Biochemical Pharmacology, Vol. 28, pp. 1843–1848.

0006-2952/79/0615–1843 $02.00/0

# ACTIONS OF THE HAEMOPOIETIC STEM CELL PROLIFERATION INHIBITOR

BRIAN I. LORD, ERIC G. WRIGHT and LASZLO G. LAJTHA

Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, England

(*Received* 7 *June* 1978)

**Abstract**—An inhibitor of haemopoietic stem cell proliferation has been prepared (a) by making extracts of haemopoietic tissue containing slowly proliferating colony-forming units–spleen (CFU–S) and fractionating by molecular ultrafiltration; and (b) by preparing "conditioned" supernatant media of such tissue. Dose–response studies of the effects of these inhibitors on the proportion of proliferating CFU-S which are synthesizing DNA have been carried out for different periods of exposure to the inhibitor. It was found that there is a minimum threshold dose for effective inhibition which varies inversely with the duration of exposure to the inhibitor. Two possible models to explain these results are suggested. First, at the threshold dose, the inhibitor is effective only at the start of the $G_1$ phase. This permits the $G_1$ cells present to progress normally into the S phase and, therefore, prevents an immediate observation of the inhibition. As the dose is increased, the effect is extended throughout the $G_1$ phase to the $G_1$–S boundary, effectively blocking the entry of cells into the S-phase and resulting in immediate inhibition. Second, the threshold dose is effective early in the $G_2$ period, which is long compared to the $G_1$ phase, but the affected cells continue their progress only up to the end of that cycle. The block, therefore, is not seen until this time has elapsed. Higher doses of inhibitor affect later $G_2$ cells, also resulting in early recognition of the block. The effects of the inhibitor are not lost simply by washing the cells, so that once it has become effective the stem cells remain in a switched off state until such time as they are switched on again by an applied proliferation stimulator.

In recent publications [1–4] we have demonstrated the presence of factors in haemopoietic tissue which are capable of modifying the proportion of haemopoietic spleen colony-forming units (CFU-S, a functional definition for the morphologically unrecognizable cells generally considered to be pluripotent haemopoietic stem cells) estimated to be synthesizing. Normal bone marrow, in which CFU-S proliferation is low (< 10 per cent are synthesizing DNA when incubated in saline for 2–4 hr, released into the medium a factor which was separated by Amicon Diaflo filters as having a molecular weight between 50,000 and 100,000 daltons [1]. This factor (NBME-IV) inhibited the incorporation of tritiated thymidine ([$^3$H]TdR) into rapidly proliferating CFU-S but did not inhibit the ultimate capacity of CFU-S to form spleen colonies i.e. the measured number of spleen colonies was unaffected. Thus, it was concluded that NBME-IV was a reversible non-cytotoxic, CFU-S proliferation inhibitor. In addition, since it had no effect on the proliferation of CFU-C (committed precursor cells of granulocyte and macrophage development and immediate descendants of the CFU-S), it also was considered to be specific for CFU-S proliferation [1]. A similarly effective inhibitor was found in medium which had been conditioned by the presence of spleen cells from phenylhydrazine (PHZ)-treated mice [2]. The active part of the PHZ spleen-derived inhibitor has been shown recently to be directly equivalent to and probably the same as that in NBME-IV [4]. Under these conditions also, splenic CFU-S are largely non-proliferating. By contrast, any haemopoietic tissue containing rapidly proliferating CFU-S (e.g. post-irradiation regenerating bone marrow or spleen, or marrow from PHZ-treated mice or foetal liver) has been found to elaborate another factor which enhances the incorporation or [$^3$H]TdR into slowly proliferating CFU-S [2]. From regenerating bone marrow, this factor was partially purified by Amicon Diaflo filtration and was retained as fraction-III (RBME-III) with a molecular weight between 30,000 and 50,000 daltons. We have considered this to be probably a stimulator of CFU-S proliferation [3]. Although there are small differences in the details these observations have been supported by complementary publications from Frindel *et al.* [5, 6].

By using these two factors in competition with one another, it was found that normal marrow CFU-S, stimulated by RBME-III to show a high [$^3$H]TdR incorporation, could subsequently be inhibited again by excess NBME-IV [3]. Conversely, proliferating bone marrow CFU-S, inhibited by NBME-IV, could subsequently be re-stimulated by the addition of excess RBME-III. The fact that the results of experiments using mixtures of cell populations containing proliferating and non-proliferating CFU-S are essentially similar to those using cell extracts [2] has encouraged us to conclude that the extract factors are probably responsible for the physiological control of CFU-S proliferation. (Pilot experiments (Lord, unpublished) indicate that NBME-IV is effective also *in vivo*.) Furthermore, the finding that both the stimulator and the inhibitor can be present in the same haemopoietic cell population [7] together with the competition experiments just outlined, led us to the conclusion that these two factors probably act in concert, their relative concentrations determining the rate of CFU-S proliferation appropriate to the requirements of the animal. In this present paper, we have attempted to investigate the mode of action of the inhibitory factor by studying its dose- and time-effectiveness and its reversibility of action.

## MATERIALS AND METHODS

Male BDF1 mice, 8- to 10-weeks-old, were used throughout. For CFU-S assays [8] ten mice/group which had been given 800 rad of 12MeV electron radiation were used as recipients. [$^3$H]TdR-kill measurements for assessment of CFU-S proliferative activity were carried out *in vitro* as described previously [9, 10] by incubating the cells with isotonic [$^3$H]TdR (200 $\mu$Ci/ml, 15 Ci/m-mole) for 30 min prior to injection for CFU-S assay.

### *Preparation of NBME-IV*

Marrow from mouse femora and tibiae was incubated at 37° in saline (conc 5 to 10 × $^6$ cells/ml) for 4 hr. The cells were then removed by centrifugation and the supernatant "conditioned" medium was fractionated on Amicon Diaflo ultra-filters. The fraction passing through the 100,000 mol. wt filter but retained on the 50,000 mol. wt filter was washed and freeze dried for storage as NBME-IV. Lowry protein estimations were made on the extracts and the dosages used were expressed as $\mu$g protein/ml.

### *Preparation of PHZ spleen conditioned medium*

Mice were injected on days 0, 1 and 3 with 60 mg/kg of phenylhydrazine and on day 7 their spleens were removed [2, 11]. Spleen cells were suspended in Fischer's medium and then separated again by centrifugation (10 min at 800 g). The cell free supernatant fraction conditioned by the cells, has been shown previously to contain the CFU-S proliferation inhibitory activity and thus has been used in this crude form [7].

### *Assay cells (proliferating CFU-S)*

A convenient source of cells for assaying NBME-IV and PHZ-spleen conditioned medium (CM) is femoral marrow from the same PHZ-treated mice. It has been shown that under these conditions the marrow CFU-S are proliferating rapidly [2, 11]. As an alternative, in some experiments, spleens were used from irradiated (800 rad) mice which had been injected with $10^6$ bone marrow cells 7 days previously. These spleens also carried rapidly proliferating CFU-S.

### *Dose–response studies*

*NBME-IV.* NBME-IV was dissolved in Fischer's

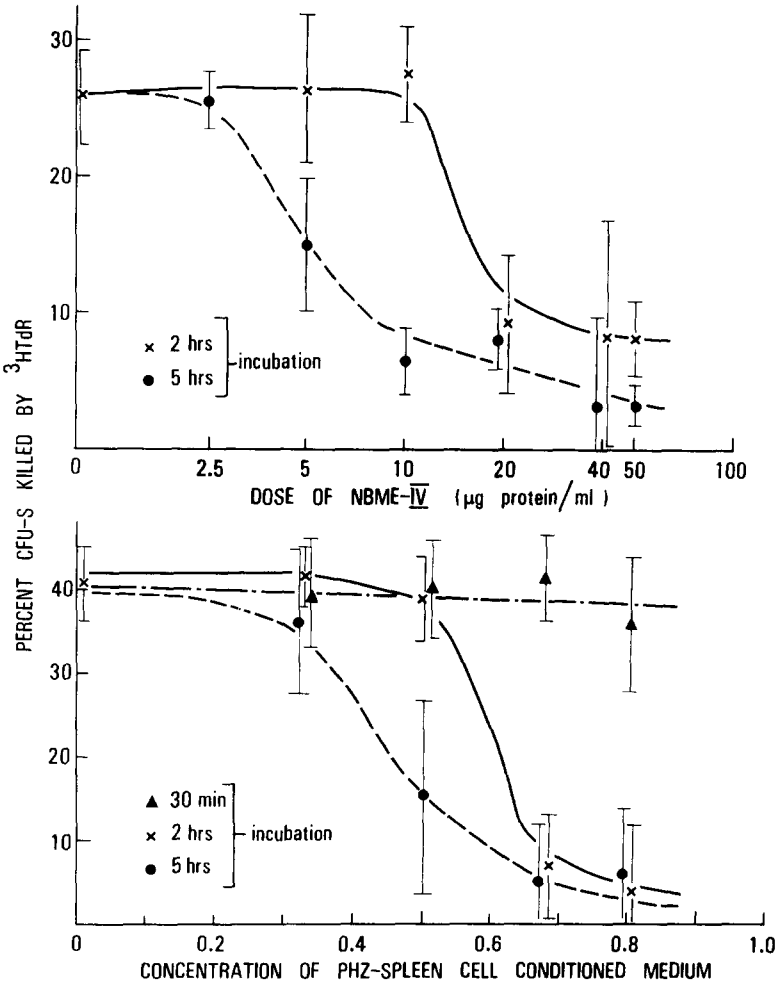


Fig. 1. CFU-S proliferation inhibitor dose–response studies. Percentage of CFU-S synthesizing DNA after a 30-min (▲), 2-hr (×) and 5-hr (●) incubation of phenylhydrazine-treated bone marrow cells with NBME-IV (top panel) or phenylhydrazine-treated spleen cell conditioned medium (lower panel).

Table 1. Per cent CFU-S killed by [$^3$H]TdR after incubation with NBME-IV or PHZ spleen conditioned medium

| Time of incubation | Treatment* | Per cent CFU-S killed by [$^3$H]TdR | No. of experiments |
|---|---|---|---|
| (2 and 5 hr pooled control) | Pre-NBME-IV<br>Pre-PHZ-spleen CM | 25.3 ± 2.3<br>41.0 ± 6.5 | 13 |
| 2 hr | NBME-IV<br>PHZ spleen CM | 6.7 ± 2.1<br>12.3 ± 7.7 | 13 |
| 2 hr (washed) | NBME-IV<br>PHZ spleen CM | 14.0 ± 5.2<br>25.3 ± 9.7 | 13 |
| 5 hr | NBME-IV<br>PHZ spleen CM | 2.0 ± 3.2<br>13.0 ± 9.9 | 10 |
| 5 hr (washed) | NBME-IV<br>PHZ spleen CM | 8.6 ± 5.4<br>16.0 ± 7.8 | 10 |

*Doses used in both cases were compatible with a 2 hr inhibition (see Fig. 1), i.e. 40 μg/ml of NBME-IV protein or a 67% concentration of PHZ spleen CM.

medium to give the required dose in 0.3 ml. A dose range of 5–50 μg protein/ml was tested. Bone marrow cells from PHZ-treated mice were suspended in Fischer's medium at a concentration of $10^7$ cells/ml. Paired incubation mixtures consisting of 0.5 ml cells 0.2 ml horse serum and 0.3 ml of extract solution (or medium for controls) were maintained in a shaking water bath at 37° for 1.5 or 4.5 hr. [$^3$H]TdR (200 μCi in 0.2 ml) was then added to one of each pair of tubes for a further 30 min of incubation. CFU-S assays and [$^3$H]TdR-kill measurements were then made for each group by injecting $7 \times 10^4$ marrow cells into irradiated mice.

*PHZ spleen conditioned medium.* A series of experiments similar to those for NBME-IV was carried out using the PHZ spleen CM. The doses used were based on the cell number initially used to make the CM. For example, 0.5 ml of assay cells at a concentration of $10^7$ cells/ml of Fischer's medium mixed with 0.5 ml CM made by suspending PHZ spleen cells in Fischer's medium at the same concentration gave a 50% concentration of CM. Similarly, an 80% concentration of CM was made up by mixing 0.5 ml of assay cells ($10^7$ cells/ml) with 0.5 ml CM made from a suspension of $4 \times 10^7$ PHZ spleen cells/ml. A dose range of 30–80% CM concentration was investigated.

*Requirement for inhibitor*

The requirement for the inhibitory factor, NBME-IV, was investigated as follows. PHZ bone marrow cells, suspended in Fischer's medium, were incubated at 37° with NBME-IV (40 μg protein/ml) or with PHZ spleen CM at a concentration of 67% (see above) for 1.5 or 4.5 hr. They were then washed three times by centrifugation (10 min at 800 *g*) and the cells were resuspended in fresh medium at their original concentration. 200 μCi [$^3$H]TdR (or an equal volume of Fischer's medium) was then added to paired samples for a further 0.5 hr of incubation prior to CFU-S assay. A parallel series of suspensions was similarly centrifuged but instead of replacing the supernatant fluid with fresh medium, the cells were resuspended in their own supernatant fluid.

In further experiments, normal bone marrow cells and PHZ spleen cells were assayed for CFU-S proliferation both before and after washing by centrifugation and resuspension, three times, in fresh medium.

## RESULTS

Dose–response curves for the effects of NBME-IV and PHZ spleen CM on the proportion of CFU-S killed by [$^3$H]TdR are shown in Fig 1. After a period of 2 hr of incubation a threshold dose of inhibitor is necessary before there is any effect on CFU-S proliferation. This is represented by a dose greater than 10 μg NBME-IV protein/ml (top panel) or a CM concentration greater than 50% (bottom panel). After a period of 5 hr of incubation much lower doses are effective. On the other hand, even an 80% concentration of CM is totally ineffective after an incubation period of 30 min (bottom panel).

Table 1 shows that simple washing does not immediately remove the inhibitory effect. Doses of NBME-IV and CM chosen from Fig. 1 gave protection from [$^3$H]TdR kill after 2 hr of incubation [$^3$H]TdR present for the last 30 min only) and for at least 5 hr: for NBME-IV a kill of 25 per cent was reduced to less than 10 per cent and, for PHZ spleen CM, a 41 per cent kill was reduced to about 12 per cent. Washing the cells after a 4.5 hr incubation did not abrogate this protection: in terms of [$^3$H]TdR kill experiments an 8.6 per cent kill is not higher than 2 per cent (NBME-IV) and 16 per cent is not higher than 13 per cent (PHZ spleen CM). Washing the cells after a 1.5 hr incubation gave a considerably less clear picture. Of the three experiments recorded, five gave a higher kill after washing than the unwashed samples, while the other eight did not. The overall result, therefore, was an apparent small loss of protection, the [$^3$H]TdR kills increasing from 6.7 to 14 per cent (NBME-IV) and from 12.3 to 25.3 per cent (PHZ spleen CM).

Washing normal bone marrow or PHZ spleen cell populations in which CFU-S proliferation is low(< 10 per cent in S) does not affect the proportion of CFU-S killed by [$^3$H]TdR.

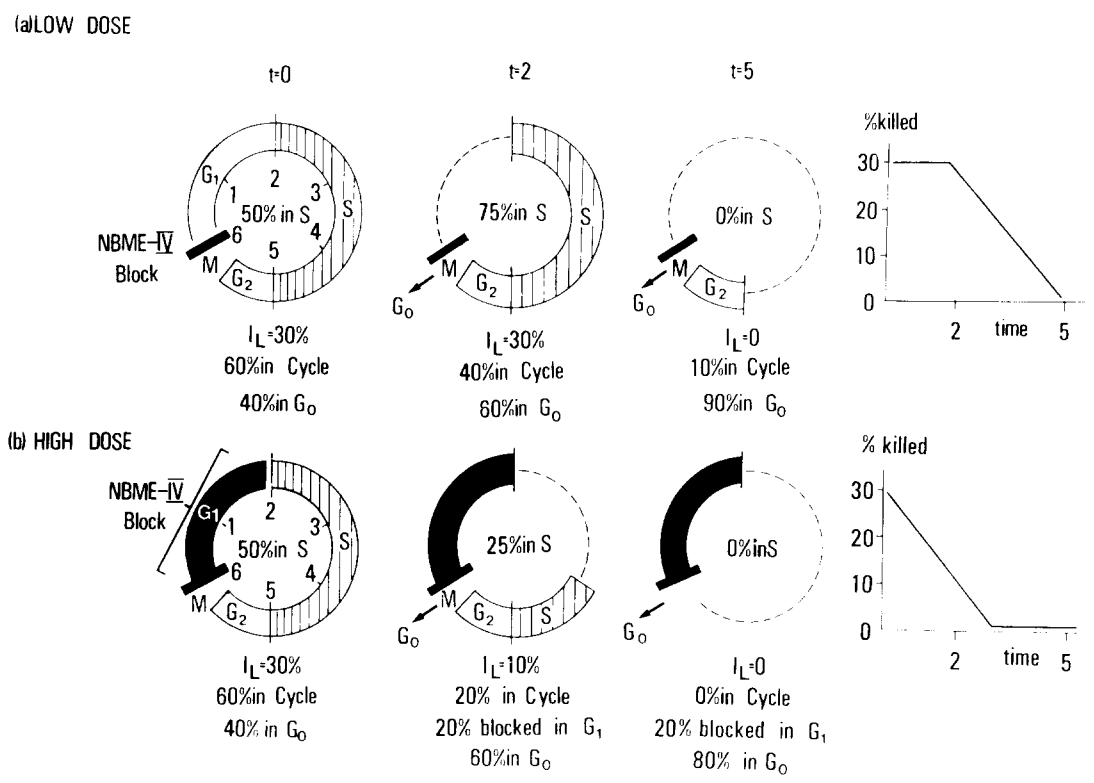


Fig. 2. Diagrammatic representation of the CFU-S cell cycle illustrating the suggested $G_1$ modes of action of the CFU-S proliferation inhibitor (a, upper row) using the 2-hr threshold dose of inhibitor (see Fig. 1) and (b, lower row) using a maximally effective 2 hr dose (see Fig. 1). The graphs on the right-hand side show the changes in the proportion of S-phase CFU-S with time expected from this model.

## DISCUSSION

In our previous publications, we reported on the effects of a 5-hr incubation with NBME-IV and of a 2-hr incubation with PHZ spleen cells or conditioned medium. The present results illustrate that the important factor in timing is the dosage of the inhibitor. Owing to the different experimental conditions, the results shown in Fig. 1 (top and bottom panels) cannot be compared directly. It is clear, however, that the dose-effectiveness of the CFU-S proliferation inhibitor is dependent upon the duration of its availability and that there is a minimum threshold dose required to produce inhibition. The threshold dose varies inversely with the time during which the inhibitor is present. These cell extracts are extremely heterogenous "soups" containing many molecular species. Any consideration of dose levels, therefore, can be only relative at this stage and we have chosen to express them in terms of the protein content of the freeze-dried materials. These observations, however, allow some speculation as to the possible points of action of the inhibitor; two possible explanations are illustrated in Figs. 2 and 3.

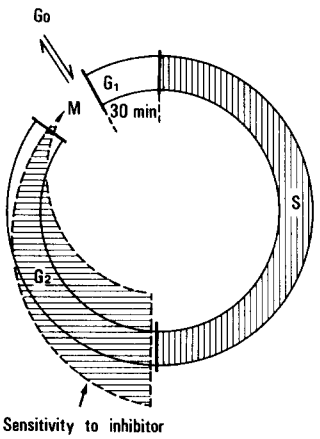


Fig. 3. Diagrammatic representation of the CFU-S cell cycle illustrating a $G_2$ mode of inhibitor action.

In the first case (Fig. 2), it has been assumed that, of the CFU-S population under these regenerating conditions, 60 per cent are in cell cycle at a given time and 40 per cent are in a $G_0$ state [12, 13]. Those in cycle have been allotted a cycle time of 6 hr with $t_{G1} = 2$ hr, $t_S = 3$ hr and $t_{G_2+M} = 1$ hr. These figures have no particular experimental basis but have been chosen to fit approximately the published estimates of cycle time and overall [$^3$H]TdR kill indices. Thus, although 50 per cent of the cycling CFU-S would be killed by [$^3$H]TdR, because of the 40 per cent in $G_0$, the net kill would be only 30 per cent, an average value under these experimental conditions. From Fig. 1 it is clear that a low dose of inhibitor (e.g. $\leqslant 10\,\mu$g NBME-IV/ml or $\leqslant$ 50% CM concentration) available for 2 hr has no

inhibitory effect. We assume that this means that cell flow into and out of S is normal during that period. There would be thus no block of the $G_1$–S transition and no direct S-phase block. By 5 hr, however, this low dose is effective, which means that the supply of new S-phase cells has been reduced, i.e. all $G_1$ cells existing at the start of treatment have entered the S-phase without being replaced. This would be most easily achieved if the inhibitor (at this low dosage) blocked only the beginning of, or entry into, $G_1$ and, possibly, the movement of $G_0$ cells into cycle also. Figure 2a illustrates this process. The ring represents an asynchronously dividing population of CFU-S and, at any one time, the proportion synthesizing DNA is represented by the shaded segment. Initially, 50 per cent of the cycling cells (30 per cent of all CFU-S) are in S-phase. The low dose NBME-IV block is effective at the start of $G_1$ only and, for 2 hr, the unaffected $G_1$ cells "empty" into S. The net number of cells in S is unaffected (and since there is no toxicity involved, the overall [$^3$H]TdR kill remains unchanged) but no $G_1$ cell remains. Beyond 2 hr therefore, there is no flow of cells into S-phase and their proportion gradually falls to zero, as cells in the S-phase continue to flow out.

Using high doses of inhibitor (e.g $\geqslant 40\ \mu$g NBME-IV/ml or $\geqslant 67\%$ CM concentration) inhibition is produced already at 2 hr, suggesting that the block is extending for the whole $G_1$ period or into the S-phase itself. A direct S-phase block, however, is unlikely in view of the fact that even very high doses are ineffective after a 30-min treatment (Fig. 1, bottom panel). This suggests that, as the dose of the inhibitor is increased, the block extends further into $G_1$ until the whole of the $G_1$ period [i.e. some process(es) on which the onset of DNA synthesis is dependent] is blocked. Thus, as shown in Fig. 2b, the block would now be not only effective at the start of $G_1$ but would act to "freeze" all the existing $G_1$ cells. There would be, therefore, no futher entry of $G_1$ cells into the S-phase, and an immediate run down of S cells would commence. By 2 hr (based on these figures) only one-third of the original S-phase cells would remain, representing 10 per cent of the total CFU-S. In terms of [$^3$H]TdR suicide experiments, this is a non-significant value and, therefore, represents complete inhibition. By 4 hr, only "frozen" $G_1$ cells would remain.

An alternative explanation of the results is that the inhibitor may establish a block which becomes effective only after a finite delay. Figure 3 illustrates the CFU-S cycle in which, in contrast to Fig. 2, the $G_2$ period is relatively long but the $G_1$ period is short (15–30 min). The $G_0$ state exits between mitosis and $G_1$, as before. If the cells were highly sensitive to the inhibitor in early $G_2$ but were allowed to continue normally through $G_2$ and M into $G_0$ but not beyond, the same results would be obtained. A low dose would affect only the early $G_2$ cells so that for a period of 2 hr, for example, no block would be seen. At this time, the cells affected by the inhibitor 2 hr earlier which are entering the M-($G_0$)-$G_1$ transition will be retained in $G_0$ and prevented from entering $G_1$. By 5 hr, the block on flow through $G_1$ and S will have caused S to empty and thus will demonstrate complete inhibition. A high dose, on the other hand, projects its effects through to late, as well as early, $G_2$. In this case, all subsequent cells entering the M-($G_0$)-$G_1$ transition will be retained in $G_0$ and, apart from those

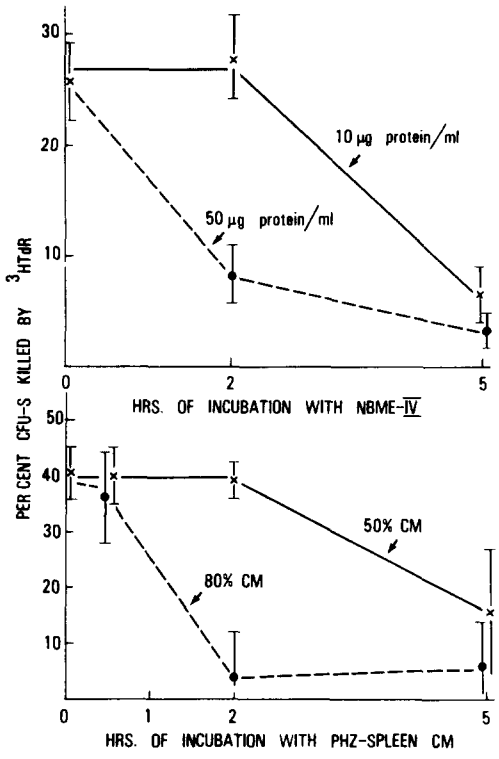


Fig. 4. Changes in the proportion of S-phase CFU-S in the phenylhydrazine-treated bone marrow with duration of exposure to inhibitor at the 2-hr threshold and maximally effective doses: (top panel, NBME-IV); (lower panel, phenylhydrazine-treated spleen cell condtioned medium). The experimental points are redrawn from Figure 1.

cells in the 30-min $G_1$ phase (and even the high dose has no inhibitory effect on CFU-S within 30 min), there will be an early and continuous run down of S-phase cells, i.e. rapid and continuously increasing inhibition.

Comparisons of the 2–5 hr kill patterns obtained from either model (Figs. 2 and 3) with the re-drawn experimental data taken from Fig. 1 show very acceptable agreement (Fig. 4). At this stage we are attempting to determine which, if either, of these models may be applicable.

From the washing experiments it is clear that, given sufficient time for it to act, a simple washing out (or off) of the inhibitory factor does not result in a quick reversion to a rapid cycling state. The effect of the factor is, however, reversible since (a) CFU-S treated with inhibitor produce spleen colonies normally, i.e. in a stimulatory environment, requiring eventual rapid CFU-S proliferation [1], and (b) competition experiments between NBME-IV and RBME-III result in on/off or off/on changes in CFU-S proliferation [2, 3]. Thus, NBME-IV does satisfy the reversibility criterion required for physiological proliferation inhibitors. On the other hand, its effect is not as simple as that of other inhibitors in haemopoietic tissue. For example, extracts of granulocytes (GCE) and erythrocytes (RCE) inhibit the proliferation of their immediate precursors, the myelocytes and normoblasts respectively, very rapidly in their normal milieu (or media) [14–16]. Equally quickly the cells resume a higher rate of proliferation on

washing [17]. It appears that the effect of inhibitor on these cells was to act simply as a brake, slowing down the progress of the cells through their cycle, so that fewer amplification divisions are permitted during their transition to mature functional cells. Furthermore, the extent of the inhibition seems to be limited to reducing proliferation by not more than about 50 per cent of maximum. For stem cells, however, unlike the amplifying transit populations, it has been suggested that under normal conditions a large part of the population is held in a prolonged $G_0$ state [12, 13] and may, therefore, require a more stringent control system. It seems reasonable to consider that proliferation control in the stem cell compartment acts by extensive alteration of the $G_0 \rightarrow$ cycle transition probability, i.e. the triggering rate from $G_0$ to cycle. That some factor in addition to the inhibitor is necessary is shown by the fact that CFU-S which had been subjected to prolonged maintenance in a state of low proliferation (e.g. normal bone marrow or PHZ spleen CFU-S) or treated for at least 5 hr with NBME-IV (or PHZ spleen CM) were not triggered into synthesizing DNA by washing out their endogenous inhibitor. Only by the addition of exogenous stimulator [3] or by the natural production of endogenous stimulator [7] can DNA synthesis be induced. Prolonged exposure of CFU-S to the inhibitory factor, therefore, appears to leave CFU-S effectively in a proliferatively switched off state. It appears that, cells progress through their cycle and find their re-entry to $G_1$ inhibited, they will be delayed in $G_0$. The longer the block is maintained the greater will be the proportion held in $G_0$, a state from which they can be released only by the addition of excess exogenous stimulator. This is probably the state of CFU-S in haemopoietic tissues such as normal bone marrow and PHZ spleen which have been almost totally inhibited for some time. Hence, the two factors [stimulator (RBME-III) and inhibitor (NBME-IV)] effectively act by triggering CFU-S into cycle or driving them into $G_0$ respectively. A balance of these two factors acting in a stochastic manner could thus effect a very sensitive control over the CFU-S proliferation rate. In addition, it cannot be stressed too highly that both these factors are functionally source specific in that they are both obtained from the tissues in which they are themselves effective. In a future publication we shall explore the cellular origins of the inhibitor and the stimulator but at this stage it suffices to point out that the control of CFU-S proliferation is effected by locally produced factors.

## CONCLUSIONS

A specific inhibitor of CFU-S proliferation can be obtained from haemopoietic tissue containing slowly or non-proliferating CFU-S. It acts by blocking some process(es) either in the early part of the $G_1$ phase on which eventual DNA synthesis is dependent or, alternatively, in the early part of the $G_2$ phase on which eventual re-entry to the cycle is dependent. Increasing doses extend the inhibition further along the $G_1$ or $G_2$ phase until reaching, respectively, the G1–S or M–$G_1$ boundary. It does not inhibit cells already in the S phase, but cells completing their cycle are apparently "held" in (or switched into) the $G_0$ state from which they may be triggered into cycle only by direct stimulation.

*Acknowledgements*—We would like to thank Mrs. Lorna B. Woolford for expert technical assistance. This work was supported by grants from the Cancer Research Campaign and the Medical Research Council.

## REFERENCES

1. B. I. Lord, K. J. Mori, E. G. Wright and L. G. Lajtha, *Br. J. Haemat.* **34**, 441 (1976).
2. E. G. Wright and B. I. Lord, *Biomed. Express* **27**, 215 (1977).
3. B. I. Lord, K. J. Mori and E. G. Wright, *Biomed. Express* **27**, 223 (1977).
4. E. G. Wright and B. I. Lord, *Leukemia Res.* in press.
5. E. Frindel, H. Croizat and F. Vassort, *Expl. Hemat.* **4**, 56 (1976).
6. E. Frindel and M. Guigon, *Expl. Hemat.* **5**, 74 (1977).
7. E. G. Wright and B. I. Lord, *Biomedicine* **28**, 156 (1978).
8. J. E. Till and E. A. McCulloch, *Radiat. Res.* **14**, 213 (1961).
9. A. J. Becker, E. A. McCulloch, L. Siminovitch and J. E. Till, *Blood* **26**, 296 (1965).
10. B. I. Lord, L. G. Lajtha and J. Gidali, *Cell Tissue Kinet.* **7**, 507 (1974).
11. N. G. Rencricca, V. Rizoli, D. Howard, P. Duffy and F. Stohlman, Jr., *Blood* **36**, 764 (1970)
12. L. G. Lajtha, R. Oliver and C. W. Gurney, *Br. J. Haemat.* **8**, 442 (1962).
13. L. G. Lajtha, *J. Cell comp. Physiol.* **62**, (suppl. 1) 143 (1963).
14. B. I. Lord, L. Cercek, B. Cercek, G. P. Shah, T. M. Dexter and L. G. Lajtha, *Br. J. Cancer.* **29**, 168 (1974).
15. B. I. Lord, *Boll. Ist. sieroter. milan.* **54**, 187 (1975).
16. B. I. Lord, G. P. Shah and L. G. Lajtha, *Cell Tissue Kinet.* **10**, 215 (1977).
17. B. I. Lord, L. Cercek, B. Cercek, G. P. Shah and L. G. Lajtha, *Br. J. Cancer* **29**, 407 (1974).