Biochemical Pharmacology, 1960, Vol. 5, pp. 130–142. Pergamon Press Ltd., Printed in Great Britain.

THE INACTIVATION OF FOLIC ACID ANTAGONISTS BY NORMAL AND LEUKAEMIC CELLS

W. JACOBSON* and I. A. B. CATHIE†

*Sir Halley Stewart Research Fellow, Strangeways Research Laboratory, Cambridge

†The Hospital for Sick Children, Great Ormond St., London

(*Received* 27 *May* 1960)

Abstract—(1) The folic acid antagonist aminopterin arrests cell division at the metaphase stage by interfering with the function of folinic acid. Certain cell types overcome this inhibition by inactivating the inhibitor.

(2) The capacity to convert aminopterin into an inactive form is found in chick embryo osteoblasts and fibroblasts, and in red blood cells and buffy coat cells of the adult fowl. Mouse liver can also inactivate aminopterin, but normal bone marrow and normal lymphoblasts and lymphocytes are unable to do so. Leukaemic cells of an acute lymphoblastic mouse leukaemia inactivate aminopterin.

(3) Among human cell types mature lymphocytes do not affect aminopterin but the red blood cells of twenty-one out of twenty-five normal subjects converted aminopterin into an inactive form.

(4) The leukaemic bone marrow of seventeen out of eighteen children with acute leukaemia was found to be a very effective inactivator of aminopterin. This quality resided in no case, whether normal or leukaemic, in the blood plasma or the supernatant fluid.

INTRODUCTION

SINCE folic acid antagonists are useful tools in the analysis of certain biochemical processes[1, 2] and since they can induce complete, though temporary remissions in children with acute leukaemia,[3] it was of interest to investigate further the fate of these compounds when in contact with normal and leukaemic tissue.

Experiments have been reported[4–6] showing that a variety of tissues could inactivate the folic acid antagonist aminopterin. Thus cultures of embryonic chick fibroblasts and osteoblasts and leukaemic cells from mice with acute lymphoblastic leukaemia were able to alter this compound in such a way that it no longer arrested cell division at metaphase. In contrast, normal mouse bone marrow was unable to convert aminopterin into an inactive substance, and chick embryonic intestinal mucous membrane was a rather weak inactivator. Although short-term exposure to aminopterin caused an arrest in metaphase in all tissues tested, different results were obtained when exposure was prolonged; for example, after 24 hr of treatment embryonic chick osteoblasts and fibroblasts in culture went through mitosis with little inhibition, whereas the intestinal mucous membrane *in vivo* remained severely affected for several days. Similarly the effect of aminopterin persisted for days in normal bone marrow cells of man and mouse, while the leukaemic cells almost invariably overcame the initial inhibition.

In the present paper, observations are recorded which add to the range of tissues capable of inactivating folic acid antagonists. In a separate paper[7] experiments will be described which indicate that the inactivation of aminopterin may involve an alteration of the glutamic acid moiety at the end of the side chain of this compound.

MATERIAL AND METHODS

To find whether a tissue was capable of inactivating aminopterin, the following procedure was adopted. Either finely minced tissue fragments or cell suspensions were incubated for 20–24 hr at 37 °C with the folic acid antagonist under strictly sterile conditions. At the end of the incubation period the incubated mixture was centrifuged, and the supernatant was tested for the presence of aminopterin by placing from 0·01 to 0·02 ml on a 48-hr hanging-drop culture of osteoblasts from frontal bones of 12-day chick embryos.

These tissue cultures, which served as the test system, were grown in a thinly spread clot of about 0·02 ml, made of equal parts of cock plasma and chick embryo extract; the explants contained many cell divisions. Great care was taken over two points: (1) that the small quantity of test fluid was placed directly over the area of the cells, and (2) that each culture was outside the incubator for not more than 30 sec while this manipulation was completed. Longer periods at room temperature may affect the dividing cells.

Fifteen minutes after the application of the aminopterin solutions, the cultures were fixed in methanol and stained with May–Grunwald and Giemsa's dyes, according to Jacobson (1954*a*, p. 605–606) and were mounted in a synthetic medium, like DPX (Canada balsam is not suitable).

Mitoses were counted by systematically scanning the zone of outgrowth and the explant of the hanging-drop cultures and were classified into four stages defined as follows. Prophase (P), the chromosomes were still confined within the nuclear membrane; metaphase (M), the chromosomes were in the process of arranging themselves or were already arranged in a single group; anaphase (A), the chromosome halves (the chromatids) were parting or had separated; telophase (T), the chromosome group for each daughter cell had acquired a nuclear membrane. The distribution pattern of these four mitotic phases was recorded as a percentage of all mitoses seen. In a normal culture 35–45 per cent of the dividing cells are in metaphase and 20–25 per cent in anaphase.

In each experiment the following two controls were generally made. (a) The aminopterin control: a solution of aminopterin (usually 0·5 mg/ml) incubated without tissue, in either Tyrode's solution alone or a mixture of Tyrode and serum. These aminopterin controls retained their full inhibitory power and after 15 min application to the test cultures about 60–70 per cent of all dividing cells were in metaphase with severely clumped chromosomes and 0–10 per cent in anaphase. (b) The tissue control: the supernatant of cells incubated without aminopterin was applied for 15 min. It never inhibited mitoses, and tissue cultures to which it had been added showed the normal distribution pattern of metaphases (about 40–45 per cent) and anaphases (20–25 per cent). The tissue whose inactivating power was to be investigated was incubated in a similar solution containing aminopterin, and the supernatant was tested on sister cultures to those used for the control tests (a) and (b). If the tissue had

inactivated the aminopterin, its supernatant produced no change in the normal distribution pattern of mitotic phases. Thus each experiment had its own internal standards of active aminopterin and normal controls. The details of the various experimental procedures employed in the different parts of the investigation will be found at the beginning of each section.

EXPERIMENTS

I. *The So-called "Resistance" of Certain Cell Types to Folic-acid Antagonists*

Object of experiments

As already reported (Jacobson, 1954b), after prolonged treatment with aminopterin some tissues recovered from their mitotic inhibition but others did not. There seemed to be two alternative explanations of this "resistance" to aminopterin: (a) The "resistant" type of tissue might by-pass the block established by aminopterin. Since it has been found that aminopterin replaces folinic acid, this would mean that division could proceed without the functioning of folinic acid or its derivatives. (b) The antagonist might be broken down or inactivated. Experiments were made to find which alternative was correct.

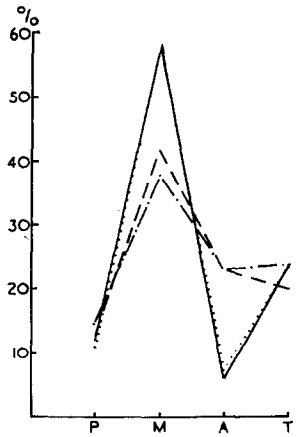

FIG. 1. Percentage distribution of mitotic phases in cultures of chick embryo osteoblasts: 15 min after application of aminopterin ————, and after 24 hr — — — —. A second application of aminopterin on the latter cultures. . . . . Saline control—·—· (N.B. aminopterin without cells retains its inhibitory power for 48 hr at 37 °C). P = prophase; M = metaphase; A = anaphase; T = telophase.

Methods

Cultures were grown in a medium containing 0·5 mg aminopterin per ml; after 48 hr of incubation, some were fixed (group II) or a small drop of a fresh solution of aminopterin (0·5 mg/ml) was deposited on the tissue for 15 min (group III). Control cultures, which had grown for 48 hr without aminopterin, were treated for 15 min with a drop of either Tyrode alone (group IV) or a fresh aminopterin solution (group I).

Results (*Fig.* 1)

In the cultures grown in the presence of aminopterin for 24–48 hr, the distribution of mitotic stages was now normal (42 per cent metaphases and 23 per cent anaphases)

as compared with controls grown for the same period without the inhibitor (38 per cent metaphases and 23 per cent anaphases). This result showed that the inhibition had been overcome. When the cultures which had grown for 48 hr in aminopterin were exposed for 15 min to a second application of aminopterin, however, they showed the typical arrest in metaphase with clumped chromosomes (58 per cent metaphases and 7 per cent anaphases) corresponding to the response obtained with the normal cultures exposed to aminopterin for the first time (58 per cent metaphase and 6 per cent anaphases) (Table 1).

TABLE 1. A SECOND APPLICATION OF AMINOPTERIN IS AS EFFECTIVE AS THE FIRST

(Chick osteoblasts, having inactivated aminopterin (II) in the course of 48 hr remain sensitive to a second application of the inhibitor (III). No "resistance" to aminopterin has developed. 48-hr-old cultures were used throughout. Groups (I) and (IV) were grown in normal medium. Groups II and III in the same medium + 0·5 mg aminopterin per ml.

	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	Number of mitoses
I. Aminopterin 15 min	12	58	6	24	491
II. Aminopterin 48 hr	13	42	23	22	588
III. Aminopterin 48 hr, followed by aminopterin 15 min	11	58	7	24	1009
IV. Tyrode control 15 min	15	38	23	24	584

Group III shows the same aminopterin effect caused by a second application as Group I, which has been exposed to aminopterin for 15 min only.
Group II is indistinguishable from the normal controls (Group IV).
The number of cultures used in the four groups were seven, four, nine and five, respectively.

These results indicated that the cells grown for 48 hr in aminopterin had overcome the metaphase block set by this compound, but remained as sensitive as before to a fresh application of the inhibitor. This ruled out the possibility that the cells used a metabolic path independent of folinic acid, and showed that they had not become resistant to aminopterin.

II. *The Inactivation of Aminopterin by Tissues*

Object of experiments

To test the ability of different types of cells to inactivate aminopterin.

Methods

The effect of various tissues taken from normal adult and embryonic chickens, from normal and leukaemic adult mice of the FAK_1 and AK strains, and from normal and leukaemic adults and children, on aminopterin was investigated.

Cell suspensions or finely cut pieces of tissue were incubated with aminopterin in a liquid medium at 37 °C under sterile conditions. The liquid media used were serum, a mixture of serum and Tyrode, or Tyrode alone. The experiments included the two controls previously mentioned, though occasionally in the case of human bone marrows not sufficient material was available for the tissue control. The concentration of aminopterin was usually 0·5 mg/ml, though in some of the experiments with human bone marrow the concentration was higher.

Experiments were made to compare the effect of the cells of normal mouse lymph nodes (i.e. normal lymphocytes and lymphoblasts) on the one hand, and of leukaemic lymph nodes on the other. The leukaemia was of the acute lymphoblastic type. Only those nodes were selected which were almost replaced by leukaemic cells. Suspensions of normal or leukaemic lymphoid cells were prepared under sterile conditions as follows: Axillary, inguinal and mesenteric nodes were isolated and cut into small pieces with fine scissors; the tissue fragments were transferred to Tyrode with a pipette and the suspension was gently shaken, which caused most of the lymphoid cells to be washed out of the cellular reticulum. After standing for a few minutes, the small tissue fragments had sunk to the bottom, leaving a milky suspension of lymphocytes and lymphoblasts which was used for the experiment. Haematocrit determinations showed that these preparations contained about 25 mg of cells per ml. Batches of 2 and 4 ml of this type of suspension were incubated in small, flat-bottomed flasks for 24 hr at 37 °C, one of each group in Tyrode alone and the other in Tyrode containing 0·5 mg aminopterin per ml. After incubation the cell suspensions were centrifuged and the supernatants tested on tissue cultures in the way described.

Results

1. *Normal chicken tissue.* In addition to the tissues already investigated (Jacobson, 1954 *a*, *b*), fowl red blood cells and buffy coat containing a few red cells were found to inactivate aminopterin. After an extensive series of tests, beginning with 250 mg red cells per ml, progressively smaller quantities of red cells were used in these experiments, all of which showed that fowl erythrocytes inactivated aminopterin. It was found that 25 mg red blood cells per ml Tyrode converted 0·5 mg aminopterin to an inactive substance. The same quantity of red cells was capable of inactivating even 2 mg aminopterin per ml, and the same quantity of buffy coat collected after 30 min of centrifuging at 3000 rev/min had the same effect. When the supernatants were applied to test cultures for 15 min the results listed in Table 2 were obtained: experiments III and IV show clearly the inactivation of aminopterin.

These results also confirm the previous observation that chick serum has no effect on aminopterin. In view of the great capacity of fowl red cells to inactivate aminopterin this method was used extensively for the experiments to be described in a separate paper.[7]

2. *Mouse tissues.* (a) Normal liver. This tissue, finely minced, inactivated aminopterin within 24 hr, as was shown in the following experiment: 14·5 mg liver/ml, were incubated with 0·5 mg aminopterin/ml in a total volume of 12·8 ml Tyrode. The "tissue control" contained 16 mg liver/ml in a total volume of 14·6 ml Tyrode. The aminopterin control was 0·5 mg/ml in Tyrode. These three fluids were incubated for 24 hr and the supernatants tested for 15 min on the usual cultures. The results are summarized in Table 3 and show that aminopterin has lost its inhibitory power after incubation with liver. This capacity of the liver was of interest, as normal bone marrow (10 mg/ml) had previously been found unable to inactivate aminopterin.[5]

(b) Leukaemic cells from leukaemic lymph nodes. In previous experiments[4] it had been shown that mouse leukaemic cells, collected either from the area of implantation or the heavily infiltrated bone marrow, inactivated aminopterin, in contrast to the normal bone marrow which had no effect on the inhibitor. As normal mouse bone

TABLE 2. THE INACTIVATION OF AMINOPTERIN BY RED CELLS AND THE BUFFY COAT OF FOWL'S BLOOD

(Distribution of mitotic phases in 48-hr cultures of chick osteoblasts, 15 min after the application of the following test solutions. Four cultures were used for each group.)

Test solutions	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	Number of mitoses
I. Aminopterin control 2 mg/ml Tyrode	11	73	2	14	654
II. Aminopterin control 2 mg/ml serum + Tyrode, equal parts	10	74	2	14	477
III. Aminopterin 2 mg/ml Tyrode + r.b.c. 25 mg	10	39	28	23	1063
IV. Aminopterin 2 mg/ml Tyrode + buffy coat 25 mg	9	42	28	21	347
V. R.B.C. control 25 mg/ml Tyrode (no aminopterin)	10	43	23	24	440
VI. Untreated cultures	13	41	27	19	470

Aminopterin has retained its inhibitory power after incubation for 24 hr with fowl serum (II) but has lost its inhibitory effect after incubation for 24 hr with fowl red cells (III) or buffy coat (IV).

TABLE 3. INACTIVATION OF AMINOPTERIN BY NORMAL MOUSE LIVER

(Distribution of mitotic phases in 48 hr cultures of osteoblasts, 15 min after the application of the test solutions.)

	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	Number of mitoses
Aminopterin control	9	74	2	15	340
Liver + aminopterin	7	52	19	22	601
Liver in Tyrode	7	51	20	22	558

Five, five and four cultures, respectively, were used for the three groups.
Aminopterin incubated with liver has lost its inhibitory power.

marrow contains probably no lymphoblasts and only a small percentage of lymphocytes (up to 10 per cent), it was decided to compare the inactivating capacities of normal and leukaemic cells from lymph nodes, as this would be a more significant experiment.

The control supernatants of the normal and of the leukaemic lymphoid cells incubated in Tyrode without aminopterin had no effect on cell division, as was to be expected, and the ratio of metaphases to anaphases was 46 per cent and 23 per cent in the test cultures (group II, Table 4). There was, however, a striking difference between the normal and the leukaemic cells in their action on aminopterin. The normal lymphocytes and lymphoblasts failed to inactivate this compound, which retained its

full inhibitory power after incubation with these cells; in the test cultures the metaphase count rose to 68 per cent and the anaphases count fell to 1 per cent (group III, Table 4). The leukaemic cells, on the other hand, converted the aminopterin into an

TABLE 4. INACTIVATION OF AMINOPTERIN BY LEUKAEMIC CELLS FROM LYMPHNODES OF MICE WITH ACUTE LYMPHOBLASTIC LEUKAEMIA (GROUP IV) AND INABILITY OF NORMAL LYMPHOID CELLS TO INACTIVATE AMINOPTERIN (GROUP III)

(Distribution of mitotic phases 15 min after application of the test solutions.)

	Prophase (%)	Metaphase (%)	Anaphase (%)	Telophase (%)	Number of mitoses
I. Aminopterin control (0·5 mg/ml)	10	70	1	19	297
II. Normal and leukaemic lymphoid cells (25 mg/ml)	9	46	23	22	235
III. Normal lymphoid cells (23 mg/ml) + aminopterin (0·5 mg/ml)	14	68	1	17	573
IV. Leukaemic lymphoid cells (8·2 mg/ml) + aminopterin (0·5 mg/ml)	11	46	20	23	478

Group III is indistinguishable from group I; in group IV no aminopterin effect can be discerned. In the four groups four, three, five and four cultures respectively were used.

inactive substance so that the supernatant had no inhibitory effect on cell division in the test cultures (group IV, Table 4).

3. *Human tissues.* (a) Normal blood. Aminopterin in concentrations of 0·5 or 1·0 mg/ml was incubated for 24 hr at 37 °C under sterile conditions with either whole blood (0·5–2·0 ml) or with the washed red cells from these volumes of blood, suspended in Tyrode's salt solution. The wet weight of red cells in the incubation fluids varied between 25 and 200 mg/ml. Serum or heparinized plasma (0·01 mg/ml) either undiluted or diluted with Tyrode, was also investigated for its effect on aminopterin (0·5 or 1·0 mg/ml). The blood of twenty-five normal humans was examined and the results are based on mitotic counts of 233 test cultures.

Plasma or serum, diluted or undiluted, had no effect on aminopterin which retained its full inhibitory action on cell division when applied to the test cultures. A total of sixty-one cultures were treated with aminopterin previously incubated with plasma or serum; in all cultures, metaphases were between 72 and 58 per cent and anaphases between 0 and 9 per cent among over 5000 mitoses; the results were indistinguishable from those obtained with aminopterin incubated in Tyrode (the aminopterin controls) and tested on twenty-six cultures. Serum, plasma or the supernatant or red cell suspensions in the absence of aminopterin, served as the "tissue" controls and had no effect on cell division in the test cultures (thirty-one cultures).

Whole blood or red cells resuspended in Tyrode, converted aminopterin into an inactive compound in twenty-one of twenty-five cases, In the twenty-one cases which

TABLE 5. FAILURE OF NORMAL HUMAN RED BLOOD CELLS TO INACTIVATE AMINOPTERIN

(In four of twenty-five cases aminopterin (1 mg) retained its inhibitory power after exposure to r.b.c. (400–450 mg) for 24 hr. Distribution of mitotic phases 15 min after application of the test solutions.)

	R.B.C. + aminopterin						Serum + aminopterin						Serum without aminopterin					
	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures
1	12	63	4	21	239	4	12	70	2	16	134	3						
2	12	72	0	16	218	4	13	71	0	16	472	4	17	41	20	22	592	4
3	11	61	7	21	290	4	14	69	4	13	251	4						
4_1	11	60	8	21	442	4	12	70	5	13	555	4	12	43	17	28	514	4
4_2	15	57	8	20	341	4	15	59	7	19	286	4						
4_3	11	57	8	24	523	4	15	62	7	16	245	4	13	43	22	22	283	4

In 4_2 and 4_3 aminopterin (1 mg) was exposed to 900 mg r.b.c. but retained most, if not all of its inhibitory action.

inactivated aminopterin the test cultures showed values well within the normal range, varying from 36–46 per cent metaphases and 18–28 per cent anaphases in eighty cultures with over 11,000 mitoses. However, in the four cases which failed to inactivate aminopterin (Table 5), the test cultures (twenty-four) had 60–72 per cent metaphases and 0–8 per cent anaphases, thus indicating an undiminished activity of the inhibitor. In all four cases at least 400–450 mg red cells failed to affect 1 mg aminopterin and in one of the four, during one of the re-tests with 900 mg red cells/ml, aminopterin retained most if not all of its inhibitory power (Table 5). No explanation can be offered for the difference between these four subjects and the twenty-one others all chosen at random.

(b) Lymphocytes. To determine whether the mature lymphocytes are responsible for the inactivation of aminopterin by human blood, three samples of 2 ml cerebrospinal fluid with high lymphocyte counts were incubated with 1 mg aminopterin. The fluid was sampled from two patients with meningitis (due to *Myco. tuberculosis* and *H. influenzae*) and one with cerebral degeneration; the content of lymphocytes was 1 mg, 0·5 mg and 0·2 mg per ml, respectively. Aminopterin was not inactivated by any of these, and test cultures showed the typical aminopterin effect. The ratios of meta- to ana-phases found in the three tests were: 71 per cent M/2 per cent A, 70 per cent M / 2 per cent A, and 73 per cent M / 3 per cent A, respectively.

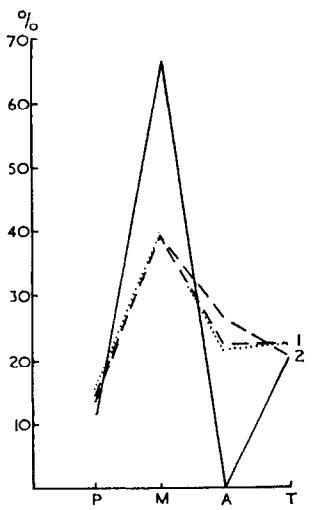

FIG. 2. Percentage distribution of mitotic phases in test cultures exposed for 15 min to: aminopterin ———; aminopterin previously incubated for 24 hr with the leukaemic bone marrow of case 1— — —; aminopterin incubated previously for 24 hr with the leukaemic bone marrow of case 2 — —; serum control (of case 1) . . . . Abbreviations as in Fig. 1.

(c) Leukaemic bone marrow. Samples of bone marrow from eighteen children with acute leukaemia were incubated for 20–24 hr with 1 mg aminopterin. The amount of leukaemic cells in these samples varied widely as is usual in sampling human bone marrow; it ranged from 1 mg (three cases) to 110 mg (three cases of 100 mg or more), with an average of 21 mg leukaemic cells. In seventeen cases aminopterin was inactivated (see Fig. 2) and in only one case did it retain its inhibitory power (Table 6).

TABLE 6. THREE CONSECUTIVE MARROW SAMPLES OF A CHILD WITH ACUTE LEUKAEMIA (CASE 18)

(The first two, taken before a remission, failed to inactivate 1 mg aminopterin; the third sample was taken in remission, during aminopterin treatment, and there is an indication that aminopterin is now partially inactivated. Distribution of mitotic phases 15 min after application of the test fluids.)

	Total white cells (mg)	Leuk. cells (mg)	R.B.C. (mg)	Bone marrow + aminopterin						Serum + aminopterin						Serum without aminopterin					
				P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures	P (%)	M (%)	A (%)	T (%)	No. of mitoses	No. of cultures
18_1	2	1·7	16	11	63	9	17	520	5							11	50	19	20	1005	4
18_2	2	1·3	50	11	64	9	16	432	5							10	50	20	20	315	2
18_3	2	0·1	20	10	53	15	22	849	4	12	64	5	19	409	3	9	47	24	20	370	3

The aminopterin inactivated by the seventeen marrows of leukaemic children was tested on sixty-six cultures with over 9000 cell divisions and was found to be unable to arrest mitoses in metaphase. The values obtained in the test cultures ranged between 40 and 48 per cent metaphases and between 17 and 27 per cent anaphases. Controls of aminopterin incubated with serum (plus Tyrode) from the samples retained their full inhibitory action on cell division in test cultures: 67–73 per cent metaphases and 0–1 per cent anaphases among 3000 mitoses. Serum plus Tyrode, without aminopterin, had no effect on cell division in the test cultures which showed a normal distribution pattern of the mitotic phases: 39–47 per cent metaphases and 18–24 per cent anaphases among over 4000 dividing cells. One of these seventeen cases may have been a borderline case of only partial inactivation of aminopterin (56 per cent M / 15 per cent A) by 22 mg leukaemic cells; however, a repeat marrow sample with only 7 mg leukaemic cells caused inactivation of aminopterin by the bone marrow cells. It is of interest that such small quantities of cells as used in these experiments were capable of converting 1 mg aminopterin into an inactive form. It is evident that the first two bone marrow samples of the eighteenth case (Table 6) failed to convert aminopterin during the first part of the disease and this patient responded subsequently to aminopterin treatment. The third sample taken towards the end of the remission, indicates that part of the aminopterin had been inactivated by incubation with marrow cells; the compound had lost some of its inhibitory qualities: 53 per cent M / 15 per cent A, when compared with the aminopterin controls 64 per cent M / 5 per cent A or the serum controls 47 per cent M / 24 per cent A.

Thus seventeen of the eighteen bone marrows of children with acute leukaemia had the capacity to convert aminopterin into an inactive compound. In the eighteenth case the capacity to inactivate aminopterin developed in the course of the disease.

DISCUSSION

(1) *The aminopterin effect on cell division*

Folic acid antagonists cause arrest of dividing cells in the metaphase stage. This effect has been observed in chick embryo fibroblasts and osteoblasts growing in tissue culture, where the cells can be exposed directly to the inhibitor, and also in the bone marrow of leukaemic children, and in normal and leukaemic marrow of mice and in their intestinal epithelium, whether the antagonist is given parenterally or by mouth. The lesions which these compounds can cause in the human intestinal mucosa are so strikingly similar to those produced experimentally in the mouse, that the human intestinal mucous membrane with its numerous cell divisions appears to be affected in the same way. Thus the arrest of cell division in metaphase by folic acid antagonists can be viewed as a general phenomenon.

Though many other quite unrelated compounds can cause a similar arrest of cell division in metaphase, the particular interest of the inhibition caused by folic acid antagonists lies in the fact that it can be prevented completely and over a considerable range of concentrations by the simultaneous application of folinic acid. The crucial experiment can only be performed on cells growing *in vitro*, where the conversion of folic to folinic acid can be precluded. In this way it was possible to demonstrate that folinic, and not folic acid, functions for a period of a few minutes during the step from meta- to ana-phase. The inhibition analysis, by exposing cells simultaneously

and directly to both the inhibitory analogue and the physiological compound, established that folinic acid has an important part to play in cell division. It appears to be the first instance of a defined chemical substance to which such a specific function during mitosis can be allocated.

For this reason, and in view of the clinical use of the folic acid antagonists in the treatment of acute leukaemias in childhood, it was relevant to investigate the so-called resistance to these compounds. They exert a striking effect initially, particularly in tissue cultures where mitotic arrest occurs within a few minutes, but finally become ineffective after longer exposure to all leukaemic cells and some normal cells.

Since cells growing *in vitro* show an easily observable response on exposure to folic acid antagonists, they provided a suitable test system for the activity of these compounds. Test cultures treated with aminopterin or other folic acid antagonists for 15 min, show not only an accumulation of metaphases and a decrease of anaphases, but also a severe clumping of the metaphase chromosomes in contrast to the control cultures treated with saline, serum, tissue fluids or inactivated aminopterin. Because of these distinctive features, and since no overlap occurred in the percentage values of the mitotic pattern of cultures treated with folic acid antagonists and those treated with the inactivated compound, a subsidiary reliance on statistical evaluation of the results was not required.

As to the specificity of the test the following points are to be mentioned: (1) Folinic acid prevents the inhibition caused by aminopterin over a range of concentrations. Folic acid fails to do this. (2) Aminopterin after exposure to some tissues retains its inhibitory qualities, but after exposure to other tissues it loses its inhibitory action. (3) Inactivated aminopterin, after treatment with mild acid, for example, regains its capacity to inhibit cell division in the test cultures. These three points provided the basis for considering the test system of cells growing *in vitro* as satisfactory and sufficiently specific.

2. *The inactivation of folic acid antagonists*

It was surprising to find that some normal human red blood cells can convert aminopterin (and A-methopterin as found in experiments to be reported later) into an inactive form, and that this quality of normal human red blood cells was not shared by all subjects. Attempts to correlate these observations with other, probably enzymic qualities of the red cells should be an interesting task. It was equally surprising that rather small quantities of leukaemic human bone marrow were very effective in inactivating aminopterin and A-methopterin. It was unfortunately not possible at this stage of the investigation to correlate the *in vitro* inactivation of aminopterin with the clinical response, but this will be attempted. The very small quantities of human bone marrow usually available did not allow the separation of white and red cells and it is notorious that attempts to increase the yield of leukaemic marrow usually lead to dilution with blood; but the proportion of the two types of cells in some of the marrows indicates that the leukaemic cells are at least as effective as the red cells in converting aminopterin into an inactive form. The experiments with mouse leukaemic tissue allowed a better separation of the two types of cells. Whenever the preparations of leukaemic cells were checked for contamination with red cells this was found to be very low. The cell-free fluids, whether serum, plasma or tissue fluids,

were always found to be without any effect on aminopterin. This has been found previously for a Tyrode extract of crushed chick embryo osteoblasts and was repeatedly confirmed with the supernatant of human leukaemic marrow, which by necessity must always contain some dissolved material from cellular débris, especially after a 24 hr incubation period.

Acknowledgements—The writers gratefully acknowledge the interest and help of Dr. H. B. Fell, F.R.S., Director of the Strangeways Research Laboratory, and thank Mrs. June Buscall for her skilful assistance throughout this work.

The generous supply of folic acid antagonists by Dr. J. Smith, Jr., Director of the Research Division of the American Cyanamid Co. is acknowledged with gratitude.

The expenses of this work were defrayed by the Medical Research Council.

REFERENCES

1. J. M. BUCHANAN, J. G. FLAKS, S. C. HARTMAN, B. LEVENBERG, L. N. LUKENS and L. WARREN, *The Chemistry and Biology of Purines* pp. 233–255. Ciba Foundation Symposium, J. & A. Churchill, London (1957).
2. R. G. GREENBERG and L. JAENICKER, *The Chemistry and Biology of Purines* pp. 204–232. Ciba Foundation Symposium, J. & A. Churchill, London (1957).
3. S. FARBER, R. TOCH, E. M. SEARS and D. PINKEL, *Advances in Chemotherapy of Cancer in Man. Advances in Cancer Research* Vol. 4, pp. 1–71. Academic Press, New York (1956).
4. W. JACOBSON, *J. Physiol.* **123**, 603, 618 (1954*a*).
5. W. JACOBSON, *The Chemistry and Biology of Pteridines* pp. 329–355. Ciba Foundation Symposium, J. & A. Churchill, London (1954*b*).
6. W. JACOBSON, *The Evaluation of Drug Toxicity* pp. 76–103. J. & A. Churchill, London (1958).
7. W. JACOBSON and I. A. B. CATHIE, *Biochem. Pharmacol.* **5**, 143 (1960).