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GLYCOLYTIC MECHANISMS IN EHRLICH ASCITES TUMOR CELLS—EFFECTS OF DISULFIDE DERIVATIVES OF PYRIDINE*

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Abstract—A study was made of the effects of 6,6′-dithiodinicotinic acid (DTDN) and 2,2′-dithiodipyridine (DTDP) on mouse Ehrlich ascites tumor cells. Both drugs inhibited glucose utilization and lactate production under aerobic conditions, DTDP being effective in lower concentrations than DTDN. Both drugs decreased the proportion of utilized glucose that is converted to lactate. This may indicate increased diversion of glucose into the phosphogluconate pathway. DTDN in concentrations inhibiting lactate production to a minimal degree greatly decreased the levels of dihydroxyacetone phosphate and increased those of glyceraldehyde-3-phosphate occurring in the cells after addition of glucose. This effect was not seen with DTDP. The effect of DTDN on the glycolytic intermediates may be due to inhibition of triosephosphate isomerase. Lack of the effect with DTDP may be due to more potent inhibition of an enzyme at a step prior to formation of fructose-1,6-diphosphate. Both drugs inhibit rabbit muscle triosephosphate isomerase *in vitro*. The drugs decreased the fall of extracellular pH due to lactic acid formation but did not lead to any unusual relationship between extracellular and intracellular pH. Both drugs caused loss of cellular K^+ but only in concentrations higher than are required for complete suppression of glycolysis.

GRASSETTI and his colleagues[1–5] have published a series of reports on the effects on Ehrlich ascites tumor cells of sulfhydryl and disulfide derivatives of pyridine. The compounds most thoroughly studied were 2,2′-dithiodipyridine (DTDP)† and 6,6′-dithiodinicotinic acid (DTDN). DTDP was reported to inhibit respiration and lactate production under both aerobic and anaerobic conditions, whereas DTDN was said to belong to a class of compounds differing in that they caused moderate or negligible inhibition of anaerobic glycolysis and of respiration, accompanied by apparent stimulation of aerobic glycolysis.[3] DTDN increased the production of CO_2 from the C in the 1-position of glucose and inhibited that from the C in the 6-position.[4] This was interpreted as indicating stimulation of the phosphogluconate pathway. The effects of both compounds were attributed to reaction with cellular sulfhydryl groups to produce disulfide bonds between two cellular groups or mixed disulfide bonds between a cellular group and half of the drug molecule. Comparison of the extent of reaction of the drugs with intact and homogenized Ehrlich ascites cells led to the conclusion that DTDN penetrates the cells poorly, reacting principally

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† Abbreviations used: DTDN, 6,6′-dithiodinicotinic acid; DTDP, 2,2′-dithiodipyridine; DMO, 5,5-dimethyl-2,4-oxazolidinedione; F-di-P, fructose-1,6-diphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; TIM, triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, 5.3.1.1); GDH, glycerophosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, 1.1.1.8); GAPDH, glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase phosphorylating, 1.2.1.12); pH_e, extracellular pH; pH_i, intracellular pH.

with surface sulfhydryl groups, whereas DTDP penetrates readily and reacts with interior as well as exterior sulfhydryl groups.[4]

Because these pyridine disulfides represented a new type of drug affecting glycolysis and because of the puzzling report of qualitatively different effects of DTDN and of DTDP on glycolysis, we considered these effects to merit further study. The present report concerns an investigation of the effects of these two drugs on glycolytic mechanisms in Ehrlich ascites tumor cells. The study includes measurements of glucose, lactate, fructose-1,6-diphosphate, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate as well as measurements of extracellular and intracellular pH and cellular K^+.

MATERIALS AND METHODS

6,6′-Dithiodinicotinic acid was prepared in this laboratory by Dr. Kenneth H. Dudley by the method of Fox and Gibas.[6] 2,2′-Dithiodipyridine was purchased from Aldrich Chemical Company. Enzymes were purchased from Sigma Chemical Company.

Preparation of cells and incubations. The Ehrlich ascites tumor cells were grown in mice and harvested as previously described.[7] The preparation of the cells, their suspension in a Krebs–Ringer phosphate buffer, the conditions of aerobic incubation and the addition of 5,5-dimethyl-2,4-[^{14}C]oxazolidinedione (DMO) and [carboxyl-^{14}C]-inulin for measurement of intracellular pH were the same as in an earlier study.[8] The packed cell volumes as percentages of the suspensions are noted in the figure legends. DTDN or DTDP was added to the suspension 15 min before addition of glucose. Stock solutions of DTDN with 2 equiv. NaOH in buffer were prepared 10 times the final concentrations desired, and 0·1 ml was added/ml of suspension. In the control preparations, 0·1 ml of buffer was added/ml of suspension. Stock solutions of DTDP 100 times the final concentrations desired were prepared in ethanol, and 0·01 ml was added/ml of suspension. In the control preparations, 0·01 ml of ethanol was added/ml of suspension. Glucose was added as a 0·11 M solution in buffer, 0·1 ml/ml of suspension. For each drug, in any given experiment, all concentrations as well as controls were studied on portions of the same cell suspension.

Calculation of intracellular pH (pH_i). The procedures for the measurement of extracellular pH (pH_e) and for the use of distribution of [^{14}C]DMO between intracellular and extracellular water for calculation of pH_i in tumor cells *in vitro* are described in an earlier report.[9]

Analytical methods. The enzymatic methods used for determination of glucose, lactate, fructose-1,6-diphosphate (F-di-P), glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) have been cited in earlier publications.[9,10] Intracellular K^+ was determined by flame photometry as previously described.[11]

Study of inhibition of triosephosphate isomerase (TIM) in vitro *by DTDN and DTDP.* The rate of conversion of GAP to DHAP by TIM was measured by coupling this reaction to the reduction of DHAP to α-glycerophosphate by NADH, enzymatically catalyzed by glycerophosphate dehydrogenase (GDH). The reaction was followed by observing the disappearance of NADH. GDH was used in such a large amount that the isomerization reaction was the rate-limiting step. This is a system described by Meyer–Arendt *et al.*[12] and subsequently used by a number of other workers to study the rate of this isomerization.

All solutions and incubations were in a buffer containing 0·4 mole of triethanolamine, 0·144 mole of NaOH, and 0·04 mole of EDTA/liter (pH 7·6).

Solutions of crystalline rabbit muscle TIM containing 8 ng of protein/ml and different concentrations of DTDN or DTDP were incubated at 37° for 30 min. At the end of these incubations, the solutions were diluted with 2 volumes of buffer. Solutions of NADH, GDH and DL-GAP were then added rapidly, the final concentrations being: TIM, 2 ng of protein/ml; GDH, 2·2 μg of protein/ml; NADH, 5×10^{-5} M; DL-GAP, 2×10^{-3} M. At this stage the concentration of DTDN or DTDP was 28 per cent of that with which TIM had originally been incubated. Immediately after the final additions, absorbancy at 340 nm was followed in the recording spectrophotometer, the cuvette being at room temperature (about 25°). The rate of oxidation of NADH was calculated from the initial rate of decline of absorbancy and the molar extinction coefficient of NADH ($6{\cdot}22 \times 10^3$).

When identical incubations were prepared except that TIM was omitted and DHAP to a concentration of 10^{-3} M was added as substrate rather than GAP, the NADH was completely oxidized in less than half a min. This is over 15 times the highest rates observed with GAP as substrate. This shows that the isomerization reaction rather than the reduction of DHAP is rate limiting. With no concentrations of either drug does any possible inhibition of GDH make the latter reaction rate limiting.

Attempts were made to study the rate of isomerization in the opposite direction with DHAP as substrate, the GAP produced being oxidized by NAD^+ in the presence of arsenate and catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The inhibitory effects of the two drugs on TIM could not be studied with this system because they are both strongly inhibitory to GAPDH. This enzyme is known to be sensitive to sulfhydryl reagents.

Statistical methods. Because there is no reason to believe that the quantitative measurements obtained by this study would follow normal distributions and because it is not feasible to accumulate sufficient observations to gain knowledge of the forms of the distributions and adequate measures of their dispersions, the use of parametric tests of significance would be inappropriate. To test the significance of differences between groups of measurements with different treatments, we have used the non-parametric ranking test of Wilcoxon,[13] as further developed by Mann and Whitney.[14] This test entails no assumption of any particular form of distribution. Under the null hypothesis of identity of two variables, x and y, all ordered sequences of the observed values of x and y have equal probability. The values of the x's and y's are ranked in order of magnitude. A low value of the probability of the observed sequence and all others in which there are smaller numbers of times in which a y precedes an x is the basis for rejection of the null hypothesis. We shall refer to a difference as significant if the value of P (two-tail) from this test is less than 0·05. For two groups of five each, the ordered sequences indicating this level of probability of a stochastic difference between the variables x and y are:

$$x_1, x_2, x_3, x_4, x_5, y_1, y_2, y_3, y_4, y_5;$$
$$x_1, x_2, x_3, x_4, y_1, x_5, y_2, y_3, y_4, y_5;$$
$$x_1, x_2, x_3, x_4, y_1, y_2, x_5, y_3, y_4, y_5;$$
$$x_1, x_2, x_3, y_1, x_4, x_5, y_2, y_3, y_4, y_5.$$

RESULTS

Glucose utilization and lactate production. Concentrations of about 5 mM of DTDN and concentrations of DTDP of about one-third this amount were capable of completely inhibiting glucose utilization and lactate production in Ehrlich ascites tumor cells. The effects of these concentrations were irreversible in that the capacity of the cells to glycolyze could not be restored in any degree by washing or by washing followed by treatment with cysteine, glutathione or dithiothreitol. Concentrations of the drugs less than those required for complete inhibition could produce partial inhibition of glycolysis. The graded effects on glycolysis produced by three concentrations of DTDN are shown in Fig. 1 and by three concentrations of DTDP in Fig. 2. For equivalent degrees of inhibition, DTDN was required in a concentration about three times that of DTDP.

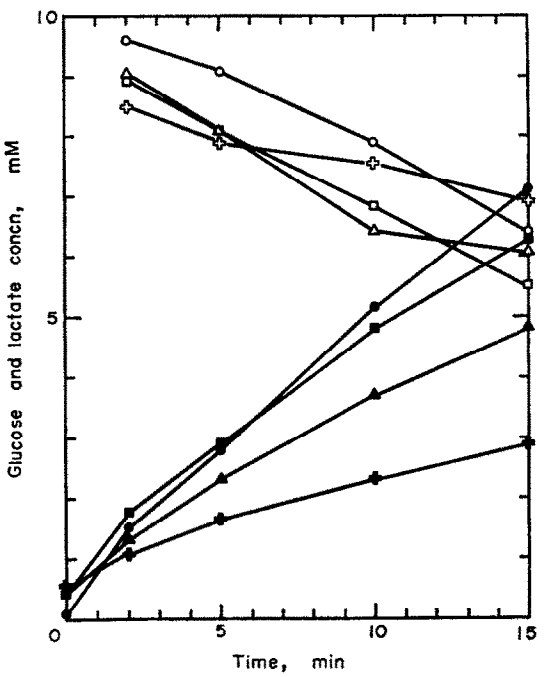

FIG. 1. Effects of 6,6′-dithiodinicotinic acid on glucose utilization and lactate production in suspensions of Ehrlich ascites tumor cells in Krebs–Ringer phosphate buffer. The values plotted are means from five experiments in which the suspensions were 12–16 per cent cells by volume. The cells were incubated with various concentrations of the drug for 15 min before addition of glucose. Glucose to a final concentration of 11 mM was added immediately after sampling at zero time. Concentrations of glucose (open symbols) and lactate (closed symbols) were measured in the extracellular phase. Drug concentration (mM): 0 = circle; 1·67 = square; 2·31 = triangle; and 3·20 = cross.

The amounts of glucose utilized and of lactate produced can be estimated from the changes in the concentrations of those substances in the extracellular phase on the assumptions that glucose is entirely extracellular and that lactate is in equal concentrations extracellularly and intracellularly. Whether or not the cells had been treated with a drug, the glucose that disappeared before the sampling at 2 min could be

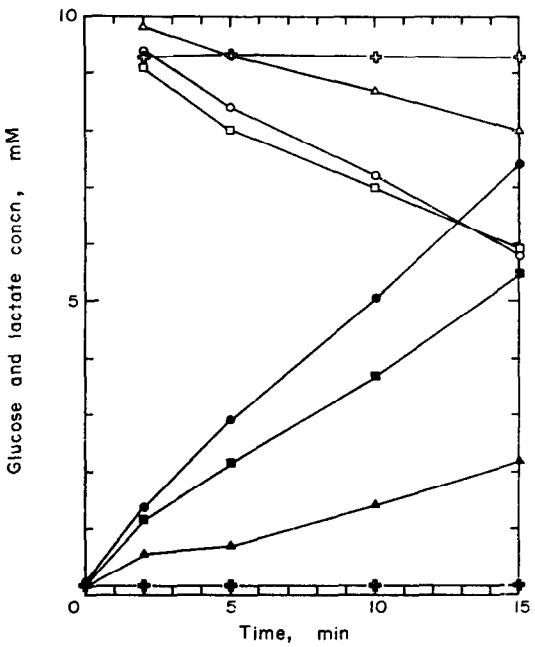

FIG. 2. Effects of 2,2′-dithiodipyridine on glucose utilization and lactate production in suspensions of Ehrlich ascites tumor cells in Krebs–Ringer phosphate buffer. The values plotted are means from five experiments in which the suspensions were 16–20 per cent cells by volume. The cells were incubated with various concentrations of the drug for 15 min before addition of glucose. Glucose to a final concentration of 11 mM was added immediately after sampling at zero time. Concentrations of glucose (open symbols) and lactate (closed symbols) were measured in the extracellular phase. Drug concentration (mM): 0 = circle; 0·60 = square; 1·00 = triangle; and 1·67 = cross.

accounted for only in part by the lactate produced. Glucose utilization and lactate production between 2 and 15 min were calculated for each of five experiments with each drug, the same experiments as those of Figs. 1 and 2. The averages of these values are tabulated in Table 1 as well as the averages of the glucose/lactate ratios. The statistically significant differences are indicated in the table. The glucose utilized between 2 and 15 min by cells untreated with drug was replaced with approximately the theoretical yield of 2 moles of lactate. With increasing concentrations of either drug, there was a progressive decrease in the lactate/glucose ratio.

Glycolytic intermediates. DTDN had a pronounced effect on the concentrations of the phosphorylated glycolytic intermediates, F-di-P, DHAP and GAP, occurring in the cells after administration of glucose. These effects, which are shown in Fig. 3, were seen even with the concentration of 1·67 mM, which had only a small effect on overall lactate production. The values in Fig. 3 are means from five experiments, but the same pattern was seen consistently in each of the experiments. The effects that are statistically significant are noted in the legend of Fig. 3. The drug in the lowest concentration used, 1·67 mM, caused a considerable increase in the concentration of F-di-P. The effect decreased with increasing drug concentration. There was a marked effect of the drug in decreasing DHAP. This effect increased with increasing drug

TABLE 1. EFFECTS OF 6,6'-DITHIODINICOTINIC ACID (DTDN) AND 2,2'-DITHIODIPYRIDINE (DTDP) ON GLUCOSE UTILIZATION AND LACTATE PRODUCTION BY EHRLICH ASCITES TUMOR CELLS DURING THE PERIOD BETWEEN 2 AND 15 min AFTER ADDITION OF GLUCOSE TO THE CELLS*

Drug	Drug concn (mM)	Glucose utilized (μmoles/ml)	Lactate produced (μmoles/ml)	Lactate/glucose
DTDN	0	20·8	40·8	1·97
	1·67	21·1	36·3	1·72
	2·31	18·7	26·2†	1·41†
	3·20	11·7†	13·0†	1·25†
DTDP	0	16·7	33·2	2·04
	0·60	14·7	24·2†	1·67
	1·00	8·0†	10·1†	1·25†
	1·67	0†	0†	

* Calculations are from the concentrations of glucose and lactate in the extracellular phase and are based on the assumptions that glucose is entirely extracellular and that lactate is in equal concentrations extracellularly and intracellularly. Data for the calculations come from the same experiments as of Figs. 1 and 3 for DTDN and of Figs. 2 and 4 for DTDP. Values are expressed as micromoles of glucose utilized or lactate produced per milliliter packed cell volume. Glucose utilization, lactate production and the lactate/glucose ratios were calculated separately for each of the five experiments with each drug. Averages of the sets of five values are tabulated above.

† Statistically significant effects in comparison of treated with untreated cells.

concentration but was very prominent with the lowest concentration used. GAP, which in the absence of drug is in concentrations too low to measure with the method used here, accumulated to greatly increased levels under the influence of the drug.

As shown in Fig. 4, the effects of DTDP on the intermediates were quite different from those of DTDN. There was no accumulation of GAP. The levels of F-di-P were decreased almost to zero by the highest concentration. The lower concentrations had smaller effects in decreasing Fi-d-P, and these effects decreased with time. There was some decrease in DHAP concentrations, but this was not comparable to the effects of DTDN at concentrations having less effect on lactate production. The only concentration of DTDP having an effect on DHAP as great as that of DTDN was the highest of Figs. 2 and 4, a concentration completely inhibiting glucose utilization, lactate production and F-di-P appearance. The effects of the drug that are statistically significant are noted in the legend of Fig. 4.

Extracellular and intracellular pH values. Measured values of pH_e and calculated values of pH_i after addition of glucose are shown for cells treated with DTDN in Fig. 5 and for those treated with DTDP in Fig. 6. The relationship between pH_e and pH_i was not greatly different in cells treated with either drug from that in untreated cells. When the fall of pH_e was decreased by treatment with a drug, the gradient between pH_e and pH_i was diminished. It should be noted that the cell suspension in the experiment of Fig. 6 was heavier than that in the experiment of Fig. 5. The production of lactate and consequent fall of pH_e were accordingly greater in untreated cells in the experiment of Fig. 6.

Intracellular K^+. Figures 7 and 8 show changes in intracellular K^+ after addition of glucose to cells treated with DTDN and DTDP respectively. Neither drug caused

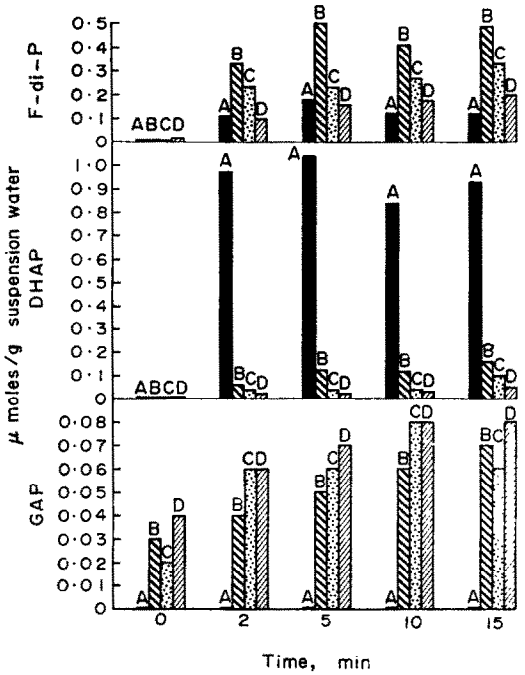

FIG. 3. Effects of 6,6′-dithiodinicotinic acid on concentrations of fructose-1,6-diphosphate (F-di-P), dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP) in Ehrlich ascites tumor cells. The values shown are means from the same five experiments as those of Fig. 1. The times indicated are the intervals after addition of glucose to the cell suspensions. The four lettered bars at each time interval represent increasing concentrations of the drug (A, no drug; B, 1·67 mM; C, 2·31 mM; D, 3·20 mM). Statistically significant effects of the drug: elevation of GAP at all times with all concentrations; lowering of DHAP at all times after addition of glucose with all concentrations; elevation of F-di-P with 1·67 mM at all times after addition of glucose. The concentrations of F-di-P in cells treated with 3·20 mM are significantly lower than those in cells treated with 1·67 mM.

TABLE 2. EFFECT OF INCUBATION OF RABBIT MUSCLE TRIOSEPHOSPHATE ISOMERASE *in vitro* WITH 6,6′-DITHIODINICOTINIC ACID (DTDN) OR 2,2′-DITHIODIPYRIDINE (DTDP) ON THE SUBSEQUENT RATE OF OXIDATION OF NADH AFTER ADDITION OF GLYCERALDEHYDE-3-PHOSPHATE AND GLYCEROPHOSPHATE DEHYDROGENASE*

Drug	Drug concn (moles × 10^{-4} l^{-1})	Rate of NADH oxidation (moles × 10^{-6} l^{-1} min^{-1})	Rate of NADH oxidation % of Control
DTDN	0	6·6	100
	1·0	5·2	79
	2·0	4·4	67
	4·0	3·1	47
	8·0	1·8	27
DTDP	0	7·1	100
	0·5	5·6	79
	1·0	4·5	64
	2·0	3·0	42
	4·0	1·6	23

* The concentration of drug shown is that with which the isomerase was originally incubated.

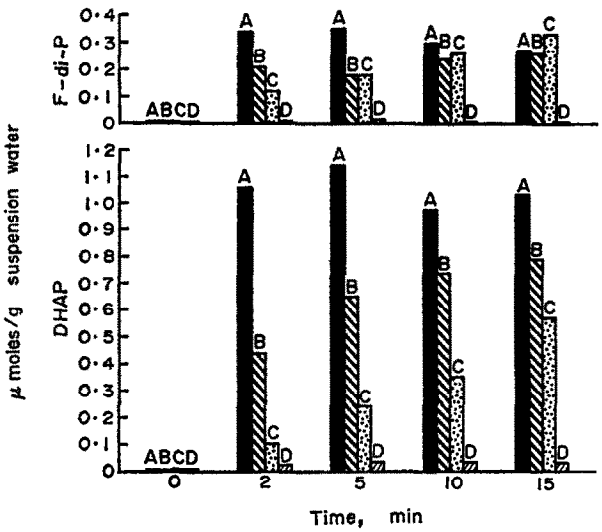

FIG. 4. Effects of 2,2′-dithiodipyridine on concentrations of fructose-1,6-phosphate (F-di-P) and dihydroxyacetone phosphate (DHAP) in Ehrlich ascites tumor cells. The concentrations of glyceraldehyde-3-phosphate were all too low for measurement. The values shown are means from the same five experiments as those of Fig. 2. The times indicated are the intervals after addition of glucose to the cell suspensions. The four lettered bars at each time interval represent increasing concentrations of the drug (A, no drug; B, 0·60 mM; C, 1·00 mM; D, 1·67 mM). Statistically significant effects of the drug: lowering of DHAP with 0·60 mM at 2 and 5 min, with 1·00 mM at 2, 5 and 10 min, and with 1·67 mM at all times after addition of glucose; lowering of F-di-P with 0·60 mM at 5 min, with 1·00 mM at 2 and 5 min, and with 1·67 mM at all times after addition of glucose.

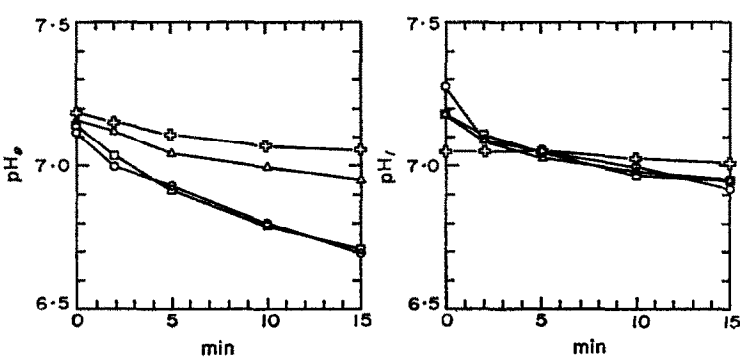

FIG. 5. Extracellular pH (pH_e) and corresponding intracellular pH (pH_i) values in suspensions of Ehrlich ascites tumor cells (approximately 15 per cent by volume) in 25 mM Krebs–Ringer phosphate buffer. The cells were incubated with three different concentrations of 6,6′-dithiodinicotinic acid for 15 min before addition of glucose. Glucose to a final concentration of 11 mM was added to all incubations immediately after sampling at zero time. Symbols denoting drug concentrations: circle, no drug; square, 1·67 mM; triangle, 2·31 mM; cross, 3·20 mM.

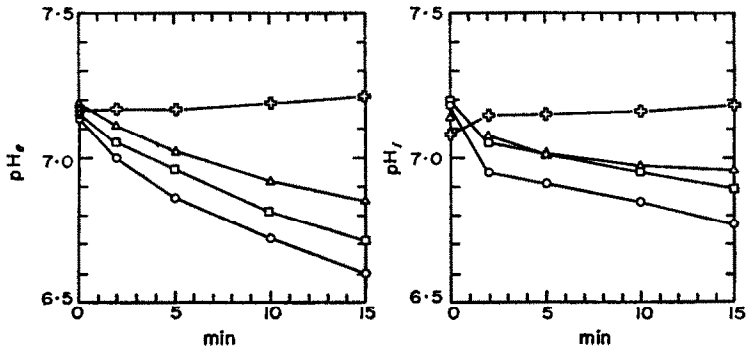

FIG. 6. Extracellular pH (pH_e) and corresponding intracellular pH (pH_i) values in suspensions of Ehrlich ascites tumor cells (approximately 20 per cent by volume) in 25 mM Krebs–Ringer phosphate buffer. The cells were incubated with three different concentrations of 2,2′-dithiodipyridine for 15 min before addition of glucose. Glucose to a final concentration of 11 mM was added immediately after sampling at zero time. Symbols denoting drug concentrations: circle, no drug; square, 0·6 mM; triangle, 1·0 mM; cross, 1·67 mM.

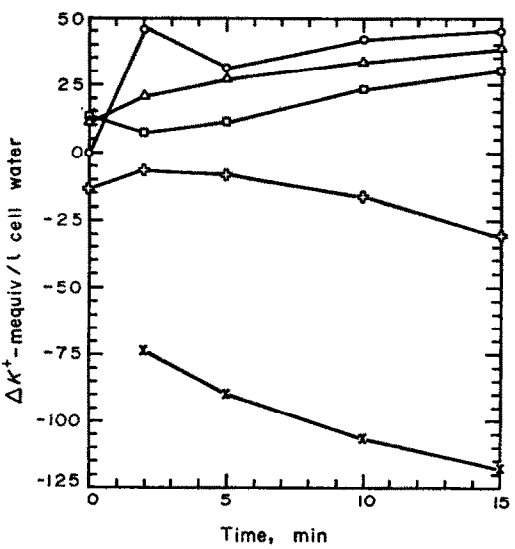

FIG. 7. Effect of 6,6′-dithiodinicotinic acid on intracellular content of K^+ in Ehrlich ascites tumor cells. The cells had been incubated for 15 min with different concentrations of the drug before addition of glucose to a final concentration of 11 mM at zero time. The value of ΔK^+ plotted is the difference between the concentration of K^+ measured in a sample and that in the zero time sample of cells untreated with the drug. Symbols designating drug concentrations: circle, no drug; square, 1·67 mM; triangle, 2·31 mM; cross, 3·20 mM; x, 5·00 mM.

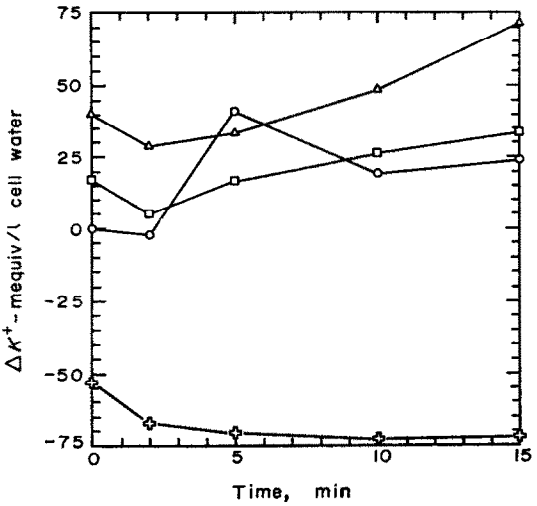

FIG. 8. Effect of 2,2′-dithiodipyridine on intracellular content of K^+ in Ehrlich ascites tumor cells. The cells had been incubated for 15 min with different concentrations of the drug before addition of glucose to a final concentration of 11 mM at zero time. The value of ΔK^+ plotted is the difference between the concentration of K^+ measured in a sample and that in the zero time sample of cells untreated with the drug. Symbols designating drug concentrations: circle, no drug; square, 0·60 mM; triangle, 1·00 mM; cross, 1·67 mM.

significant loss of cellular K^+ except in concentrations that completely inhibited lactate production. These high concentrations caused a profound loss of K^+.

Inhibition of triosephosphate isomerase (TIM) in vitro. As shown in Table 2, rabbit muscle TIM is inhibited *in vitro* both by DTDN and DTDP. For equivalent degrees of inhibition, DTDN was required in a concentration about two times that of DTDP. The concentrations of both drugs sufficing to inhibit the enzyme *in vitro* are of the order of one-tenth those required in suspensions of Ehrlich tumor cells for inhibition of lactate production.

DISCUSSION

Grassetti *et al.*,[3] estimating lactate production from the manometric measurement of CO_2 evolution from a bicarbonate buffer, concluded that DTDN in a concentration of 5×10^{-3} M stimulates aerobic glycolysis in Ehrlich tumor cells. We have found this concentration of DTDN to produce complete inhibition of glycolysis. It also causes profound loss of intracellular K^+ (Fig. 7). We have observed that a large proportion of cells treated with this concentration of DTDN lose the ability to exclude eosin. Eosin exclusion has been widely used as a criterion of viability of the Ehrlich tumor cell.[15] At no lower concentration of DTDN have we found any stimulation of lactate production.

Our work provides no support for the separation of DTDN and DTDP into qualitatively different classes so far as effects on overall lactate production are concerned. The two drugs do differ quantitatively, however, in the concentrations required to inhibit glycolysis. DTDP is effective in lower concentrations than is DTDN.

This cannot be entirely accounted for as failure of DTDN to penetrate cellular membranes. The effects of DTDN on glycolytic mechanisms indicate an intracellular site of action. The carboxyl groups of DTDN are only partially ionized at physiological pH values, and it might be expected that DTDN, like other weak organic acids, could permeate the plasma membrane in the undissociated form, the final concentrations inside and outside the cell being determined by the pH gradient across the membrane. It might be, however, that the anionic forms of DTDN are less active in inhibiting enzymes than the uncharged form. This might explain the lesser activity of DTDN relative to DTDP both in inhibiting glycolysis *in vivo* and inhibiting TIM *in vitro*.

Only in concentrations higher than are required for complete suppression of glycolysis does action of these drugs on the cell surface become apparent. At these high concentrations, permeability to eosin and loss of cellular K^+ give evidence of damage to the plasma membrane.

From the lactate/glucose ratios close to 2 for cells untreated with drug, as shown in Table 1, it may be inferred that, after the initial phase of uptake, practically all of the glucose removed from the medium by the cells is utilized in the production of lactate. The progressive decrease in the lactate/glucose ratio as cells are treated with increasing concentrations of either drug indicates that a progressively greater proportion of the glucose is being utilized in pathways other than that of glycolysis. The drugs may be causing increased diversion of glucose into the phosphogluconate pathway. This was the interpretation advanced by Grassetti *et al.*[4] in explanation of their experiments.

The rate constants in the two directions for the isomerization catalyzed by TIM, as obtained from various sources, are such that the ratio of DHAP to GAP is about 19 at equilibrium.[16] In Ehrlich tumor cells untreated with drugs, GAP is present in concentrations too low for measurement by the method used here. DTDN, even in concentrations having minimal effects on overall lactate production, had a striking effect in lowering the levels of DHAP and raising those of GAP attained after addition of glucose. The ratios of the concentrations of these two intermediates became more nearly of the order of unity rather than the normal ratio. It seems likely that this effect may be due to an inhibition of the isomerase. Inhibition of rabbit muscle TIM *in vitro* has been demonstrated with concentrations of DTDN considerably lower than those used in the cell suspensions. Presumably the inhibition of TIM produced by these drugs is due to reaction with sulfhydryl groups of the enzyme.

Irreversible inactivation of TIM *in vitro* has been demonstrated with several compounds. Iodoacetate reacts with cysteine, methionine and histidine residues in the enzyme.[17] Inactivation has been attributed to the reaction with histidine. Diazotized sulfanilic acid inactivates TIM, possibly by reaction with a lysine residue.[18] Haloacetol phosphates inactivate the enzyme by esterification of a glutamyl residue in the active site.[19–23] Glycidol phosphate also inactivates TIM by reaction with a glutamyl residue.[24,25] Competitive inhibition of TIM is produced by a number of phosphate esters, the most potent being 2-phosphoglycollate, as well as by the anions of carboxylic, arsenic, phosphoric and sulfuric acids.[16,26,27]

If our interpretation of the effect of DTDN on the glycolytic intermediates is correct, it would indicate that lactic acid production can proceed at almost a normal rate despite strong inhibition of TIM. At concentrations comparable in inhibiting

lactate production, DTDP did not have an effect comparable to that of DTDN in lowering DHAP levels and did not raise GAP concentrations to measurable levels. Even though DTDP is more active than DTDN in inhibiting rabbit muscle TIM *in vitro*, the differences between the two drugs in affecting the measured glycolytic intermediates might be explained if DTDP were even more potent in inhibiting an enzyme at a step prior to formation of F-di-P. Our experiments at the present stage do not furnish evidence sufficient to localize the step or steps in the glycolytic pathway at which either drug inhibits lactate production.

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