# THE TIME OF SYNTHESIS AND THE CONSERVATION OF MITOSIS-RELATED PROTEINS IN CULTURED HUMAN AMNION CELLS

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ABSTRACT

*p*-Fluorophenylalanine (PFPA), an analogue of phenylalanine which may be incorporated into proteins, increases the duration of mitosis. In the present experiments, based upon quantitative analyses of time-lapse cinemicrographic films, brief treatments of cells with PFPA are shown to affect the duration of metaphase in only those cells which enter division during or shortly after treatment. The offspring of cells with prolonged metaphases also tend to have prolonged metaphases. Analyses of the kinetics of the appearance of prolonged metaphases indicate that some protein specifically associated with mitosis is synthesized primarily during a period which corresponds closely to $G_2$. The manner in which the defect is passed on to daughter cells indicates that the protein involved is conserved and reutilized by daughter cells for their subsequent divisions. Comparable experiments performed with low concentrations of puromycin indicate that the major effect of PFPA is due to its incorporation into protein rather than its ability to inhibit protein synthesis. The fact that puromycin-induced effects can also be passed on to daughter cells is interpreted to mean that cells make only specific amounts of some mitosis-associated proteins and that if a cell "inherits" a deficiency in such protein it is not able to compensate for the deficiency.

## INTRODUCTION

In the somatic cells of higher organisms, there is nearly always a gap in time [the so-called $G_2$ period (1)] between the completion of DNA synthesis and the entry of cells into mitosis. This suggests that the $G_2$ period is a time when the cell must finally fulfill certain metabolic requirements if it is to be properly prepared for a normal division.

It has been known for some time that both RNA and proteins are synthesized during $G_2$ (2–5) and it has recently been shown that mammalian cells which are inhibited from synthesizing these classes of compounds during this period will not enter mitosis (6–11). The purpose of the present study was to determine whether proteins specifically related to the process of cell division are synthesized in the period just prior to mitosis and to determine the span of time during which such proteins are made.

The experiments presented are based to a large extent on the effects of *p*-fluorophenylalanine (PFPA). This compound is an analogue of phenylalanine which can become incorporated into newly formed proteins in place of phenylalanine (12–21). In some instances the incorporation of the analogue has little effect on the activity of the resultant protein, while in others the effect can be profound (16, 19, 20). One case which may be quite perti-

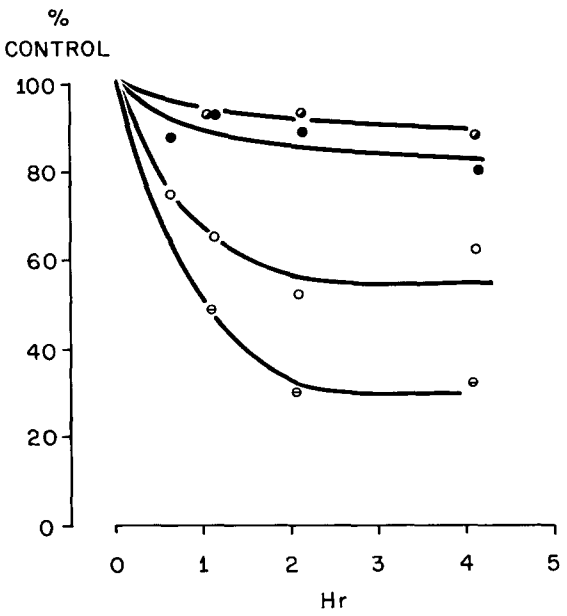


FIGURE 1 Effects of PFPA on the gross rate of protein synthesis at various times after the beginning of treatment. ◑——◑, 0.5 mM; ●——●, 1.0 mM; ○——○, 5 mM; ⊖——⊖, 10 mM. Each point represents the mean value derived from two to three separate experiments.

nent to problems involving mitotic proteins is that of regenerating bacterial flagella; the presence of PFPA does not seriously inhibit the formation of flagella, but the flagella which do form in the presence of PFPA are not functional (22). Since flagella and the mitotic apparatus are in some ways related, it appeared that PFPA might be useful for studies of the synthesis of mitotic proteins. In fact, Biesele and Jacquez (23) have shown that PFPA increases the duration of mitosis in cultured tumor cells and suggested that spindle behavior or the centromere-spindle relationship is affected. The experiments reported here, which confirm the findings of the previous authors, utilize this analogue along with puromycin for studies on the time of synthesis of mitosis-associated protein. A preliminary report of some of this work has been presented (24).

## MATERIALS AND METHODS

All experiments were performed on the Fernandes line of human amnion cells (25). The culture medium, culture procedures, and time-lapse cinemicrographic techniques have been described (26, 27). For experiments involving cinemicrography, cells were seeded in Rose chambers (28) and appropriate changes in media were made with hypodermic needles inserted through the silicone rubber gaskets.

In our analysis of the time-lapse films, we define the beginning of metaphase as that time when chromosomes are first observed to be lined up on the equatorial plate of the spindle, the beginning of anaphase as that when chromosomes first begin to separate, and the completion of anaphase as that when separation of daughter cells is first seen to be complete except for the midbody. (It will be noted that our criterion of the completion of anaphase does not conform strictly to the classical one, in that it defines anaphase completion as completion of cytokinesis.) Since photography was carried out at the rate of one frame per min, our timing of the above stages is accurate to within 1–2 min.

Measurements of the effects of PFPA and puromycin on the rate of protein synthesis were performed on cells grown on cover slips in Leighton tubes. The cover slip preparations were placed into tubes of normal medium (for controls) or of normal medium plus PFPA or puromycin for appropriate periods of time, transferred to aliquots of the same media containing tritiated leucine (2 or 5 μc/ml, 5 c/mmole) for 15 min, and then fixed in ethanol–acetic acid (3:1) and air-dried. The cover slip preparations were then rinsed twice in ice-cold 5% trichloroacetic acid and three times in water (10 min each) and again air-dried. Each cover slip preparation was then crushed in a counting vial, to which 1 ml of Hyamine base was then added. The vials were shaken in a 37°C water bath for 1.5 hr; scintillation fluid was added, and the radioactivity was assayed in a liquid scintillation spectrometer.

For measurements of the spindle, cells were grown on Leighton tube cover slips. After appropriate treatment, the cover slip preparations were fixed with osmium tetroxide fumes, washed in water, stained

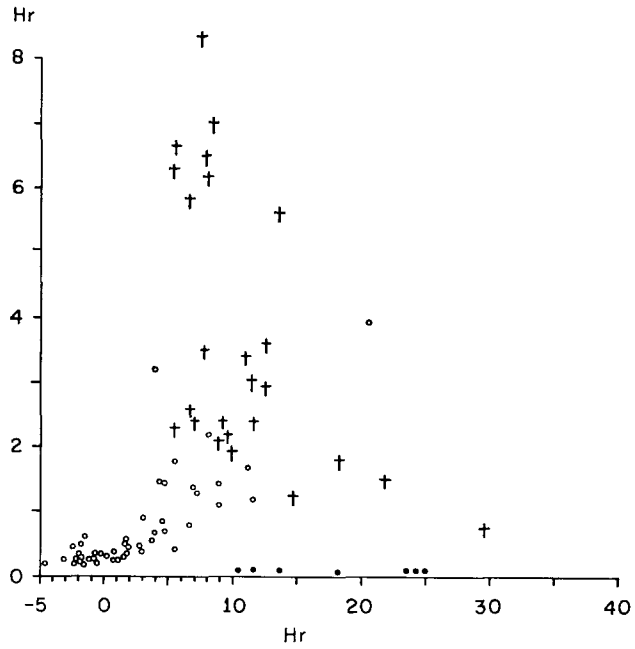


FIGURE 2 Effects of PFPA on mitosis. Ordinate shows duration of metaphase; abscissa, duration of treatment starting at time zero. ○, duration of metaphase in those cells which were able to enter anaphase; †, the time that some cells remained in metaphase before they died; ●, time of death of individual interphase cells.

very lightly with azure B, dehydrated in graded ethanols, passed through two baths of xylol, and and mounted in immersion oil.

## RESULTS

### *Effects of p-Fluorophenylalanine*

#### PROTEIN SYNTHESIS

The experiments summarized in Fig. 1 were performed to determine the effects of various concentrations of PFPA on the gross rate of protein synthesis. In the range between 0.5 and 10 mM the effect depends upon both concentration and time, with maximum inhibition at the higher concentrations occurring by the second hour. At lower concentrations more time may be required to reach maximum inhibition.

#### MITOSIS

Most of the remaining data on the effects of PFPA were obtained from analyses of time-lapse cinemicrographic films.

EFFECTS OF CONTINUOUS TREATMENT: The first effect observed after the beginning of continuous treatment with 1.0 mM PFPA is an increase in the duration of metaphase (Fig. 2), which becomes fairly obvious at 1–2 hr after the beginning of treatment.

All cells which entered metaphase during the first 5 hr after the beginning of treatment were capable of completing mitosis even though most of them required more than the usual amount of time. However, two-thirds of those which entered mitosis after the fifth hour of treatment remained blocked in metaphase for periods of up to 8 hr and then died. Reversion to interphase did not occur. The death of cells which have been blocked in metaphase for prolonged periods of time has also been observed to occur as a result of treatment with Colcemid (29) and high temperatures (unpublished observations) and appears to be related to the time the cells remain in metaphase rather than to the agent causing the blockage.

The duration of anaphase was also increased (Fig. 3), but this increase did not appear to be significant until about the fifth hour after the beginning of treatment. As indicated by the frequency of metaphases at different times after the beginning of the experiment (Fig. 2), no significant decrease in the rate at which cells entered metaphase occurred during the first 12 hr of treatment, suggesting that late interphase cells are relatively unaffected by this treatment. A few interphase cells died but only after the 11th hour of treatment (Fig. 2).

So far as could be determined by phase contrast microscopy of osmium tetroxide–fixed material.

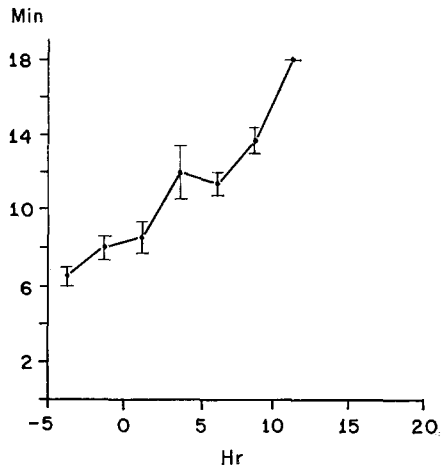


FIGURE 3 Effects of PFPA on the duration of anaphase in those cells shown in Fig. 2 which were able to enter anaphase. Ordinate shows duration of anaphase in minutes; abscissa, time since beginning of treatment.

the general appearance of PFPA-treated spindles was not different from that of control spindles. However, measurements of the distances between centrioles indicated a slight but significant decrease in size of the treated spindles (Table I).

EFFECTS OF SHORT TREATMENTS: In this series of experiments, colonies of cells whose mitotic histories and mitotic times were continuously recorded by time-lapse cinemicrography were treated for 1–3 hr with medium containing various concentrations of PFPA. Following treatment, cells were rinsed twice with normal medium and then reincubated in normal medium for the duration of the experiment. Both the normal medium and the medium with PFPA contained 0.10 mM phenylalanine. Fig. 4 shows that a 3 hr exposure to 1 mM PFPA causes the mean duration of metaphase to increase to about 30–40 min in those cells which enter division within about 10 hr after the beginning of treatment. This first increase in metaphase times will hereafter be termed the first peak. Metaphases which occur between 10 and 15 hr after the beginning of treatment are normal. A second peak occurs at about 25 hr after time zero, followed by a second decline and then a third peak at about the 50th hour. The controls, in which the over-all mean value for metaphase time was 18.1 ± 0.3 min, showed some variability but revealed no peaks which corresponded in time or magnitude to those in the treated populations.

In this type of experiment, death was rare among mitotic and interphase cells and not more frequent than among controls. Also, as shown in Table II, the generation times of treated cells and controls were essentially identical, indicating again that other parts of the cycle are relatively unaffected by this treatment.

Since even in untreated controls long metaphases were occasionally observed, we also present histograms showing the percentage of metaphases which were "abnormally" long in the various time classes. We define an abnormally long metaphase, or, as later used, an "abnormal metaphase," as one whose duration is more than two standard deviations above the mean of the control as determined separately for each experiment. A normal metaphase is defined as one whose duration is within two standard deviations of the mean of the control. As shown (Fig. 4), there are peaks in the histogram of the treated cultures which correspond to the peaks in the continuous curve. This pattern has been observed in many experiments with various combinations of treatment times and concentrations of PFPA. Another example of this pattern is presented in Fig. 5, which shows the effects of a 1 hr treatment with 10 mM PFPA. In this case the first peak is much narrower but there is the typical return to the control level, followed by a second peak a generation time later and a suggestion of a third peak two generation times later.

Unfortunately, in any single experiment or pair of experiments, the sizes of the samples are relatively small, necessitating the grouping of the data

TABLE I

*Effects of p-Fluorophenylalanine (1 mM) on Spindle Length in Fernandes' Human Amnion Cells after Varying Times of Treatment*

| Treatment | Time | Spindle length* | SE |
|---|---|---|---|
| | *hr* | | |
| PFPA | 1 | 5.2 | ±0.10 |
| PFPA | 2 | 5.0 | ±0.07 |
| Control | 2 | 5.7 | ±0.09 |
| PFPA | 4 | 4.7 | ±0.10 |
| PFPA | 6 | 4.8 | ±0.09 |
| PFPA | 8 | 4.7 | ±0.05 |
| PFPA | 10 | 4.6 | ±0.08 |
| PFPA | 12 | 4.6 | ±0.11 |
| Control | 12 | 6.1 | ±0.07 |

* In micrometer units; 1 unit = 1.48 μ.

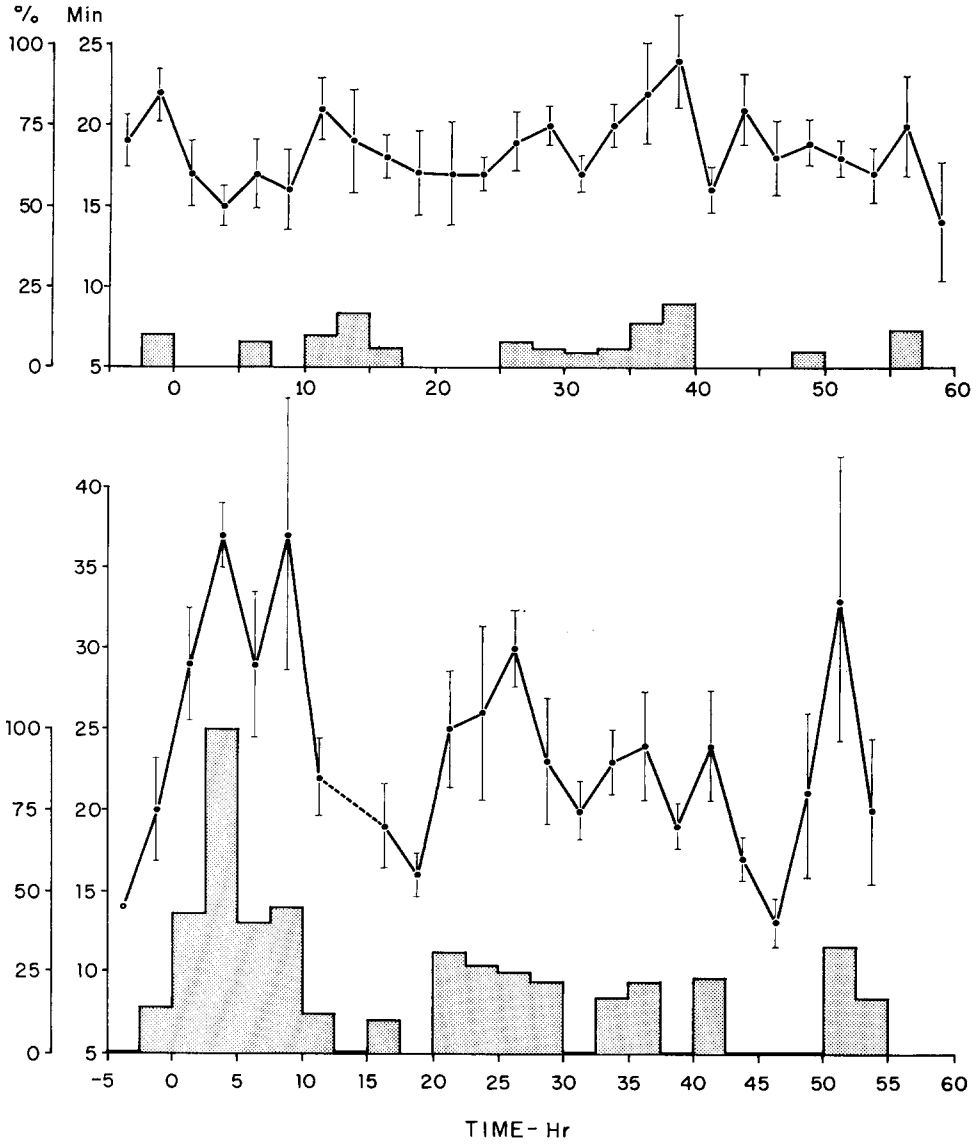


FIGURE 4 The effects of a 3 hr treatment with 1.0 mM PFPA on the duration of metaphase as a function of time since beginning of treatment. Continuous lines indicate mean metaphase times for cells grouped in 2.5 hr time classes. Histograms show the percentage of metaphase cells which had abnormally long durations as defined in the text. Upper graphs are controls; the lower graphs are for PFPA-treated cells. Total measurements: treated, 187; controls, 285.

into 2.5 hr time classes. While this grouping allows the demonstration of the major effect of PFPA, it does not permit a very good chronological resolution.

In order to increase the size of the samples, we grouped first-peak data from six experiments in which a 1 hr treatment with 10 mM or 5 mM PFPA (three experiments for each) was given. A second group was made up of the combined data from five experiments in which a 1 hr treatment with 0.5 mM PFPA was given. For controls we grouped data from four separate control experiments. These three groups represent data on samples of 132, 215, and 126 cells, respectively,

TABLE II

*Generation times (GT) of PFPA-Treated and Control Cells Born at Different Times Relative to the Beginning of Treatment*

Data are derived from the time-lapse films of the experiment shown in Fig. 4.

| | Treated | | Controls | |
|---|---|---|---|---|
| Time of birth | Mean GT | ± SE | Mean GT | ±SE |
| *hr* | *hr* | | *hr* | |
| −5–0 | 17.8 | 0.58 | 18.8 | 0.93 |
| 0–5 | 19.4 | 1.36 | 18.0 | 1.30 |
| 5–10 | 17.3 | 0.35 | 17.0 | 0.56 |
| 10–15 | 16.3 | 0.56 | 18.0 | 0.46 |
| 15–20 | 19.7 | 0.87 | 16.8 | 0.48 |
| 20–25 | 18.2 | 0.77 | 17.6 | 0.49 |
| 25–30 | 17.4 | 0.55 | 19.2 | 0.65 |
| 30–35 | 19.9 | 1.06 | 17.9 | 0.35 |
| Over-all | 18.1 | 0.28 | 17.9 | 0.18 |

which were divided into ten 1-hr age classes. The data are presented in terms of both the mean metaphase times (Fig. 6 *a*) and the percentage of abnormal metaphases in each age class (Fig. 6 *b*).

The first point to be considered is the time between the beginning of treatment and the appearance of an effect on metaphase cells. As indicated, the mean metaphase time of the first age class at the higher concentrations is significantly greater than in the controls. In fact, four cells were observed to enter metaphase within 15 min after the initiation of treatment and all four had metaphase times between 21 and 31 min, well above the mean for the controls. This is interpreted to mean that there may be less than a 15 min lag between the exposure of cells to PFPA and the appearance of an increase in metaphase times. On the other hand, production of an effect at low concentrations required the 1 hr treatment plus an additional period of about 1 hr, during which further incorporation of PFPA into protein from an endogenous pool may have taken place.

These data also show that the peak at the higher concentrations is reached between 1 and 2 hr after the beginning of treatment and that mainly those cells which enter division within about 4.5 hr after the beginning of treatment are affected. Since the effect is almost immediate and since the duration of treatment is 1 hr, it is suggested that PFPA affects mainly those cells which are within a 3.5 hr period just prior to the beginning of metaphase at the time of exposure to the agent. When it is considered that in these cells the average $G_2$ + P time is between 2 and 2.5 hr, with some cells requiring 3.5 hr or more to get through this period (30), and that the distribution of $G_2$ + P times may have the same general form as the curves shown in Fig. 6 (30, 31), the data indicate that PFPA may affect metaphase in only those cells which were in $G_2$ + P when the agent was available to them.

The spacing between the primary and secondary peaks (Figs. 4 and 5) suggested that the daughters of the first-peak cells are the cells which are responsible for the second peak. Since, in experiments like these, we can trace individual cells from one generation to the next, it was possible to study directly whether there is a tendency for abnormal cells to pass on this trait to their daughters. Two kinds of analyses were performed. The first was simply to examine the tendency for abnormal first-peak cells to pass on this trait to their daughters. Table III shows that, at all concentrations and treatment times tested, daughters of abnormal cells are much more frequently abnormal than daughters of normal cells. The second approach was to find out what fraction of second-peak cells was derived from first-peak mothers and what fraction of abnormal second-peak cells was derived from abnormal first-peak mothers. As shown in Table IV, most of the cells which divided in the second peak were derived from mothers which divided during the first peak, and about two-thirds of the abnormal second-peak cells had abnormal first-peak mothers. We conclude, therefore, that the offspring of cells which divide at the time of the first peak are generally responsible for subsequent peaks and that abnormal second-peak cells tend to be derived from abnormal first-peak mothers. The fact that some cells which divided in the first peak were normal but gave rise to abnormal duaghters will be considered later in this paper.

DOUBLE TREATMENT: The pattern of a single brief treatment followed immediately by a peak in mean metaphase times and then by another peak a generation time later was observed in experiments in which cells were treated with concentrations of PFPA equal to or greater than 1.0 mM. At 0.5 mM, however, the second peak tends to be either low or absent.

The following experiment was performed to see

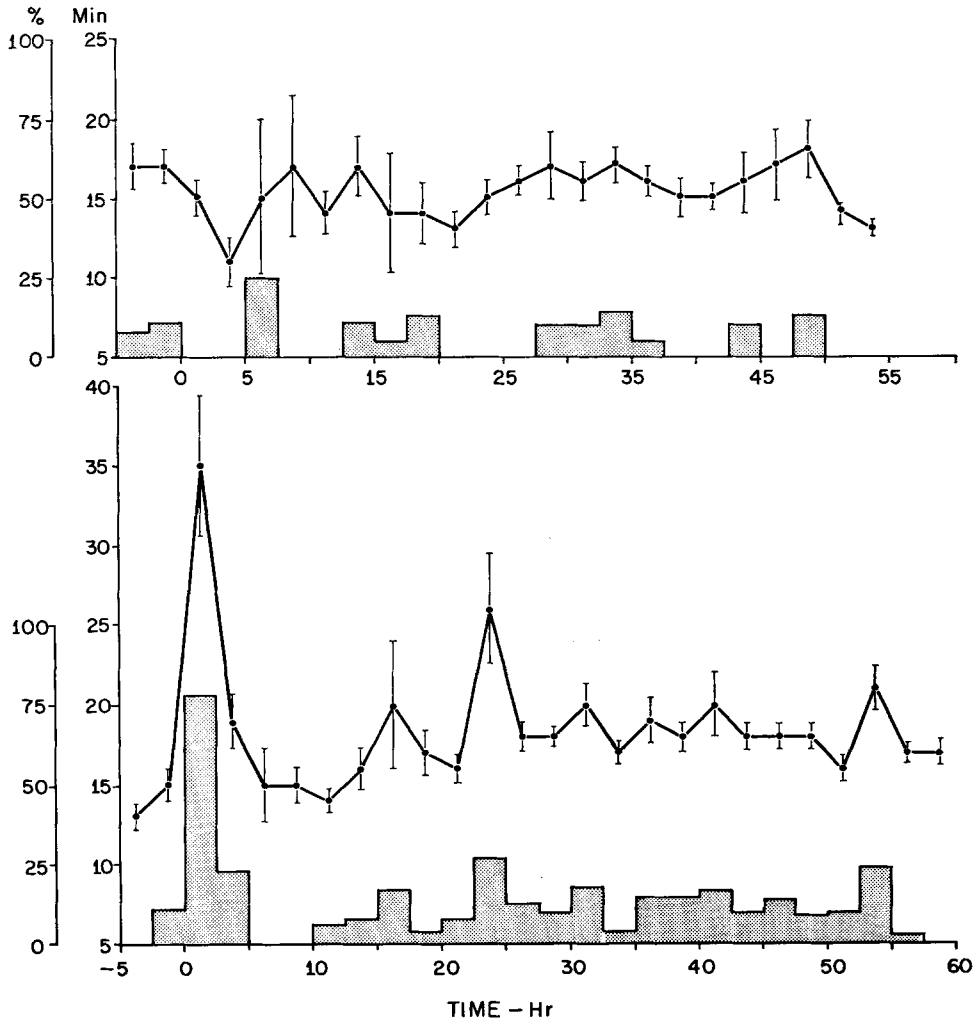


FIGURE 5 Same as Fig. 4 except that treatment is with 10 mM PFPA for 1 hr. Total measurements: treated, 766; controls, 307.

whether two 1 hr treatments with 0.5 mM PFPA spaced 20 hr apart (approximately one generation time) would enhance the appearance of a peak one generation time after the second treatment. As a control, a sister culture received only a change in growth medium at the time of the first treatment and a 1 hr exposure to medium with 0.5 mM PFPA at the time of the second treatment.

Fig. 7 *a* shows that in the control the only prominent peak occurred immediately after the single treatment with PFPA whereas in the culture which was exposed twice to PFPA we observed peaks following each treatment and, in addition, a prominent third peak one generation time after the second exposure.

### *Effects of Puromycin*

The next question we considered was whether the effect of PFPA is due to its ability to inhibit protein synthesis or to its incorporation into some division-associated protein which is thereby rendered defective. To do this we studied the effects on mitosis of concentrations of puromycin which gave us levels of inhibition of protein synthesis comparable to those levels produced by the concentrations of PFPA used in the previous experi-

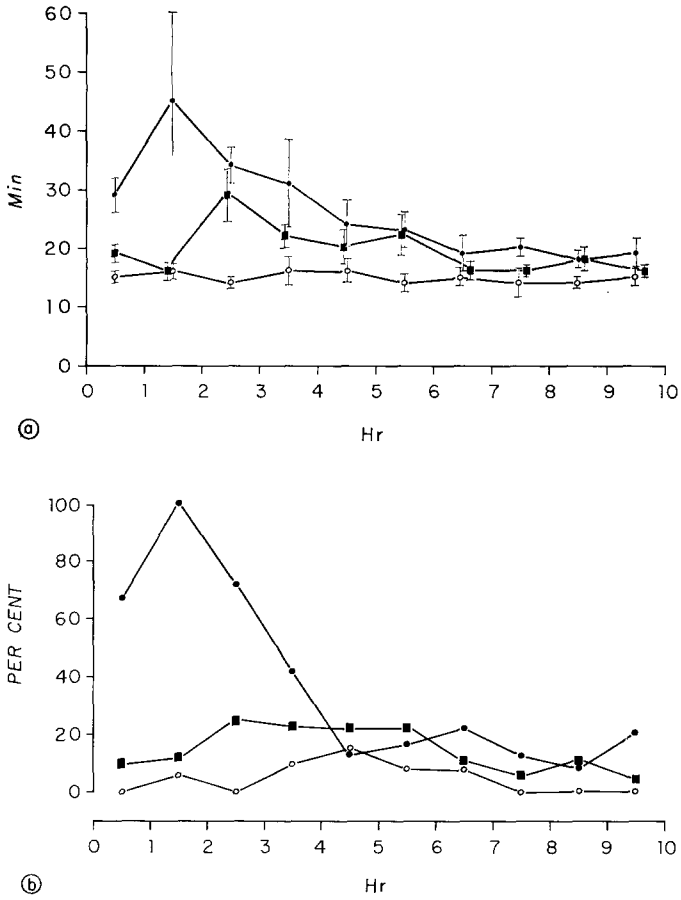


FIGURE 6 Grouped first-peak data from 1 hr treatments with PFPA. ●——●, data grouped together from three 10 mM and three 5 mM PFPA experiments; total cells, 132. ■——■, data grouped together from six 0.5 mM experiments; total cells, 215. ○——○, data grouped together from four control experiments; total cells, 126.

FIGURE 6 *a* Mean duration of metaphase for each age class.

FIGURE 6 *b* Percentage of abnormal metaphases in each age class.

ments. Table V compares the effects of different concentrations of puromycin and PFPA on the gross rate of protein synthesis at 1 hr after the beginning of treatment and indicates that in our system 1 μg/ml and 2.5 μg/ml puromycin inhibit protein synthesis to about the same extent as 5 mM and 10 mM PFPA, respectively.

The effects of 1 hr exposures to these concentrations on the duration of subsequent metaphases are shown in Figs. 8 and 9. As can be seen, neither of these concentrations produces a prolongation of metaphase at a time comparable to the first peak produced by PFPA. However, starting at about a generation time after treatment, both the mean metaphase times and the percentage of abnormal metaphases in individual age classes are increased. First-peak abnormalities can be produced by puromycin, however, if the concentration is high enough. In Fig. 10 we see that 25 μg/ml puromycin does produce a prolongation of those metaphases which appear shortly after treatment and that this characteristic is carried over into subsequent divisions of these cells, producing second and third peaks. Table VI shows that, as in the case of PFPA, cells with puromycin-induced prolonged metaphases have a greater tendency

TABLE III

*Tendency for Cells with Abnormally Long Metaphases to Pass on This Trait to Their Offspring*

Normal metaphases are defined as those whose durations are within two standard deviations of the mean of the controls. Abnormal metaphases are those whose durations are longer than two standard deviations above the mean for the controls. The mother cells are those which divided within 5 hr after the beginning of various *p*-fluorophenylalanine treatments.

| Treatment | Abnormal mothers | | Normal mothers | |
|---|---|---|---|---|
| | No. of daughters | Abnormal | No. of daughters | Abnormal |
| | | % | | % |
| 1 mM, 1 hr | 6 | 17 | 31 | 3 |
| 1 mM, 3 hr | 37 | 35 | 31 | 19 |
| 5 mM, 1 hr | 14 | 57 | 24 | 21 |
| 10 mM, 1 hr | 12 | 42 | 13 | 8 |
| Over-all | 69 | 40 | 99 | 13 |

to produce offspring with prolonged metaphases than normal cells in the same age class.

## DISCUSSION

The results of previous work (6, 7, 9–11, 32, 33) and of that presented in this paper are all consistent with the hypothesis that one or more proteins specifically related to the process of division are synthesized just prior to mitosis. In the work presented here we tried to determine the duration of synthesis of this unidentified, mitosis-associated protein. These experiments were based upon (*a*) the observation that PFPA has a relatively selective effect upon the duration of mitosis, (*b*) the probability that the major mode of action of PFPA is due to its incorporation into newly formed protein, thereby rendering the protein defective, and (*c*) the assumption that PFPA affects the duration of mitosis in only those cells which were synthesizing some mitosis-associated proteins at the time the analogue was available. A study of the kinetics of the appearance of prolonged divisions in a random population of cells after a short exposure to PFPA should tell us at what time during the mitotic cycle the affected mitosis-associated proteins were being synthesized.

The experiments (i.e. Figs. 4 and 5) yielded the expected kinds of curves, and an analysis of first-peak data (Fig. 6) suggests that only cells in that

TABLE IV

*Tendency for Second-Peak Cells to be Derived from Mothers Which Divided during the First Peak Following Various Treatments with p-Fluorophenylalanine*

Normal and abnormal are defined as in Table III.

| Treatment | Total No. of second-peak cells with traceable mothers | Derived from first-peak mothers | No. of abnormal second-peak cells with traceable mothers | Abnormal first-peak mothers |
|---|---|---|---|---|
| | | % | | % |
| 1 mM, 3 hr | 38 | 100 | 12 | 67 |
| 5 mM, 1 hr | 16 | 87 | 4 | 100 |
| 10 mM, 1 hr | 18 | 83 | 6 | 50 |
| Over-all | 72 | 93 | 22 | 68 |

part of the cycle which corresponds fairly closely to $G_2$ + P at the time of exposure to PFPA are affected. In other words, whatever protein is involved in the effects we observe, the initiation of its synthesis occurs at about the time of completion of DNA synthesis, and synthesis of this protein continued to within at least 15 min prior to metaphase.

These findings are not in agreement with the conclusions of Mueller and Kajiwara (10, 34), who studied synchronized HeLa cells. They found that continuous treatments with 0.5 mM PFPA inhibited cell division but only if cells were treated prior to a 4–6 hr period after DNA synthesis was released from blockade. They interpreted this to mean that the 4–6 hr interval after the initiation of DNA synthesis "... marks the end point for synthesis of a protein essential for division" (34). Since, in their material, metaphase begins 6–8 hr after release, this would put the end point at about 2 hr before metaphase. The differences between the two sets of experiments may be due to differences between the cell lines, between synchronized and random cells, or to the fact that, as shown for amnion cells (Fig. 6), a 2 hr lag may occur before a concentration as low as 0.5 mM shows an effect on mitosis of HeLa cells.

The fact that subsequent peaks are produced by the daughters of the cells which divided at the time of the first peak and that the abnormal daughters in the second peak generally are derived from abnormal mothers in the first peak argues

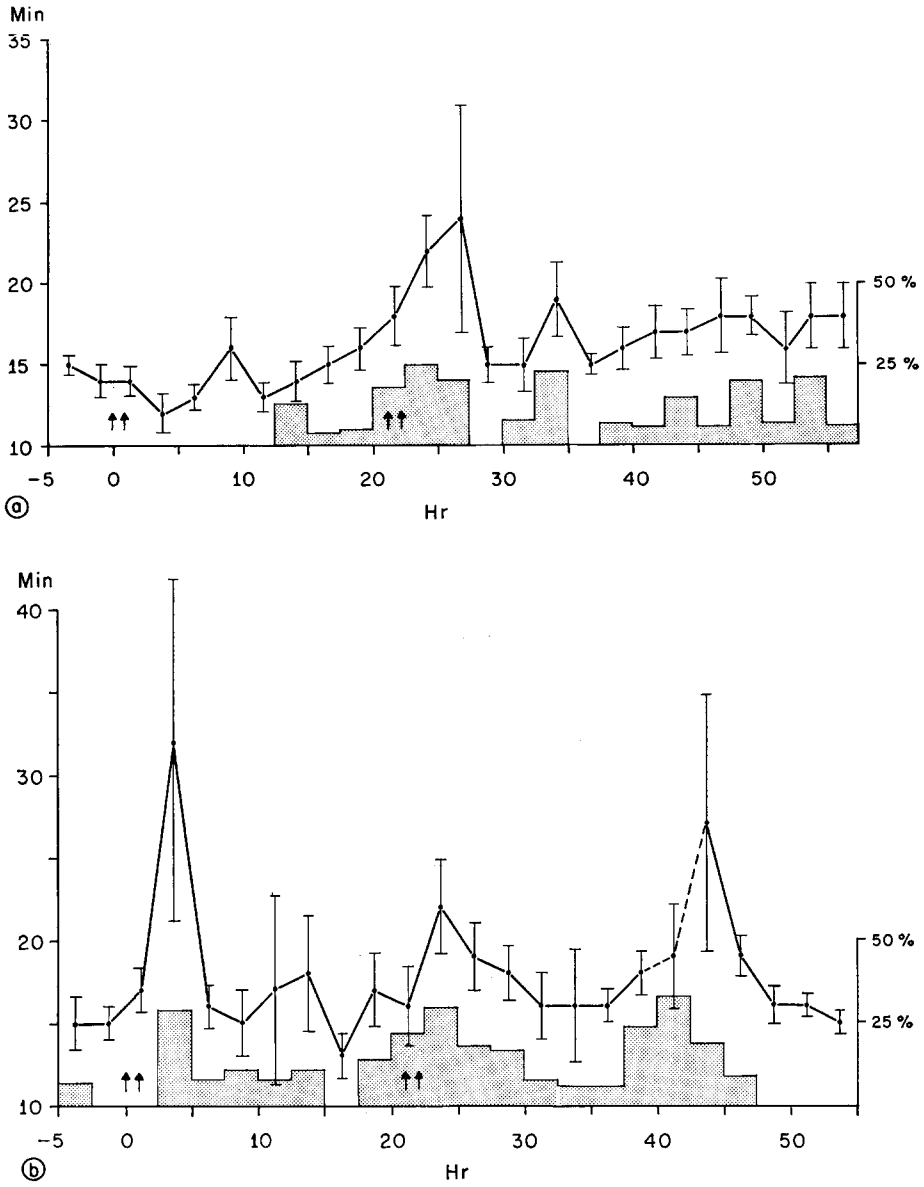


FIGURE 7 Effect of double treatment with 0.5 mM PFPA on metaphase times. Continuous lines show mean metaphase durations. Histograms show percentage of abnormal cells in the individual age classes.

FIGURE 7 *a* First pair of arrows indicates a 1 hr exposure to fresh medium; the second pair of arrows indicates a 1 hr exposure to 0.5 mM PFPA. Total measurements, 362.

FIGURE 7 *b* Both pairs of arrows indicate 1 hr exposures to 0.5 mM PFPA. Total measurements, 308.

that the lesion was induced only in a specific segment of the population of cells and that the lesion is a stable one which is passed on to daughter cells. Further support for this comes from the experiment in Fig. 8, which shows that a cumulative effect of a low concentration could be built up by short treatments over two successive generations, and the presence of third peaks in some of the single-

treatment experiments (Figs. 4, and perhaps 5), which also show that defects may be inherited from grandmothers.

Two observations which need to be explained are that (*a*) not all the offspring of abnormal mothers are abnormal and (*b*) mothers defined as normal sometimes give rise to abnormal daughters. If our general interpretations of the data are correct, the explanations for both of these phenomena may reside in the amount, distribution between daughter cells, and functional role of the PFPA-containing protein. If daughter cells inherit approximately half their mitosis-associated protein from their mothers, treatment of cells during one cycle can lead to cells in which only half of the protein contains analogue and, a generation time later, to cells in which only about a fourth of the protein contains analogue. Depending upon the above mentioned parameters, this amount of PFPA-containing protein may or may not be enough to cause daughter cells to be abnormal. To explain how normal mothers may give rise to abnormal daughters is a little more difficult but, considering the variability of metaphases and, again depending upon the above-mentioned parameters, one might, on statistical grounds, expect this phenomenon to occur at a finite frequency.

TABLE V

*Effects of Various Concentrations of p-Fluorophenylalanine and Puromycin on the Gross Rate of Protein Synthesis at 1 hr after the Beginning of Treatment*

| *p*-Fluorophenylalanine | | Puromycin | |
|---|---|---|---|
| Concentration | Inhibition | Concentration | Inhibition |
| *mM* | % | *μg/ml* | % |
| 0.5 | 6 | 1.0 | 25 |
| 1.0 | 11 | 2.5 | 55 |
| 5.0 | 33 | 25.0 | 75 |
| 10.0 | 51 | | |

The puromycin experiments allow some insight into the mechanism by which PFPA has its effects and give further support to the concept of the conservation and reutilization of mitosis-associated proteins. As indicated by the experiments shown in Figs. 8 and 9, the concentrations of puromycin which inhibit protein synthesis to about the same extent as the concentrations of PFPA utilized do not produce any detectable prolongation of metaphase of those cells which enter division immediately after treatment. This strongly suggests that the effects produced by PFPA are due to its actual incorporation into proteins rather than its ability to inhibit protein synthesis. On the other hand, prolongation of metaphases of puromycin-treated cells does occur a generation time or more after treatment. This means that puromycin produced a deficiency in some mitosis-associated protein which did not show up on the first division but did show up in later generations. In addition to supporting the concept of the conservation and reutilization of mitotic proteins, these data bring

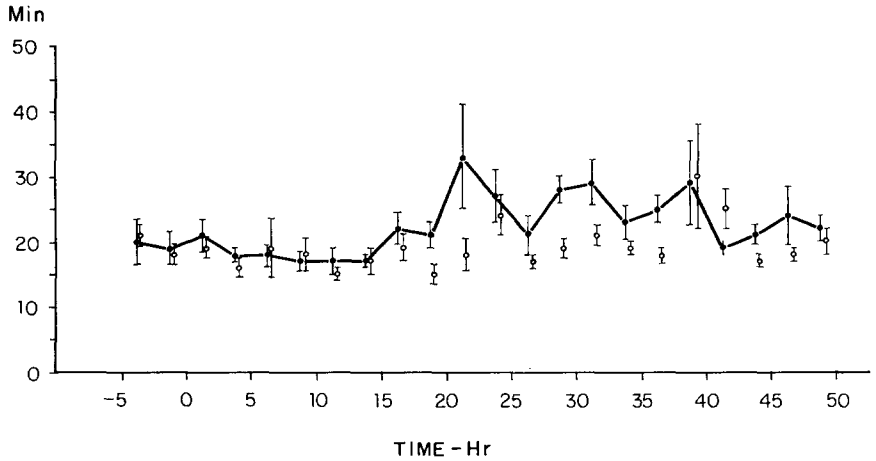


FIGURE 8 Effects of a 1 hr treatment with 1 μg/ml puromycin on the duration of metaphase. Continuous line shows mean metaphase duration for treated cells; O, mean metaphase duration for controls. Total measurements: treated, 587; controls, 490.

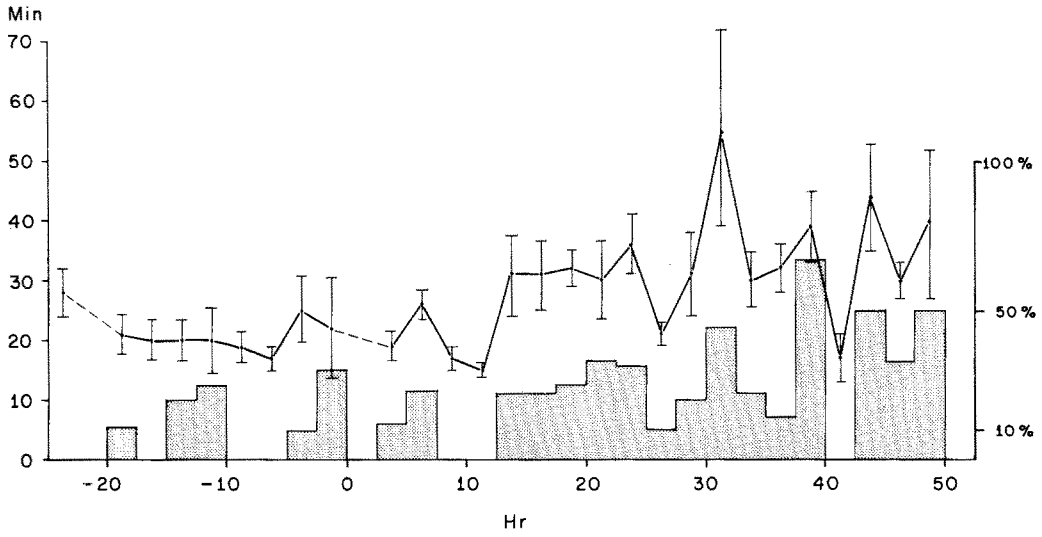


FIGURE 9 Effects of 1-hr treatment with 2.5 μg/ml puromycin on the duration of metaphase. Continuous lines indicate mean metaphase durations. Histograms indicate percentage of abnormal cells in the various age classes. Total measurements, 209.

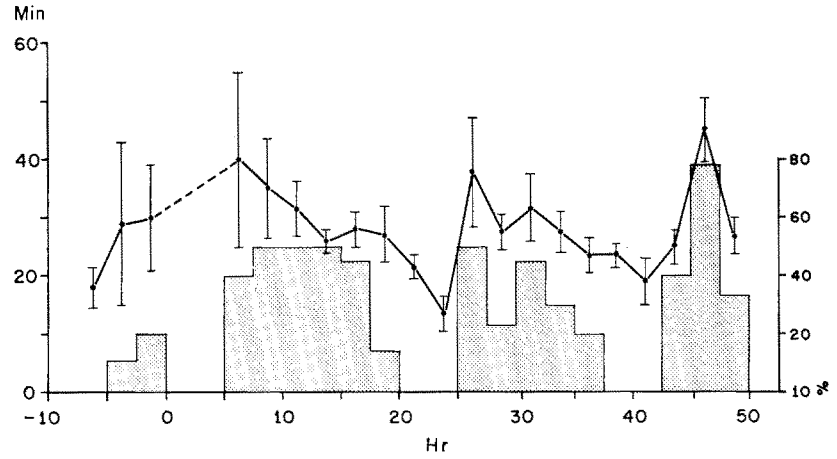


FIGURE 10 Same as Fig. 9 except that the concentration of puromycin was 25 μg/ml. Total measurements, 157.

out two further points. The first is that a normal division may take place even though the cell has a deficiency which is great enough to show up in daughter cells a generation later (Figs. 8 and 9). The second important point is that cells apparently make only specific amounts of some mitosis-associated proteins during each cycle and that if they "inherit" a deficiency from their mother they are unable to compensate for it. This is also supported by the data in Fig. 10, which indicate that, if the inhibition of protein synthesis is drastic, cells can be made deficient enough so that the immediately ensuing division will be prolonged, and that the effect can be passed on to daughter and granddaughter cells which are not capable of compensating for this deficiency.

One of the problems in this kind of study is that we do not know the nature of the protein and cannot tell whether we are dealing with one protein or several proteins. The data only indicate

TABLE VI

*Tendency for cells with abnormally long metaphases to pass on this trait to daughter cells*

The mother cells are those which divided within 5 hr after the beginning of a 1 hr treatment with puromycin. Normal and abnormal cells are defined as in Table III.

| Treatment | Abnormal mothers | | Normal mothers | |
|---|---|---|---|---|
| | No. of daughters | Abnormal | No. of daughters | Abnormal |
| | | % | | % |
| 1.0 μg/ml | 1 | 100 | 30 | 3 |
| 2.5 μg/ml | 6 | 50 | 27 | 11 |
| 25.0 μg/ml | 12 | 25 | 16 | 6 |
| Over-all | 19 | 36 | 73 | 7 |

that protein is involved in determining the duration of metaphase and anaphase, that it is synthesized in specific amounts during a specific part of the mitotic cycle, and that it is passed on to daughter cells and is conserved and reutilized by them for their own subsequent divisions. This protein may be a structural protein of the mitotic apparatus or it could be, in part at least, enzymatic protein similar to that postulated by Taylor (6). The only evidence that it might be a structural protein of the mitotic apparatus is the observation that treatment of cells with PFPA leads to a slight decrease in the mean pole-to-pole distance of the mitotic spindle. However, since PFPA treatment prolongs the duration of mitosis, this finding might be due to a normal shortening of the spindle in late metaphase, as was reported in untreated cultured newt cells by Taylor (35), who also showed that PFPA had no effect on the length or rate of formation of the spindle.

A final point to be mentioned is that we may now be approaching the position of being able to give the $G_2$ period of the mitotic cycle a chemical definition. Until now $G_2$ has been defined as a time period between the completion of DNA synthesis and the beginning of cell division. While $G_2$, as mentioned above, is known to be a period during which metabolic preparations for cell division are taking place, and while Kishimoto and Lieberman (7) have speculated that a possible relationship exists between DNA synthesis and $G_2$ events, there has been no evidence of any unique metabolic step which occurs exclusively during $G_2$ and whose beginning and end coincide with the boundaries of $G_2$. If our interpretations are correct, the findings presented in this paper indicate that such a metabolic system may exist.

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