of the drug. At even higher dose levels the direct effect of the drug on the tumour becomes sufficiently large to overcome the enhanced growth occurring as the result of the removal of the immune response. Since the tumours used in these experiments cannot be regarded as syngeneic, removal of the immune response should allow considerable growth stimulus, as has been found.

The treatment of animals with cytotoxic drugs prior to tumour transplantation has been shown to cause enhanced tumour growth.[1-4] Schmael and Sattler[4] suggested that this effect was dose dependent, a high dose of cytotoxic agent being required. Schmid, Schmid and Sugiura[3] also used high doses and concluded that the effect depended upon lymphocidal activity. The present findings differ considerably in that they have usually been obtained with a single small dose of cytotoxic agent. In fact, in some cases, the doses are too small to have produced any detectable lymphopenia.

In contrast to these findings is the suggestion[5] that the successful treatment of the Burkitt lymphoma by, often quite small, doses of various cytotoxic drugs is the result of immunity responses together with the drug action. Whilst this may mean that there are differences in the responses of mice and men to cytotoxic agents it does lead to the overall conclusion that further investigations are required in this field.

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Tetrahydrofolate-dependent enzymes in Sarcoma 180 cells sensitive and resistant to amethopterin

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AMETHOPTERIN-resistant sublines of mouse Sarcoma 180 cells (S-180) have been developed *in vitro* in the presence of hypoxanthine (AH/67) or thymidine (AT/174).[1] Both of these resistant sublines contain greatly increased amounts of folate reductase (tetrahydrofolate dehydrogenase), which by many different criteria is identical with the enzyme in the parent cells.[1, 2] The purpose of the present study was to compare the activity of several tetrahydrofolate-dependent enzyme systems in these cells. This was done to determine whether amethopterin resistance might be associated with changes other than the increase in folate reductase. Thymidylate synthetase activity in these cells had been studied earlier and was found to vary widely and independently from folate reductase.[3]

The origin of the parent S-180 cells, the development of the amethopterin-resistant sublines (AH/67 and AT/174), their maintenance media and harvesting have been previously described.[1] The media now used contain 5 per cent instead of 10 per cent of horse serum.

The cell pellets were stored at —75° and homogenized in a Potter–Elvehjem homogenizer in ice-cold 0·05 M K-maleate buffer, pH 7·0, containing 0·1 M 2-mercaptoethanol. The homogenate

was centrifuged in a Spinco model L ultracentrifuge at 105,000 *g* for 1 hr, and the supernatant (3–5 ml) containing 8–17 mg protein/ml was dialyzed for 18–20 hr at 4° against 2 × 500 ml of the K-maleate buffer. The dialyzed extracts were divided into small aliquots and stored at —75°. All of the enzyme assays were made using these dialyzed cell extracts. Protein was determined by the method of Lowry *et al.* with bovine serum albumin as the standard.[4]

The formate activating enzyme, N^{10}-formyltetrahydrofolate synthetase [formate:H_4-folate ligase (ADP) (EC 6.3.4.3)], was assayed by the method of Rabinowitz and Pricer[5] by measuring the increase in absorbance at 350 mμ after 2·5, 5 and 10 min of incubation at 37°. The blank was devoid of formate. Calculation was based on $\Delta E_{350} = 25{,}100$.[6]

5, 10-Methenyl tetrahydrofolate cyclohydrolase [5, 10-methenyl-H_4-folate-5-hydrolase (decycling) (EC 3.5.4.9)] was assayed by the method of Greenberg[7] with the exception that pH 7·0 was chosen to avoid the precipitation of protein that occurred at pH 6·5. The decrease in absorbance at 355 mμ was recorded during 5 min at room temperature. Calculations were based on $\Delta E_{355} = 25{,}100$.[6]

Hydroxymethyl tetrahydrofolate dehydrogenase [10-hydroxymethyl-H_4-folate: NADP oxidoreductase (EC 1.5.1.5)] was assayed by the procedure of Scrimgeour and Huennekens[8] by using NADP as the hydrogen acceptor and by recording the increase in absorbance at 340 mμ during 30 min at room temperature. Calculations were based on $\Delta E_{340} = 7{,}100$.[6]

Serine hydroxymethyl transferase [L-serine:H_4-folate-10-hydroxymethyl transferase (EC 2.1.2.1)] was assayed by isolating the radioactive glycine formed from uniformly labeled L-serine. The reaction mixture (0·35 ml) had the following composition: 5μmole L-serine; 2 μmole DL-tetrahydrofolate; 20 μmole potassium phosphate buffer, pH 7·5; 10 μmole pyridoxal phosphate; 15 μmole 2-mercaptoethanol and 0·25 μc ^{14}C-L-serine. The reaction (1 hr at 37°) was stopped by adding 0·01 ml concentrated HCl. The precipitated protein was centrifuged at 2000 rpm for 10 min and a 0·05-ml aliquot of the supernatant was applied to Whatman 3 MM paper. Glycine and serine were separated by using a high-voltage electrophoresis apparatus (Savant Instruments, Inc. Hicksville, N.Y.). In 0·75 M formic acid, pH 2·2 1 hr was needed for separation (50–100 mA, 2200–2300 V). Serine could be located on the paper by radioautography, but to locate glycine, a control with ninhydrin staining was necessary. The ^{14}C-content of the serine and glycine spots in 20 ml of POPOP*–toluene was then counted by using a Packard TriCarb 3002 scintillation counter. The molar quantity of glycine formed in the reaction was estimated, taking into account the loss of one carbon of serine.

For all of these enzymes, except serine hydroxymethyl transferase, the specific activities were based on the estimation of the initial velocities. These reactions were linear within the time intervals indicated. A linear relationship was also demonstrated between the amount of the dialyzed cell extract and the rate of the reaction for each of the four enzymes.

The results are based on the analysis of two separate sets of cell extracts for the three cell lines. Each extract, which originated from 15–20 Roux cultures in their respective maintenance media, was analyzed 2–4 times. For each enzyme activity the extracts of the three cell lines were always assayed at the same time. The specific activities in Table 1 for E_1, E_3 and E_4 are averages of the different assays, while the values for E_2 represent only one most active set of assays. For the latter enzyme, up to 2-fold variation in absolute values was noted between different experiments. However, the relative activities in the three cell lines for E_2 were always the same. Since the purpose of the present study was the comparison of the three cell lines, no further effort was expended to perfect the E_2-assay.

H_4-folate was purchased from General Biochemicals, Corp., Chagrin Falls, Ohio. Fresh solutions in K-maleate buffer (see above) were made just before use. 5, 10-Methenyltetrahydrofolate was prepared from Ca-leucovorin (Lederle Laboratories Division, Pearl River, N.Y.) by the procedure of Greenberg.[7] Uniformly labeled L-serine containing 84 mc/m-mole was purchased from Schwarz Bioresearch, Inc., Orangeburg, N.Y. Pyridoxal phosphate (2-methyl-3-hydroxy-4-formyl-5-pyridylmethyl phosphoric acid) was the product of Nutritional Biochemicals Co.

Fig. 1 presents the enzymes involved in this study in their functional sequence. The specific activities of these enzymes in the dialyzed extracts of the three sublines of S-180 cells are listed in Table 1. It is noteworthy that each of the three cell lines, whether sensitive or resistant to amethopterin, had the same serine hydroxymethyl transferase activity. These cells, like mammalian cells in general, synthesize serine from glucose *de novo* and utilize it as the source of glycine and C_1-units. Therefore, in order to overcome an inhibition by amethopterin, cultures of these cells must be supplemented

* POPOP = 1,4-*bis*-2-(4-methyl-5-phenylopanzolyl) benzene.

with glycine but not with serine.[9, 10] One would not expect large differences in serine hydroxymethyl transferase activity in these cells because all of them are dependent on a supply of C_1-units. It is true, however, that AT-cells, which must synthesize purines, have a greater need for C_1-units than AH-cells, which need them only for the synthesis of thymidylate. It has been pointed out that the

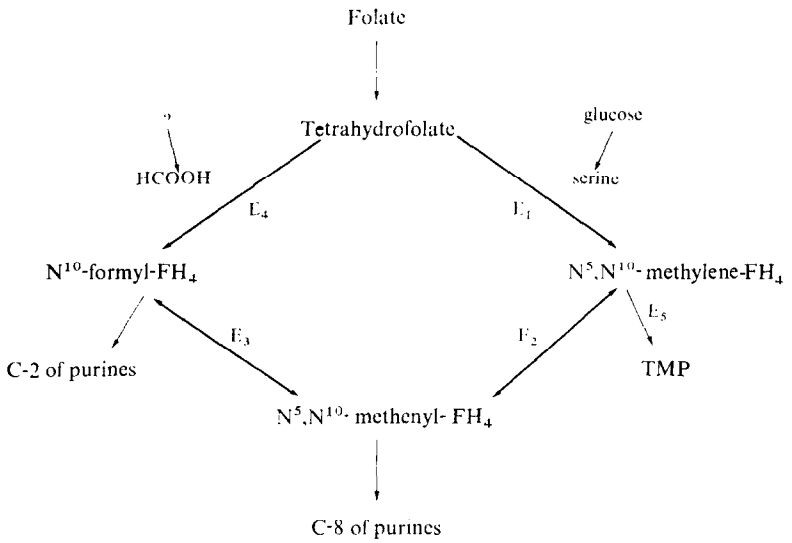

FIG. 1. Functional sequence of the tetrahydrofolate-dependent enzymes in sublines of S-180 cells.

TABLE 1. ACTIVITIES OF TETRAHYDROFOLATE-DEPENDENT ENZYMES IN SUBLINES OF S-180 CELLS

Enzyme	Code on Fig. 1	Sp. act. (mμmole/mg protein/hr) Sensitive parent	Resistant AH/67	Resistant AT/174
Serine hydroxymethyl transferase	E_1	40	39	39
5, 10-Methylenetetrahydrofolate dehydrogenase	E_2	117	40	261
5, 10-Methenyltetrahydrofolate cyclohydrolase	E_3	24	24	78
N^{10}-Formyltetrahydrofolate synthetase	E_4	40	20	56
Thymidylate synthetase (ref. 3)	E_5	7·7	10·0	0·28
Folate reductase, μmole/kg cells (ref. 16)		0·38	17·3	25·8

formation of one liver cell requires 15 times more adenine than thymidine.[11] When expressed as a requirement for C_1-units, the difference becomes 30-fold.

The second enzyme which might also be considered as a gateway for C_1-units, but whose role in mammalian cells is obscure, is N^{10}-formyl-H_4-folate synthetase. The activity of this enzyme, unlike that of serine hydroxymethyl transferase, did change and is much lower in AH-cells and slightly higher in AT-cells than in the parent S-180 cells. The present study does not permit an interpretation of the mechanism of this change. In bacteria, however, it was shown that extracellular adenine repressed the synthesis of N^{10}-formyl-H_4-folate synthetase in amethopterin-resistant *S. faecalis*.[12] Thus, it is possible that hypoxanthine in AH-cell cultures has acted as a repressor of this enzyme also.

If one assumes that the source of C_1-units in mammalian cells is serine, then those cells which are relieved of the necessity to synthesize purines (AH-cells) have no need for hydroxymethyl-H_4-folate dehydrogenase (E_2) or 5, 10-methenyl-H_4-folate cyclohydrolase (E_3). Indeed, the former enzyme (E_2) is quite low in AH-cells as compared with the parent. In AT-cells, which need N^{10}-formyl- and

N^5, N^{10}-methenyl-H_4-folate for purine synthesis, these enzymes (E_2 and E_3) are greatly elevated.

The present study reveals a total lack of correlation between folate reductase content and the activities of several tetrahydrofolate-dependent enzyme systems in S-180 cells grown *in vitro*. This is in agreement with results of other investigators who have studied cells grown *in vivo*. Thus, Bertino *et al.*[13] studied human leukocytes which differed up to 30-fold in their content of dihydrofolate reductase without any correlation with the activities of E_1, E_2, E_3 and E_4 (see Fig. 1). In Ehrlich ascites carcinoma cells, which differed by 14-fold in dihydrofolate reductase content, Sartorelli *et al.*[14] observed no difference in E_1, E_2 and two additional tetrahydrofolate enzymes. On the other hand, Sotobayashi *et al.*[15] found large differences in the content of several tetrahydrofolate cofactors in two rat tumors which were similar in folate reductase content but differed in their sensitivity to amethopterin.

The present study on mammalian cells grown *in vitro* offers new information which would be difficult to obtain by using systems *in vivo*. The resistant AT-cells which must synthesize purines, not thymidine, are very low in TMP-synthetase (E_5)[3] and quite high in enzymes required for purine synthesis (E_2 and E_3). On the other hand, the resistant AH-cells which must synthesize thymidine, not purines, have undergone a change in the reverse direction, having a high TMP-synthetase content and low E_2. Earlier studies of these cells have shown that the effect of thymidine on TMP-synthetase activity in AT-cells is not a temporary one which could be relieved when thymidine is removed from the growth medium.[3] Instead, it seems that the TMP-synthetase activity in these cells is a stable, heritable characteristic and apparently the result of cell selection which occurred early during the development of these sublines. The present study makes it very clear that thymidine or hypoxanthine, when present in the culture medium during the development of the amethopterin resistance, can have a striking effect on several tetrahydrofolate-dependent enzymes.

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