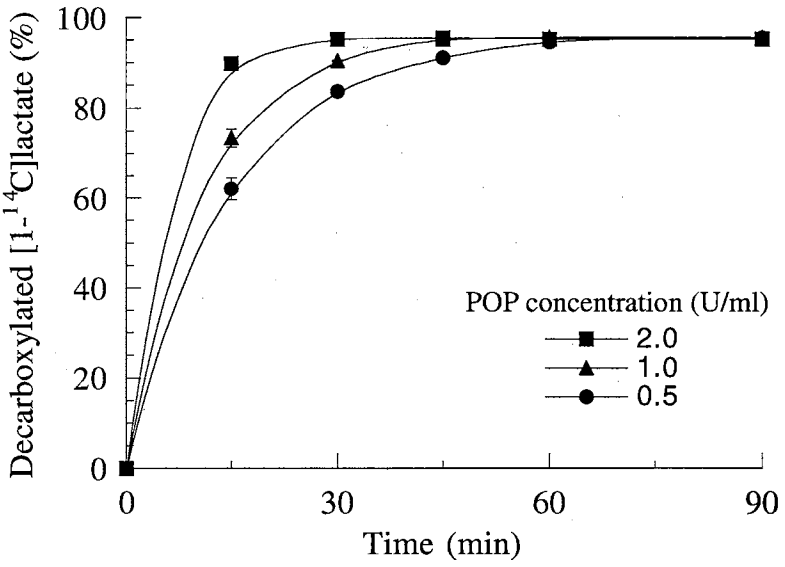
Attempts to remove pyruvate by H_2O_2 treatment (Schadewaldt *et al.*, 1984) were abandoned when we found that this treatment resulted in the concomitant decarboxylation of [1-^{14}C]pyruvate and [1-^{14}C]lactate, in contrast to the report of Schadewaldt *et al.* (1984).

Kinetics of [1-^{14}C]lactate decarboxylation

The kinetics of [1-^{14}C]lactate decarboxylation with different concentrations of POP are shown in Fig. 2 for our assay system. After 90 min of incubation, 95.4 ± 0.1% of the radioactivity of [1-^{14}C]lactate was recovered as $^{14}CO_2$ radioactivity at all POP concentrations tested. A POP concentration of 1.0 U/ml and a 90 min incubation period were chosen for our routine assay conditions. Doubling the concentration of LOD from 0.5 U/ml to 1.0 U/ml did not alter the kinetics of the assay (data not shown), indicating that the rate-limiting step of the coupled reactions is the decarboxylation of pyruvate by POP. Similarly, pre-treatment of the $HClO_4$ extract with ALT did not affect the kinetics of the assay (data not shown). The extent of [1-^{14}C]lactate decarboxylation was essentially independent of the total lactate concentration in the range tested (94.8 ± 0.2% and 95.5 ± 0.1% of [1-^{14}C]lactate decarboxylated at assay concentrations of 2.5 and 500 μM lactate, respectively, $n = 3$).

The radiochemical purity of the [1-^{14}C]lactate and [1-^{14}C]pyruvate used in this study was ca. 99 and 98.0%, respectively, as specified by the suppliers. Based on the

FIG. 2. Kinetics of [1-^{14}C]lactate decarboxylation with different concentrations of pyruvate oxidase (POP). Measurements were performed as described under Materials and Methods. Results are expressed as the percentage of [1-^{14}C]lactate decarboxylated. Symbols and error bars represent means ± SD of triplicate measurements. Error bars ≤ 1.1% are covered by the symbols.


Decarboxylated [1-^{14}C]lactate (%)
100
80
60
40
20
0
0
30
60
90
Time (min)
POP concentration (U/ml)
2.0
1.0
0.5

kinetic constants of LOD and POP, and the thermodynamic equilibria of the reactions they catalyze, a nearly complete (100%) transformation of their limiting substrates (lactate and pyruvate) is expected under our assay conditions. Since the [1-^{14}C]lactate we used was prepared from unlabeled acetaldehyde, it should have 100% of its label on carbon-1. However, as we have seen, when [1-^{14}C]lactate was used as a substrate, the percentage of substrate radioactivity recovered as $^{14}CO_2$ radioactivity was only 95.4 ± 0.1% (Fig. 2) compared to 97.4% ± 0.2% (n = 4) when [1-^{14}C]pyruvate was used as a substrate. The lower than expected percentage of [1-^{14}C]lactate decarboxylation may reflect that our enzymatic determination is more specific than the thin layer chromatography analysis used by the [1-^{14}C]lactate supplier.

Temperature of the [1-^{14}C]lactate determination

Determinations were performed at 37°C since the initial rates of pyruvate decarboxylation by POP were several-fold lower at 25°C (data not shown). This temperature dependence was much smaller with near-saturating concentrations of pyruvate, indicating that the thermal effect was primarily on the K_m of this reaction. Rates of lactate transformation by LOD varied only slightly when the temperature was decreased from 37 to 25°C (data not shown).

Precision of the determination of [1-^{14}C]lactate specific radioactivity

As mentioned earlier, the results presented above were calculated from the ratio of $^{14}CO_2$ radioactivity to total radioactivity ($^{14}CO_2$ + reaction mixture). However, when other radiolabeled compounds are present in addition to the analyte, only the radioactivity of $^{14}CO_2$ is measured. This decreases the precision of the determination by introducing pipetting errors. When only $^{14}CO_2$ radioactivity was measured, the precision of the determination decreased from 0.1% (Fig. 2) to 2.2% (n = 5) and 1.5% (n = 6) with and without ALT treatment, respectively. This precision is comparable to the precision of a total L-lactate determination with lactate dehydrogenase and ALT (2.3% relative SD; Noll, 1984). We estimated the precision of the determination without ALT treatment since, under most experimental conditions, the [^{14}C]lactate/[^{14}C]pyruvate concentration ratios in tissue extracts are so high that removal of [^{14}C]pyruvate prior to [^{14}C]lactate determination may not be necessary.

Determination of [^{14}C]pyruvate specific radioactivity

The method we present can be used to indirectly determine the specific radioactivity of [^{14}C]pyruvate by subtracting the radioactivity of [^{14}C]lactate (determined after ALT treatment) from that of [^{14}C]lactate plus [^{14}C]pyruvate (determined without ALT treatment). Unfortunately, because of the subtractive nature of the determination,

[1-^{14}C]pyruvate can only be accurately determined when the [^{14}C]lactate/[^{14}C]pyruvate concentration ratio is low.

Critique of the method

The advantages and limitations of specific radioactivity determinations of metabolic intermediates by enzymatic decarboxylation have long been recognized (Strang, 1971). Advantages include specificity, speed (especially with multiple determinations), simplicity, sensitivity, high sample recovery, small sample size, and no need for intermediate isolation or specialized equipment. Limitations include the requirement that the original metabolite be radiolabeled on the carbon that is to be decarboxylated and that the extent of labeling on that carbon be known, the somewhat expensive cost of enzymes (the current cost of POP is about $0.75 per unit), and the susceptibility of enzymatic determinations to substances that interfere with enzyme activity.

A major advantage of our determination over the only other enzymatic determination of L-[^{14}C]lactate specific radioactivity (McCormick, 1991) is its insensitivity to anions commonly found in extracts of animal tissues. Results show that increasing the extract volume to 20% of the total assay volume does not affect our determination (95.6 ± 0.1% of [1-^{14}C]lactate decarboxylated, n = 3).

In conclusion, the enzymatic method we present offers an attractive alternative to the methods currently available to determine L-[^{14}C]lactate specific radioactivity in complex biological samples containing other radiolabeled compounds.

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CHAPTER V

ASSESSMENT OF CELL MICROPERMEABILIZATION BY MEASUREMENTS OF LACTATE PRODUCTION

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PREFACE

While searching for a suitable permeabilized cell system to study glycolysis *in situ*, we discovered that permeabilization of the plasma membrane with α-toxin completely abolishes the capacity of cultured mammalian cells to produce lactate in the absence of exogenously added cofactors, but not in their presence. Being painfully aware of the critical need for simple and convenient methods to assess the permeabilization of cells to ions and small molecules (micropermeabilization), we proceeded to develop a novel assessment method based on measurements of lactate production in the absence and presence of exogenously added cofactors. In this chapter, we present this method which should greatly facilitate the assessment of cell micropermeabilization by both novices and experts in cell micropermeabilization techniques. Furthermore, given the misleading literature on the (micro)permeabilization of cells with α-toxin (see chapter VI), our method may provide inexperienced α-toxin users with an invaluable tool having the potential to turn an otherwise frustrating research endeavor into a gratifying one. Finally, it should be noted that although the research described in this chapter should prove very useful for our planned study of glycolysis *in situ*, the permeabilized cell system presented was optimized for the assessment of cell micropermeabilization and is therefore *not* intended for the study of glycolysis *in situ* without modifications and additional controls.

ABSTRACT

We present a method for the routine assessment of the permeabilization of cells to ions and small molecules (micropermeabilization). The method, which uses loss and recovery of cellular lactate production as an indicator of micropermeabilization, is simple, convenient, and requires only conventional lactate determinations and cell counts. Rat basophilic leukemia cells permeabilized with α-toxin from *S. aureus* were used as a model micropermeabilization system. The data we present also show that most metabolic studies using permeabilized cells would likely benefit from supplying the cells with an exogenous source of P_i, and that cells made permeable to Mg^2 and ATP can nevertheless retain a high concentration of intracellular ATP and actively produce lactate in the absence of exogenous Mg^2 and ATP.

INTRODUCTION

Micropermeabilization of the plasma membrane can facilitate the study of intracellular processes by permitting the manipulation of the ion and small molecule composition of the intracellular milieu without affecting the macromolecule composition, in contrast to macropermeabilization. Because the extent of micropermeabilization and the pore sizes achieved often vary with the technique, permeabilization conditions, cell type, and less understood factors that can cause variations from one preparation of cells to another (e.g., plasma membrane composition), the results of micropermeabilization

procedures must be assessed routinely. Simple and convenient methods to assess micropermeabilization are therefore needed. Furthermore, because of the ongoing explosion of research using micropermeabilization techniques, the need for such methods is increasing rapidly.

The methods currently available to assess cell micropermeabilization can be classified in three broad categories: 1) radioactive methods based on the uptake or release of radioisotopes (e.g., $^{86}Rb^{+}$) and small radiolabeled molecules (e.g., ^{32}P–ATP) (Anhert-Hilger *et al.*, 1989; Bauldry *et al.*, 1992); 2) visual methods based on the uptake of dyes (e.g., trypan blue) and the uptake or release of fluorescent molecules (e.g., lucifer yellow, fluorescein diacetate) (Steinberg and Siverstein, 1989; Bauldry *et al.*, 1992); and 3) methods based on the leakage of intracellular ions (e.g., the determination of K^{+} and Na^{+} with flame photometry; Sung *et al.*, 1985) and small molecules (e.g., the determination of ATP with luciferase (Stuart *et al.*, 1994)).

Radioactive methods present the disadvantages associated with the use of radioisotopes (health hazard, cumbersome handling procedures, high cost, etc.). In addition, the resolution of methods based on the release of pre-loaded radioactive markers is generally limited by leakage of the markers from the intact cells used as a zero reference for micropermeabilization. This leakage represents a serious problem if it is critical to establish that all treated cells are micropermeabilized. This is unfortunate since quantitative data is more readily obtained from release than uptake experiments.

Disadvantages of visual methods include the relatively large size of the dyes and fluorophores, difficulty in obtaining a crisp distinction between negative and positive results, and the lability of micropermeabilized cells under certain staining conditions. Visual methods also tend to be time consuming and therefore inconvenient for the routine processing of numerous samples. Finally, methods based on the leakage of intracellular ions and small molecules require specialized equipment. In addition, some of these methods, like ATP determinations with luciferase, are also delicate and sensitive to artifacts (Ford and Leach, 1998).

To overcome these disadvantages, we have developed a simple and convenient method for the routine assessment of cell micropermeabilization. The method, which uses loss and recovery of cellular lactate production as an indicator of micropermeabilization, requires only conventional lactate determinations and cell counts.

MATERIALS AND METHODS

Materials

PC12 cells (Greene and Tischler, 1976) an the 2H3 subline (Barsumian *et al.*, 1981) of RBL cells were obtained from the American Type Culture Collection (ATCC). Stock *Staphylococcus aureus* strain Wood-46 producing a high titer of α-toxin (see Harshman *et al.*, 1988) was a generous gift from the late Dr. Sidney Harshman (Vanderbilt University School of Medicine). Defined fetal bovine serum and Water For

Injection (WFI) quality water were from Hyclone Laboratories. Heat-inactivated donor grade horse serum was from Atlanta Biologicals. Liquid Hank's balanced salt solution without Ca^{+2} and Mg^{+2}, Eagle's minimum essential medium, RPMI 1640 medium, tissue culture grade water, and solutions of $MgCl_2$, non-essential amino acids, and sodium pyruvate were from Biofluids/Biosource International. Tryptic soy broth (Catalog No. 0370-01-1) was from Difco. Phenyl Sepharose Cl-4B (for α-toxin isolation) was from Amersham Pharmacia Biotech. Enzymes, coenzymes (highest quality available), fatty acid free BSA (Catalogue No. 100 069), and the ATP Bioluminescence Assay Kit CLS II were from Boehringer-Mannheim. Microselect grade glutamic acid was from Fluka Chemical Co. $1,N^6$-ethenoadenosine 5'-triphosphate (ε-ATP) was from Molecular Probes. *myo*-[2-^{3}H(N)]inositol (in sterile water) was from American Radiolabeled Chemicals. Ultima Gold scintillation cocktail was from Packard. Solutions for SDS-PAGE and the Silver Stain Plus kit were from Bio-Rad. All other chemicals and biochemicals were purchased from either Sigma Chemical Co. or Fisher Scientific. Tissue culture grade water was used to prepare all solutions used with cells, WFI water was used for the determination of ATP, and water purified with a NANOpure ultrapure water system (Barnstead) was used to prepare all other solutions.

Isolation of α-toxin

S. aureus was grown as described by Harshman (1988), but in tryptic soy broth. α-Toxin was isolated from the culture fluid as described by Hohman (1988). Except for the presence of a minor unidentified impurity of $M_r \approx 18{,}000$, the purified α-toxin was essentially free of impurities as judged by analytical SDS-PAGE (12.5% polyacrylamide slab gels; Laemmli *et al.*, 1970) and silver staining with a Silver Stain Plus kit. Protein concentration was determined with the Biuret method (Gornall *et al.*, 1949).

Culture and preparation of RBL 2H3 cells

RBL 2H3 cells were maintained as described by Hohman (1988) except that the growth medium contained 4 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM MEM non-essential amino acids. Cells grown to a density of ca. 2×10^5 cells per cm^2 of substrate were detached for experimental procedures by exposure to trypsin/EDTA (0.05%/0.02%) pre-warmed to 37°C. The trypsin was neutralized with 2 volumes of complete growth medium pre-warmed to the same temperature. The cells were then washed twice in complete growth medium, twice in ice-cold buffered saline (40 mM MOPS, 110 mM NaCl, 5 mM KCl, and 1% (w/v) BSA, pH adjusted to 7.1 at 25°C with NaOH), once in ice-cold permeabilization buffer (40 mM MOPS, 140 mM glutamic acid,

0.1 mM EGTA, and 1% (w/v) BSA, pH adjusted to 7.1 at 25°C with KOH), and resuspended to 5×10^6 cells/ml.

Culture and preparation of PC12 cells

PC12 cells were maintained as described by Greene *et al.* (1987). Cells in exponential growth phase were detached for experimental procedures by exposure to trypsin (0.1%) pre-warmed to 37°C. The trypsin was neutralized with 2 volumes of complete growth medium pre-warmed to the same temperature. Clumped cells were dissociated by repeated passage through a 21G × 3.5 inch spinal needle. The cells were washed in complete growth medium and reseeded at a density of ca. 4×10^6 cells/ml. Approximately 18 hours after reseeding the cells were detached and dissociated as before. The cells were further washed and resuspended as described for RBL 2H3 cells.

Permeabilization

Stock suspensions of α-toxin were centrifuged (20 min at 16,000g at 4°C) and the protein was redissolved in permeabilization buffer. Cell suspensions (5×10^6 cells/ml in permeabilization buffer) were pre-warmed to 37°C and incubated 30 min at this temperature (unless otherwise specified) in the presence of the indicated concentrations of α-toxin. The concentration of $(NH_4)_2SO_4$ introduced by the α-toxin solution was less than 0.5 mM at [α-toxin] = 8 μg/ml, as determined with the Nessler method (Hawk *et al.*,

1954) with slight modifications (to be described elsewhere). The incubation was terminated by centrifugation (4 min at 300g at 4°C) of the cell suspensions. The cells were immediately washed by resuspension (5×10^6 cells/ml) in ice-cold permeabilization buffer, centrifuged as above, and resuspended in the same buffer.

Loss and recovery of lactate production

Aliquots (250 µl) of intact and permeabilized cells in suspension (5×10^6 to 8×10^6 cells/ml in permeabilization buffer) were pre-warmed to 37°C and mixed with an equal volume of pre-warmed permeabilization buffer containing, unless otherwise specified, 20 mM glucose, $MgCl_2$ at 4 mM in excess of the concentration of ATP, 60 mM potassium phosphate, 2 mM NAD^+, and 6 mM ATP. When the permeabilization buffer contained potassium phosphate, the concentration of glutamic acid in the buffer was adjusted to maintain the final concentration of potassium constant. The concentrations of glucose, $MgCl_2$, potassium phosphate, NAD^+, and ATP used were those experimentally found to be near optimal for maximum lactate production. The cells were incubated 30 min at 37°C with continuous shaking. The incubation was terminated by adding 250 µl of ice-cold 3.5 M $HClO_4$ to each aliquot of cell suspension, except to aliquots of intact and α-toxin-treated cells kept for trypan blue staining and cell counting. Lactate was extracted by $HClO_4$ treatment and determined enzymatically with lactate

dehydrogenase and alanine aminotransferase, as previously described (Lehoux *et al.*, 1997).

[^{3}H]myo-inositol release from pre-loaded cells

The release of pre-loaded [^{3}H]*myo*-inositol by PC12 cells exposed to α-toxin was measured as described by Hohman (1988) except that total releasable [^{3}H]*myo*-inositol was determined by incubating the cells with 160 μg of α-toxin per ml for 30 min at 37°C. Under these conditions all PC12 were permeabilized (lysed) as indicated by trypan blue staining.

Fluorescence cell staining

Aliquots of intact and permeabilized cell suspensions (ca. 5×10^6 cells/ml in permeabilization buffer containing only 0.1% BSA) were incubated 30 min at 37°C in the presence of 25 μg/ml of ε-ATP or 10 μg/ml of 1,N^6-ethenoadenine nicotinamide dinucleotide (ε-NAD). The cells were washed twice with 2 vol of ice-cold permeabilization buffer and resuspended in the same buffer to 5×10^6 cells/ml. Immediately, aliquots of cell suspensions were placed on a glass slide and examined under an epifluorescence microscope equipped with UV optics. Extracellular hydrolysis of ε-ATP was negligible as judged by determinations of extracellular ATP with luciferase.

Determination of ATP with luciferase

ATP was determined with an ATP Bioluminescence Assay Kit CLS II and a Lumac/3M Biocounter. Controls were performed as recommended by Ford and Leach (1998). Intracellular ATP was measured from neutralized $HClO_4$ extracts of intact and α-toxin-treated RBL 2H3, prepared as described above. Suspensions of intact and α-toxin-treated cells were centrifuged through a hydrocarbon mixture into 7% (v/v) $HClO_4$ and the upper layer was used without delay to determine extracellular ATP (Stuart *et al.*, 1994).

Determination of cell concentration and permeability to trypan blue

Cell concentration and the percentage of cells excluding the dye trypan blue (M_r 961) were determined by counting cells in an improved Neubauer hemocytometer in the presence of 0.1% (w/v) trypan blue in BSA-free buffered saline or permeabilization buffer. Cells that stained dark blue (in phase contrast microscopy) within 2 min were considered permeable to the dye. All cell counts were performed at least in duplicate and ca. 400 cells were counted each time. At least 97% of the cells (intact and permeabilized) excluded trypan blue in all our experiments.

Data presentation

All data are presented as mean ± SD. Lactate productions were compared using Student's t-tests (two-tailed) with a 0.05 level of significance. Unless otherwise specified, lactate production is expressed in percentage using lactate production by intact cells supplied with glucose and P_i as an arbitrary 100% reference point. Lactate production is expressed in percentage to reduce experiment to experiment fluctuations in the results. The contribution of intracellular lactate present at the start of the incubations with glucose was negligible, unless otherwise specified.

RESULTS AND DISCUSSION

Micropermeabilization system

The use of cellular lactate production as an indicator of micropermeabilization was investigated using rat basophilic leukemia cells (subline 2H3) exposed to α-toxin from *S. aureus*. α-Toxin (also known as α-hemolysin) is an extracellular protein of M_r 33,400 that binds to the plasma membrane of eucaryotic cells and self-associates to form a transmembrane pore-complex that can allow the passage of ions and small molecules (for review see Bhakdi *et al.*, 1993; Bhakdi and Tranum-Jensen, 1991). We used α-toxin as a micropermeabilizing agent because of the significant advantages it

offers and its widespread use (for reviews see Bhakdi *et al.*, 1993; Bhakdi and Tranum-Jensen, 1991; Felix, 1982).

Assessment of micropermeabilization

Exposure to α-toxin decreased the production of lactate by RBL 2H3 cells supplied with glucose, but none of the essential cofactors of glycolysis (ATP, Mg^{2+}, NAD^+, and P_i). As shown in Fig. 1 (lower curve, closed symbols), the decrease was dose-dependent with lactate production being essentially abolished by exposure to [α-toxin] ≥ ca. 4 μg/ml. Supplying the cells with Mg^{2+} and ATP in addition to glucose only marginally increased lactate production (0.5 ± 0.5% vs. 5.0 ± 0.5% in the absence and presence of Mg^{2+} and ATP; Fig. 2, columns #6 and #9, respectively). In contrast, supplying the cells with P_i in addition to glucose, Mg^{2+}, and ATP increased lactate production from 5.0 ± 0.5% to 101 ± 1% (Fig. 2, columns #9 and #15, respectively). This impressive recovery of lactate production indicates that, in the absence of exogenous P_i, there is a depletion of cytoplasmic P_i in the α-toxin-treated cells. Since this depletion must result from the leakage of P_i through α-toxin pore-complexes, cellular lactate production can be used as an indicator of permeabilization to P_i.

Loss and recovery of lactate production can also be used to assess permeabilization to ATP and possibly NAD^+. As shown in Fig. 2, supplying α-toxin-permeabilized RBL 2H3 cells with MgATP in addition to glucose, P_i, and Mg^{2+} increased

FIG. 1. Effect of α-toxin from *S. aureus* on the production of lactate by RBL 2H3 cells. The cells were incubated 30 min at 37°C in permeabilization buffer containing α-toxin at the indicated concentrations. The α-toxin-treated cells were washed and incubated 30 min at 37°C in permeabilization buffer containing 10 mM glucose (●); 10 mM glucose, 2 mM $MgCl_2$, and 30 mM P_I (○); and 10 mM glucose, 5 mM $MgCl_2$, 30 mM P_i, and 3 mM ATP (□). Lactate production was determined and expressed as a percentage of the lactate production of intact cells incubated in permeabilization buffer containing 10 mM glucose and 30 mM P_i. Symbols and error bars represent means ± SD of triplicate measurements. Error bars < 1 % are covered by the symbols.

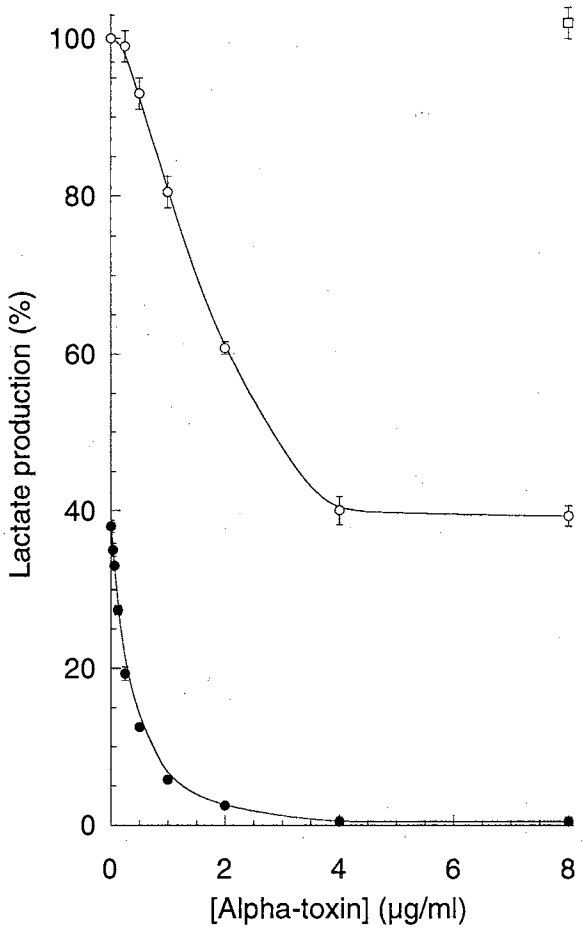
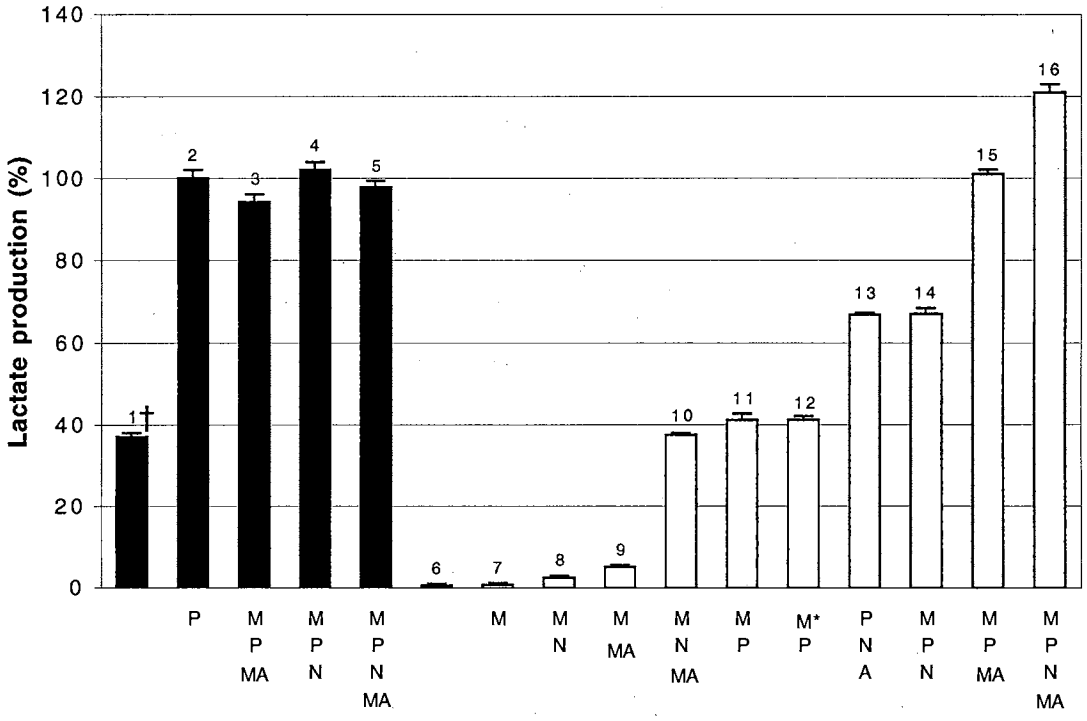

100
80
60
40
20
0
Lactate production (%)
0
2
4
6
8
[Alpha-toxin] (µg/ml)

FIG. 2. Production of lactate by intact (closed bars) and α-toxin-permeabilized (open bars) RBL 2H3 cells incubated 30 min at 37°C in permeabilization buffer containing 10 mM glucose, and where indicated, 2 mM $MgCl_2$ (M), 5 mM $MgCl_2$ (M*), 1 mM NAD^+ (N), 3 mM ATP (A), 3 mM MgATP (MA), and 30 mM P_i (P). Lactate production was determined and expressed as a percentage of the lactate production of intact cells incubated in permeabilization buffer containing 10 mM glucose and 30 mM P_i. The cells were permeabilized as described in Fig. 1 with 8 μg/ml of α-toxin. Columns and error bars represent means + SD of triplicate or tetraplicate measurements. Columns are numbered for reference. †The production of lactate by intact cells incubated with glucose, but without P_i, was decreased from ca. 90% to ca. 50% by the 30 min incubation at 37°C – no decrease was observed when the cells were maintained on ice.

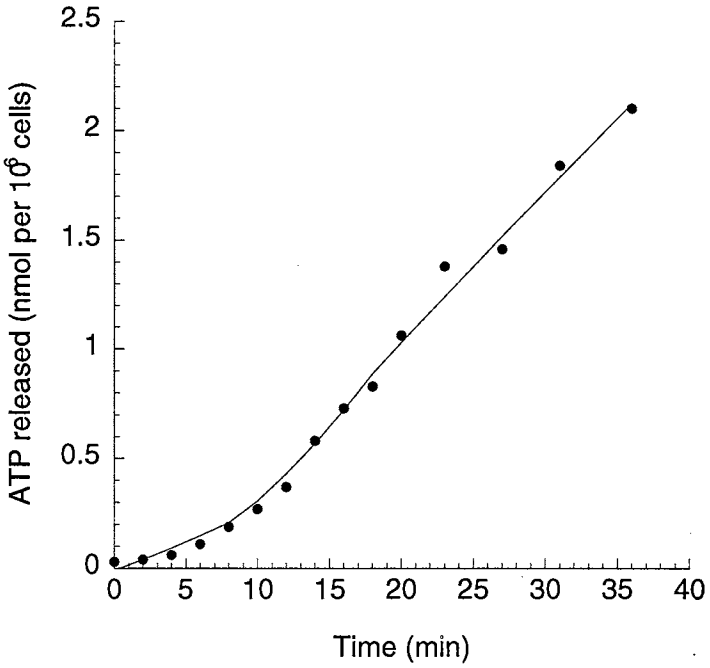

Lactate production (%)
140
120
100
80
60
40
20
0
1†
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
P
M P MA
M P N
M P N MA
M
M N
M MA
M N MA
M P
M* P
P N A
M P N
M P MA
M P N MA

the production of lactate from 41 ± 1% to 102 ± 2% (columns #11 and #15, respectively). This increase in lactate production cannot be attributed to the Mg^{2+} accompanying ATP since Mg^{2+} had no significant effect on the production of lactate in the absence of ATP (41 ± 1% vs. 41 ± 1% in the absence and presence of an extra 3 mM of Mg^{2+}, respectively; Fig. 2, columns #11 and #12, respectively). To control for the possibility that the effect of MgATP on lactate production was a response to extracellular ATP (or MgATP), intact cells were incubated with glucose, P_i, Mg^{2+}, and NAD^+ with and without MgATP. Lactate production was slightly lower in the presence than in the absence of MgATP (98 ± 2% vs. 102 ± 2%, respectively; Fig. 2, columns #5 and #4, respectively). These results strongly suggest that the observed effect of MgATP on the production of lactate in α-toxin-permeabilized cells was due to MgATP entering the cells. However, before a definite conclusion is reached, the unlikely possibility that the observed effect is due to ADP or AMP (i.e., hydrolyzed ATP) entering the cells must be eliminated — this is an unlikely possibility since (1) a relatively low [ATP] was used, (2) the ATP used was 100% pure, and (3) luciferase determinations of ATP failed to detect ATP hydrolysis in the supernatant of permeabilized cell suspensions. Evidence that Mg^{2+} entered the cells is provided by the observation that the production of lactate in permeabilized cells decreased from 121 ± 2% to 67 ± 1% when Mg^{2+} was omitted from medium containing all the essential glycolytic cofactors (Fig. 2, columns #16 and #13).

The production of lactate by RBL 2H3 cells supplied with glucose, Mg^{2+}, and P_i, reached a minimum of ca. 40% at [α-toxin] ≤ 4 μg/ml (Fig. 1, upper curve, opened symbols). Not surprisingly, this is the same [α--toxin] needed to permeabilize the entire population of RBL 2H3 (lower curve, closed symbols). However, the relatively high minimum production of lactate by cells supplied with glucose, Mg^{2+}, and P_i, but no ATP requires some explanation. Determinations of intracellular ATP showed that the concentration of ATP remained high inside RBL 2H3 cells after permeabilization with 8 μg/ml of α-toxin (2.55 ± 0.07 nmol/10^6 cells after permeabilization vs. 3.55 ± 0.05 nmol/10^6 cells immediately before permeabilization and 3.48 ± 0.12 nmol/10^6 cells immediately after harvesting, n = 3 in all cases). Determinations of extracellular ATP also showed massive leakage of ATP during the incubation with α-toxin (Fig. 3). However, ATP leakage did not show signs of plateauing even after 35 min of incubation. This result suggests that the leakage of ATP was slowed either by microcompartmentation or limited permeability of the plasma membrane. Our results also show significant synthesis of ATP during the incubation with α-toxin (> 1.5 nmol of ATP per 10^6 cells was released after 30 min of incubation with α-toxin, but the concentration of intracellular ATP decreased by only ca. 1 nmol/10^6 cells).

The results of the ATP determinations raised an important question. Was the high concentration of intracellular ATP remaining after permeabilization due to some cells

FIG. 3. Release of ATP from RBL 2H3 cells during permeabilization with 8 μg/ml of α-toxin from *S. aureus*. The cells were permeabilized as described in Fig. 1, aliquoted at the indicated times, centrifuged through a hydrocarbon mixture into 7% (v/v) $HClO_4$, and ATP was determined in the upper layer. Each data point represents a single measurement.


ATP released (nmol per 10^6 cells)
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
40
Time (min)

being permeabilized to ATP while others were not (an unlikely possibility given the micropermeabilizing agent used), or was it due to the micropermeabilized cells retaining intracellular ATP in spite of sufficient permeabilization to ATP to permit egress of the molecule from the cells? To answer this question, α-toxin-permeabilized cells were stained with 1,N^6-ethenoadenosine 5'-triphosphate (ε-ATP), a fluorescent analog of ATP. All the permeabilized cells, but none of the intact cells, were fluorescent after exposure to ε-ATP (data not shown), strongly suggesting that all the permeabilized cells were permeable to ATP. The possibility that the fluorescence originated from ε-ADP or ε-AMP (i.e., hydrolyzed ε-ATP) that had entered the cells has not yet been excluded. However, this is an unlikely possibility since permeabilized cells exposed to ε-AMP at a concentration equal to ca. 2% of the concentration of ε-ATP used for staining were not fluorescent. Therefore, our results strongly suggest that RBL 2H3 cells made permeable to ATP can nevertheless retain, at least temporarily, a high concentration of intracellular ATP and actively produce lactate in the absence of an exogenous source of Mg^2 and ATP.

Supplying α-toxin-permeabilized RBL 2H3 cells with NAD^+ in addition to glucose, P_i, Mg^{2+}, and MgATP increased the production of lactate from 101 ± 2% to 121 ± 2% (Fig. 2, columns #15 and #16, respectively). In contrast, the same treatment had essentially no effect on intact cells (compare columns #3 and #5, Fig. 2). These

results suggest that NAD^+ is entering the permeabilized cells. However, the opposite is suggested by the observation that the permeabilized cells were not stained by ε-NAD (data not shown). This apparent discrepancy probably reflects that the lactate production method is more sensitive than the staining method — only a weak signal was obtained with ε-ATP under experimental conditions identical to those used with ε-NAD, and the quantum yield of ε-NAD is ca. 20 fold lower than that of ε-ATP.

Quantitative assessment of the extent of micropermeabilization.

Micropermeabilization can be quantitatively assessed by comparing the production of lactate by intact and α-toxin-treated cells incubated with glucose, but none of the essential glycolytic cofactors. For example, the production of lactate by RBL 2H3 cells exposed to 4 μg of α-toxin per ml was only 2 ± 2% of that of intact cells incubated with glucose only (see Fig. 1, lower curve). This indicates that ≤ 2 ± 2% of the cells remained intact at 4 μg of α-toxin per ml. In other words, ≥ 98 ± 2% of the cell population was permeabilized to P_i at this α-toxin concentration (since leakage of P_i through α-toxin pore-complexes causes a depletion of this essential cofactor). Because it cannot be excluded that some permeabilized cells can retain sufficient endogenous P_i (and Mg^{2+}, NAD^+, and ATP) to contribute significantly to the production of lactate (especially when very low concentrations of α-toxin are used), the percentage of

micropermeabilized cells determined using this approach must be considered a minimum value.

As shown in Fig. 4, lactate production by intact RBL 2H3 cells was linear over the wide range of cell concentrations tested ($0.1 - 10 \times 10^6$ cells/ml). This shows that within this concentration range, the percentage of micropermeabilized cells cannot be overestimated (or underestimated) as a result of variation in lactate production with varying concentration of intact cells. It also indicates that our method can be conveniently used with a wide range of cell concentrations.

Finally, our quantitative assessment of the extent of micropermeabilization of RBL 2H3 cells by α-toxin is in excellent agreement with assessments made by measuring the leakage of [^{3}H]*myo*-inositol from pre-loaded cells: lactate production and release of [^{3}H]*myo*-inositol reached their minimum and maximum, respectively, at 4 μg/ml of α-toxin [$\geq 98 \pm 2\%$ (this study) vs. $> 93\%$ (Hohman, 1988), respectively]. Moreover, preliminary results obtained with PC12 cells show an excellent agreement between results obtained with the method we present and the well established method of release of [^{3}H]*myo*-inositol (Fig. 5.).

FIG. 4. Effect of cell concentration on the production of lactate by intact RBL 2H3 cells incubated 30 min at 37°C in permeabilization buffer containing 10 mM glucose. The data was corrected for the intracellular lactate present at the start of the incubation (1.5 ± 0.1 nmol lactate/10^6 cells, n = 3). Symbols and error bars represent means ± SD of triplicate measurements. Error bars < 15 nmol are covered by the symbols.

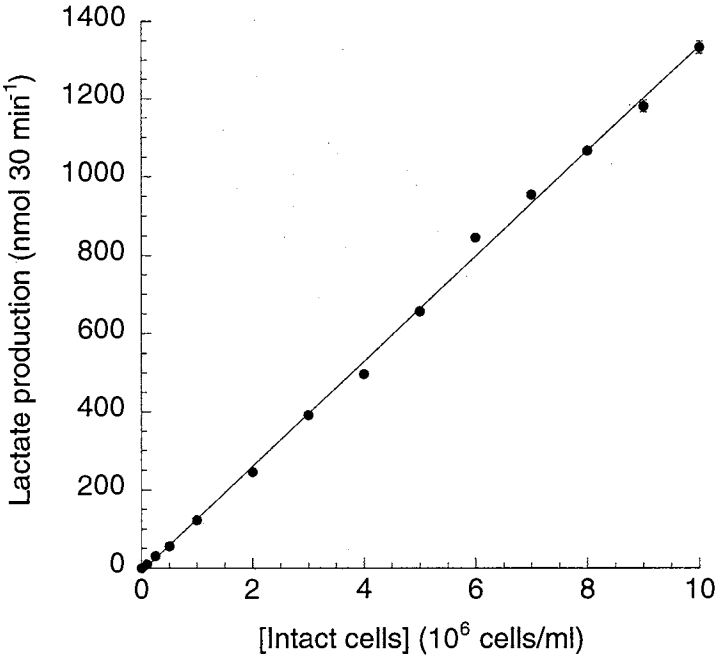
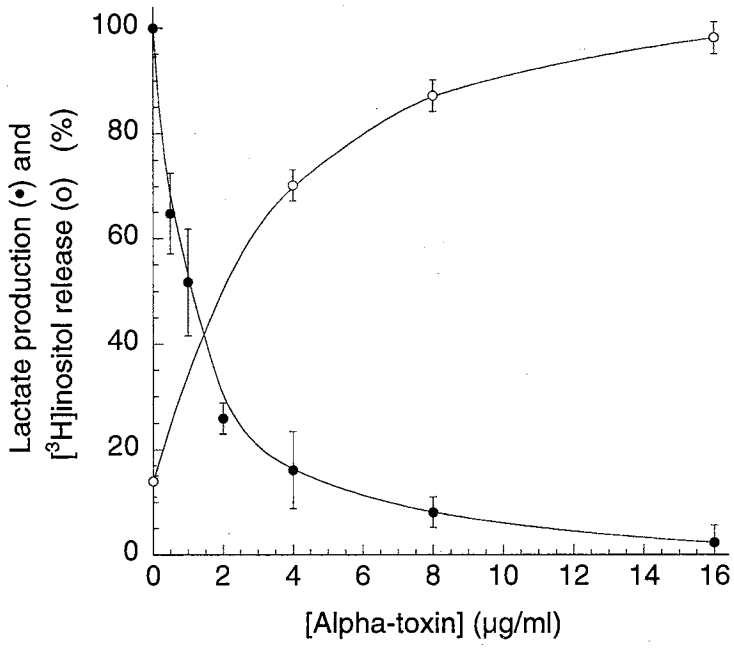

1400
1200
1000
800
600
400
200
0
Lactate production (nmol 30 min-1)
0
2
4
6
8
10
[Intact cells] (10^6 cells/ml)

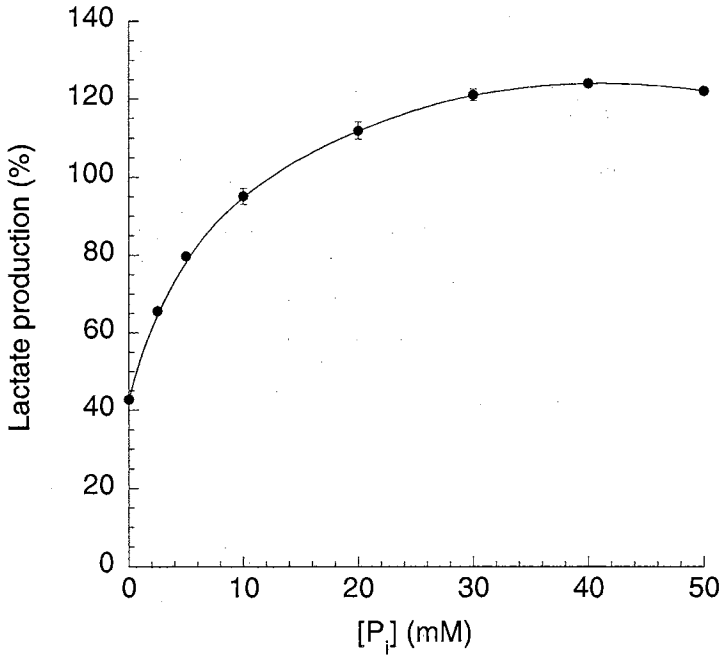
FIG. 5. Effect of α-oxin from *S. aureus* on the production of lactate (•) by, and the release of pre-loaded [^{3}H]*myo*-inositol (○) from, PC12 cells. The cells were incubated 30 min at 37°C in permeabilization buffer containing α-toxin at the indicated concentrations. For the lactate production measurements, the α-toxin-treated cells were washed and incubated 30 min at 37°C in permeabilization buffer containing 10 mM glucose. To facilitate comparison with the measurements of [^{3}H]*myo*-inositol release, lactate production is expressed as a percentage of the production of lactate by intact cells incubated in permeabilization buffer containing 10 mM glucose, but *no* P_i. The percentage of the total releasable [^{3}H]*myo*-inositol released from the pre-loaded cells at the end of the 30 min incubation with α-toxin was determined as described under materials and methods. Symbols and error bars represent means ± SD of triplicate measurements.


Lactate production (•) and
[3H]inositol release (o) (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
[Alpha-toxin] (µg/ml)

Inorganic phosphate and glycolysis in permeabilized cells

Fig. 6 shows the effect of P_i on lactate production in α-toxin-permeabilized RBL 2H3 cells incubated with glucose, ATP, Mg^{2+}, and NAD^+. Although the absolute requirement of glycolysis for P_i is well known (P_i is a cosubstrate of glyceraldehyde-3-phosphate dehydrogenase), metabolic studies using permeabilized cells are often performed without an exogenous source of this cofactor. One likely reason is that the medium used in some pioneering studies (e.g., Hohman, 1988) did not include P_i, and the choice of medium tends to follow tradition. Glucose, ATP, and Mg^{2+} (but not NAD^+) are often included in media where P_i is omitted. However, as shown in Fig. 2 (column #9), in the absence of exogenous P_i and NAD^+ the production of lactate in α-toxin permeabilized RBL 2H3 cells is marginal (5.0 ± 0.5%; the active production of lactate observed in the absence of P_i, but presence of ATP, Mg^{2+}, and NAD^+ is probably due to the release of P_i from the hydrolysis of ATP and NAD^+). Given the role of glycolysis in providing cells with ATP, NADH, and metabolic intermediates, it appears that most studies would benefit from supplying permeabilized cells with sufficient exogenous P_i to promote vigorous glycolysis. Although we have not yet conclusively established that the lactate produced by permeabilized RBL 2H3 cells is exclusively generated from the exogenously added glucose, the absence of lactate production (< 1%, n = 3) in the absence of exogenous glucose, but presence of glycolytic cofactors, strongly suggests a

FIG. 6. Effect of exogenous P_i on the production of lactate by RBL 2H3 cells permeabilized with 8 μg/ml of α-toxin from *S. aureus*. The cells were permeabilized as described in Fig. 1 and incubated 30 min at 37°C in permeabilization buffer containing 10 mM glucose, 5 mM $MgCl_2$, 1 mM NAD^+, 3 mM ATP, and the indicated concentrations of P_i. Other details as in Fig. 1.


Lactate production (%)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
[P$_i$] (mM)

purely glycolytic origin for the lactate. Finally, it should noted that because of ATP compartmentation, some intracellular processes fueled by ATP produced endogenously may not be supported by ATP from an exogenous source (e.g., Hoffman, 1997; Hardin *et al.*, 1992).

Critique of the method

The method we present was designed for the routine assessment of cell micropermeabilization. Its advantages include simplicity and convenience. The only measurements required are cell counts and conventional lactate determinations that can be conveniently performed using commercially available kits (e.g., Sigma Chemical Co., Cat. No. 826-A).

Our method, which provides precise quantitative data on the extent of permeabilization, can also be used to assess permeabilization to ATP. Given the current interest in introducing ATP and GTP (GTP differs from ATP by the presence of a single amino group on C_6 of the purine ring) into micropermeabilized cells, this represents a significant advantage. Assessment of permeabilization to ATP also provides useful information on the size-exclusion limit of the micropermeabilized cells. Unfortunately, the semi-quantitative nature of this assessment can be a limitation. Another limitation of our method is that it provides a direct assessment of permeabilization to P_i and ATP, only. However, permeabilization to other ions molecules can be inferred from this

assessment, and the basic principle of our method (the use of the production of metabolic end products to assess micropermeabilization) can, in principle, be extended to pathways other than glycolysis, thereby permitting the direct assessment of permeabilization to various molecules of interest. In principle, our approach could also be used to assess permeabilization to intermediates of the glycolytic pathway.

It should be noted that a simple and convenient enzymatic method based on the uptake of substrates of selected NAD^+ and $NADP^+$ utilizing enzymes has been successfully used to assess the permeability of macropermeabilized cells to various small molecules such as ATP (for review see Felix, 1988). Unfortunately, this method is unlikely to work well with many (and possibly all) micropermeabilized cell systems for two reasons: 1) it requires sufficient permeabilization for the rapid equilibration across the plasma membrane of the oxidized and reduced forms of NAD^+ (M_r 663) or $NADP^+$ (M_r 743); 2) the intracellularly reduced nucleotides are likely to be reoxidized locally before escaping from the cells, thereby avoiding detection.

Finally, because our method involves measurements of lactate production, it has the significant advantage of providing information on the general health status of micropermeabilized cells: healthy micropermeabilized cells should actively produce lactate when adequately supplied with glucose, P_i, Mg^{2+}, NAD^+, and ATP. In conclusion, the method we present offers significant advantages over the methods currently available to assess micropermeabilization.

ACKNOWLEDGEMENTS

We thank Drs. Stephen C. Dreskin and Motoni Kadowaki for critical comments, Dr. Robert J. Hohman for helpful advice on the isolation of α-toxin, the late Sidney Harsman for a generous gift of stock *S. aureus* and a sample of purified α-toxin, and Kimberly S. Blythe, C. Eldon Wagner, Jeremy A. Bush, and Tad D. Seinfert for technical assistance. This research was supported by NSF grant MCB-9513613, Oklahoma Center for the Advancement of Science and Technology grant HR98-061, and OAES Project OKLO-1393. E.A.L. was recipient of a F.C.A.R. scholarship from Québec (Canada).

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CHAPTER VI

THE UNDISCOVERED COUNTRY

"The compartmentation of small molecules in eucaryotic cells is easy to believe, but hard to prove."

R. H. Davis (1980)

INTERACTION BETWEEN αGDH AND GAPDH[1]

In the course of the Hummel-Dreyer association study described in chapter I, we discovered a weak interaction ($K_d \approx 5$ μM) between αGDH from rabbit muscle and GAPDH from *Bacillus stearothermophilus* (GAPDH was a generous gift from Dr. Guy Branlant, Université Henri Poincaré). This interaction is compatible with the report that random collisions between these two enzymes result in a greater perturbation of the conformation of GAPDH than does the collision of other dehydrogenases with GAPDH (Gabellieri and Strambini, 1994). Because these two enzymes were isolated from phylogenetically distant species, the physiological relevance of this interaction is currently uncertain. However, comparison of the amino acid sequence of GAPDH (GenBank accession number P00362) from *B. stearothermophilus* and rabbit muscle with the Blast algorithm (Altschul *et al.*, 1990) revealed 54% identity and 70% similarity. In addition, the backbone fold of GAPDH from *B. stearothermophilus* and other sources overlap with low r.m.s. (Zeljko Svedruzic's Ph.D. thesis). This suggests that αGDH from rabbit muscle will interact with GAPDH from the same tissue. To investigate this

[1] The research presented in this section was performed with Sharon M. Baker.
Zeljko Svedruzic is acknowledged for his intellectual contribution.

exciting possibility, we have begun analytical ultracentrifugation studies aimed at quantifying this association. Although αGDH and GAPDH are not expected to channel NADH directly (because they are of the same chiral type; Srivastava and Bernhard, 1987), the potential importance of an interaction between these enzymes is considerable.

Under anaerobic conditions, cytoplasmic NADH cannot be oxidized in the mitochondria. However, in order for glycolysis to proceed, a high ratio of NAD^+ to NADH is needed in the cytoplasm to sustain the reaction catalyzed by GAPDH [reaction I]. Quantitatively, the most important mechanism for the reoxidation of cytoplasmic NADH is the reduction of pyruvate to lactate by LDH [reaction II]. However, the reaction catalyzed by αGDH [reaction III] is likely to also play an important role in the reoxidation of cytoplasmic NADH during the initial stages of anaerobiosis (Klingenberg and Bücher, 1960). Therefore, by helping to coordinate the activity of αGDH and GAPDH, an interaction between these enzymes could play a significant role in the regulation of the cytosolic redox state of nicotinamide adenine dinucleotide during the initial stages of anaerobiosis.

D-glyceraldehyde 3-phosphate + NAD^+ + P_i →
1,3-bisphosphoglycerate + NADH + H^+ [I]

L-pyruvate + NADH + H^+ → L-lactate + NAD^+ [II]

dihydroxyacetone phosphate + NADH + H^+ → glycerol 3-phosphate + NAD^+ [III]

In the later stages of anaerobiosis, competition between αGDH and LDH for cytoplasmic NADH could be potentially detrimental since, under anaerobic conditions, αGDH activity would reduce the glycolytic yield of ATP when it is highly needed. In contrast, under aerobic conditions, significant LDH activity would reduce the glycolytic flow of carbon and hydrogen to the mitochondria (Guppy and Hochachka, 1978). Therefore, minimizing the simultaneous function of αGDH and GAPDH in the later stages of anaerobiosis seems essential, and such a control is clearly achieved *in vivo* (Guppy and Hochachka, 1978). Product inhibition of αGDH has been proposed as an important mechanism to achieve this control (Guppy and Hochachka, 1978). A weak interaction between αGDH and GAPDH offers another potential mechanism to modulate the activity of αGDH.

In addition, because αGDH can oxidize both NADH and NADPH, an interaction between αGDH and GAPDH could play a role in the regulation of the [NADH]/[NADPH] ratio in the cytoplasm by modulating the relative affinity of αGDH for NADH and NADPH. Several factors are already known to affect the nucleotide affinity of αGDH *in vitro* (e.g., pH, concentration of Mg^{2+}, and NADH channeling with LDH and malate dehydrogenase; Prabhakar *et al.*, 1998; Fahien *et al.*, 1999).

Finally, it should be noted that an interaction between muscle αGDH and GAPDH makes sense metabolically. These enzymes are colocalized in the I-band of sarcomeres

(Arnold *et al.*, 1969; Sigel and Pette, 1969) and are closely coupled functionally, αGDH producing dihydroxyacetone phosphate which is transformed by triose phosphate isomerase to glyceraldehyde 3-phosphate, a cosubstrate of GAPDH. Furthermore, as discussed in the introduction, Wojtas *et al.* (1997) recently showed that the mislocation of αGDH in *Drosophila* flight muscle results in the mislocation of GAPDH (and aldolase) and appears to be responsible for rendering the animal flightless. This strongly suggests an interaction between muscle αGDH and GAPDH *in vivo*.

REEVALUATION OF THE SIZE-EXCLUSION LIMIT OF α-TOXIN PORES IN NUCLEATED CELLS[2]

As stated in chapter I, the ultimate goal of our research with α-toxin was to develop a micropermeabilized cell system to study glycolysis *in situ*. In the pursuit of this goal, we characterized the size-exclusion limit of α-toxin pores in RBL 2H3 and PC12 (rat pheochromocytoma; Greene and Tischler, 1976) cells by monitoring the uptake and/or release of M_r markers. Our results, summarized in Table I, point to inadequacies in the current literature on α-toxin permeabilization.

[2] The research presented in this section was performed with Sharon M. Baker in collaboration with Dr. Biao Ding and Asuka Itaya.

Table I. Uptake and/or release of M_r markers by RBL 2H3 and PC12 cells permeabilized with 8–64 μg/ml and 16 μg/ml of α-toxin, respectively. [a]?, inconclusive results. [b]—, not determined. *See text. †Personal communication from Stephen C. Dreskin.

M_r Marker (M_r)	Uptake/release of M_r marker	
	RBL 2H3	PC12
Trypan Blue (960)	No	No
NAD^+ (663)	?[a]	?
Fura-2 (637)	Yes	—[b]
Eosin B (580)	No*	No*
ATP (505)	Yes	Yes
Lucifer Yellow (443)	Yes	—
Fluorescein diacetate (416)	—	?
Propidium Iodide (414)	No	No
5(6)Carboxyfluorescein (376)	Yes	—
Azure A (256)	No*	No*
myo-Inositol (180)	Yes†	Yes

The data presented in chapter V show that RBL 2H3 cells treated with 8 μg of α-toxin per ml become permeable to ATP (M_r 505) and possibly NAD^+ (M_r 663), but remain impermeable to the dye trypan blue (M_r 960). Additional data collected in our lab show that α-toxin-treated RBL 2H3 cells become permeable to 5(6)-carboxyfluorescein (M_r 376), lucifer yellow (M_r 443), and fura-2 (M_r 637), but not to propidium iodide (M_r 414; propidium iodide is bulkier than the other fluorophores) even at α-toxin concentrations up to 64 μg/ml. Together, these results indicate that NAD^+ is near the size-exclusion limit of α-toxin pores in RBL 2H3 cells. Other results obtained in our lab (see Table I) suggest a similar size-exclusion limit in PC12 cells carefully treated with 16 μg of α-toxin per ml. Our results with PC12 cells are in disagreement with the report that α-toxin pores in PC12 cells are sufficiently large to permit the influx of trypan blue (Anhert-Hilger *et al.*, 1985). More generally, our results with RBL 2H3 and PC12 cells are also in disagreement with the view that α-toxin pores large enough to permit the transmembrane flux of cations are sufficiently large to allow the influx of propidium iodide and trypan blue (Bhakdi *et al.*, 1993).

Increasing dosage of α-toxin has been reported to increase the size of α-toxin pores in human keratinocytes (Walev *et al.*, 1993), human T lymphocytes (Jonas *et al.* (1994), and Lettre cells (Korchev *et al.*, 1995; also see Bashford *et al.*, 1996). From their work on Lettre cells, Korchev *et al.* (1995) concluded that, as with other pore-forming

agents such as complement or hemolytic Sendai virus, the apparent size or stability of α-toxin pores increases with increasing dosage. Similarly, in a mini review on the use of pore-forming toxins for the controlled permeabilization of cell membranes, Bhakdi *et al.* (1993) noted that often, high concentrations of α-toxin (50-200 μg/ml) must be applied to generate the larger α-toxin pores that permit transmembrane flux of Ca^{2+} and small molecules such as nucleotides and propidium iodide. As mentioned in chapter I, reports that larger pores are formed at high α-toxin concentrations have lead to the suggestion that small pores may form when the toxin binds to high affinity specific sites on the cells, whereas α-toxin molecules absorbed non-specifically to membranes might be responsible for creating larger pores (Jonas *et al.*, 1994). However, no evidence for increases in pore size with increasing α-toxin concentrations has been presented, other than the reported transmembrane flux of larger ions and molecules (some heterogeneity in the number of α-toxin monomers comprising oligomers in deoxycholate has been reported by Tobkes *et al.*, 1985). Therefore, an alternative explanation for the apparent increase in the size of α-toxin pores with increasing dosage is that exposure to high concentrations of α-toxin creates membrane lesions *between* pore-complexes as a result of overpermeabilization. Surprisingly, to the best of our knowledge, no one has proposed this simple explanation. One possible deterrent may have been the observation that some cells exposed to high concentrations of α-toxin permit the passage of larger molecules with little or no leakage

of high M_r markers such as lactate dehydrogenase (e.g., McEwen and Arion, 1985; Anhert-Hilger *et al.*, 1985). However, permeability to small molecules, but not macromolecules, could simply reflect that the lesions are larger than (small) α-toxin pores, but smaller than macromolecules such as lactate dehydrogenase (M_r 144,000).

Results obtained in our lab clearly show that some cell lines such as PC12 cells are very sensitive to lysis, especially after treatment with α-toxin. Indeed, extreme care is required to obtain α-toxin permeabilized PC12 cells that exclude trypan blue. We also find that lysis-insensitive cells such as RBL 2H3 become sensitive to lysis after exposure to high concentrations of α-toxin (e.g., 32-64 μg/ml), especially under staining conditions. In their pioneering study, McEwen and Arion (1985) reported that rat hepatocytes became permeable to trypan blue when treated with α-toxin at 37°C, but not at 25°C. Since α-toxin permeabilization is most efficient at room temperature (Bhakdi *et al.*, 1993), the hepatocytes treated at 25°C were almost certainly permeabilized by α-toxin. Therefore, the reported uptake of trypan blue after treatment at 37°C probably did not result from the formation of α-toxin pore-complexes or larger pores-complexes, but rather from the formation of lesions beyond α-toxin pores. This interpretation is in excellent agreement with our repeated observations that α-toxin permeabilized cells are more sensitive to lysis at 37°C than at 25°C. These results together with the report that PC12 cells permeabilized with α-toxin are permeable to trypan blue (Anhert-Hilger *et al.*,

1985), in contrast to our results (above), lead us to postulate that the reported uptake of trypan blue by nucleated cells is in many (possibly all) cases due to the formation of lesions beyond α-toxin pores.

In summary, our results suggest that the size-exclusion limit of α-toxin pores in many (possibly all) nucleated cells has been overestimated due the occurrence of cell lysis, i.e., the formation of lesions beyond α-toxin pores. We are currently pursuing experiments, in collaboration with Dr. Biao Ding's lab, to better evaluate the permeability of PC12 (and perhaps Lettre) cells treated with low and high concentrations of α-toxin. We are also planning to estimate the size of the pores formed by *our* α-toxin in rabbit erythrocytes using the colloid-osmotic lysis protection approach used by Krasilnikov *et al.* (1988). Our results will be compared with the well established size of α-toxin pores in these cells (effective diameter ca. 13 nm; see Krasilnikov *et al.*, 1988). The purpose of this experiment is to confirm that our α-toxin (under our permeabilization conditions) does not behave abnormally with regard to pore size when compared with α-toxin used by other research groups. This confirmation is important since we will be reporting smaller pore sizes for nucleated cells. The results of this experiment will also be useful for our planned study of glycolysis *in situ* (see below).

The research we are pursuing in collaboration with Dr. Ding's lab is important for several reasons. Pore size is a major determinant of pathogenicity of α-toxin (for review

see Bhaki *et al.*, 1996). Proper characterization of the pore size(s) of α-toxin is required to better understand the mechanism of this important protein. Overestimation of the size-exclusion limit of α-toxin pores in nucleated cells may lead to undesirable overpermeabilization of cells by researchers using α-toxin as a tool. Paradoxically, it may also lead researchers to abandon α-toxin permeabilization because of a failure to recognize permeabilization. This latter problem may be amplified by the abundance of recent publications on α-toxin still referring to the outdated and oversimplified concept of a homogeneous size-exclusion limit of M_r 1,000 to 3,000. If "small" lesions other than α-toxin pores are formed at high α-toxin concentrations, they may permit the flux of molecules considerably larger than is currently expected on the basis of the concept of larger α-toxin pores. Since some cells treated with high concentrations of α-toxin have been shown to become permeable to larger molecules such as trypan blue, but remain impermeable to LDH, the creation of 'small' lesions by α-toxin could prove to be very useful for metabolic studies by permitting the transmembrane flux of molecules that are too large to pass through α-toxin pore-complexes. Finally, this research will help determine the suitability of α-toxin-permeabilized cells for the study of substrate channeling in the glycolytic pathway (see below).

Note added in proof.

Data recently collected in our lab clearly show that RBL 2H3 and PC12 cells permeabilized with 8 and 16 μg of α-toxin per ml, respectively, are not stained by the dyes eosin B (M_r 580) and Azure A (M_r 256), in contrast to lysed cells. The most likely explanation for this result is that, under staining conditions, molecules of eosin B and azure A exist almost exclusively as aggregates that are too large to pass through α-toxin pores (because of their flat hydrophobic surfaces, dye molecules have a tendency to stack up in a hydrophilic environment; Förster and König, 1957). Single molecules of the dyes probably do enter the cells, but not in large enough numbers to generate a good signal (stain). This explanation is compatible with our observation that α-toxin-permeabilized RBL 2H3 cells are stained with some fluorophores that are structurally similar to eosin B and azure A.

In their influential review "Poration by α-toxin and steptolysin O: an approach to analyze intracellular processes", Anhert-Hilger *et al.* (1989) recommended the use of eosin B and azure A to test the permeability of α-toxin-treated cells. In light of this, our recent results support our conclusion that the size-exclusion limit of α-toxin pores in many (possibly all) nucleated cells has been overestimated due the occurrence of cell lysis. Furthermore, our results highlight the urgent need to address this question since researchers using eosin B and azure A to test the permeability of α-toxin-treated cells

may fail to detect existing permeabilization or erroneously conclude that their cells are permeable only to ions or molecules smaller than azure A (M_r 256).

REGULATION OF GLYCOLYSIS *IN SITU* IN α-TOXIN-PERMEABILIZED CELLS

In our search for a suitable permeabilized cell system to study glycolysis *in situ*, we tested a number of permeabilizing agents including α-toxin from *S. aureus,* Brij 58, Dextran sulfate 500, and Saponin. Although the results obtained with Dextran sulfate 500 were promising, α-toxin was selected as the most promising agent because of the advantages it offers over Dextran sulfate 500 (for a summary of advantages see chapter II). However, as explained in the preceding section, we uncovered evidence that the size-exclusion limit of α-toxin pores in nucleated cells is considerably smaller (ca. 50%) than generally acknowledged. As a result, it is currently unknown if nucleated cells treated with α-toxin can be made sufficiently permeable to allow measurements of substrate channeling *in situ* using the isotopic dilution method. In contrast, the research presented in chapter III clearly shows that α-toxin-treated RBL 2H3 cells are sufficiently permeable to permit the study of glycolytic flux regulation *in situ.* The research presented in that chapter also shows that α-toxin permeabilization is easily reproducible and that the permeabilized cells are stable and can actively produce lactate. The absence of lactate production in the absence of exogenous glucose, but presence of essential cofactors of

glycolysis, also suggests that the lactate is exclusively produced from glucose added exogenously. Preliminary results obtained with PC12 cells permeabilized with 8 μg of α-toxin per ml also indicate that the production of lactate is linear with time over a period of at least 1 hour. Together, these results strongly suggest that α-toxin-permeabilized cells can provide a suitable micropermeabilized cell system for the study of glycolytic flux regulation *in situ*.

RBL 2H3 cells were used to develop the method described in chapter III because they are unusually resistant to lysis and permeable to ATP after treatment by α-toxin. However, providing that they are sufficiently stable, α-toxin-permeabilized erythrocytes should offer a considerably superior system for the study of glycolytic flux regulation *in situ*. Glycolysis has been extensively characterized in erythrocytes (but not in RBL 2H3 cells), and erythrocytes, unlike RBL 2H3 cells, have an untransformed phenotype.

Furthermore, stable α-toxin-permeabilized erythrocytes should provide a better system for the study of substrate channeling since the size-exclusion limit of α-toxin pore-complexes is larger in this cell type than in any other cell type studied (see preceding section). A larger size-exclusion limit favors rapid equilibration of glycolytic intermediates across the plasma membrane — an absolute requirement for measurements of substrate channeling in the glycolytic pathway using the isotopic dilution method. We are in the process of investigating the stability of α-toxin-permeabilized erythrocytes.

FINAL WORDS

Nature does not easily reveal Her secrets. The research presented in this dissertation is the fruit of 7 years of labor. To use a famous quote inverted for this occasion, this dissertation is a giant step for one man, but a [very] small step for mankind. Nonetheless, in its own modest way, this dissertation answers important questions, presents new ideas, raises exciting new questions, and offers new tools that we hope will prove useful as we venture deeper and deeper into the undiscovered country.

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