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TARGET AND NON-TARGET METABOLIC EFFECTS OF AMINOFOLATES AND OF A 5-METHYLQUINAZOLINE ANTIFOLATE IN MOUSE CELLS

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Abstract—Target and non-target response of mouse L-cells to aminofolates, aminopterin and amethopterin, as well as to a 5-methylquinazoline antifolate CB 3703 were compared. When added into the culture medium for 48 hr, each of those antifolates strongly inhibited the cell proliferation; the respective $I_{0.5}$ values were of the order of 10^{-8} M for aminopterin and of the order of 10^{-7} M for amethopterin and the quinazoline antifolate. However, even at concentrations which did not limit cell growth, each of the antifolates accumulated intracellularly far above the level of dihydrofolate reductase, and this accumulation was concentration dependent. Simultaneously, the activity of the target enzyme, dihydrofolate reductase, decreased considerably with a concomitant decrease in the activity of lactate dehydrogenase and in lactate content, whilst the content of pyruvate increased in the cells. Consequently, the ratio of lactate to pyruvate decreased.

At growth-limiting concentrations, the antifolates did not only reduce the activity of dihydrofolate reductase nearly to zero, but also decreased to a smaller extent the activities of other folate-related enzymes such as methionine synthetase and serine hydroxymethyltransferase, and changed those of thymidylate synthetase and formyltetrahydrofolate synthetase to a greater or lesser degree and in a differentiated manner. Thus, the quinazoline antifolate substantially diminished the activity of thymidylate synthetase, whose activity was apparently enhanced by both aminofolates as compared with the respective controls. On the other hand, the activity of formyltetrahydrofolate synthetase was elevated by the quinazoline antifolate, being greatly decreased by aminopterin and only slightly lowered by amethopterin. Each of the antifolates also caused a significant decrease in the activity of glucose-6-phosphate dehydrogenase and a sharp decrease in that of lactate dehydrogenase. A simultaneous decrease in lactate content and an increase in pyruvate content led to a significant decrease in the ratio of lactate to pyruvate, indicating an increase in intracellular NAD^+ content and glycolysis impairment in the L-cells. In conclusion, we suggest considering the ratio of lactate to pyruvate as a kind of simple measure of the non-target effects of antifolates in mammalian cells.

Only one year had passed after the chemical synthesis, in 1947, of aminopterin, an aminoanalogue of folate [1], when its application in the chemotherapy of cancer was reported [2]. Since that time, quite a number of folate analogues have been synthesized and tested as potential cytostatics, both in animals and animal cells cultured *in vitro*. But only aminopterin itself and its *N*-methyl derivative, amethopterin, further designated as aminofolates, have been used in practice. The mechanism of aminofolate uptake and accumulation in animal cells, their strong inhibitory effects on the activity of dihydrofolate reductase, their metabolic consequences, such as purine and thymidine depletion, and their inhibition of DNA synthesis have been widely investigated and discussed. So far, however, our understanding of the side-effects of these antifolates produced in mammalian cells is still insufficient. Thus, the effect of these antifolates on the activity of non-target folate enzymes and folate-unrelated dehydrogenases [3–7], as well as on the rate of glycolysis, and energy charge, are still being studied in various laboratories (e.g. Refs. 8–11).

Our knowledge of the metabolic effects of quinazoline analogues of folate is much scantier, although since 1968 they have been considered to be potential antitumour drugs [12–15]. Their advantage over aminofolates seems to be their ability to inhibit the activity of thymidylate synthetase [16–18] besides that of dihydrofolate reductase [19–23].

This paper provides comparative data concerned with some target and non-target metabolic effects of the accumulation of antifolates in mouse cells, as expressed by impairment of the activity of some folate enzymes and folate-unrelated dehydrogenases, and by a change in the lactate to pyruvate ratio.

MATERIALS AND METHODS

Cells and cell growth. Mouse L-cells with a doubling time of about 30 hr were routinely grown as monolayers in the Eagle's essential medium with calf or bovine heat-inactivated serum and with antibiotics at 37° [24, 25]. In searching for antifolate effects, the cells were subcultured for 24 hr into the standard medium, then the medium was renewed in the control cultures and changed for one with an antifolate at a concentration ranging from 10^{-10} to 10^{-5} M in

the experimental cultures and the cells were allowed to grow for another 48 hr. Then the cells were processed for estimation of their viability and metabolic response to the given antifolate.

Cell count. Cells to be checked for their viability were washed out of the medium, trypsynized and routinely collected; the trypan blue test was applied and the viable cells were counted in a Neubauer haematocytometer. The numbers of cells in experimental cultures were expressed as percentages of the number of cells in the control ones. $I_{0.5}$ was defined as the concentration of antifolate required to reduce the viable cell count by 50 per cent after 48 hr of contact.

Enzyme assays. The cells to be used for enzyme assays were washed out of the medium and from detached dead cells, then either trypsinized or scraped off the glass with a rubber policeman and centrifuged. The enzyme extracts were prepared by sonication of the cells suspended in an appropriate buffer, followed by a centrifugation of the sonicate at 20,000 *g* for 20 min to remove cell debris. All the procedures were performed at 0–4°.

The activity of the following enzymes in crude extracts was estimated by standard methods: serine hydroxymethyltransferase (2.1.2.1), formyltetrahydrofolate synthetase (6.3.4.3), vitamin B12 dependent methionine synthetase (2.1.1.13), thymidylate synthetase (2.1.1.45), lactate dehydrogenase (1.1.1.27), glucose-6-phosphate dehydrogenase (1.1.1.49) [26–30]. Because of the low activity of dihydrofolate reductase (1.5.1.4) in crude extracts, they were acidified to pH 5.1 to eliminate the bulk of cellular proteins and readjusted to pH 7.5 before being used for the enzyme assays [24, 26]. The content of dihydrofolate reductase in L-cells grown for 72 hr in the standard medium was also estimated by a titration method with amethopterin [31].

Protein content. Since protein estimation is much more precise than cell amount, we routinely determined protein content in all samples of our cellular material by the method of Lowry *et al.* [32]. By this method, the 10^6 L-cells were found to contain 377 ± 9.1 μg protein.

Determination of the intracellular contents of the antifolates, lactate and pyruvate. Cells grown for 48 hr, in the presence of an antifolate, were trypsynized, centrifuged and washed out of the medium with cold phosphate-buffered saline (twice). Then the cell pellet was suspended in distilled water, sonicated for 45 sec, boiled for 5 min and centrifuged for 10 min at 20,000 *g*. The resulting supernatant fraction was used for determination of the antifolate content in a titration assay with a partially purified rat liver dihydrofolate reductase, according to the method originally described for amethopterin by Bertino and Fischer [33].

The lactate and pyruvate contents were estimated by suitable methods [34, 35]. The intracellular contents of aminofolates, lactate and pyruvate were then expressed as respective units per 10^6 cells and, if necessary, also as molar concentration in the cells, taking as L-cell volume the value 3.65×10^3 μm^3, as measured by Ishiguro *et al.* [36].

Reagents. The reagents were purchased from the following sources: methionine, glycine, homocysteine–thiolactone, pyridoxal phosphate, folic acid and aminopterin from the Sigma Chemical Co., St. Louis, MO, U.S.A.; amethopterin from Lederle Laboratories Division, American Cyanamid Company, Pearl River, NY, U.S.A.; sodium pyruvate, NADH, NADP$^+$, NADPH, glucose-6-phosphate and ATP from Boehringer Mannheim GMBH, West Germany; Triton X-100, Dowex 1 × 8 Cl$^-$, 200–400 mesh, Norit A from Serva Feinbiochemica, Heidelberg, F.R.G.; *S*-adenosyl-L-methionine from Calbiochem, San Diego, CA, U.S.A.; vitamin B12, cyano form from Merck, Darmstadt, F.R.G.; Tris-(hydroxymethyl)-aminomethan from Fluka-AG, Buchs SG, Switzerland; paraformaldehyde from British Drug Houses, Poole, U.K.; and acetylacetone from Searle, Hopkin and Williams, Chadwell Heath, Essex, U.K. All reagents were of analytical grade.

Radioisotopes were purchased from the Radiochemical Centre, Amersham, U.K.

Crystalline dihydrofolate was prepared by the dithionite method of Futterman [37], as modified by Blakley [38] and crystalline tetrahydrofolate was obtained by the catalytic reduction of folate according to Hatefi *et al.* [39] and Slavik *et al.* [40]. 5-Methyltetrahydrofolate was synthesized from tetrahydrofolate and formaldehyde by the method of Sakami [41].

Quinazoline *N*-(*p*-{[2,4-diamino-5-methyl-6-quinazolinyl) methyl] amino}benzoyl) -L-glutamic acid (CB 3703) was synthesized by Dr T. Jones in the Department of Biochemical Pharmacology, Institute of Cancer Research, Belmont, Surrey, U.K., according to the method of Davoll and Johnson [42] and kindly given to us by Dr K. R. Harrap.

RESULTS

Growth of L-cells in the presence of antifolates. As we reported elsewhere [43], amethopterin, even at the concentration 10^{-5} M, did not increase the percentage of dead cells in culture during 24 hr of contact and only slightly increased it after 48 hr contact; concomitantly, the population density of the cells was reduced by up to 75 per cent of that in the respective control culture of L-cells. In this comparative study on the effects of aminofolates and quinazoline antifolates on L-cell growth and metabolism, we added aminopterin, amethopterin or 5-methyl quinazoline antifolate CB 3703 into the culture medium in a range of concentrations from 10^{-10} to 10^{-5} M.

The concentration curves for the cell growth in the media with the antifolates tested are shown in Fig. 1, Chart A. It was not surprising that each of the antifolates strongly inhibited the growth of the cells, provided its concentration in the medium was high enough. The respective $I_{0.5}$ values, read from the curves, were 9×10^{-9} M for aminopterin, 9×10^{-8} M for amethopterin and 7×10^{-8} M for the quinazoline antifolate. At an antifolate concentration in the medium as high as 10^{-5} M, the number of viable cells as shown by the trypan blue exclusion test was still 40 per cent of the respective control when the antifolate was the quinazoline derivative, and 20 per cent when it was one of the aminofolates.

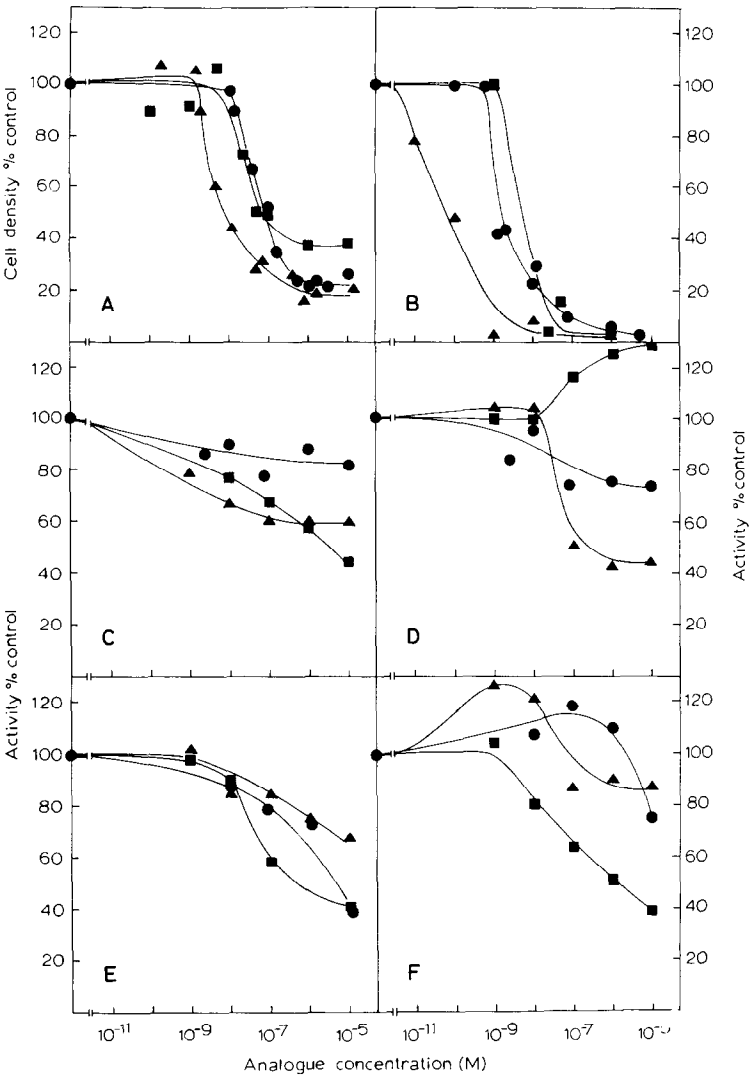

Fig. 1. Concentration curves for cell growth (Chart A) and activity of dihydrofolate reductase (Chart B), serine hydroxymethyltransferase (Chart C), formyltetrahydrofolate synthetase (Chart D), vitamin B12-dependent methionine synthetase (Chart E) and thymidylate synthethase (Chart F) in L-cells grown in the presence of antifolates. After subculturing, the cells were grown in the standard medium for 24 hr, then the medium was either renewed in the control cultures or changed for one with aminopterin (▲), amethopterin (●) or quinazoline antifolate CB 3703 (■) at the concentrations indicated, for another 48 hr. After washing off the medium, the cells were processed for estimation of cell density or for enzyme assays. Cell density (Chart A) in experimental cultures and activity of the enzymes (Charts B–F) are expressed as percentages of the controls. The respective values for activity of the enzymes per mg protein and per hour as determined 72 hr after subculturing were 0.4 μmole for dihydrofolate reductase, 3.6 μmoles for serine hydroxymethyltransferase, 0.24 μmole for formyltetrahydrofolate synthetase, 2 nmoles for vitamin B12-dependent methionine synthetase and 0.36 nmole for thymidylate synthetase. Each value is the mean of triplicate estimations in at least three experiments.

Intracellular content of dihydrofolate reductase and antifolates. As estimated by titration with amethopterin, the intracellular content of dihydrofolate reductase in L-cells grown for 72 hr in the standard medium was as low as 2.40 pmoles per 10^6 cells; whereas in the cells grown in any of the experimental media the intracellular contents of any of the antifolates tested were many times higher (Table 1), as determined by titration with partially purified rat liver dihydrofolate reductase. Even when the antifolate concentration in the culture medium was only 2.5×10^{-9}M and the cell proliferation apparently normal (Fig. 1, Chart A), the intracellular content of the antifolates exceeded that of dihydrofolate reductase, being approximately twice higher in cells exposed to quinazoline antifolate CB 3703, four times higher in those exposed to amethopterin, and five times higher in response to aminopterin. When the concentration of the antifolates was 10^{-6}M, their intracellular contents were about thirty times higher than that of dihydrofolate reductase. Thus, it is evident that the accumulation of the antifolates in L-cells is a concentration-dependent process.

Activity of folate enzymes in L-cells grown in the

Table 1. Intracellular content of antifolates in L-cells cultured in the medium containing antifolates*

Antifolate in the medium [M]	pmoles/10^6 cells ± S.E.		
	Aminopterin	Amethopterin	Quinazoline antifolate CB 3703
2.5×10^{-9}	12.8 (3)	9.0 ± 1.2 (5)	5.2 (3)
1×10^{-8}	25.1 (3)	13.3 (4)	10.7 (3)
1×10^{-6}	90.4 (3)	78 ± 15.7 (5)	67.7 ± 15.0 (6)

* The cells were grown in the standard medium for 24 hr, then for another 48 hr in the medium with an antifolate at the concentration indicated, before processing for estimation of the antifolate contents. Each value represents the mean of the results obtained in duplicate in at least three separate experiments. The number of experiments is given in parentheses.

presence of antifolates. The concentration curves for the activity of dihydrofolate reductase, serine hydroxymethyltransferase, formyltetrahydrofolate synthetase, vitamin B12 dependent methionine synthetase and thymidylate synthetase in L-cells grown in the media with antifolates are depicted in Fig. 1, Charts E–F.

The activity of dihydrofolate reductase in the cells fell to a very low level, even when the antifolate concentrations in the media were still not growth-limiting (Fig. 1, Charts A and B). Thus, 10^{-9}M aminopterin and 2×10^{-8}M amethopterin and the quinazoline antifolate made the enzyme activity practically undetectable in the cells.

Serine hydroxymethyltransferase (Fig. 1, Chart C) is the enzyme whose intracellular activity only slightly decreased with increase of the antifolate concentrations in the medium. The activity of formyltetrahydrofolate synthetase (Fig. 1, Chart D) was practically unaffected until the concentration of any of the antifolates in the medium reached 10^{-8}M, and became differentiated as the concentration increased. Thus, the effects of amethopterin on the activity of this synthetase was rather small in the whole range of concentrations applied; aminopterin, when 10^{-7}M, caused a decrease in the enzyme activity by about 60 per cent, whereas the quinazoline antifolate, at this concentration in the medium, caused an apparent increase in its activity as compared with the respective controls. The activity of vitamin B12-dependent methionine synthetase (Fig. 1, Chart E) decreased substantially but only in the cells from the media with the antifolates at concentrations over 10^{-8}M, when they greatly decreased cell growth. Finally, the activity of thymidylate synthetase (Fig. 1, Chart F) decreased significantly only in the cells grown in the media with the quinazoline antifolate CB 3703. Both aminofolates when present in the culture media caused some increase in thymidylate synthetase activity, as expressed as percentages of the respective controls.

Table 2 shows the inhibition characteristics for aminopterin, amethopterin and the quinazoline antifolate, as found for folate-related and unrelated enzymes when assayed in extracts of L-cells grown under the control conditions. It is easy to see that, aside from dihydrofolate reductase, thymidylate synthetase is the only enzyme whose activity was inhibited to a substantial extent by the quinazoline antifolate.

Thus, I_{50} for dihydrofolate reductase for aminopterin was 1.7×10^{-8}M, that for amethopterin was 1.3×10^{-8}M and that for the quinazoline analogue was 6.8×10^{-9}M. The $I_{0.5}$ value for thymidylate synthetase and the quinazoline was 7×10^{-6}M. The respective values for the same quinazoline antifolate and the enzymes of L1210 cells were reported to be of the same order for dihydrofolate reductase, and of one order smaller for thymidylate synthetase [14]. Of the antifolates tested, only amethopterin could inhibit methionine synthetase *in vitro* by about 50 per cent when at a 10^{-4}M concentration, whereas aminopterin and the quinazoline antifolate hardly affected its activity at this concentration. This was also the case with the activity of serine hydroxymethyltransferase and of formyltetrahydrofolate synthetase for each of the antifolates tested.

Activity of folate-unrelated dehydrogenases in L-cells grown in the presence of antifolates. The concentration curves for the activity of lactate dehydrogenase and glucose-6-phosphate dehydrogenase assayed in L-cells after 48 hr contact with antifolates and expressed as percentages of the control values are presented in Fig. 2.

As shown by the shape of the curves, amethopterin and the quinazoline antifolate, when present in the culture medium, began to decrease the activity of lactate dehydrogenase (Fig. 2, Chart A) at concentrations below those that suppressed growth of the cells (Fig. 1, Chart A), whereas aminopterin caused a nearly parallel fall in the activity of this enzyme and in the cell growth. The phenomenon of depression of the activity of lactate dehydrogenase by amethopterin was the strongest. The maximum decrease in the activity of lactate dehydrogenase by amethopterin was as high as 80 per cent of the control values, and was already observed in the cells grown in the medium with this aminofolate at 10^{-8}M. At this concentration, aminopterin and the quinazoline antifolate depressed lactate dehydrogenase activity by about 40 per cent; the maximum depression they produced when 10^{-7}M in the media was of the order of 70 per cent of the respective controls.

At concentrations of 10^{-8}M or higher, each of the

Table 2. Inhibition by antifolates of target and non-target enzymes of L-cells*

Antifolate	$I_{0.5}$ [M]		Per cent inhibition at 10^{-4}M				
	Dihydrofolate reductase	Thymidylate synthetase	Methionine synthetase	Serine hydroxymethyltransferase	Formyltetrahydrofolate synthetase	Lactate dehydrogenase	Glucose-6-phosphate dehydrogenase
Aminopterin	1.7×10^{-8} (3)	$>10^{-3}$ (3)	13 (3)	13 (3)	10 (3)	0 (4)	0 (4)
Amethopterin	1.3×10^{-8} (3)	6×10^{-4} (3)	60 (4)	7 (3)	5 (3)	0 (4)	0 (4)
Quinazoline antifolate CB 3703	6.8×10^{-9} (3)	7×10^{-6} (3)	17 (3)	16 (3)	5 (3)	0 (4)	0 (4)

* The appropriate extracts of the cells cultured under the standard conditions were prepared as described in Materials and Methods, and used for enzyme assays and estimations of antifolate inhibition. Each value represents the mean of the results obtained in duplicate in at least three experiments. The number of experiments is given in parentheses.

antifolates also affected the activity of glucose-6-phosphate dehydrogenase (Fig. 2, Chart B), but the maximum decrease in its activity was not higher than about 50 per cent, even in the cells surviving in the media with 10^{-5}M antifolates.

Intracellular contents of lactate and pyruvate in L-cells grown in the presence of antifolates. The intracellular contents of lactate and pyruvate were estimated in the cells cultured for 72 hr in the standard medium, or in cells which after 24 hr growth in the standard medium were transferred for another 48 hr into media, each containing one of the antifolates at one of two chosen concentrations, 2.5×10^{-9}M and 10^{-8}M. A high content of lactate (40.6 μmoles per 10^6 cells) and a rather low level of pyruvate (3.5 μmoles per 10^6 cells) characterized L-cells from the standard medium (Table 3). Consequently, the ratio of lactate to pyruvate for the control cells was of the order of 10. In the cells cultured under the experimental conditions, the lactate content decreased to about 1/2 or 1/3, whereas the content of pyruvate approximately doubled. This led to a decrease in the intracellular ratio of lactate to pyruvate and this decrease was more pronounced in cells from the media with antifolates at the higher concentration (10^{-8}M) than in those from the media with antifolates at the lower concentration (2.5×10^{-9}M).

Since an efflux of lactate from animal cells is rather a common phenomenon, we estimated the content of this metabolite in the culture media. As was to be expected, in the fresh standard medium we did not find any lactate since the Eagle's essential medium contains no lactate and the amount of lactate added with calf serum was negligible. In contrast, in the conditioned media after 48 hr growth of the cells, the contents of lactate per ml reached 6–8 μmoles, both in the control and experimental media (Table 4). Similarly, lactate dehydrogenase activity in the control and experimental media remained at the same very low level.

DISCUSSION

In mouse L-cells cultured in the presence of aminopterin, amethopterin or a 5-methylquinazoline antifolate CB 3703, we found a concentration-dependent accumulation of the drug applied. Of the antifolates tested in this respect, aminopterin was accumulated most efficiently in L-cells (this paper), L_{1210} cells [44–46] and HeLa cells [47]. Amethopterin was accumulated less efficiently in all these three cell systems, whereas accumulation of the quinazoline antifolate was the least efficient, as was found for methasquin in L_{1210} cells [44] and 5-methylquinazoline antifolate CB 3703 in L-cells (this paper). In contrast, the latter quinazoline antifolate accumulated with much higher efficiency than amethopterin in L_{1210} cells [14]. Such a differentiation in accumulation efficiency seems to be related to the properties of the antifolate molecules themselves and probably also to those of their cellular carriers.

Accumulation of antifolates in mouse L-cells and L_{1210} cells was found to be a very effective process because the intracellular contents of aminopterin, amethopterin and the quinazoline antifolates,

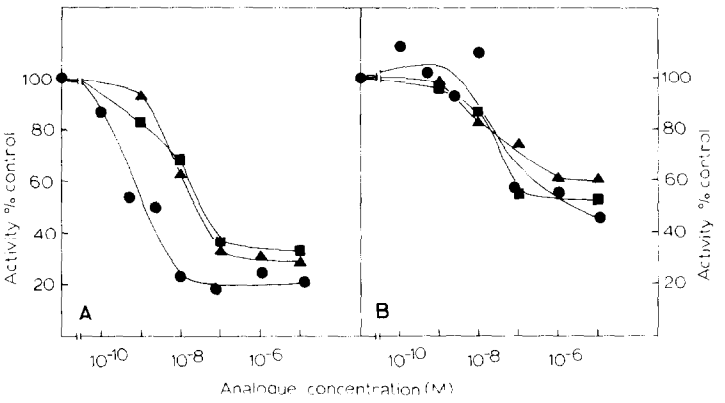

Fig. 2. Concentration curves for activity of lactate (Chart A) and glucose-6-phosphate (Chart B) dehydrogenases in L-cells grown in the presence of folate analogues. After subculturing, the cells were grown in the standard medium for 24 hr, then the medium was either renewed in the control cultures or changed for one with aminopterin (▲), amethopterin (●) or quinazoline antifolate CB 3703 (■) at the concentrations indicated. After washing off the medium, the cells were processed for enzyme assays. The activity of the enzymes was expressed as a percentage of the controls. The respective values for activity of the enzymes per mg protein and per hour, as determined 72 hr after subculturing were 0.6 mmole for lactate dehydrogenase and 30 μmoles for glucose-6-phosphate dehydrogenase. Each value is the mean of triplicate estimations in at least three experiments.

CB 3703 or methasquin, easily exceeded that of dihydrofolate reductase (this paper, [44]). Even at the lowest external concentration of the antifolates at which they did not yet influence L-cell growth, the intracellular contents of any of them were at least twice as high as that of dihydrofolate reductase after 48 hr exposure. We suppose, therefore, that antifolates in their free forms are responsible for metabolic impairment in L-cells, as was found for amethopterin in different cell systems (e.g. Refs. 45, 48–52).

In a number of animal cells cultured *in vitro*, a rapid conversion of amethopterin into its polyglutamates has been demonstrated (e.g. Refs. 44, 53–59). The preliminary results from this laboratory also suggest the possibility of such a conversion of amethopterin into polyglutamates in L-cells. As far as we know, there is still no report of conversion of aminopterin or quinazoline antifolates into their polyglutamate forms. However, the easy accumulation of both aminofolates and of the quinazoline antifolate, as well as an obvious dependence of this accumulation on external concentrations of the drugs, suggests that such a conversion in L-cells is very likely. This is the more so, because in L_{1210} cells [53] and in cells of a rat hepatoma line, the yield of amethopterin conversion into polyglutamates depends on external concentrations of the drug [57]. The easily observed but differentiated effects of amethopterin, aminopterin and the quinazoline antifolate present in the culture media on the activity of folate-related enzymes in L-cells which we now report are also consistent with our supposition of polyglutamate formation, since some polyglutamates

Table 3. Intracellular content of lactate and pyruvate in L-cells cultured in the presence of antifolates*

Antifolate in the medium [M]	μmoles/10^6 cells ± S.E.				Lactate/ pyruvate ratio
	Lactate		Pyruvate		
None	40.6 ± 0.53	(8)	3.5 ± 0.25	(8)	11.6
Aminopterin					
2.5×10^{-9}	16.2	(3)	5.5	(3)	2.9
1×10^{-8}	11.9	(4)	7.9	(4)	1.5
Amethopterin					
2.5×10^{-9}	19.0	(3)	6.0	(3)	3.2
1×10^{-8}	15.9	(4)	7.0	(4)	2.3
Quinazoline antifolate CB 3703					
2.5×10^{-9}	19.4	(3)	5.3	(3)	3.7
1×10^{-8}	14.0	(4)	8.9	(4)	1.6

* The cells were grown in the standard medium for 24 hr, then for another 48 hr in medium with an antifolate at the concentrations indicated, before processing for estimation of the metabolite assayed. Each value represents the mean of results obtained in duplicate in at least three separate experiments. The number of experiments is given in parentheses.

Table 4. Lactate dehydrogenase activity and lactate content in the control and experimental media*

Enzyme activity and metabolite content	Control medium	Experimental media with		
		Aminopterin	Amethopterin	Quinazoline antifolate CB 3703
Lactate dehydrogenase mean ± S.E. (μmoles of oxidized NADH.min^{-1}.ml^{-1})	0.60 ± 0.10 (5)	0.90 ± 0.07 (5)	0.30 ± 0.12 (5)	1.00 ± 0.06 (5)
Lactate mean ± S.E. (μmoles/ml)	7.60 ± 0.30 (6)	6.8 ± 0.60 (6)	5.9 ± 0.20 (6)	8.3 ± 0.40 (6)

* The cells were grown in standard medium for 24 hr, then for another 48 hr either in the standard medium (controls) or in medium with an antifolate at 10^{-8} M. Afterwards, the medium was decanted, centrifuged to remove floating dead cells or cell debris and processed for estimations of lactate dehydrogenase activity and lactate content. The activity of lactate dehydrogenase in the standard medium, which contained no lactate, was 1.5 ± 0.2 μmole of oxidized NADH.min^{-1}.ml^{-1} medium. Each value represents the mean of the results obtained in duplicate in at least three separate experiments. The number of experiments is given in parentheses.

of amethopterin appeared in fact to be as strong inhibitors of animal dihydrofolate reductase as is the drug itself [53, 57, 58, 60].

The metabolic effects of accumulation of amethopterin, aminopterin and the quinazoline antifolate which we looked for in this study by means of comparison were the fluctuations in intracellular content of pyruvate and lactate, as well as changes in activity of the folate-related enzymes and folate-unrelated dehydrogenases, whose activity in L-cells was affected to a substantial extent by amethopterin [3, 4, 6]. Therefore, this time we did not assay either 5-10-methylenetetrahydrofolate dehydrogenase (1.5.1.5), whose activity was kept constant at the control level, or the activity of isocitrate dehydrogenase (1.1.4.42) which was only slightly lowered by amethopterin [4, 6].

Of the three antifolates tested aminopterin exhibited the most rapid and strong fall in dihydrofolate reductase activity; aminopterin was also most effective as a growth suppressor in L-cells. Aminopterin also, more than amethopterin, diminished the activity of formyltetrahydrofolate synthetase, whereas the quinazoline antifolate apparently caused it to increase, probably because of stabilization of its enzymic protein. This quinazoline antifolate strongly depressed thymidylate synthetase activity, whereas both aminofolates caused its apparent increase, presumably also because of stabilization of enzymic proteins and prolongation of their life times, as was supposed earlier for amethopterin [6]. In R.P.M.I. cells a similar increase in the activity of thymidylate synthetase and dihydrofolate reductase (and thymidylate kinase, not estimated in this research) was reported after growth in the media with aminopterin, amethopterin or with a chlorinated quinazoline antifolate [7].

The effect of aminopterin, amethopterin and the 5-methyl quinazoline antifolate CB 3703 on other folate-related enzymes and glycolysis-linked dehydrogenases in L-cells were nearly parallel, but different for particular enzymes. The most remarkable was the great fall in the activity of lactate dehydrogenase. The activity of this dehydrogenase was especially strongly influenced by amethopterin, and this event was already seen in cells growing in the media with any of the drugs at concentrations still not growth-limiting. However, none of the antifolates tested had any effect on the activity of lactate dehydrogenase when assayed *in vitro*. Thus, the great fall in the activity of this dehydrogenase which we observed in L-cells and in L_{1210} cells (data for L_{1210} not included into this paper), was an indirect effect, whose mechanism remains to be investigated. We already found, however, that in L-cells exposed to any of the analogues, the intracellular ratio of lactate to pyruvate decreased. This was the result of a decrease in the lactate content and an increase in the pyruvate content. However, the actual intracellular pyruvate concentration, although it increased from 1×10^{-6}M in cells from the control medium to 2.5×10^{-6}M in cells from the media with antifolates, was still more than two orders of magnitude too low to be responsible for the fall in the lactate dehydrogenase activity, since the $I_{0.5}$ for pyruvate, when assayed in enzyme extracts, was as high as 5×10^{-4} M.

The intracellular contents of pyruvate and lactate depend not only on the rates of their formation and further metabolic transformation, but also on their efflux from cells into the surrounding medium [61]. The lactate efflux from animal cells is a rather common phenomenon ([8, 9, 36], this paper). Moreover, in some cells it could be enhanced by amethopterin [8, 9], but that was not the case in L-cells. Since the lactate to pyruvate ratio is believed to reflect the cytosolic NAD^+ oxidation–reduction potential (e.g. Refs. 61–63), a decrease in this ratio in L-cells exposed to any of the antifolates tested indicates an increase in intracellular concentration of the oxidized nicotinamide adenine dinucleotide. This in turn suggests that even in L-cells still growing at their normal rate in the medium with aminopterin, amethopterin or the 5-methyl quinazoline antifolate CB 3703, these antifolates impaired glycolysis. In conclusion, we propose that the ratio of lactate to pyruvate be considered a kind of simple measure of non-target effects of antifolates in mammalian cells.

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