Addition of serum to quiescent cultures of 3T3 cells markedly enhances the specific activity of phosphofructokinase assayed in centrifuged homogenates. The effect depends on both serum concentration and the time of exposure to serum. Mixing experiments, measurements of the enzyme at different pH values and persistence of the activation after Sephadex chromatography render unlikely the possibility that activators or inhibitors play a significant role in the stimulation of phosphofructokinase by serum.

In addition to serum, epidermal growth factor and insulin also enhance the activity of phosphofructokinase. The activation occurs in the presence of 30 μg/ml of cycloheximide. It is not the result of a high glycolytic flux because drugs (dinitrophenol and oligomycin) that interfere with ATP synthesis and are potent stimulators of glycolysis in intact cells fail to increase phosphofructokinase activity and because the growth-promoting factors increase the enzyme activity in glucose-free medium. Thus, the activation of phosphofructokinase activity appears specifically related to the action of growth-promoting factors and it may offer an experimental system to investigate the chemical signals or cellular conditions, or both, that lead to rapid cell proliferation.

In the accompanying paper we report that addition of serum or defined growth-promoting molecules, such as epidermal growth factor and insulin, rapidly increases the rate of lactic acid production by density-inhibited cultures of 3T3 cells (1). The stimulation of glycolysis precedes by many hours the onset of DNA synthesis seen in serum-stimulated cultures and is not prevented by inhibitors of RNA or protein synthesis (1). A special feature of this study was the finding that the stimulation of phosphofructokinase by serum persists after homogenization. These findings raise important questions concerning the molecular nature of the mechanism responsible for maintaining the high rate of aerobic glycolysis.

In the present paper we report that serum increases the activity of phosphofructokinase, a key enzyme in the regulation of glycolysis (2-4). The effect depends on both serum concentration and the time of exposure to serum. The growth-promoting factors, epidermal growth factor and insulin, cause a similar stimulation of phosphofructokinase activity. The stimulation neither requires de novo protein synthesis nor the presence of glucose in the medium. Drugs that interfere with ATP synthesis and are potent stimulators of glycolysis in intact cells fail to increase phosphofructokinase activity. Such drugs are also potent inhibitors of cell proliferation. The results suggest that the stimulation of phosphofructokinase activity demonstrated in cell-free systems is related to the action of the growth-promoting factors and results from the activation of pre-existing inactive homogenates and not from the synthesis of new catalytic molecules.

"Addition of serum, epidermal growth factor, and insulin increases the activity of phosphofructokinase in a protein synthesis-independent manner.*"

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MATERIALS AND METHODS

Cell Culture—The cultures were maintained as described in the previous paper (1). Two methods were used to ensure that confluent 3T3 cell cultures were arrested in G1 before the addition of growth stimulators. In early experiments the confluent cultures were shifted to Dulbecco’s modified Eagle’s medium containing 0.5% fetal bovine serum for 24 h. In later experiments the conditioned medium in which the cells were growing was diluted with serum-free medium to a final serum concentration of 0.77% for 24 h. The latter method gave more reproducible control values of phosphofructokinase activity (see below). Then, the cultures were either exposed to medium containing 15% fetal bovine serum or to synthetic medium alone for the indicated period of time.

Preparation of Cellular Homogenates—The Petri dishes were placed on ice and the cells were washed three times with 0.14 M NaCl at 4°. The cells were then removed with a rubber policeman and pelleted by centrifugation at 750 × g for 3 min. Twenty-five microliters of 10 mM Tris/HC1 (pH 7.2), 2 mM MgSO4, and 10 mM KCl/10° cells was added to the cell pellet and after dispersion of the pellet the cells were allowed to “swell” for 10 min and were then disrupted with 100 strokes in a Dounce homogenizer. This material was centrifuged at 20,000 × g for 20 min and the enzyme was assayed in the supernatant.

Assay of Phosphofructokinase—The enzyme was assayed by measuring the disappearance of NADH spectrophotometrically (5) at 340 nm at room temperature. The conditions were 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, pH 7.4), 2 mM ATP, 1 mM MgCl2, 0.02% BSA, 0.1 M NaCl, 0.125 mg/ml pyruvate, and 1 mg/ml glucose, pH 7.4, 340 nm.

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2 mM MgSO$_4$, 5 mM (NH$_4$)$_2$SO$_4$, 1 unit of aldolase, 3 units of α-glycerophosphate dehydrogenase, 7 units of triosephosphate isomerase, 0.15 mM NADH, and the indicated concentration of fructose-6-P in a final volume of 0.4 ml. The auxiliary enzymes were obtained from Sigma (aldolase, 14 units/mg of protein; α-glycerophosphate dehydrogenase and triosephosphate isomerase, 110 and 1260 units/mg, respectively) and were dialyzed for 6 h against two changes of 1,000 volumes of 20 mM Tris buffer (pH 7.5). Neither increasing the aldolase activity 5-fold nor dialyzing the auxiliary enzymes for over 24 h influenced the measurements of phosphofructokinase activity. For studies of initial rates, the fall in optical density was plotted for the first minute after the addition of supernatant. The time course of these reactions was not linear, there being a progressive decrease in activity which was particularly evident in the control (no serum) homogenates. The reaction rates tended toward linearity after about 5 min. Accordingly, in some assays the cellular supernatant was incubated with the assay mixture without substrate at room temperature for 10 min before the sequential addition of increasing amounts of substrate. Blanks were estimated by omission of the substrate or of the supernatant, or both. The reaction rates were calculated from the linear sections of the recorded curves. Units of phosphofructokinase activity are defined as micromoles of fructose-6-P phosphorylated per min under the conditions described above. Protein was determined colorimetrically (5) using crystalline serum albumin as standard.

RESULTS

Effect of Serum Stimulation on Initial Rates of Phosphofructokinase Activity – The dependence of initial rates of phosphofructokinase activity as a function of fructose-6-P concentration is plotted in Fig. 1. Serum treatment of the cells markedly enhances the specific activity of phosphofructokinase in the homogenates. Such difference is seen even at very high concentrations of fructose-6-P (26 mM, not shown). Mixing homogenates from maximally stimulated cells with control homogenates resulted in additive activity of phosphofructokinase according to the theoretical predictions. Furthermore, initial rates of enzyme activity were proportional to the amount of cellular supernatant used (data not shown).

Time Course of Phosphofructokinase Activation by Serum – The dependence on time of serum activation of phosphofructokinase activity is shown in Fig. 2. Cultures were treated with 15% fetal bovine serum and homogenates were prepared at various times. The resulting preparations were incubated for 10 min in the incubation mixture lacking fructose-6-P and then exposed to increasing concentrations of fructose-6-P. The experiment shows that there is a detectable change in the response of phosphofructokinase to fructose-6-P after 1 h of exposure to fresh serum and such change becomes dramatic after 3 h of serum treatment.

Dependence of Phosphofructokinase Activation on Serum Concentration – The effect of different concentrations of serum on phosphofructokinase activity is presented in Fig. 3. A striking feature of this experiment is that the response of the phosphofructokinase appears to have a sharp threshold for serum since medium containing no serum or 1.25% serum failed to stimulate phosphofructokinase while 2.5% serum was fully active. It is worth pointing out that the proliferative response of these cultures to serum (as assessed by autoradiography of cultures exposed to [3H]thymidine and different levels of serum) also shows a marked sigmoid dependence on serum concentration. In an experiment in which we tested the efficiency of two different lots of fetal bovine serum we found one of them was more efficient in both increasing phosphofructokinase activity (after 3 h of addition) and in stimulating DNA synthesis (after 40 h of addition).

Influence of pH and Sephadex Filtration on Phosphofructokinase Activity – The effect of different concentrations of serum on phosphofructokinase activity is presented in Fig. 4. A striking feature of this experiment is that the response of the phosphofructokinase appears to have a sharp threshold for serum since medium containing no serum or 1.25% serum failed to stimulate phosphofructokinase while 2.5% serum was fully active. It is worth pointing out that the proliferative response of these cultures to serum (as assessed by autoradiography of cultures exposed to [3H]thymidine and different levels of serum) also shows a marked sigmoid dependence on serum concentration. In an experiment in which we tested the efficiency of two different lots of fetal bovine serum we found one of them was more efficient in both increasing phosphofructokinase activity (after 3 h of addition) and in stimulating DNA synthesis (after 40 h of addition).
Phosphofructokinase in 3T3 Cells

Phosphofructokinase Activity - The above-mentioned mixing experiments render unlikely the possibility that activators or inhibitors play a role in the stimulation of phosphofructokinase activity by serum. It is known that the influence of the many allosteric effectors of this enzyme is markedly reduced at alkaline pH (2). Accordingly, the enzyme from 3T3 cells shows a marked increase in apparent affinity for fructose-6-P as the pH increases from 7.0 to 8.2, but the large increase in activity brought about by addition of serum to quiescent cells remains unchanged (Fig. 4). Furthermore, the difference in specific activity and in the time course of the reaction between extracts from control and serum-stimulated cells is clearly seen after chromatography on Sephadex G-25 under conditions that maintain the enzyme activity (Table I). All these results indicate that there is a stable modification of pre-existing molecules or an increase in the number of enzyme molecules as a result of de novo synthesis.

Dependence of Phosphofructokinase Activity on Protein Synthesis - To determine whether the increase in enzyme activity after serum treatment requires continuous protein synthesis we exposed cells to serum in the presence or absence of cycloheximide (30 \(\mu g/ml\)). This concentration of inhibitor reduced the incorporation of \(^{14}\)C]leucine into heat-stable, acid-precipitable material in cultures of 3T3 cells by 95% (results not shown). Cycloheximide did not prevent the stimulation of phosphofructokinase activity brought about by serum (Fig. 5), suggesting that such effect is independent of de novo protein synthesis. This result is consistent with the findings in intact cells and homogenates presented in the accompanying paper (1).

Activation of Phosphofructokinase Activity by Insulin and Epidermal Growth Factor - Although serum is required to support continuous cell proliferation of 3T3 cells, its chemical complexity leaves open the possibility that some of its early effects are produced by molecules unrelated to the mitogenic response. To evaluate this possibility, we tested the chemically defined hormones, epidermal growth factor and insulin, for their effect in leading to an increased phosphofructokinase activity (Fig. 5). The results clearly demonstrate that both peptides are potent stimulators of enzyme activity. As observed with serum, addition of cycloheximide (30 \(\mu g/ml\)) does not prevent the stimulation of phosphofructokinase activity (Fig. 5).

Influence of Inhibitors of ATP Synthesis and of the Presence of Extracellular Glucose on Phosphofructokinase Activity - In addition to growth-promoting molecules, glycolysis can be

![Figure 3](http://www.jbc.org/)

**Fig. 3.** Dependence of phosphofructokinase activity on the concentration of fetal bovine serum present in the medium. The cultures were exposed to each serum concentration for 3 h and the assays were as described in the legend to Fig. 2. Fructose-6-P, fructose-6-P; mU PFK, milliunits of phosphofructokinase per mg of protein.

**TABLE I**

| Cellular supernatant | After Sephadex G-25 filtration |
|----------------------|-----------------------------|
|                      | I                          | II*                      |
| mU phosphofructokinase per mg protein |                  |
| Control cells        |                             |                          |
| 1 min                | 31.6                        | 35.5                     |
| 10 min               | 15.5                        | 10.1                     |
| Serum-stimulated cells | 108.4                     | 110.0                    |
| 1 min                | 89.4                        | 89.4                     |
| 10 min               | 89.4                        | 89.4                     |

*The values shown in this column were obtained in the presence of 100 \(\mu l\) of the column equilibrating buffer described above.

![Figure 4](http://www.jbc.org/)

**Fig. 4.** Initial velocity of phosphofructokinase in cellular homogenates as a function of fructose-6-P (Fru-6-P) concentration in quiescent and serum-stimulated 3T3 cells at pH 7.0 and 8.2. The experimental conditions were as described in Fig. 1 except that 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer, pH 7.4, was replaced by 50 mM 2-(N-morpholinolethanesulfonic acid (Mes) buffer, pH 7, or 50 mM Tris/HCl buffer, pH 8.2. Note the abscissa has a log scale. mU PFK, milliunits of phosphofructokinase per mg of protein.
stimulated by interfering with the synthesis of ATP by depressing oxidative phosphorylation with oligomycin or stimulating the mitochondrial ATPase activity with dinitrophenol. Although both compounds produce a large stimulation of lactic acid by intact 3T3 cells (11), they fail to increase the activity of phosphofructokinase (Fig. 6). Furthermore, mixing homogenates from cultures treated with dinitrophenol or control, oligomycin, or dinitrophenol (DNP), 15% fetal bovine serum, or synthetic medium alone (control) for 3 h. At each condition, half of the dishes also contained 30 μg/ml of cycloheximide (O). Phosphofructokinase was assayed after 10 min incubation with the reaction mixture lacking substrate by the addition of fructose-6-P (Fr6-P) as described under "Materials and Methods." mU PFK, milliunits of phosphofructokinase per mg of protein.

**FIG. 6.** Effect of oligomycin and dinitrophenol treatment of 3T3 cells on phosphofructokinase activity. Quiescent cultures of 3T3 cells were exposed to either 0.5 μg/ml of oligomycin, 0.2 mM dinitrophenol (DNP), 15% fetal bovine serum, or synthetic medium alone (control) for 3 h. Cellular homogenates were assayed as described in the legend of Fig. 5. Homogenates of serum- and of oligomycin-treated cells and homogenates of serum- and of dinitrophenol-treated cells were mixed in equal proportions and also assayed. Actual enzyme activity is plotted with O, and the theoretical activity of the mixtures if neither homogenate influenced the activity of the other is plotted with . Fr6-P, fructose-6-P; mU PFK, milliunits of phosphofructokinase per mg of protein.

**FIG. 7.** Phosphofructokinase activity and activation in the absence of extracellular glucose. Dulbecco’s modified Eagle’s medium was prepared without glucose, and fetal bovine serum was dialyzed against 100 volumes of 0.14 M NaCl at 4 °C for 5 days, with daily changes of dialysate. Quiescent cultures of 3T3 cells were exposed to either 15% dialyzed fetal bovine serum, 0.5 μg/ml of insulin, 5 ng/ml of epidermal growth factor (EGF) or synthetic, glucose-free medium alone for 3 h. At this time the cells were prepared and enzyme assayed as described under "Materials and Methods." Fr6-P, fructose-6-P; mU PFK, milliunits of phosphofructokinase per mg of protein.

**FIG. 8.** Influence of extracellular Ca²⁺ concentration on phosphofructokinase activity and activation by epidermal growth factor (EGF). Dulbecco’s modified Eagle’s media were prepared without calcium and with calcium added to the indicated concentrations. Panel A plots the average of two separate experiments in which half the cells at each Ca²⁺ concentration were treated with 5 ng/ml of epidermal growth factor for 3 h. Cellular homogenates were then prepared and assayed for phosphofructokinase as described under “Materials and Methods.” Panel B plots the phosphofructokinase activity of cellular homogenates after the cells were incubated with medium (without serum) at the indicated Ca²⁺ concentrations for 3 h. The media used in the experiments were supplemented with 0.1 mM (Panel A) and 0.2 mM (Panel B) ethylene glycol bis(β-aminoethyl) etherN,N’-tetraacetic acid (EGTA). In all cases the phosphofructokinase activity shown is at 3.8 mM fructose-6-P. mU PFK, milliunits of phosphofructokinase per mg of protein.
oligomycin with serum-stimulated homogenates resulted in additive activity of the enzyme according to the theoretical predictions (Fig. 6). Thus, the data suggest that the increase in enzyme activity, as seen after homogenization is correlated with growth stimulation rather than with an increase in the glycolytic flux. In order to further substantiate this conclusion we studied the effect of the presence of glucose in the medium on the stimulation of phosphofructokinase activity by serum. Fig. 7 shows that incubation of 3T3 cells with diazylated serum, insulin, or epidermal growth factor in glucose-free medium resulted in increased phosphofructokinase activity. Thus, enzyme activation occurred without the entry of glucose into the cells.

Influence of Extracellular Ca²⁺ Concentration on Phosphofructokinase Activity and Activation—In the previous paper we found that Ca²⁺ ions may play a role in mediating the stimulatory effect of epidermal growth factor and insulin (1). We also investigated the effect of the presence of Ca²⁺ ions in the growth medium on the activation of phosphofructokinase activity. Omission of Ca²⁺ markedly inhibits the effect of epidermal growth factor (Fig. 8A). Furthermore, an increase in Ca²⁺ concentration from 0 to 3.6 mM resulted in a progressive increase in the specific activity of phosphofructokinase in the absence of growth-promoting factors (Fig. 8B). Dulbecco's modified Eagle's medium contains 1.8 mM Ca²⁺ which was the concentration of Ca²⁺ routinely used in these studies.

**DISCUSSION**

Since the original observation of Warburg over 50 years ago (7), many hypotheses have been put forward to explain the origin of the high rates of aerobic glycolysis seen in rapidly proliferating cells, including tumor cells. The experiments presented in the previous (1) and present paper reveal a striking parallel between the output of lactic acid produced by growth-promoting factors in intact cells and the activity of phosphofructokinase measured in cell-free supernatants. Indeed, the effect of serum, epidermal growth factor, and insulin, the time course and dose response of the stimulation after serum, the resistance of the activation to inhibition by high levels of cycloheximide, and the effect of the presence of Ca²⁺ ions in the medium clearly document the relationship between lactic acid production by intact cells and phosphofructokinase activity measured in cell-free homogenates. A similar relationship has also been found when confluent 3T3 cells are compared with their simian virus 40-transformed or polyoma virus-transformed derivatives.

An essential question is whether activation of phosphofructokinase is a primary effect triggered by the growth-promoting molecules or whether it results from the activation of glycolytic flux (8) seen after growth stimulation. Two lines of evidence support the first possibility: (a) in sharp contrast to the stimulation of glycolysis by serum, insulin, and epidermal growth factor, the increase in glycolysis brought about by drugs which interfere with ATP synthesis (oligomycin and dinitrophenol) does not persist after cell homogenization (1) and accordingly, such drugs fail to increase phosphofructokinase activity; and (b) activation is largely independent of glucose uptake since incubation of cells with growth-promoting factors in the absence of glucose resulted in increased phosphofructokinase activity. Thus, the activation of phosphofructokinase appears specifically related to the action of growth-promoting molecules. The increased activity of phosphofructokinase demonstrated in cellular homogenates is consistent with the indirect findings of a shift in the intracellular concentrations of fructose-6-P and fructose 1,6-diphosphate seen in quiescent cultures of chick embryo fibroblasts stimulated to grow (9, 10) or transformed by oncogenic viruses (11). Our findings are also consistent with studies in 3T3 cells indicating that the rate limiting step in glucose uptake is phosphorylation rather than transport (12), with the persistence of high glycolytic activity after cell homogenization (1), and with the finding that insulin acts in a glucose-free medium to stimulate glycolysis in the rat diaphragm (13). In another study, Mansour demonstrated the direct stimulation of phosphofructokinase in rabbit skeletal muscle after intravenous administration of epinephrine (14). Unlike the present study, Mansour's system required caffeine in the homogenization buffer and cAMP in the assay mixture. The most striking contrast is that the stimulation was abolished in the muscle system at alkaline pH (8.2) while it was not in the 3T3 cells. Thus, the stimulation of phosphofructokinase in skeletal muscle by epinephrine may occur via a different mechanism than the stimulation in 3T3 cells by growth factors. Indeed, growth factors are known to decrease intracellular cAMP in quiescent 3T3 cells (15, 16), whereas epinephrine increases cAMP in skeletal muscle (17).

How do the growth factors activate phosphofructokinase? This question has added importance because the activation of the phosphofructokinase may represent one of a set of metabolic changes that coordinately respond to growth stimulation (18). Thus, it seems feasible that the molecules or cellular conditions that regulate phosphofructokinase activity after growth stimulation can be of considerable general significance. It has been proposed that the activation of phosphofructokinase is caused by a small increase in intracellular pH (9, 10). More recently intracellular Mg²⁺ concentration has been proposed as a controlling system for intracellular transphosphorylation (19, 20). Neither of these hypotheses, however, would have predicted the increased specific activity of phosphofructokinase which was directly demonstrated in homogenates of growth-stimulated cells in the present study. In these assays the pH and Mg²⁺ concentration are kept constant. Thus, our results do not support these hypotheses. In agreement with this conclusion, intracellular pH is not changed by serum stimulation of quiescent cells and the difference in specific activity of phosphofructokinase is clearly seen at different pH values (Fig. 4).

An alternative possibility is suggested by our experiments on the role of Ca²⁺ as a potential mediator of the action of insulin and epidermal growth factor (1). The effect of omission of Ca²⁺ (Ref. 1 and Fig. 8), the action of the divalent cation ionophore A23187 (1) and the activation of phosphofructokinase produced by doubling the concentration of extracellular Ca²⁺ (Fig. 8) are consistent with a role of Ca²⁺ in mediating the increase in glucose catabolism. Furthermore, insulin apparently induces a mobilization of Ca²⁺ ions from intracellular compartments in a variety of cell types (21, 22). However, it is unlikely that Ca²⁺ represents the second messenger itself. Mammalian phosphofructokinases are apparently not affected by Ca²⁺ and we verified that addition of Ca²⁺ to our control homogenates does not stimulate phosphofructokinase activity (data not shown). It is still possible, however, that Ca²⁺ plays an indirect role in this stimulation.

The molecular mechanism by which the specific activity of phosphofructokinase increases in cells stimulated by growth-promoting factors is unknown. It is known that phosphofructi-
Phosphofructokinase in 3T3 Cells

Phosphofructokinase undergoes polymerization reactions which are dependent on temperature, pH, presence of substrates, and allosteric effectors and which occur at concentrations of enzyme present in cell (2-4, 23-25). It has been shown that the purified enzyme exhibits slow changes in specific activity that reflect the association of inactive dimers into active tetramers (23-25). It is reasonable to speculate that the activation of phosphofructokinase seen in intact cells after treatment with growth-promoting molecules might follow a similar path. Inactive dimers present in the resting cell may become associated into tetramers in a time-dependent process and in a cycloheximide-insensitive manner. No evidence of a diffusible activator or inhibitor of enzyme activity in homogenates from control or serum-treated cells was detected either in mixing experiments, in measurements of activity at different concentrations of homogenate, or by Sephadex chromatography. If such effectors exist in this system, we may have been unable to detect them because they are tightly bound to the enzyme or because they have already induced a stable modification of the enzyme.

Recent reports indicating that a more active form of phosphofructokinase from liver and muscle differs from a less active one because it is phosphorylated offer an intriguing possibility (26-28). However, other workers have failed to find evidence for phosphorylation of the enzyme (14) when our cellular homogenates are incubated in the presence of ATP and Mg2+ with or without fructose-6-P. They show a decrease rather than an increase in the activity of phosphofructokinase. The possibility that phosphofructokinase activity is regulated by a phosphorylation mechanism requires further experimental work.

Our results indicate that phosphofructokinase, the key enzyme of the glycolytic pathway, undergoes a rapid change in specific activity that plays a critical role in maintaining a high rate of glucose catabolism in cells which have been stimulated to grow. The change in phosphofructokinase activity may offer an experimental approach to investigate the chemical signals or cellular conditions that lead to rapid cell proliferation.

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