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Induction of glycolytic enzyme synthesis in proliferating fibroblasts

Study of phosphofructokinase, glucose phosphate isomerase and pyruvate kinase

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Specific activity of phosphofructokinase is 7–8-fold higher in exponentially growing human fibroblasts than in quiescent cells, but the difference is considerably less pronounced for two other glycolytic enzymes, glucose phosphate isomerase and pyruvate kinase. The ratio of the F-type to L-type phosphofructokinase subunits is essentially the same in growing and resting cells, 4:1. F-type-phosphofructokinase-related antigen concentration is decreased in resting cells as compared with proliferating fibroblasts, but relatively less than the enzyme activity; the ratio of the enzyme activity to the antigen concentration (immunological specific activity) is therefore lower in resting than in growing fibroblasts. Synthesis of phosphofructokinase, as a percentage of the total protein synthesis, is about 30-fold greater during the proliferative phase than in quiescent cells, but this difference is only 3–4-fold for glucose phosphate isomerase and pyruvate kinase. Modulation of the synthesis of phosphofructokinase therefore seems to be responsible for the changes of its specific activity in function of cell proliferation. The appearance of some inactive cross-reacting material in quiescent cells is probably due to post-translational alteration of the pre-synthesized molecules. Compared with other glycolytic enzymes, such as glucose phosphate isomerase and pyruvate kinase, phosphofructokinase seems to be the (or one of the) preferential target of glycolytic induction in proliferating cells.

Phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) activity increases in fibroblastic cells stimulated to proliferate by addition of serum (Singh *et al.*, 1976). The same phenomenon, preceding the increase in DNA synthesis, is observed within 24h after infection by Rous sarcoma virus (Fodge & Rubin, 1973). Inhibition of glycolysis blocks the increase of DNA synthesis that occurs after cells have been stimulated to proliferate by various factors (Fodge & Rubin, 1973). It appears therefore that stimulation of glycolysis and of phosphofructokinase, which is one of the key enzymes of this metabolic pathway, constitutes an early event in induced growth. However, the real role of these stimulations in DNA synthesis and proliferation remains controversial (Pouyssegur *et al.*, 1980).

Human phosphofructokinase exists under different forms corresponding to a tetrameric combination of three types of subunits, M, L and F (Cottreau *et al.*, 1979; Kahn *et al.*, 1979, 1980*b*; Vora & Seaman, 1980; Vora *et al.*, 1981). In fibroblasts, the F subunits (M_r 80000) are predominant; they coexist (Kahn *et al.*, 1979; Weil *et al.*, 1980; Lagrange *et al.*, 1981) with less abundant L subunits (M_r 76000). The M-type subunits appear to be very rare in these cells (Kahn *et al.*, 1979; Weil *et al.*, 1980; Lagrange *et al.*, 1981). F-type phosphofructokinase is kinetically characterized by poor regulatory properties as compared with M- and L-type enzymes (Meienhofer *et al.*, 1980).

Our goal in undertaking this work was therefore to clarify the following points: (i) which phosphofructokinase isoenzyme is preferentially stimulated by the inducers of cell proliferation?; (ii) what is the mechanism of this increase of the phosphofructokinase activity?; (iii) is phosphofructokinase a preferential target of glycolysis induced in cells stimulated to proliferate?; (iv) if enzymes other than phosphofructokinase exhibit an increased activity in stimulated fibroblasts, is the mechanism of their induction similar to that observed for phosphofructokinase?

To answer these questions, we measured activity,

Abbreviation used: SDS, sodium dodecyl sulphate.

immunological specific activity and synthesis rate of three glycolytic enzymes, phosphofructokinase, glucose phosphate isomerase (EC 5.3.1.9) and pyruvate kinase (EC 2.7.1.40), in resting and proliferating fibroblasts. In addition, the isoenzymic composition of phosphofructokinase was determined.

Materials and methods

Materials

Chemicals were from Sigma, Merck or Boehringer–Mannheim. Culture medium and sera were supplied by Gibco; acrylamide, bisacrylamide and autoradiographic X-Omat AR films by Eastman–Kodak; glutaraldehyde-activated agarose beads (Ultrogel) was from l'Industrie Biologique Française; Blue Dextran and CNBr-activated Sepharose 4B were from Pharmacia; ^{14}C-labelled amino acids and [^{35}S]methionine from Amersham; Econofluor, Protosol and En^{3}hance autoradiography enhancer from New England Nuclear. Radioactivity was measured in a LKB scintillation counter (1215 Rackbeta model) and enzyme activity in a Gilford (model 2400) spectrophotometer.

Culture methods

The fibroblast strains studied were derived from embryonic lung (L 809 strain). Cells were grown in Ham F10 medium supplemented with 10% (v/v) foetal-calf serum, kanamycin and tylosin. The cells were subcultured by trypsin treatment every 3 days.

Metabolic labelling of proteins

(i) *Protracted labelling of proliferating cells.* Cells were subcultured in the presence of a mixture of ^{14}C-labelled amino acids (100 μCi/10 ml of medium) and grown to confluency at day 3. The Petri dishes containing the cell monolayer were then washed three times with phosphate-buffered saline [10 mM-sodium phosphate (pH 7.2)/140 mM-NaCl] and the cells scraped off, then collected by centrifugation for 10 min at 2000 *g*; the pellets were kept frozen at −80°C until used.

(ii) *Protracted labelling of resting cells.* After the cells reached confluency, the medium containing 10% foetal-calf serum was replaced by a medium containing only 0.1% serum. At day 3 after confluency this medium was replaced by a similar one containing in addition 500 μCi of the mixture of ^{14}C-labelled amino acids/10 ml. The cells were harvested at day 6 as reported above.

(iii) *Short labelling of proliferating cells.* At day 2 of exponential growth, the cells were washed three times with phosphate-buffered saline, then starved for 30 min in Dulbecco's medium lacking methionine and supplemented with 8% (v/v) extensively dialysed foetal-calf serum. Then 100 μCi of [^{35}S]-methionine (1000–1300 Ci/mmol) was added to the medium, and 1 h later the cells were harvested.

(iv) *Short labelling of resting cells.* [^{35}S]-Methionine was added as reported above at day 6 after confluency, the cells being cultivated in the presence of 0.1% foetal-calf serum.

Cell lysis and determination of the radioactivity incorporated into proteins

The fibroblasts were lysed at 0°C in 20 mM-Tris/phosphate buffer (pH 7.5), containing 10 mM-KF, 10 mM-$(NH_4)_2SO_4$, 0.01 mM-fructose 1,6-bisphosphate, 0.1 mM-EDTA, 10 mM-dithiothreitol and 0.5% (v/v) Nonidet P40 (Meienhofer *et al.*, 1979). Cell debris, nuclei and the particulate fraction were eliminated by centrifugation at 130000 *g* for 20 min (in a Beckman Airfuge). Incorporation of radioactivity into cytosolic proteins was determined by pipetting 5 and 10 μl of supernatant on to small pieces of Whatman 3MM paper (1 cm^2), which were then boiled in 10% (w/v) trichloroacetic acid for 10 min. The pieces were rinsed successively with water, ethanol and acetone, and then air-dried; they were then incubated in scintillation vials with 0.5 ml of Protosol at 55°C for 30 min. Then 10 ml of Econofluor was added to each vial; the mixtures were left in ice for 10 min, and radioactivity was counted.

Enzyme-activity and protein-concentration measurements

Enzyme activities were measured as described elsewhere (Beutler, 1975; Rubinson *et al.*, 1976). The results were expressed in units (1 μmol of substrate transformed/min at 30°C, in cuvettes of 1 cm optical pathway). Proteins were assayed by the Coomassie Blue method (Read & Northcote, 1981) with a standard of crystallized bovine albumin.

Immunological characterization of fibroblast phosphofructokinase

Preparation and characterization of specific anti-M-, anti-L- and anti-F-type phosphofructokinase antisera have been previously reported (Meienhofer *et al.*, 1979).

Electroimmunodiffusion of phosphofructokinase-related antigen was carried out as previously described (Kahn *et al.*, 1974*a*,*b*, 1977) for glucose phosphate isomerase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, except that the immunoprecipitate peaks were revealed by staining for phosphofructokinase activity with a tetrazolium-salt-reduction-linked reaction (Meienhofer *et al.*, 1979).

The area of the immunoprecipitate peaks is proportional to the antigen concentration applied to the gel, and the plot of the peak areas versus the enzyme activity of the extract dilutions applied to the

gel is a straight line whose slope is proportional to the ratio of the enzyme activity to the antigen concentration. We have called this ratio 'immunological specific activity'.

The isoenzymic nature of phosphofructokinase was also investigated by immunoneutralization with each of the three specific antisera (Cottreau *et al.*, 1979; Kahn *et al.*, 1979, 1980*a*,*b*) and by studying the ability of antibody-linked agarose beads (Ultrogel) to bind the enzyme from cell extracts applied to absorbent columns (Kahn *et al.*, 1981).

Purification of fibroblast phosphofructokinase by affinity chromatography

^{14}C- or ^{35}S-labelled cytosolic fractions were mixed with 1–2 units of partially purified unlabelled fibroblast phosphofructokinase (Kahn *et al.*, 1980*b*). The enzyme was then purified by $(NH_4)_2SO_4$ fractionation and chromatography on Blue Dextran–Sepharose 4B columns with elective elution by ADP and fructose 6-phosphate (Cottreau *et al.*, 1980; Kahn *et al.*, 1980*a*,*b*). The overall yield of this purification procedure was 50–60%. After specific elution, the eluate was freeze-dried, and the product was dissolved in water containing 2% (v/v) β-mercaptoethanol, and extensively dialysed against 62.5 mM-Tris/HCl buffer (pH 6.8) containing 0.01%-β-mercaptoethanol and 0.01% (w/v) SDS. Then the sample was concentrated by ultrafiltration, made 2% in SDS and 5% in β-mercaptoethanol and heated at 98°C for 3 min. It was then directly loaded on a SDS/polyacrylamide gradient gel (Laemmli, 1970) and electrophoresed. After drying of the gel, radioactive bands were located by fluorography (Bonner & Laskey, 1974).

Purification of pyruvate kinase and glucose phosphate isomerase by immunoaffinity micro-chromatography

Pyruvate kinase and glucose phosphate isomerase of cells labelled with [^{35}S]methionine were purified by immunoabsorption on antibody-coupled Ultrogel micro-columns, as described in detail elsewhere (Kahn *et al.*, 1981). The anti-(pyruvate kinase) serum used was developed by injecting hens with rabbit muscle enzyme; it cross-reacted perfectly with human M_1- and M_2-type pyruvate kinase isoenzymes (Levin *et al.*, 1982). The enzymes bound to the absorbents were eluted at 98°C in the dissociating solution described by Laemmli (1970) containing 2% SDS and 5% β-mercaptoethanol, then analysed by SDS/polyacrylamide-gel electrophoresis as reported above.

Quantification of the labelled enzymes

After gel electrophoresis, the radioactive bands corresponding to the studied enzymes were cut from the gel, rehydrated in 100 μl of water, and then incubated in 500 μl of Protosol at 55°C for 2 h. Econofluor (10 ml) was then added and the vials were further incubated overnight at 37°C. After cooling at 0°C for a few minutes, radioactivity was counted. Radioactivity of a gel slice corresponding to the eluate from a non-immune absorbent column (for pyruvate kinase and glucose phosphate isomerase) or located outside the enzyme band (for phosphofructokinase) was subtracted from the radioactivity found for the specific bands.

Total radioactivity of labelled enzyme in the cell extract was calculated from this value, corrected for the yield of the enzyme purification (for phosphofructokinase) or the ratio of enzyme binding to the immunoabsorbent (for pyruvate kinase and glucose phosphate isomerase), and for the ratio of radioactivity recovered from the gel by the method described above (about 65%).

Results

Total protein synthesis

Total incorporation of [^{35}S]methionine into cytosolic trichloroacetic acid-insoluble material was expressed in c.p.m./10^6 cells for a 1 h incubation (Table 1). The value found for proliferating cells (12.8×10^6) was about twice that in resting fibroblasts (6.7×10^6).

Variation of enzyme specific activity in the cultured cells

Fig. 1 shows that phosphofructokinase specific activity increased about 7-fold with cell proliferation. Enzyme activity was maximum as the cells reached confluency, then decreased progressively after the serum concentration was decreased from 10 to 0.1% (v/v).

The variation of the activity of glucose phosphate isomerase and pyruvate kinase was considerably less pronounced than for phosphofructokinase (Table 2): the ratio of the activity of proliferating cells to that of quiescent cells cultured in the presence of 0.1% foetal-calf serum was 1.3 for pyruvate kinase and 1.4 for glucose phosphate isomerase.

Immunological characterization of the phosphofructokinase isoenzymes in proliferating and quiescent cells

Maximum neutralization of phosphofructokinase activity by excess anti-F-type and anti-L-type sera was similar for proliferating and quiescent cells: 85–95% with the former antiserum and 40–60% with the latter one. As discussed previously (Kahn *et al.*, 1979; Meienhofer *et al.*, 1979), these results should be interpreted in view of the tetrameric structure of the enzyme: although L-type subunits represent about 20% of the phosphofructokinase subunits in fibroblasts (Kahn *et al.*, 1979; the

Table 1. *Synthesis of total cytosolic proteins and of specific enzymes in proliferating and quiescent cells*
The values given represent the results of independent experiments, from which means ± S.D. are calculated. Abbreviation: n.d., not detectable. Labelling was performed for 1 h in the presence of 10 μCi of [^{35}S]methionine/ml as reported in the Materials and methods section.

	Protein synthesis (c.p.m. incorporated into trichloroacetic acid-precipitable material/h per 10^6 cells)	Enzyme synthesis (percentage of total protein synthesis)			
		Phosphofructokinase		Pyruvate kinase	Glucose phosphate isomerase
		F	L		
Proliferating cells (2 days after duplication	11.9×10^6	0.059	0.0135	0.080	0.070
	13.5×10^6	0.046	0.0105	0.042	0.058
	13.2×10^6			0.10	0.058
				0.055	0.047
	Mean = $12.8 (\pm 0.8) \times 10^6$			Mean = 0.069 ± 0.0026	Mean = 0.0585 ± 0.009
Quiescent cells (6 days after confluency in 0.1% foetal-calf serum)	3.13×10^6	0.0016	n.d.	0.010	0.029
	9.4×10^6	0.0018	n.d.	0.010	0.014
	7.6×10^6			0.014	0.016
				0.018	0.012
	Mean = $6.7 (\pm 3) \times 10^6$			0.024	0.017
				Mean = 0.015 ± 0.0059	Mean = 0.018 ± 0.0066
Ratio of synthesis in in proliferating to that in quiescent cells	1.9	31		4.5	3.4

Table 2. *Pyruvate kinase and glucose phosphate isomerase activity in proliferating and quiescent fibroblasts*
Proliferating cells were harvested 3 days after subcultivation in the presence of 10% foetal-calf serum, and quiescent cells 6 days after confluency in the presence of 0.1% foetal-calf serum. Means ± S.D. are given for *n* independent assays.

	Enzyme specific activity (units/mg of protein)	
Proliferating cells ($n = 11$)	4.7 ± 0.4	1.32 ± 0.2
Quiescent cells ($n = 5$)	3.5 ± 0.3	0.95 ± 0.2

present paper), many tetrameric structures contain these subunits and are therefore precipitable by anti-L-type serum. By contrast, as previously shown (Cottreau *et al.*, 1979; Kahn *et al.*, 1979), anti-M-type serum inhibited fibroblast phosphofructokinase activity by only 5–20%. Since this result is in disagreement with that reported by other authors, who found more muscle-type phosphofructokinase in some fibroblast strains (Vora, 1981; Vora & Seaman, 1980), we made further tests for the presence of this isoenzyme by different techniques. Electroimmunodiffusion revealed no immunoprecipitate peaks; even with high concentrations of anti-M-type serum in the agarose gel, phosphofructokinase activity migrated at the same speed as in a gel without antiserum (results not shown). Finally, when cytosol of fibroblasts cultured in the presence of [^{35}S]methionine (total trichloroacetic acid-precipitable radioactivity = 50×10^6 c.p.m.) was passed through an immunoaffinity column containing anti-M-type-antibody-linked Ultrogel (Kahn *et al.*, 1981), no M-type subunits could be detected in the eluate. This immunoabsorbent recognizes M-type enzyme very well, both from tissues and when it has been biosynthesized under the direction of human muscle mRNA (Kahn *et al.*, 1981). It appears therefore that the fibroblast strains used in the study did not contain a significant amount of M-type phosphofructokinase and that, in a proliferating as well as in a quiescent state, they contain F-type subunits as the major form and L-type as the minor form.

Characterization of the phosphofructokinase subunits by enzyme purification and gel analysis

Phosphofructokinase was purified from either proliferating or quiescent cells after protracted or short labelling with radiolabelled amino acids. In both conditions the ratio of the M_r-80000 to the M_r-76000 enzyme subunits did not seem to be modified in function of the proliferating state, with about 20% of the light form in both proliferating and quiescent cells (Fig. 2).

Since we have shown that M-type subunits were practically undetectable in fibroblasts, the heavy forms represent F-type and the light forms L-type subunits (Kahn *et al.*, 1980*b*; Lagrange *et al.*, 1981).

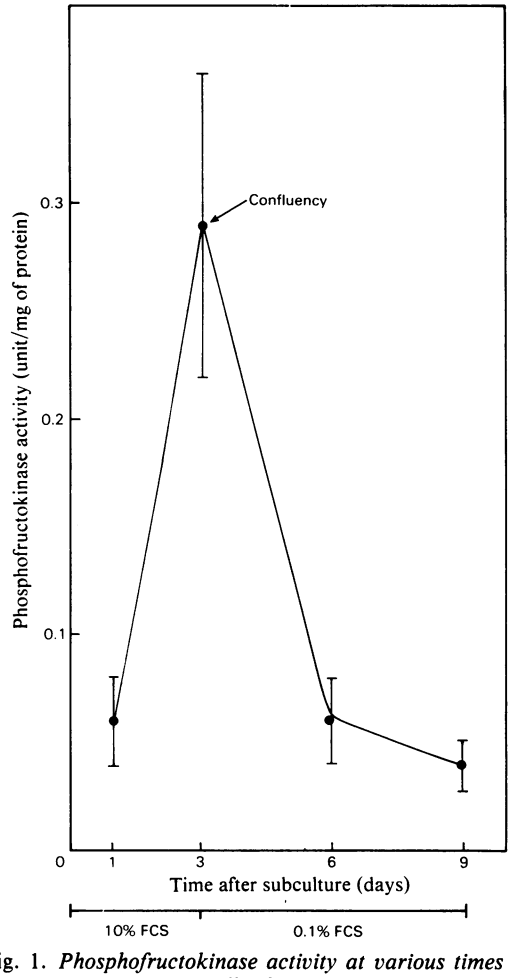

Fig. 1. *Phosphofructokinase activity at various times of cell culture*

Each point represents the mean ± S.D. for five independent measurements. '10% FCS' indicates culture in the presence of 10% foetal-calf serum (corresponding to exponential growth), and '0.1% FCS' culture in the presence of 0.1% foetal-calf serum (quiescent cells). For further details see the text.

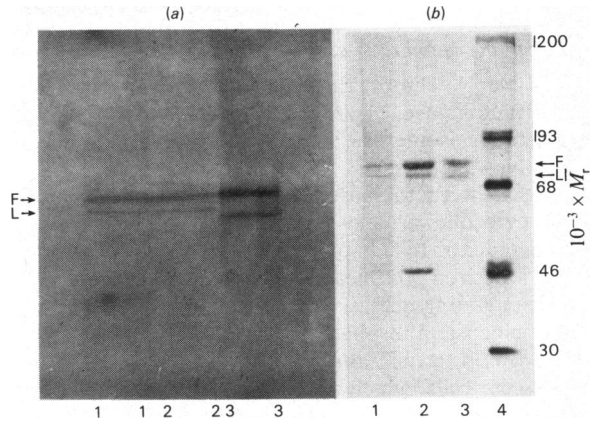

Fig. 2. *SDS/polyacrylamide-gradient-gel electrophoresis of purified fibroblast phosphofructokinase*

(*a*) Protracted (3 days) labelling with ^{14}C-labelled amino acids; (*b*) brief (1 h) labelling with [^{35}S]-methionine. Tracks: 1, quiescent cells; 2, proliferating cells; 3, ^{14}C-labelled purified platelet F/L phosphofructokinase hybrids (Kahn *et al.*, 1981); 4, molecular-weight standards. The arrows indicate positions of the phosphofructokinase subunits F and L. The L subunits from the platelet phosphofructokinase hybrids are partially proteolysed (Lagrange *et al.*, 1981), which explains their lower M_r than that of the fibroblast L-subunits. The band of M_r approx. 45000 observed in channel 2 of part (*b*) is contaminant actin (Kahn *et al.*, 1981).

Immunological quantification of phosphofructokinase-related antigen

Table 3 shows that, in two independent experiments, enzyme activity in quiescent cells was 15% and 13%, and antigen concentration 22%, of the values found in proliferating fibroblasts. The ratio of the enzyme activity to the antigen concentration, which we call 'immunological specific activity' (Table 3), was therefore decreased to 62 and 58% in quiescent as compared with proliferating fibroblasts.

Table 3. *Phosphofructokinase-F-type related antigen concentration and immunological specific activity in proliferating and quiescent cells*

Antigen concentration and the ratio of enzyme activity to antigen concentration (= immunological specific activity) are expressed in arbitrary units, the value 100 being attributable to proliferating cells. The proliferating and quiescent cells were as in Table 2. Antigen concentration was measured by electroimmunodiffusion in a 1% (w/v) agarose gel, in 0.5 M-barbital buffer (pH 8.2) with 50 μl of anti-F-type-phosphofructokinase serum per 200 ml gel. Immunoprecipitate peaks were revealed by the specific tetrazolium-salt-reduction-linked reaction (Kahn *et al.*, 1979).

Expt. no.		Enzyme activity (munits)	Specific activity (unit/mg of protein)	Antigen concn. (%)	$\frac{\text{Enzyme activity}}{\text{Antigen concn.}}$ (%)
1	Proliferating cells	2.6	0.24	100	100
	Quiescent cells	0.4	0.035	22	62
2	Proliferating cells	2.8	0.32	100	100
	Quiescent cells	0.36	0.041	22	58

Synthesis rates of phosphofructokinase, glucose phosphate isomerase and pyruvate kinase

Table 1 shows the synthesis rates of phosphofructokinase, pyruvate kinase and glucose phosphate isomerase in quiescent and proliferating fibroblasts.

Phosphofructokinase F represents about 0.05% of the cytosolic proteins synthesized in proliferating cells and 0.0017% in quiescent ones, i.e. this enzyme is synthesized 30 times more actively in proliferating than in resting cells. The values for pyruvate kinase and glucose phosphate isomerase are 0.069 and 0.058% in proliferating cells, 0.015 and 0.18 in quiescent cells, which corresponds to a difference of 4.5-fold for pyruvate kinase and 3.4-fold for glucose phosphate isomerase.

Discussion

This paper confirms that phosphofructokinase probably plays a major role in stimulation of glycolysis in proliferating cells: its activity is markedly more stimulated in exponentially growing fibroblasts compared with two other glycolytic enzymes, pyruvate kinase and glucose phosphate isomerase. Since fibroblasts contain two main types of phosphofructokinase subunits, the first question is whether enzyme induction involves one of these subunits preferentially. In fact, the ratio F/L subunits does not change with the induction of the enzyme in proliferating cells, which indicates that induction is non-preferential.

The fact that the ratio of labelled F/L subunits was similar whether labelling was performed for 3 days or only for 1 h proves that neither proliferating nor quiescent fibroblasts electively synthesize either of the subunits at any time.

The second question concerns the mechanism of this enzyme induction: are phenomena of enzyme activation–inactivation involved, or is induction due to stimulation of phosphofructokinase synthesis? Our immunological results show clearly that in quiescent cells both enzyme activity and antigen concentration are decreased, which seems to favour the latter hypothesis. However, the 'immunological specific activity' of quiescent-cell phosphofructokinase was lower than in growing cells, which could suggest that a mechanism regulating the enzyme activity might also be involved.

To solve this problem, we directly measured enzyme synthesis in both resting and growing cells. We have previously described the purification of biosynthesized enzymes by immunoaffinity microchromatography (Kahn *et al.*, 1981), a method that we have applied to numerous proteins, especially enzymic proteins (Besmond *et al.*, 1981; Kahn *et al.*, 1981; Marie *et al.*, 1981; Gregori *et al.*, 1982; Uzan *et al.*, 1982). However, to obtain good results, specific immunoglobulins purified by affinity chromatography on antigen-bound absorbent are required. This technique is difficult to apply to F-type phosphofructokinase, which is relatively rare and difficult to purify in sufficient amounts to prepare such a specific immunoabsorbent. This is why to purify fibroblast phosphofructokinase we used affinity chromatography on Blue Dextran–Sepharose 4B as previously reported (Cottreau *et al.*, 1979; Kahn *et al.*, 1980*a*,*b*).

This procedure enables the co-purification of F and L enzymes, which are eluted in the same conditions (Kahn *et al.*, 1980*a*,*b*; Lagrange *et al.*, 1981).

Determination of the radioactivity of phosphofructokinase purified from proliferating and resting cells incubated for 1 h in the presence of high-specific-radioactivity [^{35}S]methionine shows that production of phosphofructokinase is 60 times higher in the former cells than in the latter ones. Compared with total protein synthesis (which is twice as great in growing than in resting cells), the percentage of synthesized enzyme is 30 times higher in growing than in resting fibroblasts. It seems therefore that all the proliferation-associated induction of phosphofructokinase activity is due to increase of the synthesis of the enzyme. The lower immunological specific activity of resting-cell phosphofructokinase is probably explained by the post-translational inactivation of the enzyme previously synthesized at the stage of exponential growth.

Compared with phosphofructokinase, both the other glycolytic enzymes studied here are 7–8 times less induced during cell proliferation. This reinforces the concept that phosphofructokinase is one of the major targets of glycolysis stimulation in proliferating cells.

For pyruvate kinase and glucose phosphate isomerase, as well as for phosphofructokinase, the mechanism of enzyme induction seems to be stimulation of protein synthesis.

In conclusion, cell proliferation of fibroblastic strains in exponential growth is accompanied by a considerable increase in the synthesis of phosphofructokinase and, to a lesser extent, of pyruvate kinase and glucose phosphate isomerase. Both F and L subunits of phosphofructokinase are induced non-preferentially.

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