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ACTIVE TRANSPORT OF AMINOPTERIN IN YOSHIDA SARCOMA CELLS

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SUMMARY

1. It has been demonstrated that aminopterin is transported into the Yoshida sarcoma cells by an energy-dependent active process. The uptake was temperature-dependent and could be inhibited by inhibitors of energy metabolism. For 0.91 μM aminopterin in the medium the concentration ratio based on free intra-/extracellular aminopterin was 2.4.

2. 80% of the aminopterin transported into the cells has been detected in the cell sap and was partly bound to tissue components (dihydrofolic acid reductase).

3. The presence of the two inhibitors, 2,4-dinitrophenol and iodoacetate was necessary in order to demonstrate a significant decrease in the uptake of aminopterin which suggests that both oxidative and glycolytic processes contribute significantly to the synthesis of intracellular ATP. Ouabain (0.1 mM) inhibited the uptake by 50% indicating that (Na^+–K^+)-activated ATPase of the cell wall was involved in the transport mechanism.

4. High concentrations of folic acid in the medium were able to inhibit the transport of aminopterin.

5. It may be concluded that the capacity of Yoshida sarcoma cells to rapidly accumulate aminopterin is achieved through the complex enzyme carrier system. The rapid transport of the drug correlates well with the high responsiveness of this tumour to the antifolics as compared to some other tumours described in the literature (S-180 and Ehrlich ascitic tumour) in which the antifolics are transported by passive diffusion.

INTRODUCTION

The inhibitory action of aminopterin (4-aminopteroylglutamic acid) and amethopterin (4-amino-N^{10}-methylpteroylglutamic acid) on the growth of cells[1] is due to the inactivation of dihydrofolic acid reductase (5,6,7,8-tetrahydrofolate: NAD(P) oxidoreductase, EC 1.5.1.3) which converts 7,8-dihydrofolate to tetrahydrofolate[2], the active coenzyme form of folic acid.

Lack of correlation observed in normal[3] and tumour[4] tissues between levels of

dihydrofolic acid reductase activity and the toxicity of the drug, suggests that the overall responsiveness of a particular tissue to the antifolics is determined both by the concentration of dihydrofolic acid reductase as well as by the availability of the inhibitor to the enzyme. These considerations have turned the attention of the investigators to the study of transport of these antimetabolites into the tumour cells. It appears from the literature that antifolics are transported into tumour cells by different mechanisms. Studies by FISCHER[5] have shown that amethopterin is transported to L 5178 Y murine leukaemic cells by an active process mediated by a carrier system, whereas HAKALA's[6] studies on S-180 cells, also in tissue culture, indicate that the transport of amethopterin and aminopterin into these cells is by passive diffusion. According to HALL, ROBERTS AND KESSEL[7], the uptake of tritiated methotrexate by human white blood cells takes place by a process of facilitated diffusion. The present paper is a study of the kinetics of the uptake of aminopterin into Yoshida sarcoma ascites cells and shows that aminopterin is transported into these cells by an energy-dependent process against a concentration gradient.

MATERIALS AND METHODS

Preparation of Yoshida sarcoma cells

Yoshida sarcoma ascitic tumour was regularly maintained in Wistar rats by intraperitoneal transplantation of $8 \cdot 10^7$ ascites cells. The ascitic fluid was withdrawn from tumour-bearing rats 4–5 days after tumour inoculation and the Yoshida sarcoma cells were separated from red-blood cells by repeated centrifugation and washing as described by MCKEE[8]. The white tumour cells obtained were finally suspended in Tyrode solution to give a Klett reading of 100 using filter 66. The number of cells corresponding to the Klett reading was obtained from previously calibrated graphs (Klett 100 = $2.5 \cdot 10^6$ cells/ml).

Determination of transport of aminopterin

Incubation system. 50 ml cell suspension of Yoshida sarcoma cells containing $1.25 \cdot 10^8$ cells (approx. 260 mg wet wt.) were incubated in conical flasks at 37° with slow shaking in a metabolic shaker to prevent foaming. After 5 min freshly prepared aminopterin solution (1 mg/ml) in 0.05 M phosphate buffer (pH 7.4) was added and the flasks were shaken for 1 h. The control flask contained a 50-ml cell suspension without aminopterin which was used to determine the folic acid present in the cells (see below). Aerobic conditions were employed in all experiments. At the end of the incubation period the integrity of the cells was tested using lissamine green[9]. After 2 h of incubation, 95% of the cells were generally found to be intact as judged by exclusion of the dye.

Preparation of cell extracts. After incubation the Yoshida sarcoma cells were separated by centrifuging at 1300 × *g* and were washed twice with 30 ml Tyrode solution to remove traces of aminopterin adhering to the cells. The washed cells were suspended in 2 ml water, sonicated (Raytheon) for 1 min and then autoclaved for 10 min at 10 lbs pressure. The volume was then adjusted to 10 ml and the coagulated proteins were removed by centrifuging at 40000 × *g* in the Spinco ultracentrifuge. Preliminary experiments had shown that autoclaving changed aminopterin to the free form the inactive state and clear extracts suitable for aminopterin assay could be obtained.

The extracts were stored frozen until assayed. The unheated extracts were sterilized by passing through a bacteriological filter.

Folic acid assay

This was carried out with *Streptococcus faecalis* as the test organism[10]. Each determination of folic acid was carried out in duplicate in 3 different aliquots of the cell extract. A reference standard folic acid curve was plotted simultaneously with each experiment. The standard curve was linear up to 0.6 mμg/ml folic acid in the medium. Generally 0.1- to 0.2-ml aliquots of the extract were used for the assay.

Aminopterin assay

Aminopterin was assayed by the method of inhibition analysis[11]. The inhibition curve was linear in the range of 0.3 to 0.7 mng/ml of aminopterin in the medium. As the inhibition of growth of *S. faecalis* produced by aminopterin is dependent on the concentration of folic acid[12], the assay was standardized at a folic acid concentration of 0.8 mng/ml, this being the concentration of folic acid required for optimum growth of *S. faecalis*. For this reason in all experiments the concentration of residual folic acid present in the cells after incubation was first determined in the control tube, as described above. It can be seen that in experiments described in Table VII the control cell suspension was incubated with the appropriate amounts of folic acid used in the medium. Aminopterin assay tubes were then supplemented with additional amounts of folic acid where necessary to make the level constant at 0.8 mμg/ml. Due to these limitations, aliquots of cell extracts having greater than 8 mμg folic acid could not be employed. Each determination of aminopterin was carried out in duplicate with 3 different aliquots of cell extracts and always with reference to a standard aminopterin inhibition curve plotted simultaneously. Generally 0.1- to 0.2-ml aliquots of the extracts were used.

Determination of the volume of cell water in Yoshida sarcoma cells

Intracellular water = total water in cell pellet—weight of extracellular water in the pellet.

Determination of wet and dry weight of cells. Tumour cell suspension in Tyrode solution containing 10^8 cells was centrifuged at 1300 × *g* for 15 min in a tared tube. The supernatant was poured out and the tube was drained for 5 min. The traces of water adhering to the sides of the tube were wiped off with filter paper and the tube was weighed again to determine the wet weight of the cell pellet. The dry weight was obtained by heating the tube containing the pellet at 110° for 20 h or more to a constant weight. It was consistently found that under these conditions the wet weight of the cell pellet of 10^8 cells was 200–215 mg. The dry weight was approx. 18% of the wet weight of the cells. The total water in the cell pellet was calculated from the difference between the wet and dry weights of the cell pellet.

Determination of the weight of extracellular water in the cell pellet. The volume of extracellular water in the cell pellet was found by using a dye, lissamine green, which does not penetrate the intact cells[9]. To a cell pellet consisting of 10^8 cells obtained as described above, 3 ml of lissamine green solution (400 μg/ml) in Tyrode were added to give a uniform suspension. The tube was then centrifuged at 1300 × *g* for 15 min. Supernatant dye solution was poured out and the tube was allowed to

drain for 5 min. Traces of the dye solution adhering to the sides of the tube were wiped off with filter paper. The pellet so obtained was suspended in a definite volume of Tyrode solution in order to extract the dye solution held in extracellular spaces. The suspension was then centrifuged under similar conditions and the absorbance of the supernatant was determined in a Beckman DU spectrophotometer at 420 mμ (optimum for lissamine green). The blank consisted of a tube containing 10^8 cells treated in an identical way without the dye. The concentration of the dye in the fluid held in extracellular spaces in the pellet was calculated from a standard graph relating concentration to absorbance. From the original concentration of the dye solution added (400 μg/ml) the dilution factor was obtained and hence the weight of extracellular water (density was assumed to be unity). Results of several experiments showed that the weight of extracellular water was $38\% \pm 2\%$ of the wet weight of the cell pellet.

Concentration ratio of aminopterin in the cell

The total aminopterin present in cell extracts obtained from $1.25 \cdot 10^8$ cells was determined and the concentration of aminopterin present in cell water was calculated. The decrease in the concentration of aminopterin in the medium due to its uptake by the cells was neglected since it was very small compared to the aminopterin present in the medium. Concentration ratio = concentration of aminopterin in cell water/concentration of aminopterin in the medium.

Folic acid transport was studied in an identical manner as described above for aminopterin transport.

Materials

Folic acid (pteroylglutamic acid) and aminopterin (4-aminopteroylglutamic acid) were commercial preparations from Tanabe Seiyaku Co., Ltd. and L. Light and Co., respectively. Solutions were prepared in 0.05 M phosphate buffer (pH 7.4) and were used within 1 week. 2,4-Dinitrophenol (B.D.H.) was dissolved in Tyrode solution and the pH was adjusted to 7.4 with a few drops of 0.1 M NaOH. Ouabain (Sigma) was dissolved in Tyrode solution. Iodoacetic acid (B.D.H.) was dissolved in water and the pH was adjusted to 7.4 with 1 M NaOH.

RESULTS

Fig. 1 shows the uptake of aminopterin by the Yoshida sarcoma ascites cells incubated with varying amounts of the analogue in the medium. The rate of transport per h is linearly proportional up to 0.91 μM aminopterin. At this concentration apparently equilibrium is reached and no further significant increase in the total transport of aminopterin could be detected by increasing the external concentration of the drug from 0.91 to 2.3 μM.

In order to study the relationship between time of incubation and transport, Yoshida sarcoma cells were incubated in a medium containing 0.91 μM aminopterin for various lengths of time. The results given in Table I show that for the first 15 min the uptake of aminopterin expressed in terms of cell water is below the level detectable by these techniques (see MATERIALS AND METHODS). At the end of 60 min, the intracellular concentration of aminopterin reaches a constant value indicating that

TABLE I

UPTAKE OF AMINOPTERIN BY YOSHIDA SARCOMA CELLS IN RELATION TO TIME

Experimental conditions same as given in Fig. 1. Aminopterin in medium = 0.91 μM.

Period of incubation (min)	*Aminopterin in cell water (μM)*
15	—
30	1.21
45	4.44
60	6.16
90	6.67

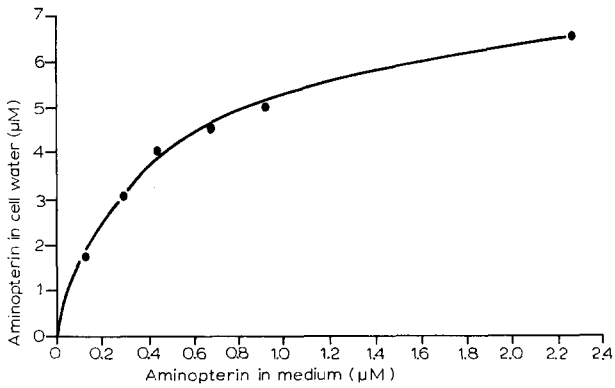

Fig. 1. Uptake of aminopterin by Yoshida sarcoma cells at various concentrations of aminopterin in the medium. ●- - - -●, aminopterin in cell water. Number of Yoshida sarcoma cells, $1.25 \cdot 10^8$; total vol., 50 ml; time of incubation, 1 h; temp., 37°; pH 7.4.

equilibrium was reached. On extending the incubation period to 90 min there is no further increase in the uptake.

Experiments in which the transport was measured at various temperatures ranging from 0° to 37° (Table II) showed that the uptake of aminopterin by these cells was considerably decreased at 22° and at temperatures below 6° no uptake could be detected. The temperature coefficient between 25° and 35° was 2.0.

TABLE II

UPTAKE OF AMINOPTERIN BY YOSHIDA SARCOMA CELLS AT DIFFERENT TEMPERATURES

Experimental conditions were same as given in Fig. 1. Aminopterin in medium = 0.91 μM.

Temp. (°)	*Aminopterin in cell water (μM)*
0	—
6	—
22	2.12
37	6.48

TABLE III

EFFECT OF INHIBITORS OF ENERGY METABOLISM ON AMINOPTERIN UPTAKE BY YOSHIDA SARCOMA CELLS

Incubation medium: 135 mM NaCl; 2.7 mM KCl; 1.1 mM $MgCl_2$; 11.9 mM $NaHCO_3$; 0.4 mM NaH_2PO_4. Number of Yoshida sarcoma cells, $1.25 \cdot 10^8$; total vol., 50 ml; time of incubation, 1 h; temp., 37°; pH 7.4. Yoshida sarcoma cells were preincubated with the inhibitors for 30 min at 37° before addition of aminopterin (0.91 μM).

Expt. No.	*Additions*	*Concn. (mM)*	*Aminopterin in cell water (μM)*
1	None	—	5.25
2	2,4-Dinitrophenol	0.01	5.36
3	2,4-Dinitrophenol	1	5.25
4	Sodium iodoacetate	0.1	6.46
5	Sodium iodoacetate	2	6.26
6	2,4-Dinitrophenol	0.5	1.41
	+sodium iodoacetate	2	
7	Ouabain*	0.01	4.06
8	Ouabain*	0.1	2.64

* Medium supplemented with 6.0 mM glucose and 1.8 mM $CaCl_2$.

Experiments designed to study the role of intracellular ATP on the transport of aminopterin (Table III) have shown that the uptake remains unaffected by 0.01 to 1 mM dinitrophenol, a well known inhibitor of phosphorylations accompanying the oxidative process. In these experiments Yoshida sarcoma cells were preincubated without glucose and Ca^{2+} to decrease the existing intracellular concentration of ATP before addition of aminopterin. Since tumours have a very active aerobic glycolysis, it was conceivable that ATP synthesised by phosphorylations at substrate level (a process which is not blocked by dinitrophenol), occurring during glycolysis was sufficient to maintain the necessary intracellular ATP. It is, however, observed that addition of iodoacetate (2 mM) which is an inhibitor of glycolysis does not affect the transport of aminopterin into the cells, whereas the combination of dinitrophenol and iodoacetate causes 80% inhibition. Ouabain (0.1 mM), a specific inhibitor of the enzyme (Na^+–K^+)-activated ATPase[13] which is closely implicated in the active transport of ions and other molecules, blocks the transport of aminopterin by 50%.

TABLE IV

DISTRIBUTION OF INTRACELLULAR AMINOPTERIN IN YOSHIDA SARCOMA CELL FRACTIONS

Experimental conditions same as given in Fig. 1. Aminopterin in medium, 0.91 μM.

Cell fraction	*Aminopterin detected (mμmole/ 10^8 cells)*	*% of total*
Cell homogenate (heated)	0.47	100
Supernatant 105 000 × *g* (heated)	0.37	79
Supernatant 105 000 × *g* (non heated)	0.19	40
Residue 105 000 × *g* (heated)	0.05	11

Most evidence in literature[14] indicates that intracellular aminopterin is present in combination with dihydrofolic acid reductase which is present in the soluble portion of the cell. In experiments designed to study the fate of transported aminopterin, the cells were harvested after the incubation period by centrifuging as usual (see MATERIALS AND METHODS). The aminopterin assays were then carried out in the total suspension of cells in Tyrode solution after disintegration with Raytheon and in the supernatant obtained by centrifuging the homogenate at 105 000 × *g* to remove the particulate elements. It is apparent from such assays (Table IV) that 80% of aminopterin transported into the cells is present in the cell sap, and 50% of this becomes available to *S. faecalis* (the test organism) only after autoclaving the extracts for 15 min at 10 lbs pressure. It may be noted that for these reasons all determinations of total aminopterin reported in this paper were routinely carried out after autoclaving the cell homogenates.

TABLE V

RELATIONSHIP OF CONCENTRATION TO FOLIC ACID TRANSPORT IN YOSHIDA SARCOMA CELLS

Number of Yoshida sarcoma cells, $1.25 \cdot 10^8$; total vol., 50 ml; time of incubation, 1 h; temp., 37°; pH 7.4.

Folic acid in medium (μM)	*Folic acid in cell water (μM)*
—	6.26
0.46	6.06
0.91	6.54
1.82	5.65
91.00	11.30

The residual folic acid contents of Yoshida sarcoma cells isolated from the ascitic fluid ranged from 0.5 to 0.8 mμmole per 10^8 cells. No loss of intracellular folic acid can be detected on incubating the cells in Tyrode solution at 37° for an hour. Experiments in which the cells were incubated in the presence of various concentrations of folic acid in the medium have shown (Table V) that no significant uptake takes place, when the concentration in the external medium is below the folic acid concentration in cell water. On increasing the folic acid in the medium to a level higher

TABLE VI

DISTRIBUTION OF INTRACELLULAR FOLIC ACID IN YOSHIDA SARCOMA CELL FRACTIONS

Experimental conditions same as given in Table V. Folic acid in medium, 91.0 μM.

Additions to medium	*Cell fraction*	*Folic acid detected (mμmoles/ 10^8 cells)*
—	Cell homogenate (heated)	1.02
—	Supernatant 105 000 × *g* (heated)	0.99
—	Residue 105 000 × *g* (heated)	0.04
Ouabain, 10 μM	Supernatant 105 000 × *g* (heated)	0.96

TABLE VII

AMINOPTERIN TRANSPORT IN YOSHIDA SARCOMA CELLS IN THE PRESENCE OF FOLIC ACID

Experimental conditions same as in Table V.

Folic acid in medium (μM)	*Aminopterin in medium (μM)*	*Aminopterin in the cell water (μM)*
—	0.91	6.26
0.91	0.91	6.46
1.82	0.91	6.06
91.00	0.91	1.10

than the intracellular concentration, some further uptake is detected. Experiments described in Table VI show that the folic acid transported into the cells is present in the cell sap and only a negligible amount can be detected in the cell residue. Under these conditions addition of ouabain to the incubation medium does not alter the uptake of folic acid. Results of experiments in which the transport of aminopterin was examined in the presence of various concentrations of folic acid in the medium are given in Table VII. The uptake of aminopterin is found to be the same in the presence or absence of folic acid, when the concentration of folic acid is twice that of aminopterin. With much higher amounts of folic acid in the medium (100 times concentration of aminopterin) the transport of the analogue is inhibited by more than 80%.

DISCUSSION

An overall analysis of the results reported in this paper suggests that aminopterin is transported into Yoshida sarcoma cells by an active process dependent on an enzyme carrier system. In dealing with cell systems such as the present one, it is necessary to consider that high concentration ratios may arise due to firm complex formation between the antifolics and dihydrofolic acid reductase[14]. Observations described here have also shown that under equilibrium conditions using 0.91 μM aminopterin in the medium and 1-h incubation period, 80% of the aminopterin transported was present in the supernatant cell fraction and 50% of this became available to *S. faecalis* only after heating, indicating that only 50% of the intracellular aminopterin was in a free state in the cell sap. However, the concentration ratio based on free intra-/extracellular aminopterin for 0.91 μM aminopterin and 60-min incubation period was above unity (2.4) and suggests that an active transport mechanism may be involved. Results showing that the uptake of aminopterin is temperature-dependent and was inhibited by lowering the ATP concentration, conform to the same view. The significant decrease in the uptake of aminopterin brought about by ouabain further points to the involvement of (Na^+–K^+)-activated ATPase (located in the cell membrane) in the transport process.

The maximum amount of aminopterin taken up by 10^8 cells at equilibrium conditions was 0.6 mμmole. The residual concentration of folic acid in the cells isolated from the ascitic fluid was of the same order (0.5–0.8 mμmole per 10^8 cells). Results obtained with 3 different lines of Yoshida sarcoma cells[15] have also shown a similarity in the optimum amount of aminopterin transported into the cells and the

intracellular folic acid content. The uptake of folic acid was not detected in a system in which external concentrations of folic acid used were lower than in the cell water, apparently because the cells were already saturated with folic acid; some transport of folic acid was observed where higher concentrations of folic acid were employed. The uptake detected under these conditions was not inhibited by inhibitors of ATPase such as ouabain, indicating that it was due to passive diffusion of folic acid into the cells. These concentrations of folic acid were also able to inhibit the transport of aminopterin possibly by competing with the transport carrier system. Although further studies are necessary in this area to demonstrate a common enzyme carrier system for the transport of folic acid and its analogues, the present findings suggest such a possibility.

Attempts to demonstrate that the transport of aminopterin into the cells was ATP-dependent have shown that both glycolysis and the oxidative process contribute significantly to the synthesis of cellular ATP in this tumour. Only by blocking both the pathways with iodoacetate and dinitrophenol, respectively, was it possible to decrease the intracellular ATP concentration sufficiently to cause a significant inhibition of aminopterin uptake into the cells.

The transport of aminopterin and amethopterin into S-180 cells in tissue culture has been demonstrated by HAKALA[16] to be due to passive diffusion. The authors conclude that the observed slow diffusion of these drugs in this tumour is due to the negative charge and low lipid solubility of these molecules, which does not favour rapid penetration through the negatively charged bimolecular lipid membrane of the mammalian cells[17]. It is of interest that the present findings show that for approximately similar concentrations of aminopterin in the medium, the uptake into Yoshida sarcoma cells was about 10-fold faster than observed by HAKALA in S-180 cells[6]. The rapid accumulation of the drug in these cells is conceivably achieved through the complex enzyme carrier system on the cell surface and may account for the high responsiveness of this tumour to the antifolics. Aminopterin and amethopterin[15] in doses of 30 μg/kg body weight of the rat could prevent the growth of tumour indefinitely whereas in S-180 tumour[18] doses of 0.2 mg/kg body weight were required to inhibit the growth by 50%. Much higher concentrations of the drug[19] (doses of 0.5 mg/kg body weight of the animal increased the survival time of mice by 100%) were also required to protect the animals against Ehrlich ascitic tumour in which the transport of aminopterin is by slow passive diffusion[20].

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