Proc. Natl. Acad. Sci. USA
Vol. 74, No. 10, pp. 4481–4485, October 1977
Cell Biology

Induction of DNA synthesis in BALB/c 3T3 cells by serum components: Reevaluation of the commitment process

(platelets/plasma/cell cycle/transitional probability)

W. J. PLEDGER*, C. D. STILES†, H. N. ANTONIADES‡, AND C. D. SCHER*

* Department of Pediatrics, Sidney Farber Cancer Institute, and The Children's Hospital Medical Center, Harvard Medical School, Boston, Massachusetts 02115; † Department of Basic Sciences, Sidney Farber Cancer Institute, and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115; and ‡ Center for Blood Research and the Department of Nutrition, Harvard University School of Public Health, Boston, Massachusetts 02115

Communicated by Arthur B. Pardee, July 20, 1977

ABSTRACT **Serum contains a growth factor derived from platelets and also growth factors derived from platelet-poor plasma. Extracts of heated (100°) human platelets function synergistically with platelet-poor plasma to induce DNA synthesis in quiescent, density-inhibited BALB/c 3T3 cells. Platelet-poor plasma alone did not induce DNA synthesis. Cells exposed to platelet extracts became competent to enter the cell cycle, but the rate of entry into the S phase depended upon the concentration of platelet-poor plasma. The time required for the induction of this competent state was a function of the concentration of the platelet extract. A 2-hr exposure to 100 μg of the platelet extract at 37° caused the entire cell population to become competent to enter the S phase. At 4° or 25° the cells did not become competent to synthesize DNA. The platelet extract-induced competent state was stable for at least 13 hr after removal of the platelet extract; however, in the absence of platelet-poor plasma, these competent cells did not progress through the cell cycle. The addition of an optimal concentration of platelet-poor plasma (5%) to these competent cells initiated cell cycle traverse with a rapid, first-order entry of cells into the S phase beginning 12 hr after addition of the plasma. The addition of a suboptimal concentration of the plasma (0.25%) did not increase the rate of cell entry into the S phase. Thus, the induction of DNA synthesis in quiescent BALB/c 3T3 cells can be resolved into at least two phases, controlled by different serum components: (*i*) competence, induced by the platelet-derived growth factor; and (*ii*) progression of competent cells into the cell cycle, mediated by factors in platelet-poor plasma.**

The growth of 3T3 cells (1), diploid fibroblasts (2), and smooth muscle cells (3) *in vitro* is controlled by the concentration of serum in the medium. Serum can be separated into two sets of components which control different cell functions. One set maintains cell viability (4), while the other stimulates replication (5). A heat-stable (100°) cationic growth factor (6) derived from platelets (7) is released into serum during the clotting process (8, 9). Human serum contains about 770 pg of this polypeptide growth factor per mg of protein, as demonstrated by radioimmunoassay (7). Defibrinogenated platelet-poor plasma, a fraction prepared from unclotted blood, contains only low levels of the growth factor and does not stimulate the replication of diploid fibroblasts or BALB/c 3T3 cells (3, 7–9). Platelet-poor plasma does, however, contain the factors that maintain cell viability (3).

The process by which resting cells become committed to enter the growth cycle remains unclear. Smith and Martin (10) have proposed that the commitment of quiescent cells to synthesize DNA is a random event characterized by a first-order rate constant, the transition probability. According to this model, the "initiation of cell replication processes is random, in the sense that radioactive decay is random" (10). Brooks (11, 12) has tactically used serum as a single component and has published data that support the transition probability model of the cell cycle. Serum was interpreted to induce cell cycle traverse by increasing the transition probability. The 12- to 14-hr lag period required for serum to induce DNA synthesis in a resting population was suggested to be the interval needed to increase the transition probability for commitment (12). The commitment of a quiescent population of cells to initiate DNA synthesis was found to occur gradually over a period of time that even exceeded the lag period for entry into the S phase (12). Single cells were felt to become committed to synthesize DNA less than 5 hr before the S phase (after a 7- to 9-hr lag period), because the withdrawal of serum before this time prevented the replication of DNA (12). The randomness, the lack of synchrony, and the delay in commitment make biochemical analysis of the cell cycle difficult.

Since serum contains both platelet-derived growth factors and plasma-derived viability factors, we have reevaluated the commitment process. Using brief exposure to heated platelet extracts followed by transfer to platelet-poor plasma, we now show that the process by which cells leave the quiescent state (G_0) and enter the cell cycle can be resolved into at least two discrete stages which are controlled by separate serum components. The first stage, termed competence, is a prerequisite for entry into the growth cycle. Competence is mediated by a polypeptide derived from platelets and may occur rapidly. The second stage, progression, controls the entry of competent cells into the growth cycle. Progression is controlled by factors in platelet-poor plasma.

MATERIALS AND METHODS

Cell Culture. BALB/c 3T3 cells (clone A31) were plated in 0.3-cm^2 microtest wells (Falcon; approximately 1000 cells per well) in Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with calf serum (Colorado Serum Co.) to 10% and incubated for 6 days at 37° to reach confluence. The spent medium was removed; serum, platelet extract, or platelet-poor plasma was added and the cells were incubated at 4, 25, or 37°, as indicated. The serum (or serum components) was removed after various time periods and the cells were rapidly washed with 28 mM 2-mercaptoethanol in modified Eagle's medium followed by a wash with modified Eagle's medium alone. Medium containing platelet-poor plasma and [^{3}H]dThd (5 μCi/ml; 6.7 Ci/mmol) (New England Nuclear) was then added and the cells were incubated at 37°. At appropriate times the cells were fixed with methanol, washed twice with 5% trichloroacetic acid, and processed for autoradiography (6). Between 500 and 1000 cells were scored for each point.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

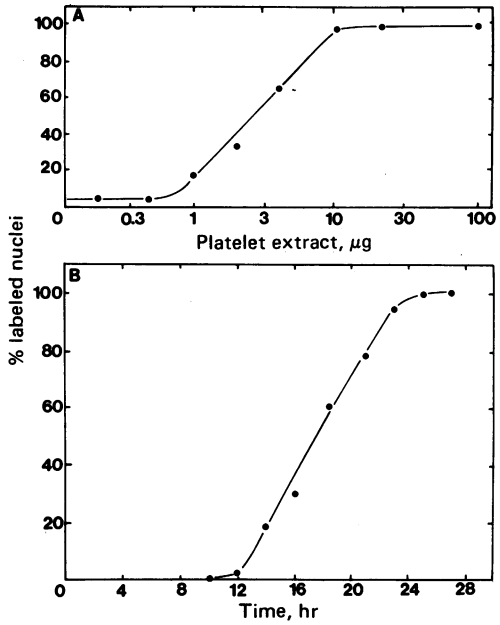

FIG. 1. Platelet extract-induced stimulation of DNA synthesis in density-inhibited BALB/c 3T3 cells. (*A*) Various concentrations of platelet extract were added to cultures in 0.2 ml of medium containing [^{3}H]dThd and 5% platelet-poor plasma. Forty hours later the cells were fixed and processed for autoradiography. (*B*) Platelet extract (10 μg) was added to cultures in 0.2 ml of medium containing [^{3}H]dThd and 5% platelet-poor plasma. At the indicated times the cultures were fixed and processed for autoradiography.

Preparation of Platelet Extract and Platelet-Poor Plasma. Outdated human platelets in ACD buffer that were not suitable for clinical use were pooled, brought to pH 7.5 with Tris (17 mM), concentrated by centrifugation, washed twice, freeze-thawed, and heated to 100° for 10 min. The supernatant (platelet extract), containing the growth factor, was clarified by low-speed centrifugation, dialyzed against 0.15 M NaCl, and lyophilized (7).

Human platelet-poor plasma was obtained from the American Red Cross (Boston, MA). Freshly drawn blood in CPD buffer was centrifuged to remove the platelets, erythrocytes, and leukocytes. The plasma, as received from the Red Cross, was centrifuged again at 28,000 × *g* for 30 min to remove any residual platelets and heated to 56° for 30 min to precipitate fibrinogen. This platelet-poor plasma was clarified by centrifugation, dialyzed against phosphate-buffered saline, centrifuged again, and passed through a 0.45-μm membrane filter (Nalgene). Only preparations of platelet-poor plasma that maintained cell viability without stimulating the growth of sparse populations of BALB/c 3T3 cells were used. Since these blood preparations represent a potential source of hepatitis virus, no mouth pipetting was done and all samples were autoclaved before they were discarded.

Quantitation of Growth Factor Concentration in Platelet Extract and Platelet-Poor Plasma by Radioimmunoassay. The radioimmunoassay for the human serum polypeptide (molecular weight 13,000; pI 9.7) with growth-stimulating activity for BALB/c 3T3 cells has been described (7). The preparation of platelet extract used in these experiments had 350 pg of growth factor antigen determinants per μg of protein.

RESULTS

Stimulation of DNA Synthesis and Cell Replication by Platelet Extract. Varying quantities of platelet extract were added to confluent BALB/c 3T3 cells (15 to 20 × 10^4 cells) placed in medium containing platelet-poor plasma (5%) and 5 μCi of [^{3}H]dThd per ml. The percentage of cells that were stimulated to synthesize DNA increased approximately linearly with the logarithm of the platelet extract concentration over a 10-fold range (Fig. 1*A*). In this experiment, approximately 10 μg of platelet extract containing 3.5 ng of the polypeptide growth factor antigenic determinants (as determined by radioimmunoassay) (7) induced DNA synthesis in 100% of the cells within 40 hr. DNA synthesis began 12–14 hr after the addition of 10 μg of the platelet extract, and the total population had entered the S phase by 24 hr (Fig. 1*B*). Thus, the initiation of DNA synthesis by platelet extract in the presence of platelet-poor plasma follows the same kinetics as serum stimulation (1, 11, 12). In cultures containing platelet-poor plasma but not platelet extract, less than 2% of the population replicated DNA.

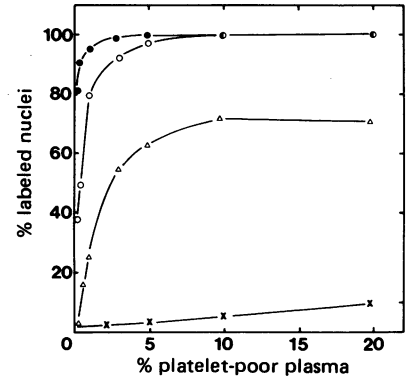

FIG. 2. Effect of concentration of platelet-poor plasma on platelet extract-induced DNA synthesis. Platelet extract (●, 100 μg; ○, 10 μg; △, 5 μg; or ×, 0 μg) was added to cultures in 0.2 ml of medium containing [^{3}H]dThd and various concentrations of platelet-poor plasma. Cultures were fixed 36 hr later and processed for autoradiography.

When confluent cultures of BALB/c 3T3 cells were incubated in medium containing 5% platelet-poor plasma, the addition of platelet extract stimulated cell replication. One hundred micrograms of the platelet extract stimulated the cells to increase their saturation density from 5 × 10^4 cells/cm^2 to 6 × 10^5 cells/cm^2 within 4 days. In addition, these stimulated cells assumed a transformed morphology (data not shown).

Effect of Platelet-Poor Plasma Concentration in Platelet Extract-Induced DNA Synthesis. Various concentrations of both platelet extract and platelet-poor plasma were added to cultures of confluent BALB/c 3T3 cells and the percentage of cells synthesizing DNA within 36 hr was determined. The percentage of cells that entered the S phase was a function of both the amount of platelet extract added and the platelet-poor plasma concentration (Fig. 2). With aliquots of platelet extract ranging from 5 to 100 μg per culture, DNA synthesis was enhanced by the presence of platelet-poor plasma. Five to ten percent platelet-poor plasma was optimal for the expression of platelet extract-induced DNA synthesis. The addition of 5 μg of platelet extract alone stimulated less than 5% of the cells to enter the S phase, but in the presence of an optimal concentration of platelet-poor plasma (5–10%) more than 60% of the cells synthesized DNA. Although 100 μg of platelet extract alone stimulated 80% of the cells to initiate DNA synthesis, the addition of platelet-poor plasma to a concentration of 5% allowed 100% of the cells to replicate DNA. The plasma alone did not appreciably stimulate DNA synthesis at concentrations up to 20%. The platelet extract used in these experiments was a crude preparation that contained many proteins, some of which may

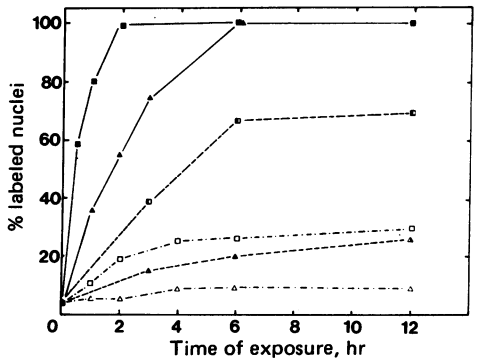

FIG. 3. Temperature dependence of platelet extract-induced commitment to DNA synthesis. Cultures were incubated with the platelet extract in 0.2 ml of medium at 37° (■, 100 μg; ▲, 50 μg), 25° (◧, 100 μg; ◭, 50 μg), or 4° (□, 100 μg; △, 50 μg). At the indicated times the platelet extract was removed and the cultures were washed with 28 mM 2-mercaptoethanol followed by medium. The cultures were placed in 0.2 ml of medium containing [^{3}H]dThd and 5% platelet-poor plasma. Cultures were fixed and processed for autoradiography at 36 hr.

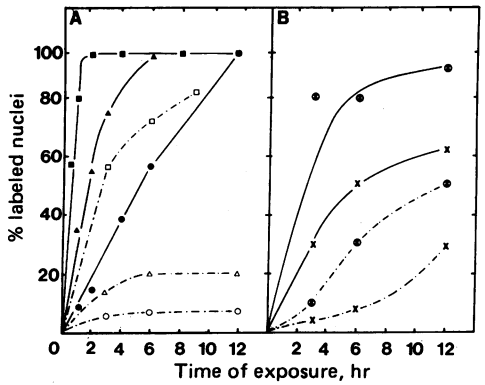

FIG. 4. The effect of the concentration of platelet-poor plasma on platelet extract- or serum-induced commitment to DNA synthesis. (*A*) Cultures were exposed to platelet extract (■ and □, 100 μg; ▲ and △, 50 μg; ● and ○, 10 μg) at 37° in 0.2 ml of medium. At the indicated times the platelet extract was removed and the cultures were washed and transferred to 0.2 ml of medium containing [^{3}H]dThd and 5% (closed symbols) or 0.25% (open symbols) platelet-poor plasma. (*B*) Cultures of cells were exposed to 10% human (⊗) or calf (×) serum in 0.2 ml of medium at 37° for the times indicated, washed, and transferred to 0.2 ml of medium containing [^{3}H]dThd and 5% (—) or 0.25% (—·—·) platelet-poor plasma. Cultures were fixed and processed for autoradiography at 36 hr.

also be present in platelet-poor plasma. Nonetheless, the data of Fig. 2 show that platelet extract was deficient in factors required for the expression of DNA synthesis, while platelet-poor plasma was deficient in the factor(s) required for the stimulation of DNA synthesis. Platelet extract and platelet-poor plasma function synergistically to induce DNA synthesis.

Platelet Extract-Induced Stimulation of DNA Synthesis. To induce cells to become competent to synthesize DNA, cultures were treated with platelet extract, washed, and shifted to medium containing an optimal concentration (5%) of platelet-poor plasma at 37°. Since the growth-stimulating activity and the antigenicity of the platelet extract are destroyed by incubation with 2-mercaptoethanol (6, 13), cultures were washed with 2-mercaptoethanol to remove any residual growth-stimulating factors and then transferred to medium containing platelet-poor plasma. The 2-mercaptoethanol wash had no effect on the kinetics of DNA synthesis or on the number of cells responding to platelet extract stimulation. Treatment of the population with the platelet extract at 37° caused the cells to rapidly become competent to synthesize DNA, while treatment at 25° or 4° did not (Fig. 3).

To exclude the possibility that incubation at 4° reduced the potential of competent BALB/c 3T3 cells to enter S phase, the following experiment was performed. Cells were treated with 50 μg of platelet extract for 10 hr at 4°, washed, and shifted to medium containing 5% platelet-poor plasma and 50 μg of extract or no extract; the cultures were then incubated at 37° for an additional 30 hr. When platelet extract was added during the 37° incubation, 100% of the cells were stimulated to synthesize DNA; only 10% of the cells entered the S phase when the platelet extract was absent during the 37° incubation. Similar results were obtained when cells were treated with platelet extract at 25° (data not shown).

These experiments show that there is a temperature-dependent step in the platelet extract-induced stimulation of DNA synthesis. In addition, they demonstrate that the washing procedure removes the growth-stimulating factors because cells treated with the platelet extract at 4°, washed, and shifted to medium containing platelet-poor plasma (and no platelet extract) at 37° did not synthesize DNA (Fig. 3).

The concentration of platelet extract determined the time required for the cells to become competent to synthesize DNA (Figs. 3 and 4*A*). Cultures were exposed to the platelet extract at 37°, washed, and shifted to medium containing 5% platelet-poor plasma. The total population of cells became competent to synthesize DNA 1–2 hr after the addition of 100 μg of platelet extract (containing 35 ng of the growth factor), while 12 hr were required for all the cells to become competent after the addition of 10 μg.

Platelet-Poor Plasma Controls Cell Cycle Entry. In order to reproduce the serum "stepdown" conditions used in previous studies of the commitment process (11), cultures were exposed to platelet extract for various lengths of time, washed, and transferred to medium containing 5% or 0.25% platelet-poor plasma. Although 10 μg of platelet extract induced 100% of the cells to synthesize DNA after transfer to 5% platelet-poor plasma, only 10% of the cells synthesized DNA in 0.25% platelet-poor plasma (Fig. 4*A*). One hundred micrograms of platelet extract induced the total population to enter the S phase after transfer to 0.25% or 5% platelet-poor plasma; however, the time required for the platelet extract to induce the cells to become competent to synthesize DNA appeared to be longer when cells were shifted to 0.25% platelet-poor plasma. Similar findings were noted when cells were shifted to various concentrations of human or calf serum after exposure to platelet extract (data not shown).

The serum-induced competence for DNA synthesis was also studied. Cells were exposed to 10% human serum or calf serum, washed with 2-mercaptoethanol, and shifted to medium containing 5% or 0.25% platelet-poor plasma. Again the expression of competence for DNA synthesis depended upon the concentration of platelet-poor plasma in the replacement medium (Fig. 4*B*). More cells synthesized DNA after transfer to 5% platelet-poor plasma than after transfer to 0.25% plasma. Thus, the expression of DNA synthesis in competent cells was dependent on factors present in platelet-poor plasma. These data show that transfer of cells to low serum concentrations after stimulation with platelet extract does not allow the optimal expression of DNA synthesis; therefore the number of competent cells cannot be accurately determined from serum stepdown experiments.

Rate of Entry into S Phase. Cells treated with high concentrations of whole serum enter the S phase at a greater rate

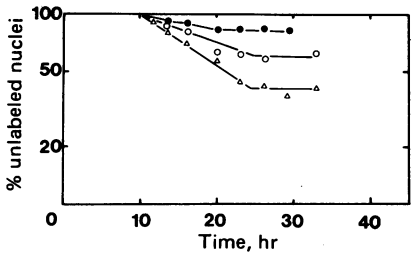

FIG. 5. Effect of concentration of platelet-poor plasma on the rate of cell entry into the S phase. Cultures were treated with platelet extract (5 μg) at 37° in 0.2 ml of medium supplemented with [^{3}H]dThd and platelet-poor plasma (●, 0.25%; ○, 2.5%; △, 5%). Cultures were fixed at the times indicated and processed for autoradiography.

than do cells treated with low concentrations (11, 12). Factors in platelet-poor plasma may control the rate of entry into S phase. To test this hypothesis, quiescent cells were treated with 5 μg of platelet extract in medium containing 0.25%, 2.5%, or 5% platelet-poor plasma. This concentration of platelet extract is limiting and induces about 60% of the population to enter the S phase (Fig. 1). The rate at which the cells entered the S phase initially followed first-order kinetics and was a function of the concentration of platelet-poor plasma (Fig. 5). Cells in 5% platelet-poor plasma entered S at a greater rate than cells in 0.25% platelet-poor plasma.

Stability of the Competent State. Quiescent cells exposed to 50 μg of platelet extract in the presence of 5% platelet-poor plasma entered the S phase 12–14 hr later at a constant rate ($K = -0.17\ hr^{-1}$) (Fig. 6*A*). In order to show that the progression through the cell cycle depends upon factors in platelet-poor plasma, quiescent cultures of cells were exposed to 50 μg of platelet extract in the absence of plasma for 5 hr, washed, and incubated in medium alone; platelet-poor plasma was then added to the cells at 0, 5, 10, or 13 hr after the removal of the platelet extract. In all cases the rapid entry of cells into the S phase occurred 12 hr after the addition of platelet-poor plasma to 5% (Fig. 6 *B–E*). The first-order rate constant for entry of cells into the S phase was (-0.18 to $-0.25\ hr^{-1}$) and was independent of the time of addition of the platelet-poor plasma. Furthermore, 85% of the cells synthesized DNA when the plasma was added at the time of platelet extract removal, while 80% synthesized DNA when the plasma was added 10 hr later.

The addition of platelet-poor plasma to 0.25% at 0, 5, 10, or 13 hr after the removal of platelet extract did not increase the rate of cell entry into the S phase. Thus, a platelet-poor plasma concentration of 0.25% did not allow the entry of competent cells into the S phase of the cell cycle, while a concentration of 5% did.

This experiment demonstrates that the entry of cells into the growth cycle depends on two sets of serum components. Platelet extract induces cells to become competent to replicate DNA, but progression through G_1 does not occur until an optimal concentration of platelet-poor plasma is added. The platelet extract-induced competence for DNA synthesis is stable for at least 13 hr after platelet extract removal. The expression of this competence (i.e., DNA synthesis) requires the addition of an optimal concentration of platelet-poor plasma.

DISCUSSION

The present study shows that entry of quiescent cells into the cell cycle can be resolved into at least two phases under separate regulatory control. For the purpose of discussion, we propose

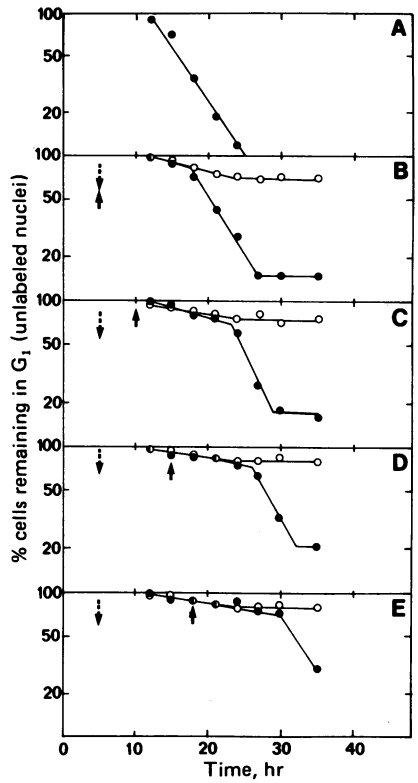

FIG. 6. The stability of the platelet extract-induced committed state. (*A*) Cultures were treated with 50 μg of platelet extract at 37° in 0.2 ml of medium containing 5% platelet-poor plasma and [^{3}H]-dThd. At the indicated times, cultures were fixed and processed for autoradiography. (*B–E*) Cultures were treated with 50 μg of platelet extract in 0.2 ml of medium for 5 hr (↓) at 37°, washed, and returned to 0.2 ml of medium containing [^{3}H]dThd but lacking platelet-poor plasma. At the times indicated by (↑) the medium was supplemented with platelet-poor plasma (●, 5%; ○, 0.25%). The cultures were fixed and processed for autoradiography at time intervals.

a terminology to depict the events that precede the onset of DNA synthesis in serum-stimulated BALB/c 3T3 cells.

The term "competence" will be used to describe the state induced by the cationic polypeptide mitogen found in whole platelets or serum (6). Competent cells are potentially able to leave G_0 and enter the cell cycle, while incompetent cells are not. The time required for the cell population to become competent is a function of the platelet extract concentration; at high concentrations the entire population rapidly becomes competent to synthesize DNA. In addition, the induction of competence is temperature dependent, occurring more quickly at 37° than at 25° or 4°.

The term "progression" will be used to describe the event(s) mediated by the serum factors found in platelet-poor plasma. Progression, which is mediated by anionic serum or plasma factors (data not shown), enables competent cells to traverse the cell cycle, while the growth of incompetent cells remains arrested. For all competent cells to progress through the growth cycle, an optimal amount of platelet-poor plasma must be present. Progression through G_1 and S requires the continual presence of platelet-poor plasma (data not shown). The concentration of the plasma determines, in part, the rate of entry of cells into the S phase. Progression follows the kinetics of a first-order process (Fig. 5). However, the mechanism of progression may be more complex than that of a single-step event.

The present experiments demonstrate that competent cells

do not progress through the growth cycle in the absence of platelet-poor plasma. These nonprogressing cells remain competent to synthesize DNA for as long as 13 hr, because the addition of an optimal concentration of platelet-poor plasma at this time induces the population to enter the cell cycle. Thus, platelet extract-induced competence is stable in the absence of progression.

The commitment of cells to enter the cell cycle and synthesize DNA is likely to be the result of a complex series of interactions. Smith and Martin (10) have suggested that the entry of cells into the cell cycle is a random event that can be characterized by a first-order rate constant, the transitional probability. From serum "pulse" and serum "stepdown" experiments, Brooks (12) has concluded that the role of serum in growth stimulation is to increase the transition probability of resting cells. In order to increase this apparent first-order rate constant for resting cells and initiate cell cycle traverse, we show that the platelet-derived competence factor and the plasma progression factor(s) must both be present.

Rutherford and Ross (3) have shown that smooth muscle cells can be stimulated to synthesize DNA after a 1- to 2-hr exposure to a platelet-derived mitogen. The present experiments extend this observation and demonstrate that entry into the cell cycle is a two-stage process, with each stage under the control of separate serum components.

A characteristic feature of normal fibroblast-like cells is the ability to enter a quiescent state in the absence of growth-stimulatory signals. Pardee (14) has suggested that disturbances in the regulation and maintenance of this quiescent state are a salient feature of the transformation process. The fractionation of serum into components with different functions will facilitate future studies on the quiescent state and on the transition from quiescence to growth.

Note Added in Proof. Transient exposure (8 hr) to 5% platelet-poor plasma followed by continual exposure to platelet extract (5 μg) does not induce DNA synthesis.

We thank Drs. H. Abelson, J. Hamlin, N. Howell, and M. Klagsburn for review of the manuscript, and D. Schneider for his help in preparing it. This work was supported by Grants CA-13472, CA-06516, and CA-15388 from the National Institutes of Health. C.D.S. was aided by a Cancer Research Scholar Award from the American Cancer Society, Massachusetts Division, Inc.

1. Todaro, G. J., Lazar, G. K. & Green, H. (1965) *J. Cell. Comp. Physiol.* **66**, 325–334.
2. Temin, H. M. (1967) in *Growth Regulating Substances for Animal Cells in Culture*, eds. Defendi, V. & Stoker, M. (Wistar Institute Press, Philadelphia, PA), *Symp. Monogr.* **7**, 103–114.
3. Rutherford, R. B. & Ross, R. (1976) *J. Cell Biol.* **69**, 196–203.
4. Paul, D., Lipton, A. & Klinger, I. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 634–648.
5. Holley, R. W. & Kiernan, J. A. (1971) in *Growth Control in Cell Cultures*, eds. Wolstenholme, G. E. W. & Knight, J. (Churchill and Livingstone, London), Ciba Foundation Symposium, pp. 3–10.
6. Antoniades, H. N., Stathakos, D. & Scher, C. D. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 2635–2639.
7. Antoniades, H. N. & Scher, C. D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1973–1977.
8. Ross, R., Glomset, J., Kariya, B. & Harker, L. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 1207–1210.
9. Kohler, N. & Lipton, A. (1974) *Exp. Cell Res.* **87**, 297–301.
10. Smith, J. A. & Martin, L. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1263–1267.
11. Brooks, R. F. (1975) *J. Cell. Physiol.* **86**, 369–378.
12. Brooks, R. F. (1976) *Nature* **260**, 248–250.
13. Antoniades, H. N. & Scher, C. D. (1977) *Natl. Cancer Inst. Monogr.*, in press.
14. Pardee, A. B. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 1286–1290.