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Permeabilization of animal cells for kinetic studies of intracellular enzymes: *In situ* behavior of the glycolytic enzymes of erythrocytes

(macromolecular environment/protein–protein interactions/metabolic control/enzyme regulation/phosphofructokinase)

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ABSTRACT **Intracellular enzymes in erythrocytes can be made accessible for *in situ* kinetic studies by treating the cells with bifunctional reagents to crosslink proteins, thus creating a network that allows subsequent permeabilization by delipidation without escape of intracellular proteins. Dimethyl suberimidate, dimethyl 3,3′-dithiobispropionimidate, and toluene-2,4-diisocyanate have been used successfully as crosslinking reagents, and digitonin has been used for delipidation. In a systematic study of the *in situ* behavior of the 11 glycolytic enzymes of rat erythrocytes, it was observed that K_m and V_{max} values for the majority of the enzymes are essentially the same *in situ* as *in vitro*. Lactate dehydrogenase (L-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) is inhibited by excess of pyruvate as much *in situ* as *in vitro*. Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) was allosterically inhibited by glucose 6-phosphate nearly as much *in situ* as *in vitro* but was not affected by 2,3-bisphosphoglycerate. The allosteric properties of 6-phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11), glyceraldehyde-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD$^+$ oxidoreductase (phosphorylating), EC 1.2.1.12], and pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) *in situ* were qualitatively similar to those observed *in vitro*, but some important quantitative differences were noticed. Particularly striking was the much greater activity of phosphofructokinase *in situ* compared to that *in vitro* at physiological concentrations of effector metabolites.**

Many intracellular enzymes are present in high concentrations (1). Protein–protein interactions, both homologous (2) and heterologous (3), could induce intracellular changes in some properties of the enzymes important for metabolic regulation. For these reasons it can be said that *in vitro* studies of kinetic parameters of potential significance for metabolic regulation, particularly with allosteric enzymes, should be considered as guidelines to studies "when techniques are available to study control of enzymes *in situ*" (4). The *in situ* approach for the kinetic study of enzymes in cells has been proposed; it could be easily accomplished in microorganisms endowed with a cell wall (5–7).

A general method for the kinetic study of enzymes in animal cells based on crosslinking with bifunctional reagents and permeabilization with digitonin is presented here. With this approach we have carried out a systematic exploration of the kinetic behavior of the 11 enzymes of glycolysis in erythrocytes. The results indicate that K_m and V_{max} are essentially the same *in situ* (in permeabilized cells) as *in vitro* and, hence, presumably *in vivo;* however, the kinetic parameters of phosphofructokinase and certain other allosteric enzymes *in situ* show important differences from those observed *in vitro*. Preliminary reports of part of this work have been presented (8, 9).

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EXPERIMENTAL PROCEDURE

Bifunctional Crosslinking Reagents. Dimethyl suberimidate and dimethyl 3,3′-dithiobispropionimidate (DTBP) were obtained from Pierce; toluene-2,4-diisocyanate was from Merck-Schuchardt (Darmstadt, Federal Republic of Germany); glucose was from May and Baker (Dagenham, England); [*U*-^{14}C]glucose was from the Radiochemical Centre (Amersham, England); digitonin and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid were from Calbiochem; Ficoll was from Pharmacia; other chemicals were obtained from Boehringer or Sigma.

Buffer Solutions. Buffer E: 115 mM NaCl/5 mM potassium phosphate, pH 7.4. Buffer I: 125 mM KCl/5 mM $MgCl_2$/5 mM NaCl/5 mM potassium phosphate, pH 7.4.

Erythrocytes. Blood was obtained from Swiss albino rats and occasionally from human volunteers; heparin was used to prevent coagulation.

Crosslinking and Permeabilization of Erythrocytes. Fresh blood (2 ml) was diluted with ≈10 ml of buffer E and centrifuged at low speed (10,000 × *g* for 5 min); the plasma and bluffy coat were removed by suction. After a second wash with buffer E, the erythrocytes were treated with one of the crosslinking reagents, as follows.

Dimethyl suberimidate. Forty milligrams of dimethyl suberimidate dihydrochloride was dissolved in 7 ml of 100 mM triethanolamine, pH 8.5/75 mM NaCl and 0.26 ml of 1 M NaOH and mixed immediately with the washed erythrocytes; the mixture was allowed to stand at 37°C for 30 min. After centrifugation at 1000 × *g* for 5 min, the supernatant solution was removed and the sediment was washed with 10 ml of buffer E. The washed cells obtained by centrifugation were suspended in a mixture of 20 ml of water and 1 ml of 1% digitonin in ethanol, allowed to stand for 5 min at room temperature, centrifuged, washed with 10 ml of buffer I, and finally resuspended in a convenient volume of the same buffer.

DTBP. Ten milligrams of DTBP dihydrochloride was dissolved in 8 ml of 50 mM Tris·HCl, pH 9.0/125 mM NaCl and mixed immediately with the washed erythrocytes obtained from 2 ml of blood. After 30 min at 37°C the cells were treated as indicated above.

Toluene diisocyanate. A fine emulsion at 0.5% in buffer E was prepared by sonication. Three milliliters of this fresh emulsion was added to 20 ml of a suspension, in buffer E at 4°C, of washed erythrocytes obtained from 2 ml of blood. The

Abbreviation: DTBP, dimethyl 3,3′-dithiobispropionimidate.

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mixture was stored for 3 min and then the process was continued as above.

ASSAY OF ENZYMES *IN SITU*

Permeabilized cells were suspended [at not more that 1–2 mg of cells (net weight) per ml, to avoid excessive turbidity] in a reaction mixture consisting of 50 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonate at pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 7% Ficoll (to maintain the cells in suspension), and substrates and cofactors as appropriate, in a total volume of 1 ml. When necessary, an auxiliary system to couple the enzyme under investigation to a pyridine nucleotide-dependent dehydrogenase was also added. The change in absorbance at 340 nm was followed in a Gilford 2.400 spectrophotometer at 37°C. Enzymes whose activity was too low to permit continuous spectrophotometric observation (below ≈0.5 unit/g), like hexokinase in this case, could be studied by a two-step method, either spectrophotometric or with an isotopically labeled substrate.

Individual Assay Methods. Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1.) was estimated by an isotopic method (10). Either permeabilized or lysed erythrocytes (20 mg) were incubated in the reaction mixture described above with 2mM [*U*-^{14}C]glucose (0.15 μCi/μmol; 1 Ci = 3.7×10^{10} becquerels) and 5 mM MgATP in a final volume of 0.4 ml; after 15-min incubation at 37°C, 50 μl was mixed with 50 μl of 1 M glucose. Half of this mixture (50 μl) was placed on a Whatman DE 81 filter paper (2.5 cm diameter), washed with 300 ml of water, dried and assayed in a liquid scintillation counter. Phosphorylation of glucose proceeded in a linear fashion during the incubation period.

All other enzymes were assayed spectrophotometrically with the above mixture plus specific additions as follows.

Glucosephosphate isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9): 2 mM fructose 6-phosphate, 0.5 mM $NADP^+$, and 1 unit of glucose-6-phosphate dehydrogenase.

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11): 0.25 mM fructose 6-phosphate, 0.75 mM glucose 6-phosphate, 1 mM MgITP, 1 mM dithioerythritol, 0.15 mM NADH, 0.5 unit of glucosephosphate isomerase, 1 unit of aldolase and glycerol-3-phosphate dehydrogenase, and 10 units of triosephosphate isomerase. Auxiliary enzymes were dialyzed for several hours against 50 mM Tris/1 mM EDTA, pH 7.5, to eliminate most of the accompanying NH_4^+.

Aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13): 1 mM fructose 1,6-bisphosphate, 0.15 mM NADH, 0.5 unit of glycerol-3-phosphate dehydrogenase, and 5 units of triosephosphate isomerase.

Triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1.): 3 mM glyceraldehyde 3-phosphate, 0.15 mM NADH, and 1 unit of glycerol-3-phosphate dehydrogenase.

Glyceraldehyde-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12]: by the linear method of Aragón and Sols (11).

Phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3): 5 mM 3-phosphoglycerate, 1 mM MgATP, 1 mM dithioerythritol, 0.15 mM NADH, and 1 unit of glyceraldehyde-phosphate dehydrogenase.

Phosphoglyceromutase (2,3-bisphospho-D-glycerate:2-phospho-D-glycerate phosphotransferase, EC 2.7.5.3): 1 mM 3-phosphoglycerate, 0.05 mM 2,3-bisphosphoglycerate, 1 mM dithioerythritol, 1 mM MgADP, 0.15 mM NADH, 1 unit of enolase, and 2 units of pyruvate kinase and lactate dehydrogenase.

Enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11): 0.5 mM 2-phosphoglycerate, 1 mM EDTA, 1 mM MgADP, 0.15 mM NADH, 0.5 unit of pyruvate kinase, and 1 unit of lactate dehydrogenase.

Pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40): 5 mM phospho*enol*pyruvate, 2 mM MgADP, 0.15 mM NADH, and 1 unit of lactate dehydrogenase.

Lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27): 2 mM pyruvate and 0.3 mM NADH.

Other Methods. Hemoglobin was estimated with Drabkin's reagent (12).

RESULTS

The use of toluene diisocyanate and DTBP to crosslink proteins in erythrocytes has been described (13, 14). Rat erythrocytes crosslinked and permeabilized by the method described with either dimethyl suberimidate or toluene diisocyanate were found to be resistant to freezing and thawing, sonication, and nonionic detergents, without liberation of either hemoglobin or lactate dehydrogenase. Moreover, sonication of crosslinked cells in a suspension of glass powder led to the appearance of large cell fragments. These results strongly suggest that, as reported by Wang and Richards (14) for DTBP, these crosslinking reagents penetrate the cells and crosslink hemoglobin molecules to membrane proteins as well as to each other, in addition to the crosslinking of membrane proteins among themselves as shown for spectrin (15).

The permeabilization procedure resulted in preparations that kept essentially all their proteins but lost essentially all their metabolites, judging from the fact that 2,3-bisphosphoglycerate and ATP were below the limits of detection (unpublished data). Insufficient crosslinking leads to a marked loss of cells during the permeabilization treatment. Adequate permeabilization can be easily checked by assaying *in situ* an enzyme with high activity and a rather large, impermeant substrate and using the latter at a subsaturating concentration. For erythrocytes, the ideal marker was found to be lactate dehydrogenase, which had the same K_m for NADH *in situ* as *in vitro* (Table 1) with a V_{max} *in situ* of ≈50% of that *in vitro*.

The effect of the concentration of pyruvate on the activity of lactate dehydrogenase was the same *in situ* as *in vitro* (Fig. 1), including the inhibition by excess pyruvate (16). This result gives direct evidence that the H isozyme of lactate dehydrogenase can be inhibited in the cells (although by unphysiological concentrations of pyruvate), despite claims to the contrary on the basis of experiments *in vitro* with high concentrations of the enzyme alone or in the presence of some other proteins (17).

V_{max} and K_m Values *in Situ*. The V_{max} of each of the glycolytic enzymes *in situ* was 30 to 60% of that *in vitro* (Table 1) in otherwise identical conditions of assay, with an average of roughly 50%. Losses of 90% or more of the activities of phosphoglycerate kinase, phosphoglyceromutase, and pyruvate kinase were observed after treatment with toluene diisocyanate, making this reagent unsuitable for kinetic studies with these enzymes. Treatment of hemolysates with the corresponding bifunctional reagents led to losses within a factor of 2 of those *in situ*, with an average loss of 43% for the 11 enzymes. These results suggest extensive chemical modification of the enzymes but indicate that the V_{max} of intracellular enzymes is essentially equal to that observable *in vitro*, at least within a factor of 2.

Each of the seven nonallosteric glycolytic enzymes exhibited the same K_m value *in situ* as *in vitro* (within an allowance of ±50% variability in our experimental conditions), for the substrate and, when applicable, for the coenzyme. In addition, some of the $S_{0.5}$ values for allosteric enzymes, particularly those

Table 1. Glycolytic enzymes of rat erythrocytes *in situ* and *in vitro*

En-zyme*	V_{max} *In vitro*, units/g	V_{max} *In situ*,† %	Substrate	$S_{0.5}$, mM *In situ*	$S_{0.5}$, mM *In vitro*
HK	0.08	33 (TDI)	Glc	0.2	0.2
			ATP	0.15; 1.5	0.3
PGI	15‡	47 (TDI)	Fru-6-*P*	0.1	0.1
PFK	8	48 (DMS)	Fru-6-*P*	0.2 (0.04)§	2 (0.025)§
			ITP	0.3	0.3
ALD	0.5	40 (TDI)	Fru-1,6-P_2	0.02	0.02
TIM	200‡	40 (TDI)	Gra-3-*P*¶	0.4	0.4
GAPDH	9	57 (DMS)	Gra-3-*P*	0.3	0.3
			NAD^+	0.18	0.05
			P_i	10	10
PGK	16‡	44 (DMS)	3-*P*-Gri¶	0.7	0.7
PGM	0.7	62 (DMS)	3-*P*-Gri	0.1	0.1
ENO	8	57 (TDI)	2-*P*-Gri	0.02	0.02
PK	6	58 (DMS)	*P-enol*py-ruvate	1.5	0.8
LDH	44	51 (DMS)	Pyruvate	0.2	0.2
			NADH	0.03	0.03

* The abbreviations correspond to the 11 glycolytic enzymes as listed in the text.

† Shown as % of value *in vitro*. The reagent used for crosslinking is shown in parentheses: TDI, toluene diisocyanate; DMS, dimethyl suberimidate.

‡ In the reverse reaction with respect to glycolysis.

§ Numbers in parentheses are the values in the presence of positive effectors.

¶ Gra, glyceraldehyde; Gri, glycerate

corresponding to a substrate or coenzyme that gives *in vitro* hyperbolic kinetics, were also essentially the same as *in vitro*. One exception was the K_m of hexokinase for ATP, which *in situ* had two values, 0.15 and 1.5 mM, instead of the 0.3 mM value obtained *in vitro*.

Allosteric Parameters *in Situ*. In contrast with the above results for nonallosteric enzymes, the allosteric effects *in situ* were quantitatively different from those *in vitro* in several instances.

Hexokinase is inhibited allosterically by glucose 6-phosphate *in vitro* (18) and *in vivo* (19). The enzyme appeared to be somewhat less strongly inhibited *in situ* than *in vitro*, with K_i values of 0.07 and 0.04 mM, respectively. The isotopic method allowed the observation in the intermediate situation of an "undiluted" hemolysate (involving only ≈50% dilution between extracellular fluid and reaction mixture), which gave an intermediate value (K_i, 0.05 mM). Nevertheless, these small differences may not be significant because of potential interference by removal of hexose 6-phosphate by phosphofructokinase which, as shown below, was found to be more active *in situ* than *in vitro*, in the conditions used for the hexokinase assay. 2,3-Bisphosphoglycerate has also been reported as an assumed physiological inhibitor of erythrocyte hexokinase (20, 21). Nevertheless, in our more physiological conditions, we have found no significant inhibition (less than 5%) either *in situ* or *in vitro*, with up to 2.5 mM 2,3-bisphosphoglycerate, a concentration in the upper range of this metabolite uncomplexed to hemoglobin in erythrocytes (22).

Phosphofructokinase is a highly sophisticated regulatory enzyme, the main control point in glycolysis (23, 24) and kinetically sensitive to the concentration of the enzyme (2). The enzyme has markedly sigmoidal kinetics with respect to fructose 6-phosphate in the absence of positive effectors. These are changed to hyperbolic kinetics by the synergistic positive effectors AMP, P_i, and NH_4^+. A smaller contrast between the two conditions was found *in situ*. The $S_{0.5}$ and n values obtained for fructose 6-phosphate were: *in vitro* without effectors, 2.2 mM and 1.6; *in vitro* with the three effectors, 0.025 mM and 1.0; *in situ* without effectors, 0.2 mM and 1.1; and *in situ* with effectors, 0.04 mM and 1.0. Treatment of a pellet of cells, after lysis by sonication, with dimethyl suberimidate indicated that the reagent could account for part of the difference observed between *in situ* and *in vitro*.

Crosslinking with DTBP resulted in a similar picture and gave the opportunity to observe the effect of the chemical modification, after the crosslink was broken with dithioerythritol. The results shown in Fig. 2 were essentially identical with those obtained with dimethyl suberimidate-treated cells or hemolysates. The remaining differences between the curves in the absence of effectors before and after lysis presumably correspond to the high concentration of the enzyme in the cell. The noninhibitory phosphoryl donor MgITP gave hyperbolic kinetics with the same K_m value *in situ* and *in vitro* (Table 1) with 0.04 mM fructose 6-phosphate, both in the absence and in the presence of the positive effectors. In the presence of MgITP, addition of MgATP inhibited the enzyme *in situ*, but

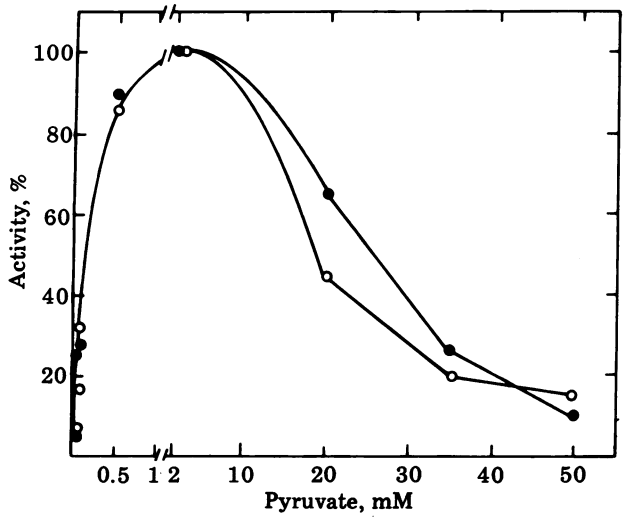

FIG. 1. Inhibition of lactate dehydrogenase by high concentrations of pyruvate *in situ* (●) and *in vitro* (○). Toluene diisocyanate was used for crosslinking.

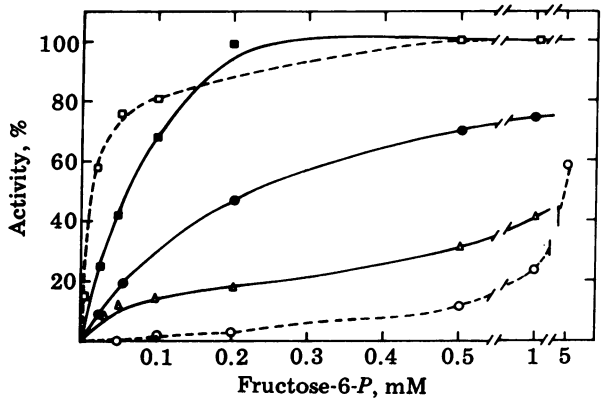

FIG. 2. Allosteric behavior of phosphofructokinase *in situ* and *in vitro* at 1 mM MgITP. Effectors were 0.1 mM AMP, 5 mM P_i, and 1 mM NH_4^+. DTBP was used for crosslinking. ●, *In situ*, no effectors; ■, *in situ*, with effectors; ○, *in vitro*, no effectors; □, *in vitro*, with effectors; △, DTBP-treated erythrocytes after the crosslinks were broken with dithioerythritol, no effectors.

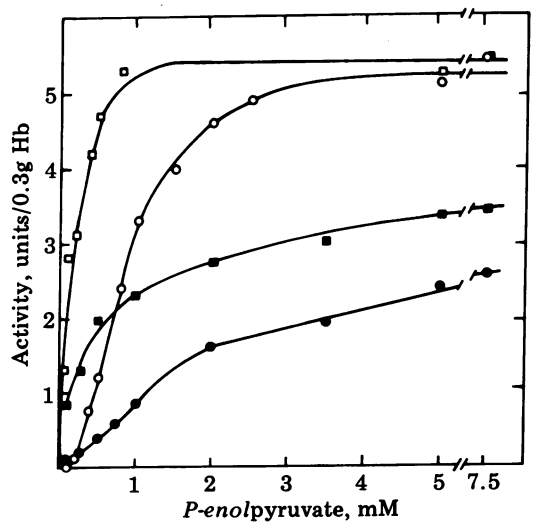

FIG. 3. Allosteric behavior of pyruvate kinase *in situ* and *in vitro*. Dimethyl suberimidate was used for crosslinking. ■, ●, *In situ* with and without 10 μM fructose 1,6-bisphosphate; □, ○, *in vitro* with and without 10 μM fructose 1,6-bisphosphate.

to a smaller extent than *in vitro*. The K_i values obtained at pH 7.4 were 0.9 and 0.4 mM, respectively, and at pH 6.9 they were 0.5 and 0.03 mM, respectively.

Glyceraldehyde-phosphate dehydrogenase represents a classical example of homotropic cooperativity (25), although it is unlikely to be involved in metabolic regulation (24). *In situ* studies of this enzyme yielded, for NAD^+, values of $S_{0.5}$ of 0.18 mM and n of 2.6, compared to $S_{0.5}$ of 0.05 mM and n of 1.6 *in vitro*.

The pyruvate kinase of erythrocytes is a slight posttranslational modification of the L isozyme (26), itself a multimodulated enzyme (23). The saturation curves for phospho*enol*pyruvate *in situ* and *in vitro* in the absence and presence of the activator fructose 1,6-bisphosphate are shown in Fig. 3. Marked differences between *in situ* and *in vitro* were observed, but their quantitative significance is open to question because this enzyme seemed to be particularly sensitive to bifunctional reagents. Treatment of hemolysates with dimethyl suberimidate led to important shifts, indicating that part of the differences observed with the *in situ* approach were due to chemical modification.

DISCUSSION

Shortly after the introduction of the *in situ* approach for kinetic studies of intracellular enzymes by permeabilization of microorganisms (5, 6) we made many attempts to permeabilize hepatocytes and erythrocytes by various mild treatments (unpublished data). These attempts were considered to be unsuccessful because either the cells were not permeable enough for kinetic studies or they disintegrated during the assays. Finally, we decided to crosslink proteins with bifunctional reagents prior to permeabilization. This approach has been successful with erythrocytes, as demonstrated in this communication, and several other types of cell. The dimethyl suberimidate-based treatment can be applied to ascites tumor cells (9) and, with minor modifications (crosslinking at 1–4°C for 1 hr), to leukocytes and enterocytes (unpublished results), but not to hepatocytes.

While we were developing this method and studying systematically the glycolytic enzymes, several methods for partial permeabilization of animal cells have been published by other investigators. Table 2 summarizes the other various approaches compared with the method described here. Faced with the dilemma between insufficient permeabilization to metabolites and incomplete retention of proteins, we decided to build up a sieve even at the expense of potential artifacts. The crosslinking of some intracellular proteins to some membrane proteins would result in the formation of some type of three-dimensional network, rather than the ideal two-dimensional one that would arise if only membrane components were crosslinked. This consideration implies the strong possibility that intracellular enzyme molecules may be crosslinked, or at least chemically modified. Therefore, caution is necessary when assessing the significance of any differences in kinetic behavior between the *in situ* and *in vitro* systems. The treatment of a cell lysate with the crosslinking reagent may confirm or eliminate the artifact in some cases but may be insufficient in others, because in this particular case, when lysis precedes the chemical treatment, the protein concentration will be diluted in comparison to the whole cell. Ultimately, the behavior of the enzyme for which a significant difference has been observed *in situ* should be studied in undiluted lysates or in concentrated solutions.

Most enzymes can be efficiently studied in many animal cells by one or another of the various approaches already available. For kinetic studies of enzymes of quantitatively major metabolic pathways, the only practical approach now available is that involving crosslinking prior to permabilization; for studies of enzymes of quantitatively minor metabolic pathways (i.e.,

Table 2. Permeabilization of animal cells

Method	Ref.	Cells	Permeability to metabolites,* μmol/g/min, at 0.1 mM	Protein retention, %	Stability	Enzymes: Activity, % of *in vivo*	Enzymes: Kinetic observability
DMS + digitonin	(this paper)	Erythrocytes†	>100	100	Unlimited	≈50	Good
Toluene	27	Hepatocytes	Presumably good	80	≈5 min	≈100	?
Filipin	28	Hepatocytes	?	?	?	70	?
Dextran sulfate	29	Ascites tumor	>0.06	100	Hours ?	<5‡	?
	30	Ascites tumor	?	95	20 min	?	Poor
Lysolecithin	31	Several types	?	>75	>1 hr	>90	?
	32	Cultured kidney	>0.02			≈100	?
Hypertonic treatment	33	Cultured kidney	>0.01 ?	>90	Few hours	≈100	?

* In the size range 100–800 daltons.
† As well as leukocytes, ascites tumor cells, and enterocytes but not hepatocytes (see text).
‡ Hexokinase (with 5 mM ATP) and glucose-6-phosphate dehydrogenase (with 0.5 mM $NADP^+$) (unpublished data).

coenzyme biosynthesis) the best approaches available seem to be those involving lysolecithin or dextran sulfate. There is as much as 10^2–10^4 difference in activity between typical enzymes of the different types of pathways.

The allosteric behavior of animal phosphofructokinase is markedly influenced by its concentration, which affects the degree of association of the oligomer in usual assay conditions (34–37). We have found marked quantitative differences in allosteric parameters *in situ* and *in vitro* (Fig. 2). Our observations indicate that part of the differences are due to chemical modification of the enzymes, as would be expected from previous observations by Lad and Hammes (38) after treatment of muscle enzyme with a large excess of dimethyl suberimidate. Part of the effect of the crosslinking could be a "freezing" of the native oligomer, which would prevent its dissociation but could also affect subunit–subunit interactions. In any case, the results indicate that the enzyme in erythrocytes indeed behaves differently from the usual assay conditions *in vitro* and suggest, but do not prove, that in its natural macromolecular environment *in vivo* the enzyme is not as strongly sensitive to allosteric effectors as it appears to be *in vitro*. Hence, it would be of great interest to study the enzyme in conditions as close as possible to those prevailing *in vivo* but in the absence of chemical modifications.

The lack of inhibition of hexokinase by 2,3-bisphosphoglycerate observed in our studies decreases the potential significance of this metabolite as a feedback regulator of glucose phosphorylation *in vivo*, as proposed originally by Dische in 1940, although it is still possible that it would operate at the level of phosphofructokinase (39).

Glycolysis is particularly relevant in non-nucleated erythrocytes because it represents the major metabolic pathway and the only one available for energy metabolism in these cells. For these reasons, revised parameters of its allosteric enzymes that more closely approach the physiological values would improve the models for overall regulation *in vivo* (40). The *in situ* approach for study of the physiological behavior of enzymes should be complemented with *in vitro* studies under more physiological conditions.

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1. Sols, A. & Marco, R. (1970) *Curr. Top. Cell. Regul.* **2**, 227–273.
2. Hulme, E. C. & Tripton, K. F. (1971) *FEBS Lett.* **12**, 197–200.
3. Hess, B. & Boiteux, A. (1972) *Protein-Protein Interactions* (Springer, Berlin), pp. 271–296.
4. Srere, P. A. (1973) *J. Biol. Chem.* **248**, 8031–8035.
5. Reeves, R. A. & Sols, A. (1973) *Biochem. Biophys. Res. Commun.* **50**, 459–466.
6. Sols, A., Reeves, R. E. & Gancedo, C. (1974) in *Metabolic Interconversion of Enzymes 1973*, eds. Fisher, E. H., Krebs, E. G., Neurath, H. & Stadtman, E. R. (Springer, Berlin), pp. 393–399.
7. Bañuelos, M. & Gancedo, C. (1978) *Arch. Microbiol.* **117**, 197–201.
8. Sols, A., Felíu, J. E. & Aragón, J. J. (1976) in *Metabolic Interconversion of Enzymes 1975*, ed. Shaltiel, S. (Springer, Berlin), pp. 191–197.
9. Sols, A., Aragón, J. J., Lazo, P. & González, J. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 1428 (abstr.).
10. Blume, K. G. & Beutler, E. (1975) *Methods Enzymol.* **42**, 47–53.
11. Aragón, J. J. & Sols, A. (1978) *Biochem. Biophys. Res. Commun.* **82**, 1098–1103.
12. Winterhalter, K. H. (1974) in *Clinical Biochemistry*, eds. Curtins, H. Ch. & Roth, M. (de Guyter, Berlin), pp. 1305–1322.
13. Kitajima, M., Sekiguchi, W. & Kondo, A. (1971) *Bull. Chem. Soc. Jpn.* **44**, 139–143.
14. Wang, K. & Richards, F. M. (1975) *J. Biol. Chem.* **250**, 6622–6626.
15. Freedman, R. B. (1979) *TIBS*, **4**, 193–197.
16. Caha, R. D., Kaplan, N. O., Levine, L. & Zwilling, E. (1962) *Science* **136**, 962–969.
17. Wuntch, T., Chem, R. F. & Vesell, E. S. (1970) *Science* **169**, 480–481.
18. Crane, R. K. & Sols, A. (1954) *J. Biol. Chem.* **210**, 597–606.
19. Rose, I. A. & O'Donnell, E. L. (1964) *J. Biol. Chem.* **239**, 12–17.
20. Dische, Z. (1941) *Bull. Soc. Chim. Biol.* **23**, 1140–1148.
21. Chiba, H. & Saski, R. (1973) *Curr. Top. Cell. Regul.* **14**, 75–116.
22. Gupta, R. K., Benovic, J. L. & Rose, Z. B. (1978) *J. Biol. Chem.* **253**, 6172–6176.
23. Sols, A. (1979) in *Covalent and Noncovalent Modulation of Protein Function*, eds. Atkinson, D. E. & Fox, C. F. (Academic, New York), pp. 27–45.
24. Sols, A. (1976) in *Reflections on Biochemistry*, eds. Kornberg, A., Horecker, B. L., Cornudella, L. & Oró, J. (Pergamon, Oxford), pp. 235–240.
25. Conway, A. & Koshland, D. E., Jr. (1968) *Biochemistry* **7**, 4011–4023.
26. Marie, J. M., Garreau, H. & Kahn, A. (1977) *FEBS Lett.* **78**, 91–94.
27. Hilderman, R. H., Goldblatt, P. J. & Deutscher, M. P. (1975) *J. Biol. Chem.* **250**, 4796–4801.
28. Joergenson, R. A. & Nordlie, R. C. (1977) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 2103.
29. Kasahara, M. (1977) *Arch. Biochem. Biophys.* **184**, 400–407.
30. Chen, J-J. & Jones, M. E. (1979) *J. Biol. Chem.* **254**, 2697–2704.
31. Miller, M. R., Castellot, J. J. & Pardee, A. B. (1979) *Exp. Cell. Res.* **120**, 421–425.
32. Castellot, J. J., Miller, M. R., Lehtomaki, D. M. & Pardee, A. B. (1979) *J. Biol. Chem.* **254**, 6904–6908.
33. Castellot, J. J., Miller, M. R. & Pardee, A. B. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 351–355.
34. Hofer, H. W. & Krysteck, E. (1975) *FEBS Lett.* **53**, 217–275.
35. Bock, P. E. & Frieden, C. (1976) *J. Biol. Chem.* **251**, 5630–5636.
36. Wenzel, K. W., Kurganov, B. I., Zimmerman, G., Yakovlev, A., Schellenberger, W. & Hofman, E. (1976) *Eur. J. Biochem.* **61**, 181–190.
37. Karadsheh, N. S., Uyeda, K. & Oliver, R. M. (1977) *J. Biol. Chem.* **252**, 3515–3524.
38. Lad, P. M. & Hammes, G. G. (1974) *Biochemistry* **13**, 4530–4537.
39. Otto, M., Heinrich, R., Jacobasch, G. & Rapoport, S. (1977) *Eur. J. Biochem.* **74**, 413–420.
40. Rapoport, T. A., Heinrich, R. & Rapoport, S. M. (1976) *Biochem. J.* **154**, 449–469.