Inhibition of Lactate Transport in Ehrlich Ascites Tumor Cells and Human Erythrocytes by a Synthetic Anhydride of Lactic Acid†

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ABSTRACT: The synthesis and some of the physical and biological characteristics of a new inhibitor of lactate transport are described. The inhibitor is isobutylcarbonyl lactyl anhydride (iBCLA). It is formed by the condensation of lactic acid and isobutylchloroformate. It inhibits lactate transport 50% at 0.5 μg/mg of protein in both Ehrlich ascites tumor cells and human erythrocytes. In contrast, 15 μg of iBCLA/mg of protein is required for 50% inhibition of phosphate transport in erythrocytes, and phosphate transport in Ehrlich ascites tumor cells is unaffected at levels as high as 50 μg of iBCLA/mg of protein. A time-dependent and concentration-dependent reversal of lactate transport inhibition took place on exposure of iBCLA-treated Ehrlich ascites cells to hydroxylamine or dithiothreitol. These data, along with the observed sensitivity of the lactate transporter to sulfhydryl reagents [Spencer, T. L., & Lehninger, A. L. (1976) *Biochem. J. 154*, 405–414], suggest that iBCLA acylates an essential sulfhydryl group on the transporter. When glycolyzing Ehrlich ascites tumor cells were treated with concentrations of iBCLA sufficient for complete inhibition of lactate transport, intracellular lactate levels increased, intracellular pH and extracellular lactate levels decreased, and overall lactate production was inhibited.

Ehrlich ascites tumor cells and human erythrocytes excrete lactate by a proton–lactate symport mechanism (Spencer & Lehninger, 1976; Dubinsky & Racker, 1978). Since tumor cells produce copious quantities of lactate via aerobic glycolysis (Warburg, 1926), compounds which arrest lactate transport could have selectively adverse effects on tumor cells due to a greater potential for lowering intracellular pH.

We have previously demonstrated that bioflavonoids inhibit lactate efflux, lower the intracellular pH, and depress glycolysis in tumor cells (Belt et al., 1979; Suolinna et al., 1975). However, rigorous comparisons of the effects of bioflavonoids on normal and transformed cells in culture have been complicated by the fact that serum albumin reverses the inhibition of glycolysis (Suolinna et al., 1975) and lactate transport (J. A. Belt and E. Racker, unpublished observation). In this paper, we report the synthesis and characterization of a specific inhibitor of lactate transport. The molecule consists of a mixed anhydride formed from the condensation of lactic acid and isobutylchloroformic acid. The molecule also appears to contain a second isobutyl carbonate moiety which has formed an ester with the α-hydroxyl group of lactic acid (Figure 1). We shall describe its effects on lactate transport, its reversibility, and its effects on glycolysis in Ehrlich ascites tumor cells.

Materials and Methods

Synthesis of Carbonic Acid Monoanhydride with Lactic Acid, Isobutyl Ester, and Isobutyl Carbonate (iBCLA).[1,2] All reagents used in the synthesis were of the highest available purity, and several additional measures were taken to ensure anhydrous conditions during the synthesis. The free acid form of L(+)-lactic acid (Type L-1, Sigma Chemical Co., St. Louis, MO) was desiccated in vacuo at −20 °C over P_2O_5 for 48 h, and tetrahydrofuran (Gold Label, Aldrich Chemical Co., Milwaukee, WI) was distilled over $LiAlH_4$ immediately prior to use. Isobutylchloroformate and *N*-methylmorpholine (Aldrich Chemical Co., Milwaukee, WI) were used without further processing. Glassware used in the synthesis was dried at 125 °C for 60 min and cooled by passing N_2 gas through inlet and ventilation tubes containing anhydrous $CaSO_4$ (Drierite).

Lactic acid (5 mmol) was dissolved in 15 mL of tetrahydrofuran, cooled to −15 °C, and flushed with dry N_2 gas. After the solution was cooled, 10 mmol of *N*-methylmorpholine was added and the solution was stirred for 15 min under a stream of N_2 gas. Isobutylchloroformate (10 mmol in 5 mL of tetrahydrofuran) was added dropwise with vigorous stirring at −15 °C. The resulting suspension was stirred for an additional 15 min at 5 °C, and the white *N*-methylmorpholine hydrochloride precipitate was removed by centrifugation in closed tubes at 400*g* for 3 min at 4 °C. The supernatant was collected, and solvents were removed by rotary evaporation under vacuum at ambient temperature for 30 min. Residual amounts of *N*-methylmorpholine hydrochloride were again removed by centrifugation at 800*g* for 5 min at 4 °C. The supernatant was collected, and the very lightly yellow colored oil (ρ = 0.982) was stored at −70 °C, where it was stable for months. Yields obtained from the synthesis were 80–85%.

Chemical and Spectroscopic Analyses of iBCLA. Determinations of iBCLA content were made by using the hydroxamic acid assay (Lipmann & Tuttle, 1945) with succinic anhydride as the standard. Integrated proton magnetic resonance spectra were obtained at ambient temperature in both

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[1] The authors express their appreciation to Kurt L. Loening, Director of Nomenclature, Chemical Abstracts Service, American Chemical Society, for assistance in naming this compound.

[2] Abbreviations used: BSA, bovine serum albumin; DTT, dithiothreitol; Hepes, *N*-2-hydroxyethylpiperizine-*N*′-2-ethanesulfonic acid; $HONH_2$, hydroxylamine; iBCLA, carbonic acid monoanhydride with lactic acid, isobutyl ester, and isobutyl carbonate; NMR, nuclear magnetic resonance.

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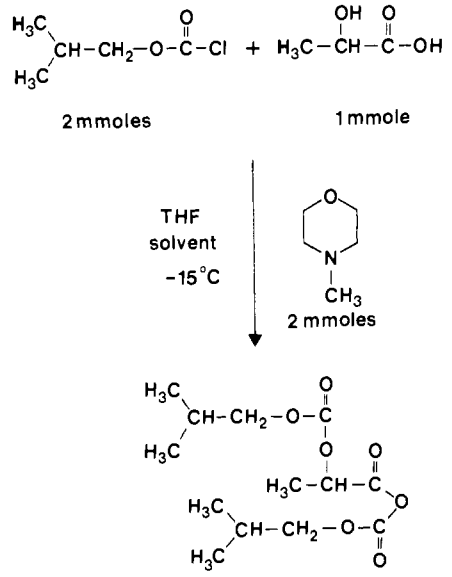

FIGURE 1: Synthesis of carbonic acid monoanhydride with lactic acid, isobutyl ester, and isobutyl carbonate (iBCLA).

deuteriochloroform and carbon tetrachloride solvents in a Varian EM 390 nuclear magnetic resonance (NMR) spectrometer operating at 90 MHz. Tetramethylsilane was used as an internal lock standard. Infrared spectra were obtained with a Perkin-Elmer 257 grating infrared spectrophotometer. The anhydride was analyzed as a neat liquid on NaCl plates (International Crystal Laboratories, Elizabeth, N.J.).

Measurements of Glycolysis, Lactate Uptake, and Phosphate Uptake in Ehrlich Ascites Tumor Cells. Ehrlich ascites tumor cells were maintained and harvested as previously described (Belt et al., 1979), except that the cells were suspended at 10 mg of protein/mL in buffer solution containing 105 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 4 mM NaP_i, and 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.4. The iBCLA was added to the cell suspension in absolute ethanol (5 μL of ethanol/mL of cell suspension) with appropriate controls. Following a 60-min incubation at 0–4 °C, the cells were washed twice in Hepes buffer containing 1 mg of defatted bovine serum albumin/mL. Assays for glycolysis and L-[^{14}C]lactate uptake have been described elsewhere (Belt et al., 1979). For phosphate uptake measurements, cells were treated as described above except that phosphate was omitted from all buffers. Cells were suspended at 2 mg of protein/mL and incubated for 5 min at 37 °C with oligomycin (8 μg/mL) and antimycin A (2 μM). Phosphate uptake was initiated by the addition of 125 μM [^{32}P]P_i (700 cpm/nmol). After 5 min, P_i uptake was measured by a modification of the method of Gasko et al. (1976). The cell suspension (0.5 mL) was passed over a 0.6 × 9.0 cm Dowex formate column (Bio-Rad AG-1-X8, 50–100 mesh) which had been treated with 0.5 mL of horse serum (Grand Island Biological Co., Grand Island, N.Y.) and 2 mL of 0.3 M sucrose containing 5% horse serum. Cells were eluted from the column with 2 mL of 0.3 M sucrose–5% horse serum and counted in 12 mL of Liquiscint (National Diagnostics, Somerville, N.J.).

Measurements in Lactate and Phosphate Uptake into Human Erythrocytes. Uptake of L-[^{14}C]lactate and [^{32}P]P_i into human erythrocytes was measured as previously described (Dubinsky & Racker, 1978).

Other Methods. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. The relative change in intracellular pH of ascites cells was determined from the distribution of [^{14}C]methylamine (Rottenberg et al., 1972) by using the modification of Belt et al. (1979).

Results and Discussion

The synthesis and proposed structure of the iBCLA molecule are outlined in Figure 1. The product is shown to be a mixed anhydride formed by the condensation of isobutylchloroformate and the lactate carboxyl group, as well as an ester formed by the attack of isobutylchloroformate on the lactate α-hydroxyl group. This formulation is based upon the following observations. Firstly, integration of the peak intensities in the proton magnetic resonance spectrum shown in Figure 2 reveals that

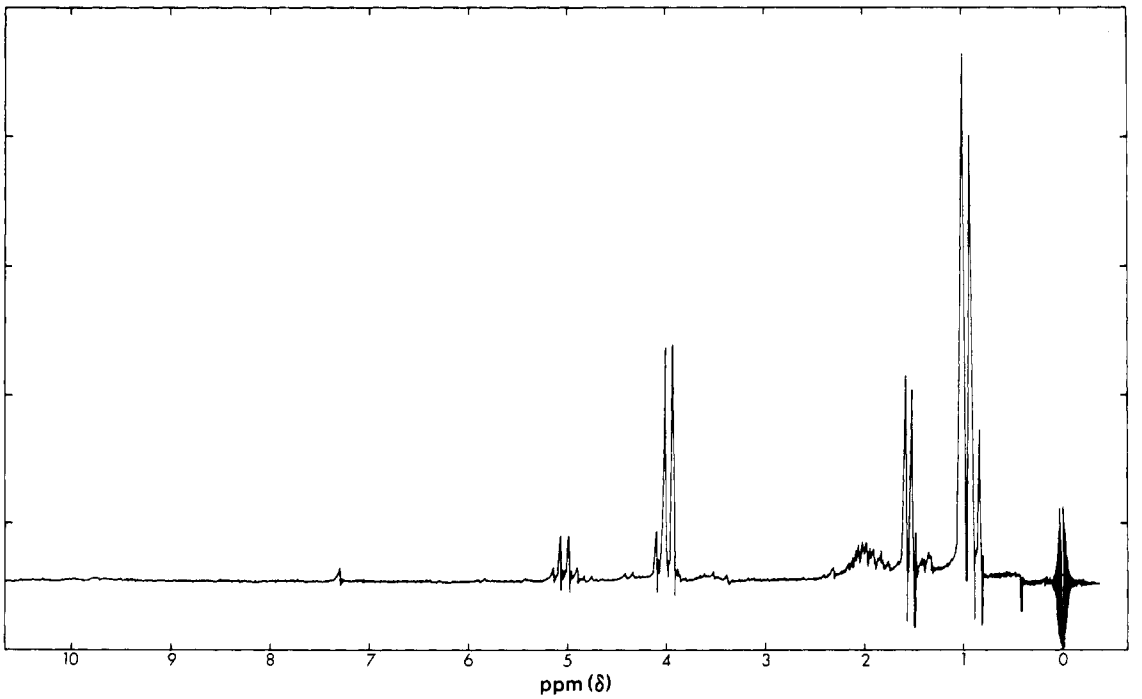

FIGURE 2: Proton magnetic resonance spectrum of iBCLA. The spectrum was obtained in a $CDCl_3$ solvent using tetramethylsilane as an internal lock standard at 90 MHz.

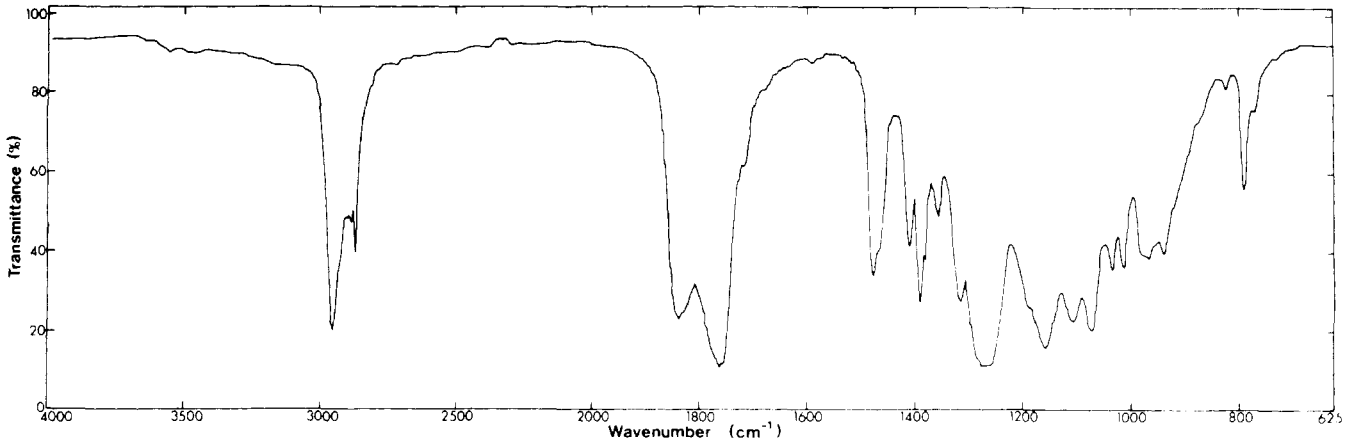

FIGURE 3: Infrared absorption spectrum of iBCLA. The spectrum was obtained with a sample of iBCLA as a neat liquid on NaCl plates.

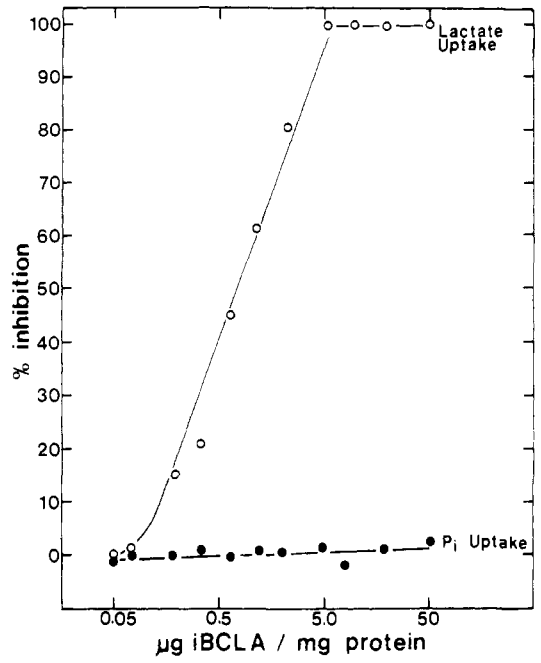

FIGURE 4: Lactate and phosphate uptake in iBCLA-treated Ehrlich ascites tumor cells. Cells were incubated in Hepes buffer, pH 7.4, containing the indicated concentration of iBCLA at 0 °C for 60 min and then washed 2 times in Hepes buffer, pH 7.4, containing 1 mg of defatted bovine serum albumin (BSA)/mL. Lactate uptake (○) and phosphate uptake (●) were assayed as described under Materials and Methods.

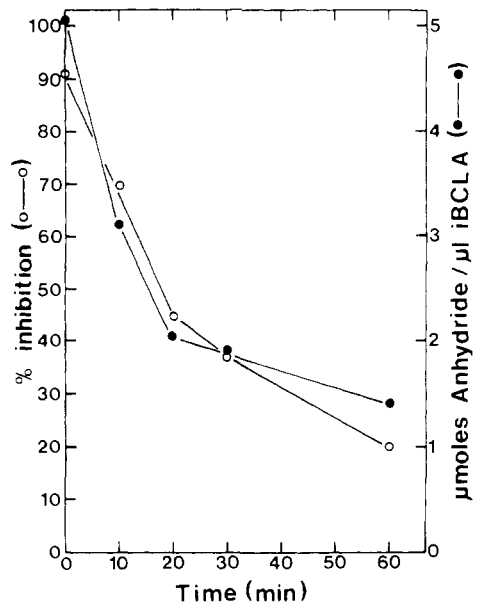

FIGURE 5: Comparison of the stabilities of iBCLA anhydride linkage and iBCLA lactate transport inhibitory activity during incubation in aqueous buffers. Ethanolic solutions of iBCLA were added to Hepes buffer, pH 7.4, at 10 μg of iBCLA/mL of buffer and incubated for the indicated times at room temperature. Following the incubation, cells (2 mg of protein/mL final concentration) were added to one aliquot of the buffer–iBCLA mixture and assayed for lactate uptake (○) as described in Figure 4. The second aliquot was used to assay for the anhydride linkage of iBCLA (●) as described under Materials and Methods.

there are two isobutyl groups per lactyl group in the molecule. Secondly, all efforts to demonstrate free hydroxyl groups or carboxyl groups with nuclear magnetic resonance or infrared spectroscopy (Figure 3) have been uniformly negative. Further, if the synthesis is carried out with 1 equiv of isobutylchloroformate and *N*-methylmorpholine per equiv of lactate, a product is obtained which quantitatively contains less anhydride and is much less effective as a lactate transport inhibitor (data not shown).

The infrared spectrum shown in Figure 3 contains two bands in the carbonyl stretching region at ~1765 and ~1828 cm^{-1} which are characteristic of acid anhydrides. Other features of the spectrum are bands at 1065 and 1105 cm^{-1} indicative of primary and secondary C–O groups, strong bands at 2950, 1475, 1385, and 1260 cm^{-1} indicative of multiple CH_3 and CH_2 groups, and the absence of bands for acid chlorides and free carboxyl and free hydroxyl groups.

Inhibition of Lactate Uptake by iBCLA in Ehrlich Ascites Tumor Cells. A titration of the effects of iBCLA on both lactate and phosphate uptake is given in Figure 4. It can be seen that 0.5 μg of iBCLA/mg of protein resulted in a 50% loss of lactate uptake while no inhibition of phosphate transport in these cells was observed at concentrations up to 50 μg/mg of protein.

Bioflavonoid-induced inhibition of lactate transport (J. A. Belt and E. Racker, unpublished results) and glycolysis (Suolinna et al., 1975) are reversed by serum albumin. In contrast, washing ascites cells with serum albumin did not relieve inhibition of lactate transport by iBCLA.

As shown in Figure 5, the anhydride content and the ability of iBCLA to inhibit lactate uptake were lost at the same rate when iBCLA was incubated in aqueous buffer at room temperature. The half-life of the anhydride linkage and lactate transport inhibitory activity was ~15 min.

Table I compares the effectiveness of iBCLA with succinic anhydride, maleic anhydride, and acetyl phosphate. Maleic anhydride and succinic anhydride inhibited lactate transport in Ehrlich ascites tumor cells, but the concentrations required

Table I: Comparison of iBCLA, Maleic Anhydride, Succinic Anhydride, and Acetyl Phosphate as Inhibitors of Lactate Uptake in Ehrlich Ascites Tumor Cells[a]

compd	μg/mg of protein	(nmol of lactate)/(min mg of protein)	% inhibn
none		21.5	
iBCLA	0.1	18.3	15
	0.5	11.6	46
	1.0	3.4	84
maleic anhydride	5.0	17.4	19
	25.0	10.3	52
	50.0	3.9	82
succinic anhydride	25.0	17.6	18
	50.0	14.6	32
	100.0	9.5	56
acetyl phosphate	25.0	23.1	0
	50.0	24.6	0
	100.0	24.0	0

[a] Compounds were added as ethanolic solutions to cells in Hepes buffer, pH 7.4, and incubated for 60 min at 0 °C. The cells were then washed 2 times in Hepes buffer, pH 7.4, containing 1 mg of defatted BSA/mL and assayed for lactate uptake as described under Materials and Methods.

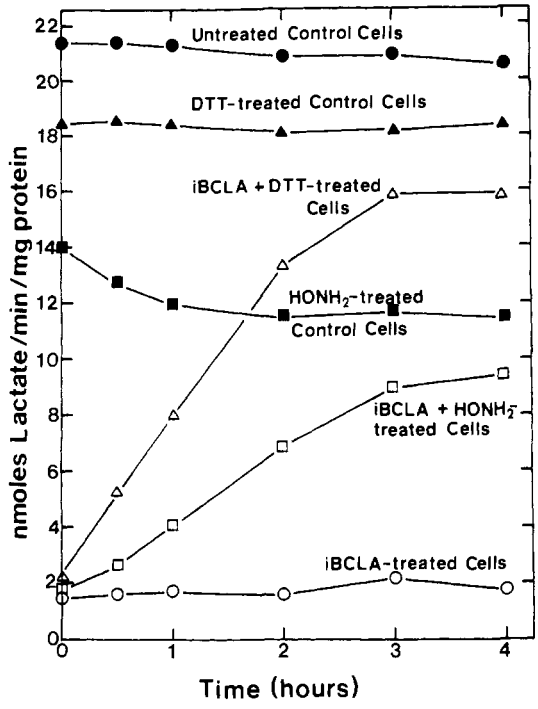

FIGURE 6: Time course of hydroxylamine and dithiothreitol reversal of iBCLA-induced lactate transport inhibition of Ehrlich ascites tumor cells. Cells were treated with 1 μg of iBCLA/mg of cell protein as described in Figure 4, washed twice in buffer containing 1 mg of defatted BSA/mL, and resuspended in buffer containing 50 mM dithiothreitol or 100 mM hydroxylamine, pH 7.4. After incubation for the indicated time intervals, the cells were rewashed 2 times in buffer containing BSA and assayed for lactate uptake. Untreated control cells (●), control cells incubated in 50 mM dithiothreitol (▲), iBCLA-treated cells incubated in 50 mM dithiothreitol (△), control cells incubated in 100 mM hydroxylamine (■), iBCLA-treated cells incubated in 100 mM hydroxylamine (□), and iBCLA-treated cells incubated in buffer (○) are shown.

for 50% inhibition were 50- and 200-fold higher, respectively, than that required with iBCLA. Inhibition by maleic and succinic anhydrides was lost at the same rate as their respective anhydride linkages were hydrolyzed in aqueous buffers. Therefore, it appears that the anhydride linkage is required for inhibition of the lactate transporter by these compounds and that the structure of iBCLA gives the molecule some degree of specificity for the transporter.

Table II: Effects of iBCLA on the Intracellular pH of Glycolyzing Ehrlich Ascites Tumor Cells[a]

treatment	relative internal pH
none	6.76
1.0 μg of iBCLA/mg of protein	6.76
5.0 μg of iBCLA/mg of protein	6.48
10.0 μg of iBCLA/mg of protein	6.45
20.0 μg of iBCLA/mg of protein	6.46

[a] Cells were treated with iBCLA at the indicated concentrations as described in Figure 4. After washing the cells and resuspending them in Hepes pH 7.24 buffer containing either [^{14}C]methylamine-[^{3}H]inulin or [^{14}C]inulin-[$^{3}H_2O$], glycolysis was initiated by the addition of 10 mM glucose. After 30 min of incubation at 30 °C, the cell suspension was passed through silicone by centrifugation as described by Belt et al. (1979).

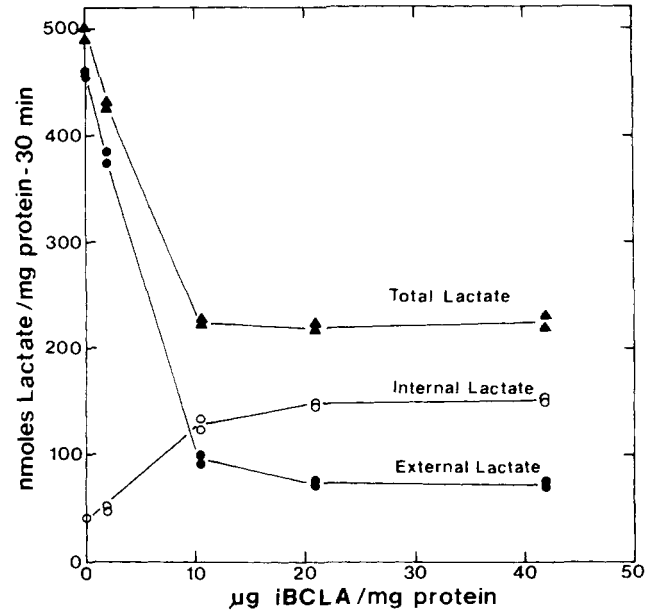

FIGURE 7: Effect of iBCLA on glycolysis in Ehrlich ascites tumor cells. Cells were treated with iBCLA as described in Figure 4 and were assayed for internal lactate concentrations (○), external lactate concentrations (●), and total lactate production (▲) as described by Belt et al. (1979).

The hydrolysis data, along with the observed sensitivity of the transporter to sulfhydryl reagents (Spencer & Lehninger, 1976), suggested that iBCLA might acylate an essential sulfhydryl group on the transporter. As a test of this hypothesis, we explored deacylation of the transporter by hydroxylamine and transacylation in the presence of dithiothreitol following treatment of the cells with iBCLA. Both hydroxylamine and dithiothreitol incubations resulted in a time-dependent (Figure 6), concentration-dependent (data not shown) reversal of transport inhibition. Incubation of control cells in hydroxylamine reduced the transport ability of the cells by ~40% indicating some deleterious effects of hydroxylamine. On the other hand, dithiothreitol had virtually no effect on control cells. In both cases, transport in control cells and "restored" transport in iBCLA cells were sensitive to inhibition by $HgCl_2$ and α-cyano-4-hydroxycinnamic acid.

Effects of iBCLA on Glycolysis in Ehrlich Ascites Tumor Cells. Lactate is excreted in Ehrlich ascites tumor cells together with a proton (Spencer & Lehninger, 1976), and we have previously shown (Belt et al., 1979) that bioflavonoids block the excretion of lactate, thereby lowering the intracellular pH. This, in turn, results in an inhibition of glycolysis. As shown in Figure 7 and Table II, iBCLA had an effect very

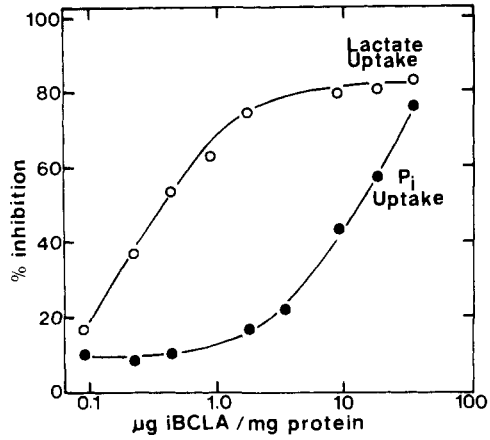

FIGURE 8: Effect of iBCLA on lactate and phosphate uptake in human erythrocytes. Erythrocytes (4 mg of protein/mL) were washed as previously described (Dubinsky & Racker, 1978) and treated with the indicated amounts of iBCLA for 60 min at 0 °C. They were then washed twice in buffer containing 1 mg of BSA/mL and assayed for lactate uptake (○) and phosphate uptake (●).

similar to that of the bioflavonoids. When glycolysis was measured in iBCLA-treated cells, lactate accumulated intracellularly (Figure 7), the internal pH decreased (Table II), and total lactate production was inhibited (Figure 7). As was observed with the bioflavonoids (Belt et al., 1979), nearly complete inhibition of lactate transport was required before an increase of internal lactate levels, a lowering of intracellular pH, and inhibition of glycolysis were observed (compare Figures 4 and 7 and Table II). This type of response further supports the conclusion of Spencer & Lehninger (1976) that the capacity of the transporter far exceeds the cellular rate of lactate production. A rigorous evaluation of the effects of iBCLA on normal and transformed cell growth in tissue culture is presently underway.

Inhibition of Lactate and Phosphate Uptake in Human Erythrocytes by the iBCLA. Erythrocytes represent a second class of cells which must excrete lactate since they undergo glycolysis to form lactate in a virtually stoichiometric manner (Barlett & Marlow, 1953). Moreover, human erythrocytes are devoid of the metabolic machinery needed for further oxidation.

The sensitivity of lactate transport in human erythrocytes to inhibitors of inorganic anion fluxes such as 4,4′-bis(isothiocyano)-2,2′-stilbenedisulfonate (DIDS) has led to the postulation of a monocarboxylic acid–inorganic anion antiport mechanism for monocarboxylic acid transport (Rice & Steck, 1976). More recently, Dubinsky & Racker (1978) have shown that the stilbene derivatives can either inhibit or stimulate lactate transport depending on the direction of the chloride flux (which creates a proton gradient). Direct measurements of lactate and proton fluxes revealed a monocarboxylic acid transport system in erythrocytes independent of the inorganic ion channel and consistent with a lactate–proton symport mechanism.

The effects of iBCLA on lactate and phosphate transport in human erythrocytes are shown in Figure 8. Both transporters were sensitive to iBCLA. However, the lactate transporter was selectively inhibited 50% at 0.5 μg of iBCLA/mg of protein while ~15 μg of iBCLA/mg of protein was required for 50% inhibition of phosphate uptake. These data reinforce the striking similarities between lactate transport in erythrocytes and ascites tumor cells which include pH dependence, activation energies of both uptake and efflux, and sensitivity to bioflavonoid inhibitors and iBCLA (Dubinsky & Racker, 1978; Spencer & Lehninger, 1976; Belt et al., 1979; Dubinsky et al., 1978). However, iBCLA might prove to be more toxic to ascites tumor cells than to erythrocytes since erythrocytes have an alternative pathway for proton excretion (e.g., SITS-sensitive chloride–bicarbonate exchange).

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