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THE NATURE OF AMINOPTERIN INACTIVATED BY NORMAL AND LEUKAEMIC TISSUES

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Abstract—(1) Inactivated aminopterin can be reactivated by a brief exposure, at room temperature, to either mild acid or alkali.

(2) The u.v. absorption spectrum of aminopterin and its inactive form are indistinguishable.

(3) A-methopterin, the N_{10}-methyl analogue of aminopterin, is also inactivated, but amino-an-fol, a weak folic acid antagonist which differs from aminopterin by a terminal aspartic instead of a glutamic acid, resists inactivation.

(4) The *in vitro* inactivation of aminopterin does not occur in the presence of relatively high concentrations of glutamate or *p*-aminobenzoylglutamate.

(5) The Mg salt of inactivated aminopterin indicates that a free carboxylic acid group has been lost in the course of inactivation.

(6) In view of the evidence listed under points (1)—(5) the tentative hypothesis is proposed that inactivation of aminopterin is caused by a ring closure of the terminal glutamic acid.

(7) The possible implications of these findings for the treatment of acute leukaemias are discussed.

INTRODUCTION

IN A previous paper[1] experiments were described which showed that the folic acid antagonist aminopterin (4-aminopteroylglutamic acid) loses its capacity to arrest cell division at the metaphase stage after incubation with a variety of normal tissues or leukaemic cells from mice or children with acute leukaemia.

Folic acid antagonists are used in the investigation of cellular processes and as therapeutic agents. They act by interfering with the function of folinic acid, which is essential for all cell divisions and for the *de novo* synthesis of purines and thymine in the two daughter cells after completion of mitosis. Furthermore, they are capable of inducing temporary remissions in a certain percentage of children with acute leukaemia. It was therefore of interest to conduct experiments which may throw light on the nature of the inactivation of folic acid antagonists, especially as the majority of children with acute leukaemia fail to respond to these compounds and those who initially responded become eventually resistant to treatment with folic acid antagonists. The results of the investigations reported here suggest that the inactivation of aminopterin may be due to a ring formation of the terminal glutamic acid of the molecule.

MATERIAL AND METHODS

Aminopterin dissolved in Tyrode's salt solution was inactivated by incubating it with either human or fowl red blood cells or with leukaemic cells of mice with an acute lymphoblastic leukaemia. The concentrations of aminopterin varied between 0·5 and 2·5 mg/ml. The quantities of cells varied between 10 and 50 mg/ml. The total volumes of the incubation mixtures ranged from 2 to 20 ml. The fluids were kept in a shallow layer for 20–24 hr at 37 °C. They were then centrifuged and the supernatant, containing the incubated aminopterin was either tested immediately or stored at 3 °C for 1 or 2 days. All solutions and materials were prepared throughout under strictly sterile conditions.

The standard procedure for testing, whether aminopterin had retained its ability to arrest mitoses in metaphase, or whether it had lost its inhibitory power, were performed on hanging drop tissue cultures, 48 hr old, of chick embryo frontal bones. These cultures provided abundant supply of cells in division. The test fluid (0·01–0·02 ml) was placed on the cultures for 15 min. They were then fixed in methanol and stained with May–Grunwald's and Giemsa's dyes. Further details of the procedure are given in a previous paper (Jacobson and Cathie, 1960).

Dividing cells were counted and classified as prophases (P), metaphases (M), anaphases (A) and telophases (T) and recorded as percentage of all dividing cells.

In each experiment control cultures were counted, so that each experiment had its own internal standards for comparison. Untreated cultures, and those exposed for 15 min to supernatants of cells incubated without aminopterin, showed a normal distribution pattern of mitotic phases (about 40–45 per cent metaphases and about 20–25 per cent anaphases). Similar values were found in cultures exposed for 15 min to fully inactivated aminopterin. In contrast, test cultures exposed for the same engthh of time to aminopterin showed a striking arrest in metaphases (about 60–70 per cent) with severe clumping of the chromosomes, and very few anaphases (0–10 per cent). Thus by comparing the metaphase (M) and anaphase (A) ratios in the tables the reader can appraise the essential part of the information. Special technical procedures are given in the corresponding sections.

RESULTS

Seeking information about the chemical nature of the inactivated aminopterin, a number of tests were undertaken which can be grouped into two classes: those that would indicate whether a partial breakdown of the molecule had taken place, and those that would show whether there had been a transformation without involving a katabolic process. Over fifty preparations of inactivated aminopterin were used for this purpose.

1. *Tests to determine whether aminopterin breaks down during inactivation*

All of the investigations in this group gave negative results.

(a) *Tests for deamination.* When 10 mg of aminopterin, inactivated by incubation with fowl or human red blood cells or mouse leukaemic cells, were placed in a Conway diffusion unit, no ammonia was liberated, and similar tests with Nessler's reagent were also negative. No sign of urea formation could be detected during the inactivation of 10 mg aminopterin with fowl or human red blood cells, when the solution containing the inactivated compound was treated with urease, followed by Nessler's reagent.

(b) *Tests for liberation of free pteridine,* p-*aminobenzoic acid,* p-*aminobenzoyl-glutamic acid, and glutamic acid in the course of inactivation of aminopterin.* After aminopterin was incubated with either human or fowl red blood cells or mouse

leukaemic cells, neither the ultra-violet abosrption sectrum nor the emission spectrum of the inactivated form showed any evidence for the liberation of a pteridine, nor could this be demonstrated by extraction with *n*-butanol into which a free pteridine might have been concentrated. The Bratton and Marshall[2] test with naphthylethylenediamine for aromatic amines was negative: there was thus no indication of the presence of either free *p*-aminobenzoic acid or free *p*-aminobenzoylglutamic acid. No free amino acid could be demonstrated with ninhydrin, so that the setting free of the glutamic acid part of the compound could be excluded.

(c) *Experiments to determine whether the inactivation of aminopterin was caused by acetylation or a similar acylation.* Inactivated aminopterin was treated with a mixture of phosphoric acid and phosphorus pentachloride, to form an acylchloride; subsequent treatment with *n*-butanol, to form butylacetate or other butyl esters, failed to show the presence of acetate or other volatile fatty acids. Distillation of the acid hydrolysate of the inactivated compound did not reveal any volatile acids. These findings exluded the possibility that acetylation of aminopterin or a similar process was the cause of inactivation.

(d) *Inconclusive attempts to distinguish aminopterin from its inactive form chromatographically.* Four different solvents were used: 3% NH_4Cl, *n*-butanol + 5N-acetic acid + water (8 : 1 : 1), 5% sodium citrate saturated with *iso*amyl alcohol, and 70% *iso*propanol in water.

In the first two solvents, both aminopterin and inactivated aminopterin gave long streaks instead of defined spots. The front of the streaks were at the same positions for both forms. This indicated that both compounds may have undergone some alteration in the course of the procedure. The third solvent (5% sodium citrate saturated with *iso*amyl alcohol) did not distinguish between the active and the inactive form (both R_f 0·20). The u.v. absorbing area of a paper chromatogram obtained from 0·5 mg aminopterin and 0·5 mg inactivated aminopterin was eluted with 1 ml Tyrode and the eluates were tested in the usual way on four cultures each: the eluate of the aminopterin chromatogram inhibited mitoses (P 9 per cent, M 67 per cent, A 6 per cent, T 18 per cent in 418 dividing cells); the eluate of the inactivated aminopterin did not inhibit mitoses (P 8 per cent, M 49 per cent, A 20 per cent, T 23 per cent in 414 dividing cells).

The two derivatives of aminopterin: N_5-formyl-, 5:6-, 7 : 8-tetrahydroaminopterin and N_{10}-formylaminopterin, kindly supplied by Dr. Slavikova,[8] could be excluded as products of inactivation as their R_f-values, under these conditions, were 0·60 and 0·52.

2. *Tests to determine whether a molecular "transformation" without breakdown of aminopterin occurs in the course of inactivation*

(a) *The ultra-violet absorption spectra of aminopterin and its inactivated form.* The u.v. absorption spectra of the two compounds in Tyrode's solution were indistinguishable (Fig. 1). This observation established that in the pteridine and benzene moieties of the two compounds no substantial alteration could have occurred. Any change in the system of conjugated double bonds, such as hydrogenation, or substitutions on certain positions of the pteridine or benzene rings, would have caused characteristic changes in the ultra-violet absorption spectrum.

(b) *The effect of mild acid or alkaline hydrolysis on inactivated aminopterin.* Brief exposure of inactivated aminopterin to very mild acid or alkali at room temperature led to re-activation of the compound, restoring its inhibitory power on cell divisions in the test cultures.

In view of the lability of aminopterin itself on treatment with acid, a series of experiments were undertaken to determine at what degree of acidity aminopterin would not be destroyed. When 0·5 mg aminopterin in 1 ml plasma + Tyrode (1 : 1) was mixed with 1 ml of 0·3N HCl at room temperature for 3 min and was then

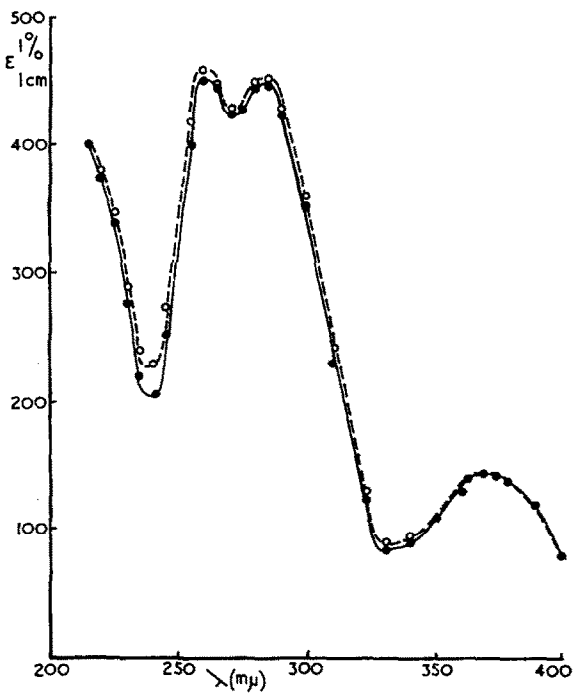

FIG. 1. U.V. absorption spectrum of aminopterin (●——●) and inactivated aminopterin (○ — — ○)

neutralized with 0·3N NaOH (this required 0.75 ml), it failed to arrest mitoses in the test cultures (Table 1, group 1). By a stepwise reduction of the amount of acid added to aminopterin solutions (0·5 mg/ml plasma + Tyrode, equal parts), it was found (Table 1) that the addition of 0·1 ml of 0·3N HCl per ml at room temperature did not diminish the inhibitory activity of aminopterin during the first 3 min (Table 1, group 4), though some reduction might occur during 30 min (Table 1, group 5). It was therefore assumed that in an attempt to reactivate the inactivated aminopterin it would be safe to expose the inactive form for short periods to 0·05 ml of 0·3N HCl per ml.

The inactivated compound used throughout these experiments was prepared by incubation of aminopterin in Tyrode with either human or fowl red blood cells or mouse leukaemic cells. If 0·05 ml of a 0·3N hydrochloric acid was added at room temperature for each ml of the solution containing 0·5 mg inactivated aminopterin

TABLE 1. DESTRUCTION OF AMINOPTERIN BY ACID AT ROOM TEMPERATURE

(0·5 mg aminopterin per ml plasma and Tyrode equal parts. The neutralized solutions are applied to two or more test cultures for 15 min.)

	Vol. of 0·3 N HCl added to each ml of aminopterin sol. (ml)	Time of exposure to acid (min)	Vol. of 0·3 N NaOH required for neutralization (ml)	% Ratio of meta- to ana-phase in test cultures (%)
(1)	1	3	0·75	45–20
(2)	0·5	3	0·34	47–22
(3)	0·2	3	0·16	53–13
(4)	0·1	3	0·05	71–2
(5)	0·1	30	0·06	63–1
(6)	0·05	20	0·02	70–1

Under the first three conditions aminopterin has lost all or most (3) of its inhibitory action. Under the last three conditions aminopterin has retained all or nearly all (5) of its inhibitory action.

per ml and this was then carefully neutralized with 0·3N sodium hydroxide to pH 7·2–7·3 with the aid of a micropipette, the inhibitory action of the inactivated compound was restored, provided the exposure to acid did not exceed 15 min. The course of the reactivation is illustrated by the following experiment (Fig. 2): to the solution containing the inactivated aminopterin, acid was added in the quantity described above.

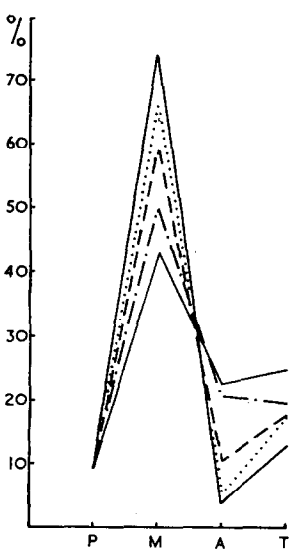

FIG. 2. Reactivation of inactive aminopterin by acid treatment. Distribution of mitotic phases in test cultures after 15 min exposure to: inactivated aminopterin ——— (metaphases, 43 per cent); inactivated aminopterin after treatment with 0·05 ml 0·3 N HCl/ml for 3 min — — — —; for 5 min curve not drawn, metaphases 64 per cent, marked X; for 15 min . . . . . ; for 30 min—·—·. Aminopterin control ——— (metaphases, 74 per cent). Abbreviations: P, prophase; M, metaphase; A, anaphase; T, telophase.

Aliquots were neutralized after 3, 5, 15 and 30 min of exposure to the acid. These four samples were applied to groups of test cultures. The following served as controls (1) inactivated aminopterin, not exposed to acid, (2) aminopterin in Tyrode, exposed to acid for corresponding periods, and (3) as the tissue control, the supernatant of red cells incubated without aminopterin (Table 2).

It can be seen that exposure of the inactive compound to acid for 3,5 and 15 min led to increasing inhibitory action on cell division. After 30 min exposure to acid,

TABLE 2. REACTIVATION OF INACTIVE AMINOPTERIN BY ACID TREATMENT

(The inhibitory action is restored increasingly up to 15 min exposure to acid. The neutralized solutions were applied to test cultures for 15 min. The mitotic ratios were as shown in the Table.)

The controls	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures
(1) 0·5 mg aminopterin per ml serum + Tyrode (1 : 1)	9	74	4	13	196	3
(2) 0·5 aminopterin + 100 mg r.b.c./ml incub. 24 hr	9	43	23	25	246	3
(3) 100 mg r.b.c./ml Tyrode, incub. 24 hr	9	44	24	23	230	2
Acid treated solution (2) = inactivated aminopterin + 0·05 ml 0·3 N HCl/ml.						
Neutralized after 3 min	9	60	11	20	127	2
Neutralized after 5 min	9	64	6	21	204	2
Neutralized after 15 min	10	66	6	18	246	2
Neutralized after 30 min	9	52	21	18	260	2

Neutralization required 0·02 ml 0·3 N NaOH/ml in each case. For acid treatment of aminopterin (incubated without r.b.c.) see Table 1.

The inactivated aminopterin (solution 2) has regained most of its inhibitory power after 15 min exposure to acid. P = prophase, M = metaphase, A = anaphase, T = telophase.

however, the inhibitory power of the compound was much reduced. The interpretation of this observation was made possible by the second control group in which the active, unaltered aminopterin was treated with the same amount of acid for corresponding periods. It was found that under these conditions aminopterin itself was partially destroyed when exposed to 0·3N hydrochloric acid for 30 min; longer exposure, e.g. 1 hr, or the use of 1 ml 0·3N HCl per ml of the aminopterin solution led to complete loss of the inhibitory action (Table 1) possibly by deamination. It can be assumed, therefore, that exposure to 0·3N HCl, 0·05 ml/ml for periods of not more than 15 min converts the inactive into the active form, but beyond this period the active form is destroyed at a rate faster than that of the reactivation process. Thus, extending the exposure to acid beyond a point when a maximum has been reached, leads to an irreversible destruction of aminopterin.

When inactivated aminopterin in Tyrode (2·5 mg/ml) was exposed to 0·3N NaOH (0·1 ml/ml aminopterin solution) for 15 min at room temperature and then carefully neutralized with 0·3N HCl, some of its inhibitory action was restored (Table 3). The inactivated aminopterin produced the mitotic ratios M, 46 per cent, A, 24 per cent, but after it had been exposed to alkali for 15 min it gave the ratios: M, 61 per cent, A, 6 per cent, and after 30 min of exposure, M, 53 per cent, A, 12 per cent. It may be concluded that alkali, like acid, reactivates the compound, but that prolonged exposure to alkali destroys some of the aminopterin formed by alkaline hydrolysis.

TABLE 3. REACTIVATION OF INACTIVE AMINOPTERIN BY TREATMENT WITH ALKALI

(The inhibitory action was restored increasingly up to 20 min exposure to alkali (solution 3). The neutralized solutions were applied to test cultures for 15 min. The mitotic ratios were as shown in the Table.)

Control	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures
(1) Inactivated aminopterin (aminopterin 2·5 mg/ml) (+ r.b.c. 50 mg/ml)	10	46	24	20	679	7
Inactivated aminopterin (= solution 1) + 0·1 ml 0·3 N NaOH/ml						
(2) Neutralized after 15 min	11	61	6	22	340	2
(3) Neutralized after 20 min	10	70	4	16	969	4
(4) Neutralized after 30 min	13	53	12	22	391	4

30 min of exposure to alkali lead already to a partial destruction of aminopterin, the inhibitory power is decreased. (N.B. aminopterin itself exposed to alkali for 1 hr loses all its inhibitory action). Abbreviations as in Table 2.

Hence 30 min of treatment with NaOH results in less inhibitory activity than 15 min. This is also borne out by an experiment in which aminopterin itself was treated for 1 hr with the same amount of NaOH, when nearly all its inhibitory power was destroyed (M, 48 per cent, A, 17 per cent).

In view of the reactivation of inactivated aminopterin by very mild treatment with acid or alkali at room temperature, it was tentatively assumed that a ring closure of the terminal glutamic acid might be the process underlying the inactivation. The validity of this assumption was tested by the following three experiments (sections c, d and e).

(c) *Inactivation of A-methopterin by red blood cells and failure of red blood cells to inactivate amino-an-fol.* If inactivation is caused by ring closure, then A-methopterin (4-amino-N_{10}-methylpteroylglutamic acid), which is an effective folic acid antagonist, should be inactivated under the same conditions as aminopterin, since both compounds have a terminal glutamic acid. On the other hand, amino-an-fol (4-aminopteroyl-aspartic acid), which is a rather weak folic acid antagonist, should retain its inhibitory power, since it carries a terminal aspartic acid, instead of a glutamic acid. Aspartic acid could not be readily cyclized, as its carbon chain would be too short (see Fig. 5). A-methopterin inhibited cell divisions in cultures of embryonic chick osteoblasts and

in human bone marrow,[3] and the chick cells growing in cultures were able to overcome this inhibition within 24 hr.

In the present experiments, when 1 mg A-methopterin was incubated with 20 mg human red blood cells it lost its effect on cell division: M, 48 per cent, A, 21 per cent (among 893 mitoses of four cultures) (Fig. 3). The same amount of A-methopterin incubated with 18 mg r.b.c. + 1 mg leukaemic cells of a child with acute leukaemia in relapse after treatment with this compound, also lost its inhibitory action on cell division: M, 45 per cent, A, 23 per cent (among 439 mitoses in four cultures). Finally 10 mg red cells plus less than 1 mg leukaemic cells of the bone marrow from a child

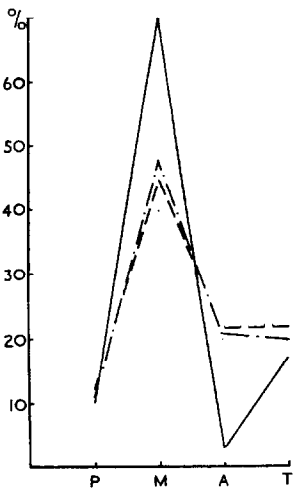

FIG. 3. Inactivation of A-methopterin by red blood cells. Distribution of mitotic phases in test cultures 15 min after exposure to: A-methopterin (2·5 mg/ml)———; A-methopterin (2·5 mg/ml), previously incubated for 24 hr with red blood cells (20 mg/ml.) —·—·—·; serum controls — — —. Abbreviations as in Fig. 2.

with acute leukaemia inactivated 1 mg A-methopterin: M, 46 per cent, A, 22 per cent (among 181 mitoses in three cultures). Thus A-methopterin is inactivated by the same tissues as aminopterin.

In contrast, when 4-aminopteroylaspartic acid (amino-an-fol) was incubated with human red cells (of the same blood as that used for the experiment with A-methopterin and with the same quantities) it did not lose its inhibitory action on cell division in test cultures: M, 65 per cent, A, 5 per cent (among 949 mitoses in seven cultures): the control, amino-an-fol incubated for 24 hr without red cells produced a similar mitotic ratio: M, 70 per cent, A, 4 per cent, (among 1108 mitoses in seven cultures) (Fig. 4).

(d) *Magnesium salts of inactivated aminopterin and of aminopterin.*[7] A ring closure of the terminal glutamic acid of aminopterin would entail a loss of the γ-carboxylic acid. This could be demonstrated by the formation of an insoluble salt with, e.g. Mg^{2+} or Ba^{2+}: either of the bivalent cations would combine with one molecule of active, and two molecules of the inactivated aminopterin. Thus the weight of Ba^{2+} or Mg^{2+} in the salt of the inactivated compound would be about half that in the aminopterin salt.

(1) Inactivated aminopterin was prepared as follows: each of two flasks received 20 ml Tyrode in which were dissolved 40 mg aminopterin and to which were added 400 mg packed red cells. The flasks were incubated for 24 hr, under sterile conditions at 37 °C, the contents were centrifuged and the clear yellow supernatant containing the incubated aminopterin was tested on cultures. It had no inhibitory action on cell division (M, 48 per cent, A, 21 per cent among 551 mitoses); untreated control cultures showed M, 47 per cent, A, 22 per cent (among 526 mitoses). The aminopterin, therefore, had been inactivated. The solution was deproteinized by shaking with chloroform, centrifuged and the clear yellow solution pipetted off. This process was repeated four times, until no precipitate with chloroform was formed. The inactivated aminopterin was stored overnight at 0 °C. A yellow, microcrystalline precipitate was collected by centrifuging at 3 °C and twice washed in a small volume of ice-cold,

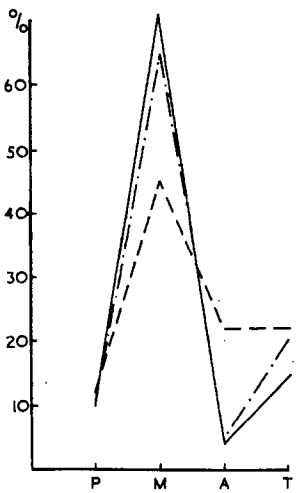

FIG. 4. Failure of red blood cells to inactivate amino-an-fol. Distribution of mitotic phases in test cultures 15 min after exposure to: amino-an-fol 2·5 mg/ml inc. 24 hr. ———; amino-an-fol, 2·5 mg/ml previously incubated for 24 hr with red blood cells 20 mg/ml, inc. 24 hr —·—·—·; serum controls — — —. Abbreviations as in Fig. 2.

glass-distilled water; care was taken to insure that the material stayed at temperatures between 0 ° and 3 °C. After the washed suspension of the precipitate had been centrifuged at 3 °C, the water was drained off and the fine yellow crystals were dried *in vacuo* over P_2O_5. To the yellow supernatant still containing a substantial part of the inactivated aminopterin, were added 3 vols. of ethanol, and this was placed at — 23 °C overnight. A further microcrystalline precipitate was collected, washed, treated in the same way as the first precipitate and pooled with it. The dried, inactivated aminopterin was retested at this stage on dividing cells in cultures: it had remained inactive throughout these procedures, M, 47 per cent, A, 19 per cent (among 594 mitoses). The magnesium salt was prepared as follows. The remainder of the crystalline inactivated aminopterin (34·0 mg) was dissolved in 17 ml 2 per cent sodium acetate, $MgCl_2$ (20 mg anhydrous, dried *in vacuo*) was added. Some precipitate appeared already at room temperature. The flasks were stored at 0 °C overnight, and the considerably increased precipitate was collected by centrifuging at 3 °C; it was washed in

a small volume of ice-cold, glass-distilled water and centrifuged in the cold, after which the water was drained off and the precipitate dried *in vacuo* over P_2O_5. To the supernatant, containing the inactivated aminopterin and magnesium chloride, were added 3 vols of ethanol; this was stored at — 23 °C overnight and the second precipitate of fine yellow crystals was collected, treated as the first precipitate and pooled with it; 29·60 mg of the inactivated aminopterin-Mg-compound was ashed. The weight of the ash was 1·50 mg as MgO, which corresponds to a Mg^{2+} content of inactivated aminopterin of 3·0 per cent. The theoretical value for inactivated aminopterin + $Mg^{2+}/2$ (m.w. 433·3) would be 2·8 per cent.

(2) The following controls were prepared: (a) Aminopterin was incubated in Tyrode without red blood cells, and treated in the same way as the inactivated aminopterin, except that it was shaken with chloroform once only. When tested on cultures it was found to have retained its inhibitory capacity at the end of these procedures, and gave the mitotic ratios: M, 63 per cent, A, 5 per cent (among 440 mitoses). When ashed as a free compound no measureable or visible ash could be detected. (b) The Mg salt of aminopterin was prepared in the same way as described above for the inactivated aminopterin. 52·14 mg of the Mg salt of aminopterin, when ashed, yielded an ash weight of 4·55 mg, which, as MgO, corresponds to a Mg^{2+} content of 5·26 per cent for aminopterin-Mg compound (m.w. 462·5). The theoretical value of the Mg^{2+} content is 5·26 per cent.

The following "blanks" were prepared: Tyrode solution, when shaken with chloroform and stored at 0 °C, did not form a precipitate; it remained clear when 3 vols. ethanol were added and when this mixture was stored at — 23 °C. A solution of 2% sodium acetate (1 vol.) + ethanol (3 vols.) + $MgCl_2$ (11·3 mg/10 ml 2% sodium acetate) gave no precipitate on storage at — 23 °C for several days. The $MgCl_2$ used gave the correct theoretical value of MgO when ashed.

Thus the difference in the Mg content of the magnesium salts of aminopterin (5·26 per cent) and of inactivated aminopterin (3·0 per cent) would not be inconsistent with the assumption that in the course of inactivation one free carboxylic acid is blocked, possibly by a ring closure of the terminal glutamic acid (Fig. 5).

(e) *The protective action of glutamate and* p-*aminobenzoylglutamate* (*p.a.b. glut.*) *against inactivation of aminopterin* in vitro. If inactivation of aminopterin involved an alteration of the terminal glutamic acid, it would be of interest to investigate the effect of relatively high concentrations of glutamate and p.a.b. glut. on this process. Loading the incubation mixture of aminopterin and blood cells with either glutamate or *p*-aminobenzoylglutamate might saturate the inactivating enzyme system to such an extent that the aminopterin molecules may be protected and thus not so readily inactivated.

The following technical points had to be considered before setting up the experiment. The addition of glutamic and *p*-aminobenzoylgluatmic acid to the incubation mixture would raise the tonicity of the solution and lower its pH unduly. This was an important point in view of the instability of aminopterin in acid solutions. It was found that 10 mg glutamic acid or 10 mg p.a.b. glut., dissolved in 0·2 ml 3·5% $Na_2HPO_4 + 12H_2O$ and brought to pH 6·5 by the addition of 0·03 ml 1·5N NaOH, could be added to 1·77 ml Tyrode to make 2 ml total volume, without affecting the

tonicity unduly. The final pH values of the incubation mixtures, containing the cells, were between 7·0–7·3.

Besides the standard aminopterin control and the tissue control (i.e. these two incubated separately and alone) the following three additional control systems had to be set up: (1) aminopterin incubated in the presence of glutamate or p.a.b. glut.; (2) leukaemic cells incubated in the presence of these two substances without

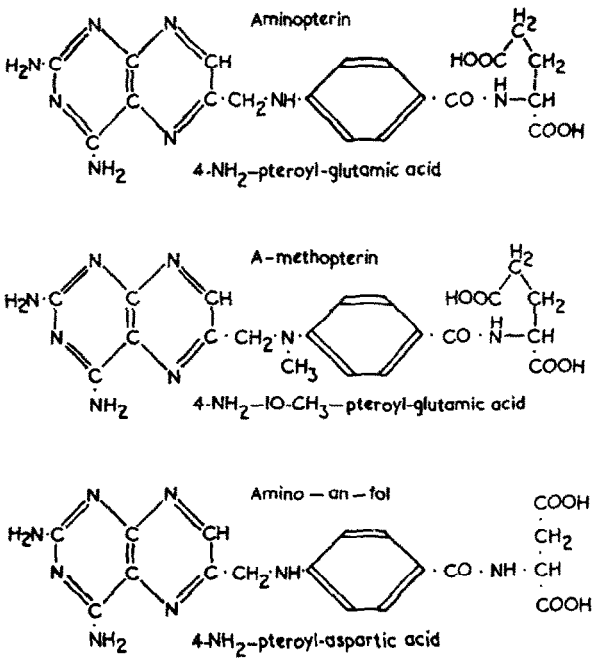

FIG. 5. The formulae of the three folic acid antagonists drawn to illustrate the hypothesis of inactivation by ring closure of the terminal glutamic acid in the case of aminopterin and A-methopterin, and to illustrate the failure to inactivate amino-an-fol.

aminopterin; (3) leukaemic cells and aminopterin incubated together in the presence of additional phosphate. This was made up of a mixture of Na_2HPO_4 and NaH_2PO_4 to give the equivalent values of added phosphate, as used in the solution for the test substances. All these controls were satisfactory: the aminopterin control (without leukaemic cells) retained its inhibitory action on dividing cells, after it had been incubated in the presence of glutamate or p.a.b. glut., and the leukaemic cells inactivated aminopterin in the presence of additional phosphate.

When aminopterin was incubated with leukaemic cells in the presence of relatively high concentrations of glutamate or p.a.b. glut. it retained its capacity to arrest mitosis in the test cultures (Figs. 6 and 7), while without these two compounds aminopterin was inactivated by the leukaemic cells. These results are summarized in Table 4, and show that either glutamate or p.a.b. glut. can protect aminopterin from being inactivated by leukaemic cells. The effect of other amino acids has not yet been tried.

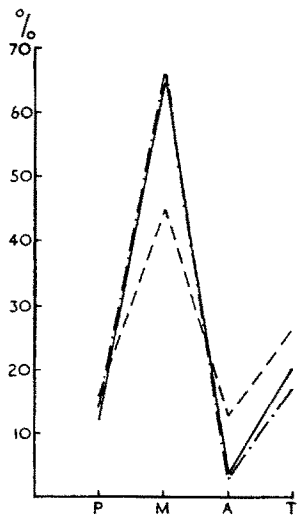

FIG. 6. Protection of aminopterin by glutamate against inactivation by leukaemic cells. Distribution of mitotic phases in test cultures 15 min after application of the following solutions previously incubated for 24 hr: aminopterin 1:500 + glutamate 1:200 ———; leukaemic cells + aminopterin 1:500 + glutamate 1:200 —·—·—·; leukaemic cells + glutamate 1:200 — — —. Abbreviations as in Fig. 2. All solutions in Tyrode for 24 hr at 37 °C.

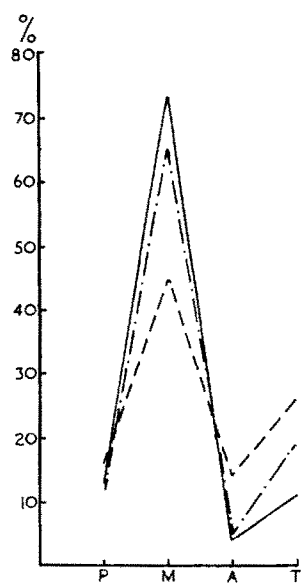

FIG. 7. Protection of aminopterin by *p*-aminobenzoylglutamate (p.a.b. glut) against inactivation by leukaemic cells.

Distribution of mitotic phases in test cultures 15 min after application of the following solutions previously incubated for 24 hr: aminopterin 1:500 + p.a.b. glut. 1:200 ———; leukaemic cells + aminopterin 1:500 + p.a.b. glut. 1:200 —·—·—·; leukaemic cells + p.a.b. glut. 1:200 — — —. Abbreviations as in Fig. 2.

TABLE 4. PROTECTION OF AMINOPTERIN BY GLUTAMATE OR *p*-AMINOBENZOYLGLUTAMATE (P.A.B. GLUT.) AGAINST THE INACTIVATION BY LEUKAEMIC CELLS
Distribution of mitotic phases in the test cultures 15 min. after application of the solutions.

	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures
(1) Tissue controls:						
(a) leuk. cells + glutamate	20	48	14	18	124	3
(b) leuk. cells + p.a.b. glut.	16	45	13	26	232	3
(2) Aminopterin controls:						
(a) aminopterin + glutamate	12	65	3	20	991	6
(b) aminopterin + p.a.b. glut.	13	73	3	11	173	2
(3) Leuk. cells + aminopterin	11	50	19	20	982	8
(4) Leuk. cells + aminopterin + glutamate	14	66	3	17	1687	16
(5) Leuk. cells + aminopterin + p.a.b. glut.	12	65	4	19	1653	15

Groups (4) and (5) show the protection of aminopterin against inactivation by leukaemic cells in the presence of glutamate or p.a.b. glut. The test cultures of these two groups show the typical aminopterin effect, i.e. arrest of mitoses in metaphase. All fluids were supplemented with phosphate and were incubated for 24 hr. The concentrations used were: aminopterin 2·0 or 2·5 mg/ml, glutamate or p.a.b. glut. 5 mg/ml, mouse leukaemic cells 25 mg/ml. Abbreviations as in Table 2.

DISCUSSION

A variety of normal tissues convert aminopterin into an inactive substance in the course of 20–24 hr, so that cells exposed to this form of aminopterin go through cell division unimpaired and can make full use of their endogenous folinic acid. Cells which have inactivated aminopterin can be arrested again in mitosis by a second application of aminopterin. This indicates that the cell still requires folinic acid for mitotic division.[1, 3–5, 7] The inactivation of aminopterin leads to an apparent resistance of cells to the action of the inhibitor. This aspect is of importance in interpreting biological and biochemical observations when the function of folinic acid and its derivatives is investigated by means of folic acid antagonists over a period of several hours or days. The case of leukaemic cells which are either resistant to folic acid antagonists like aminopterin or A-methopterin from the onset or become so in the course of treatment, makes the question of the mechanism by which this resistance is achieved particularly relevant.

As to the nature of the inactivation of aminopterin by red blood cells and leukaemic cells it must be stressed that the hypothesis advanced in this paper cannot be more than a working hypothesis, that inactivation is brought about by a ring closure of the terminal glutamic acid of the aminopterin molecule. Further work will show whether this hypothesis is tenable or not, but no evidence could be obtained for any of the well-known mechanisms of detoxication.

The following facts concerning the process of inactivation can be stated and are consistent with the assumption that the site of inactivation may be localized in the glutamic acid moiety of the aminopterin molecule. (1) The inactive form is labile and

can be reactivated by brief exposure to either mild acid or alkali at room temperature. (2) the u.v. absorption spectrum of aminopterin and its inactive form cannot be distinguished. (3) There is a significant difference in the Mg content of the magnesium salts of aminopterin and its inactive form. (4) The 4-aminopteroylaspartic acid analogue is not attacked in the same way as the two other antagonists investigated. In view of this observation, it may be worth re-investigating the clinical effectiveness of this compound which was tried about 10 years ago[6] but was superseded by the two which were more effective per mg. (5) Since glutamate and *p*-aminobenzoylglutamate can protect aminopterin *in vitro* against inactivation by mouse leukaemic cells during 24 hr of incubation, experiments will now be undertaken to find out whether a similar protective mechanism can be invoked in the treatment of acute leukaemias with aminopterin or A-methopterin.

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REFERENCES

1. W. JACOBSON and I. A. B. CATHIE, *Biochem. Pharmacol.* **5**, 130 (1960).
2. A. C. BRATTON and E. K. MARSHALL, *J. Biol. Chem.* **128**, 537 (1939).
3. W. JACOBSON, *J. Physiol.* **123**, 603, 618 (1954).
4. W. JACOBSON, *The Chemistry and Biology of Pteridines* pp. 329–355. Ciba Foundation Symposium, J. & A. Churchill, London (1954).
5. W. JACOBSON, *The Evaluation of Drug Toxicity* pp. 76–103. J. & A. Churchill, London (1958).
6. S. FARBER, *Proceedings of the Second Conference on Folic Acid Antagonists in the Treatment of Leukaemia* Edited S. Farber. *Blood* Vol. 7, pp. 97–190 (1952).
7. W. JACOBSON, *Mitt. Naturforsch. Ges. Bern.* N.F. **17**, 55 (1959).
8. K. SLAVIK, A. DVORÁKOVÁ and V. SLAVIKOVÁ, *Collection Czechoslov. Chem. Commun.* **23**, 1382, 1387 (1958).