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Occurrence of the Malate-Aspartate Shuttle in Various Tumor Types[1]

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SUMMARY

The activity of the malate-aspartate shuttle for the reoxidation of cytoplasmic reduced nicotinamide adenine dinucleotide (NADH) by mitochondria was assessed in six lines of rodent ascites tumor cells (two strains of Ehrlich ascites carcinoma, Krebs II carcinoma, Novikoff hepatoma, AS-30D hepatoma, and L1210 mouse leukemia). All the tumor cells examined showed mitochondrial reoxidation of cytoplasmic NADH, as evidenced by the accumulation of pyruvate when the cells were incubated aerobically with L-lactate. Reoxidation of cytoplasmic NADH thus generated was completely inhibited by the transaminase inhibitor aminooxyacetate. The involvement of the respiratory chain in the reoxidation of cytoplasmic NADH was demonstrated by the action of cyanide, rotenone, and antimycin A, which strongly inhibited the formation of pyruvate from added L-lactate. Compounds that inhibit the carrier-mediated entry of malate into mitochondria, such as butylmalonate, benzenetricarboxylate, and iodobenzylmalonate, also inhibited the accumulation of pyruvate from added L-lactate by the tumor cells. The maximal rate of the malate-aspartate shuttle was established by addition of arsenite to inhibit the mitochondrial oxidation of the pyruvate formed from added lactate. The capacity of the various tumor lines for the reoxidation of cytoplasmic NADH via the malate-aspartate shuttle approaches 20% of the total respiratory rate of the cells and thus appears to be sufficient to account for the mitochondrial reoxidation of that fraction of glycolytic NADH not reoxidized by pyruvate and lactate dehydrogenase in the cytoplasm.

INTRODUCTION

Since the early studies of Boxer and Devlin (1) and other investigators (8, 12, 13), there has been considerable interest in the extent and the rate of the mitochondrial oxidation of glycolytic NADH generated in the cytosol of cancer cells, as well as the identity of the NADH shuttle system(s) used. The glycerol phosphate shuttle system does not appear to be present in most tumors (1, 8, 13, 14), but several reports indicate that the enzymes and transport systems required in the operation of the malate-aspartate shuttle do occur in Ehrlich ascites tumor cells (4, 6, 7). However, Kovacevic (6) has concluded that the aspartate concentration in Ehrlich ascites cells is too low to allow the malate-aspartate shuttle to function effectively in Ehrlich cells, because of the relatively high K_M of aspartate transaminase. Since the malate-aspartate shuttle now appears to be the major mechansim for transfer of reducing equivalents from cytosol NADH into the mitochondria in heart and liver and other normal tissues (15, 16, 19, 20), it appears desirable to collect further evidence regarding the possible function of this important shuttle system in malignant cells.

In this paper we report evidence that each of 6 types of experimental tumors that we have examined contains the enzymes and transport systems required for the operation of the malate-aspartate shuttle. Moreover, we have also established what appears to be the maximum potential rate at which the malate-aspartate shuttle can function in several types of tumor cells.

MATERIALS AND METHODS

The AS-30D rat ascites hepatoma was obtained from Dr. E. F. Walborg, Department of Biochemistry, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas (17); Dr. A. C. Griffin of the same institution supplied the MDA strain of Ehrlich mouse ascites tumor (EMAT) and the Novikoff rat ascites hepatoma. Dr. E. L. Coe of the Department of Biochemistry, Northwestern University Medical School, Chicago, Ill., supplied the NU strain of Ehrlich ascites tumor; Dr. J. M. Colvin of the Oncology Center, The Johns Hopkins Hospital, Baltimore, Md., supplied the L1210 mouse leukemia (ascites form); and Dr. F. Reynolds, of the same department, supplied the Krebs II carcinoma (ascites form). The tumors were transplanted by weekly (or biweekly for L1210) i.p. injection in appropriate animals. These were Sprague-Dawley albino rats (Sprague-Dawley, Inc., Madison, Wis.) for both the AS-30D and Novikoff hepatomas, Swiss albino mice (Buckberg Lab Animals, Inc., Tomkins Cove, N. Y.) for both the MDA and NU strains of Ehrlich ascites tumor; C57BL/6J × DBA/2J F_1 (hereafter called $BD2F_1$) mice from Cumberland View Farms, Clinton, Tenn., for the L1210 leukemia; and CD-1 Swiss albino mice from Charles River Breeding Laboratories, Wilmington, Mass., for the Krebs II carcinoma. After growth of the cells the ascitic fluid was collected and filtered through 3 to 4 layers of cheesecloth to remove clumps, and the cells were recovered by centrifugation. They were washed twice in a medium of 150 mM NaCl, 5 mM KCl, and 10 mM Tris-Cl, pH

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7.4. Incubations were performed aerobically with shaking at 80 to 100 cycles/min at 30° in the same medium plus 10 mM sodium phosphate buffer (pH 7.4); other additions were as indicated in the tables and figures. Protein concentrations were 10 to 20 mg cell protein per ml in the incubation medium. The reactions were terminated with perchloric acid (final concentration, 3.5% w/v), and the supernatant fractions were carefully neutralized with KOH in the presence of 0.05 M 2-(*N*-morpholino)ethanesulfonic acid-0.10 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer to a pH of about 6.5 to 6.8. Pyruvate was determined enzymatically by a coupled spectrophotometric method involving the oxidation of NADH in the presence of lactate dehydrogenase (3). Oxygen uptake was measured polarographically with a Clark electrode in a thermostatted (30°) 2-ml vessel equipped with a magnetic stirrer. A combination pH electrode connected to a Beckman Model SS-2 Expandomatic pH meter could also be inserted when necessary to monitor the pH of the suspension. Both pH and oxygen uptake were recorded on a Sargent Scientific Equipment Co. Model DSRG dual-channel recorder. Protein content was determined by the biuret method of Szarkowska and Klingenberg (18).

Certain inhibitors (rotenone, antimycin A) were dissolved in 95% ethanol and added in very small volumes.

Lactate dehydrogenase, NADH, 2-(*N*-morpholino)ethanesulfonic acid, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, and aminooxyacetate were obtained from Sigma Chemical Co., St. Louis, Mo. 2-*n*-Butylmalonic acid and *p*-iodobenzylmalonic acid were obtained from Aldrich Chemical Co., Metuchen, N. J. 1,2,3-Benzenetricarboxylic acid was obtained from ICN/K+K Laboratories, Plainview, N. Y. All other chemicals were reagent grade.

RESULTS

We have assayed the capacity of intact ascites tumor cells of the 6 strains listed to promote the formation of pyruvate from externally added lactate, according to the principle described by Dionisi *et al.* (4). They have shown that aerobic incubation of lactate with Ehrlich cells leads to the formation of pyruvate, an event that results in the reduction of cytosol NAD^+ to NADH since lactate dehydrogenase is present only in the cytosol of these cells. The accumulation of pyruvate is evidence for the mitochondrial reoxidation of cytosol NADH, since pyruvate formation from added lactate was blocked by respiratory inhibitors. That cytosol NADH was reoxidized via the malate-aspartate shuttle (or some pathway involving transamination) was shown by the finding that aminooxyacetate, a potent inhibitor of aspartate transaminase (5, 12), almost completely inhibited the aerobic formation of pyruvate from added lactate.

Chart 1 shows the effects of a 10-min preincubation of the tumor cells with aminooxyacetate (0.2 mM) on the aerobic pyruvate production from added lactate in 6 different types of ascites tumor cells. Each of the 6 showed substantial aerobic pyruvate accumulation from lactate, ranging from about 1 to 6 nmoles/mg protein in 10 min. The effect of preincubation of the tumor cells with aminooxyacetate,

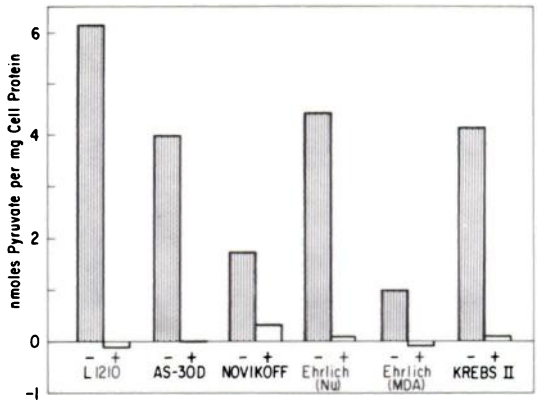

Chart 1. The effect of aminooxyacetate on the net accumulation of pyruvate from lactate. Washed tumor cells of the type indicated were preincubated aerobically for 10 min at 30°, with or without 0.2 mM (final concentration) aminooxyacetate, as described in "Materials and Methods." L-Lactate was added at zero time at a final concentration of 20 mM. At zero time and at 10 min, perchloric acid was added and cell debris was then centrifuged out at 50,000 × g_{min}. The supernatant fraction was neutralized to pH 6.5 to 6.8 with KOH in the presence of 50 mM 2-(*N*-morpholino)ethanesulfonic acid-100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid. The neutralized supernatant fraction was assayed for pyruvate. − or + signs refer to the absence or presence of 0.2 mM aminooxyacetate.

however, in all but 1 case, caused essentially complete inhibition of aerobic pyruvate production from lactate. This strongly indicates that transaminase activity is essential for the aerobic production of pyruvate from lactate in these cells and is consistent with the view that these tumors transport reducing equivalents from cytoplasmic NADH into the respiratory chain of mitochondria via the malate-aspartate shuttle. Tests of the effect of lactate concentration indicate that addition of 10 to 20 mM lactate gives maximal rates of pyruvate formation.

In confirmation of the findings of Dionisi *et al.* (4), we have found that pyruvate formation by tumor cells incubated with lactate requires electron transport to oxygen, since addition of rotenone + antimycin A or cyanide blocks pyruvate formation (data not shown). Moreover, rotenone alone very strongly inhibited pyruvate accumulation. This effect of rotenone supports the view that the tumor cells use the malate-aspartate shuttle, which results in electron transfer through all 3 sites, and use the glycerol phosphate shuttle (which is rotenone insensitive since it utilizes only Sites II and III) very little, if at all.

The rates of aminooxyacetate-sensitive pyruvate formation recorded by Dionisi *et al.* (4) and shown in Chart 1 do not necessarily represent maximal potential rates of NADH oxidation via the malate-aspartate shuttle, since it is probable that some of the pyruvate formed from external lactate undergoes further oxidation, presumably via acetyl-CoA and the tricarboxylic acid cycle. In order to establish the upper limit of the rate of aerobic pyruvate formation from lactate, and thus the upper limit of the rate of the malate-aspartate shuttle in these tumors, we have used arsenite to block the oxidative removal of pyruvate by the pyruvate dehydrogenase complex. As can be seen in Chart 2, arsenite greatly increases the accumulation of pyruvate from lactate, the maximum effect being achieved above 5 mM arsenite. In the AS30D tumor, arsenite produced nearly a 4-fold increase in pyruvate accumulation, whereas pyruvate accu-

mulation was enhanced 2.5-fold in L1210 cells. Furthermore, as Chart 2 also shows, the accumulation of pyruvate from lactate in the presence of arsenite is completely blocked by aminooxyacetate. Clearly, then (as indicated in Chart 3), the true capacity of the malate-aspartate cycle system is in great excess of the rate indicated by the pyruvate accumulation assay in the absence of arsenite for the tumors tested.

The malate-aspartate shuttle requires, in addition to cytosolic and mitochondrial aspartate transaminase and malate dehydrogenase, the operation of mitochondrial membrane transport systems for malate. The dicarboxylate transport system, capable of exchanging malate with certain other dicarboxylates or phosphate, is inhibited by *n*-butylmalonate (10, 11); the tricarboxylate carrier, which promotes malate-citrate exchange, is blocked by 1,2,3-benzene tricarboxylate (10, 11), although these inhibitors are much less active and less specific than aminooxyacetate. As shown in Table 1, both of these compounds inhibit the aerobic accumulation of pyruvate from lactate in tumor cells, although not as extensively as does aminooxyacetate, confirming the probable participation of the malate-aspartate shuttle in NADH transport. These observations may be compared with the findings of Williamson *et al.* (19), who have shown that *n*-butylmalonate inhibits oxidation of cytosol NADH in the perfused liver. 1,2,3-Benzene tricarboxylate is a more effective inhibitor of pyruvate accumulation in tumor cells than is butylmalonate. This may be accounted for either by a sizable flux of malate via the tricarboxylate carrier or, alternatively, that the 1,2,3-benzene tricarboxylate may be more effective than butylmalonate in penetrating the plasma membrane. The malate-aspartate shuttle as postulated would utilize, in part, the malate-α-ketoglutarate exchange system (2, 9). Table 1 shows that iodobenzylmalonate, which is more active than butylmalonate in inhibiting the malate-α-ketoglutarate exchange (10), also inhibits aerobic pyruvate accumulation from lactate and in an additive manner with butylmalonate plus benzene tricarboxylate. The apparent difference in effectiveness of iodobenzylmalonate *versus* butylmalonate + benzene tricarboxylate may be due either to differences in permeability to these agents or to differences in the utilization by the malate-aspartate cycle of different malate transport systems for the required malate flux across the mitochondrial membranes. The less than complete inhibition of apparent cycle flux most probably represents the reduction of cytoplasmic oxaloacetate to malate at the expense of aspartate. In any case these obser-

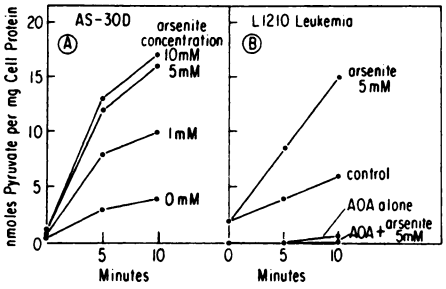

Chart 2. *A*, the effect of arsenite on apparent malate-aspartate shuttle activity. Incubations with 20 mM L-lactate were performed as described in the legend to Chart 1, with the indicated final concentrations of sodium arsenite added 1 min before lactate addition. *B*, the effect of aminooxyacetate (*AOA*) on arsenite-stimulated pyruvate accumulation. Incubations in the presence or absence of 0.2 mM aminooxyacetate and the presence or absence of 5 mM sodium arsenite were performed as in the legend to Chart 1. The final L-lactate concentration was 20 mM and preincubation times were 5 min for aminooxyacetate and 1 min for arsenite.

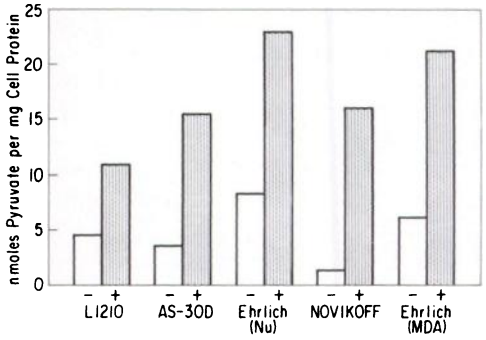

Chart 3. The stimulation of apparent malate-aspartate shuttle activity by arsenite. The incubations (10 min) were as described in the legend to Chart 1, in the presence (+) or absence (−) of 5 mM sodium arsenite, preincubated with the cells for 1 min.

Table 1

The apparent malate-aspartate shuttle activity and the effect of mitochondrial malate transport inhibitors

The indicated tumors were incubated in the presence of the inhibitors and assayed for pyruvate accumulation as in the legend to Chart 1. Both preincubation and incubation times were 10 min. Values in nmoles pyruvate per mg cell protein. The final concentration of inhibitors were 10 mM each except AOA,[a] which was used at 0.2 mM final concentration.

Experiment	Tumor	Control	AOA	BU	BT	BU + BT
1	L1210	9.11	−2.72	7.76	6.25	6.73
2	AS-30D	3.81	0.00	2.54	2.24	2.01
3	EMAT/NU	5.86	0.28			3.72
4	Novikoff	2.22	0.60	1.97	1.79	
				+ IBM	+ BU + BT	+ IBM + BU + BT
5	AS-30D	6.64	6.64	5.37	2.76	1.72

[a] The abbreviations used are: AOA, aminooxyacetic acid-HCl; BU, 2-*n*-butylmalonic acid; BT, 1,2,3-benzene tricarboxylic acid; IBM, *p*-iodobenzylmalonic acid.

vations strongly indicate that in the tumors tested there is an active shuttle mechanism for the reoxidation of cytosolic NADH that is dependent both on transaminase activity and on transport systems capable of carrying malate across the mitochondrial membrane.

The question now arises as to how the rate of malate-aspartate shuttle activity as given by the type of assay described compares with the rate of respiration of tumor cells. Table 2 shows the rates of oxygen consumption and malate-aspartate shuttle activity (as assayed in the presence of arsenite), in comparable terms of nmoles or ng atoms/min/mg protein. First, the shuttle activity of the various tumors is about 15 to 20% of the rate of oxygen consumption, which is in turn the sum of the oxidation of pyruvate that could be derived from glucose by glycolysis and the oxidation of fatty acids, which probably represents the bulk of the total oxidative activity. Theoretically, the NADH shuttle activity should be one-sixth of the oxygen consumption due to glucose oxidation alone, which was not measured in this investigation. However, if less than one-third of the oxygen consumption of the tumor cells is due to pyruvate oxidation, a generous estimate in view of the usually low respiratory quotient (~0.75) of tumor cells respiring in the presence of glucose, then it would appear that the malate-aspartate shuttle activity as measured by the procedure used would be adequate to account for the cytosol NADH generated during the production of pyruvate by glycolysis and its steady-state oxidation to CO_2. Since the true rate of the malate-aspartate shuttle might be much higher than the values given in Chart 1, as indicated by the fact that the presence of arsenite yields much higher values for shuttle activity (Table 2), it would appear that the malate-aspartate shuttle activity of the tumors tested is potentially capable of transferring all the reducing equivalents of cytosol NADH generated during pyruvate formation from glucose into the respiratory chain of mitochondria.

Table 2

A comparison of the rates of malate-aspartate shuttle activity and oxygen consumption

Rates of oxygen uptake were measured with a Clark electrode at 30° and expressed as ng atoms of oxygen per min per mg cell protein. Malate-aspartate shuttle activity rates were determined from the "arsenite-stimulated" data of Chart 5 and are expressed as nmoles of pyruvate produced per min per mg cell protein.

Tumor	Malate-aspartate shuttle activity	Oxygen uptake rate
L1210	1.10	9.49
AS-30D	1.55	10.4
Novikoff	1.60	10.3
EMAT/NU	2.30	7.31
EMAT/MDA	2.20	12.6

DISCUSSION

The experiments reported here firmly support the view that the enzymes and transport systems required in the malate-aspartate shuttle for NADH occur in all the types of tumors studied and thus greatly extend the earlier information on Ehrlich ascites tumors. Moreover, the data also indicate that shuttles operating only by electron transport through Sites II and III of the respiratory chain, such as the glycerol phosphate shuttle, are not operative in these tumors. In particular, comparisons of the rate of malate-aspartate shuttle activity by the assay procedure used with the rate of oxygen consumption by these tumors strongly indicate, but do not prove, that the malate-aspartate shuttle activity is sufficiently high to account for the transfer of the reducing equivalents of all the cytosol NADH equivalent to the pyruvate formed by glycolysis and oxidized to completion in each of the 6 tumor strains examined. Our data thus support the conclusions of Dionisi *et al.* (4) and of LaNoue *et al.* (7). Moreover, our data disprove the conclusion of Kovacevic (6) that the malate-aspartate shuttle cannot function at a sufficiently high rate because of the low concentration of aspartate in tumor cells. Our data clearly indicate that whatever amino acid actually participates in the required transamination step is present in sufficient concentration to yield an overall rate of shuttle activity that could account for the rate required when glucose is utilized by tumor cells.

A possible objection to this conclusion is that the conditions of the assay used to measure shuttle activity, specifically, the presence of 20 mM lactate in the medium, are unphysiological and yield a rate reflecting shuttle activity only under the special circumstance of a high external lactate concentration. However, the concentration of lactate in the ascitic fluid of mice bearing growing Ehrlich ascites tumor cells has been found in other work in this laboratory to be about 20 mM (T. Spencer, unpublished observations). The external lactate concentration in our assay system for the malate-aspartate shuttle is thus unlikely to induce an abnormal balance of metabolic reactions in Ehrlich cells.

It is clear, however, that further quantitative measurements are required in order to evaluate more quantitatively the participation of the malate-aspartate shuttle in the metabolism of tumor cells, particularly data on the actual rate of utilization of glucose; the rate of formation of pyruvate, lactate, and CO_2; and the respiratory quotient. Such experiments have been carried out on an extensive basis and will be reported elsewhere.

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