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Reversion of transformed glycolysis to normal by inhibition of protein synthesis in rat kidney cells infected with temperature-sensitive mutant of Rous sarcoma virus

(aerobic glycolysis/malignant transformation/*src* gene product)

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ABSTRACT **Normal rat kidney cells infected with a temperature-sensitive mutant (LA23) of Rous sarcoma virus exhibit the transformed phenotype when grown at 33° and the normal phenotype at 39°. We have previously shown [Ash, J. F., Vogt, P. K. & Singer, S. J. (1976) *Proc. Natl. Acad. Sci. USA* 73, 3603–3607] that the addition of protein synthesis inhibitors to LA23-infected cells grown at 33° causes them to revert, over a period of 12 hr, to the normal phenotype with respect to morphological and cytoskeletal characteristics. We now show that reversion of the metabolic characteristics of the transformed phenotype to those of the normal also occurs under these conditions. LA23-infected cells show an increased rate of aerobic glycolysis at 33° compared to that at 39°. They also show a different sensitivity of that rate to dinitrophenol and oligomycin at 33° compared to 39°. Such cells grown at 33° in the presence of cycloheximide or abrin rapidly recover the aerobic glycolysis characteristics of the normal phenotype. These results support the thesis that transformation by the *src* gene of the Rous sarcoma virus is a pleiotypic and reversible process, such as is involved in a pleiotypic enzymic modification reaction and its reversal.**

In a previous study (1), we investigated certain of the characteristics of malignant transformation in a particularly favorable experimental system, utilizing a normal rat kidney (NRK) cell line infected with a temperature-sensitive mutant (LA23) of Rous sarcoma virus (2). At 33° (the permissive temperature) these infected cells exhibit the transformed phenotype, and at 39° (the nonpermissive temperature) the cells appear normal; they can be rapidly switched (within hours) from one state to the other by changing the growth temperature. With such cells, we found (1) that certain structural features of the transformed phenotype would revert to normal if the cells were grown *at the permissive temperature* in the presence of inhibitors of protein synthesis. These structural characteristics included overall cell morphology, the degree of organization of intracellular myosin filaments, and the mobilities of concanavalin A receptors in the cell surface. These results seemed to us of great interest because they indicated not only that protein synthesis was required to maintain the transformed state in these cells but also that protein synthesis was not required to revert from the transformed to the normal phenotype, at least with respect to structural characteristics (i.e., some of the components involved in determining those structural characteristics might be altered, but not irreversibly, upon transformation). There is substantial evidence that transformation by the Rous sarcoma virus is effected by the product of a single viral gene, the *src* gene (3). We therefore suggested that our results were consistent with the hypothesis that the *src* gene product "is directly or indirectly involved in a reversible chemical modification" of such structural components in the course of induction of the transformed state.

If this hypothesis were correct, then such reversibility to the normal phenotype on inhibition of protein synthesis in these cells might be pleiotypic and might be true not only for structural characteristics but also for all other features of the normal phenotype, such as its metabolic characteristics. As an indicator of the metabolic state of the cell, we elected to study aerobic glycolysis. There were at least two reasons for this choice. First, aerobic glycolysis is a major route of metabolism even in normal cells under tissue culture conditions (4). Second, the rate of aerobic glycolysis is known generally to be increased upon transformation (5–8), although there is as yet no consensus as to the underlying mechanisms involved.

We report in this paper that LA23-infected NRK cells grown at the permissive temperature (transformed phenotype) do indeed have an enhanced rate of aerobic glycolysis, and an altered sensitivity of that rate to dinitrophenol and to oligomycin (8–10), than when the cells are grown at the nonpermissive temperature (normal phenotype). A shift from nonpermissive to permissive temperature rapidly induces these glycolytic changes, indicating that they are tightly coupled to the transformation process. Furthermore, addition of the protein synthesis inhibitors cycloheximide or abrin to these cells growing at the permissive temperature reverts the glycolytic characteristics of the transformed phenotype, as well as the structural characteristics, to normal. A preliminary report of these findings has been presented (11).

MATERIALS AND METHODS

Abrin, 3-*O*-methylglucose, and ouabain were purchased from Calbiochem (La Jolla, CA). 3-*O*-[^{3}H]Methylglucose (60–90 Ci/mmol), 2-deoxy[^{3}H]glucose (30–60 Ci/mmol), and Aquasol were obtained from New England Nuclear (Boston, MA). Cycloheximide, oligomycin, 2-deoxyglucose, and lactate assay kit 826-UV were obtained from Sigma (St. Louis, MO). Dimilume-30 was bought from Packard (Downers Grove, IL).

Normal and Virus-Infected Cell Cultures. The source and growth properties of NRK and LA23-infected NRK have been described (2). Cells were cultured in Ham's F-12 medium containing 10% fetal calf serum and antibiotics under a 10% CO_2/90% air atmosphere. Cells dissociated with trypsin were replated at a density of 15,000–30,000 cells per 35-mm dish. Cells were grown for 3–4 days at the indicated temperature before experiments. Growth medium was replenished 16–24 hr prior to experimental treatments. Stock cultures of cells were maintained at the nonpermissive temperature (39°).

Treatment with Protein Synthesis Inhibitors. At the indi-

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Abbreviations: NRK, normal rat kidney; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

cated time, either cycloheximide (15 μg/ml) or abrin (200 ng/ml) was added to the dishes from concentrated stock solutions (10 mg/ml in ethanol or 2 mg/ml in medium without serum, respectively). After the contents of the dishes were gently swirled, the dishes were immediately returned to the appropriate incubators for the indicated times for experimental determinations. In all experiments with protein synthesis inhibitors, the cells were given fresh medium 2–4 hr prior to addition of the inhibitors. As noted previously (1) in the experiments with the reversible inhibitor cycloheximide, the cells remain completely viable for up to 24 hr.

Measurement of Aerobic Glycolysis. Assays were done in triplicate by first washing the plates three times with Hanks' balanced salts solution without glucose but with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer at pH 7.4. Dishes were then preincubated for 10 min with gentle shaking with 1 ml of this buffer plus the indicated drugs. The assay was initiated by the addition of 25 μl of 1 M glucose to give a final concentration of 25 mM (all steps at 37°). After 30 min, the solution was removed from the cells and immediately chilled to 0°, and 0.1- or 0.2-ml aliquots were assayed for lactate content by using the Sigma lactate assay kit.

3-*O*-Methylglucose and 2-Deoxyglucose Transport Assays. Assays were done in triplicate essentially as described (10) by washing the cells three times (1 ml per wash) at room temperature in Hanks' balanced salts solution without glucose but with 20 mM Hepes buffer at pH 7.4. The reaction was started by adding either 3-*O*-[^{3}H]methylglucose or 2-deoxy[^{3}H]glucose (1 M stock solution at 0.2 mCi/ml). Time points were taken at 5-sec intervals up to 30 sec. Protein was analyzed on control dishes taken through the same procedure. If care was taken at the initial plating of the cells to give each dish an equivalent amount of cells, there was less than 5% variation in the protein determinations on the 10 control dishes used for each batch of cells.

Hexokinase and Phosphofructokinase Assays. Cells were grown for 3–4 days on 100-mm dishes and then washed three times with 0.25 M sucrose/1 mM EDTA/1 mM 2-mercaptoethanol, pH 8.2 at 0°. They were scraped off the plate and Dounce-homogenized (25 strokes with tight pestle) in 2 ml of this buffer plus 3 mM ATP. Aliquots (0.1 or 0.2 ml) were quickly assayed for activity. Hexokinase activity was determined by the spectrophotometric method of Grossbard and Schimke (12) and phosphofructokinase activity, according to Schneider *et al.* (13). Both assays were done at room temperature.

Protein Determinations. Cells were dissolved with 1 ml of 2.5% deoxycholate for at least 4 hr (usually overnight). The entire liquid phase was removed and the dishes were further washed with 0.5 ml of water. The protein was analyzed by the biuret method (14).

RESULTS

Two sets of experiments, carried out at widely separated times, gave qualitatively similar but quantitatively different results; each set was internally reproducible. These experiments are therefore reported separately, as sets A and B. In set A, without any additions, the rate of lactate production was greater by about 50–60% for LA23-infected cells at the permissive temperature than for the same cells at the nonpermissive temperature or for uninfected NRK cells at either temperature (Table 1). Assays made with added ouabain, an inhibitor of Na^+,K^+-ATPase, showed that cells were all equally inhibited by about 30–40% whether or not they were virus-infected or transformed. When the mitochondrial uncoupler dinitrophenol was added to the assay, all cells showed an increased rate of glycolysis over controls. In order to standardize the latter data, we have also expressed them as the percentage, control rate/dinitrophenol rate. This quantity is 82% for the transformed cells but is only 55–65% for normal cells or for cells infected but not transformed. This represents a significant and reproducible difference. The most striking results involve the addition of the mitochondrial ATPase inhibitor oligomycin. Whereas NRK cells or LA23-infected cells grown at the nonpermissive temperature showed little effect of oligomycin on their glycolytic rates, LA23-infected cells at the permissive temperature had their rates markedly inhibited (>70%).

Table 1. Aerobic glycolytic rates of NRK cells and NRK cells infected with a temperature-sensitive Rous sarcoma virus (LA23)

	Rate of lactate production* at different temperatures					
	Normal cells			Infected cells		
	33°	33°	39°	33°	39°	33°
Set A experiments						
Cycloheximide (15 μg/ml)	−	+	−	−	−	+
Additions to assay:						
None (control)	1.00	0.83	0.99	1.62	1.11	0.93
Ouabain (0.75 mM)	0.69	0.55	0.66	1.01	0.63	0.64
Dinitrophenol (0.1 mM)	1.58	1.54	1.52	1.98	1.74	1.65
Oligomycin, (8 μg/ml)	1.12	0.79	1.05	0.44	1.06	0.83
Control/DNP rate	63%	54%	65%	82%	64%	56%
Oligomycin/control rate	112%	95%	106%	27%	95%	89%
Ouabain inhibition	31%	34%	33%	38%	43%	31%
Set B experiments						
Abrin (200 ng/ml)	−	+		−	−	+
Additions to assay:						
None (control)	0.71	0.65		1.34	0.75	0.66
Ouabain (0.75 mM)	0.79	0.76		1.38	0.75	0.69
Dinitrophenol (0.1 mM)	1.10	1.08		1.59	1.46	1.08
Oligomycin (8 μg/ml)	0.83	0.80		1.33	0.86	0.80
Control/DNP rate	65%	60%		84%	51%	62%
Oligomycin/control rate	117%	123%		99%	115%	121%
Ouabain inhibition	0%	0%		0%	0%	0%

* μmol of lactate/30 min per mg of cell protein measured at 37°; assayed in triplicate; SE, ±0.03.

When set A transformed cells grown at the permissive temperature were treated with cycloheximide, the rate of glycolysis without any other additives was decreased to normal values; the ratio control rate/dinitrophenol rate returned to normal, and the oligomycin sensitivity of the rate was eliminated. In other words, in those aspects in which the rate of lactate production was shown to be different for the transformed compared to the normal phenotype, cycloheximide treatment of the transformed cells caused them to revert to the rate characteristics of the normal phenotype.

Set A experiments also included a study of the time course of the glycolytic rate changes upon changing LA23-infected cells from the nonpermissive to the permissive growth temperature. Within 4 hr after the temperature shift, changes were already detected in the control glycolytic rate and in the effects of dinitrophenol and oligomycin on that rate (Fig. 1). These changes were as rapid as any that have been observed after the

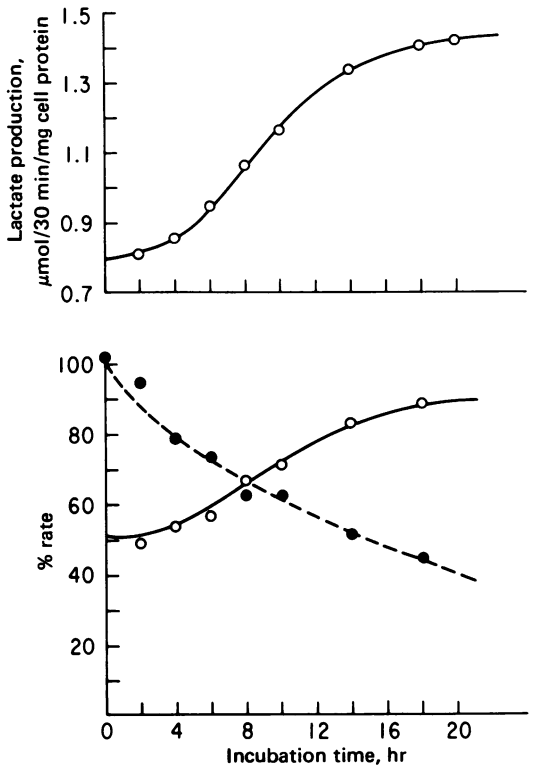

FIG. 1. Time course of changes in aerobic glycolysis characteristics after shift of LA23-infected NRK cells from the nonpermissive (39°) to the permissive (33°) temperature in set A experiments. (*Upper*) O, Control rate of aerobic glycolysis. (*Lower*) O, Control/dinitrophenol; ●, oligomycin/control.

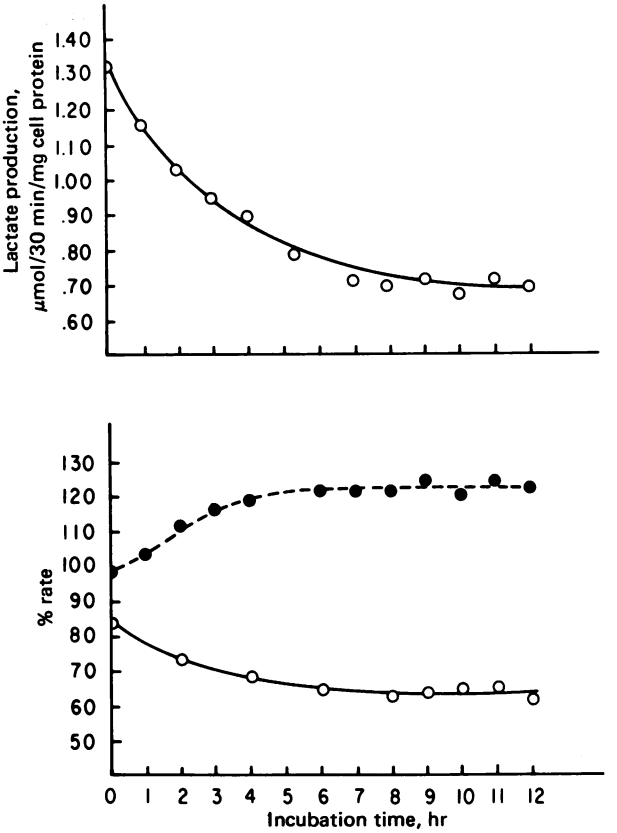

FIG. 2. Time course of changes in aerobic glycolysis characteristics of LA23-infected NRK cells maintained at the permissive temperature after the addition of the protein synthesis inhibitor abrin in set B experiments. (*Upper*) O, Control rate of aerobic glycolysis. (*Lower*) O, Control/dinitrophenol; ●, oligomycin/control.

shift to permissive temperature. Our experiments showed that noticeable changes in myosin filament organization and in cell ruffling over the nucleus (15, 16) occurred by 6–8 hr after the shift with these cells. This suggests that these changes in aerobic glycolysis characteristics are not secondary effects but are closely associated with the process of transformation in these cells.

At this point, the LA23-infected cell line used in the set A experiments was lost. Eventually, the cell line was reestablished from the same stock of infected cells, but with a different lot of fetal calf serum. Although the morphological features found in the experiments of set A were reproduced with the new cells (set B), their aerobic glycolysis characteristics were different although reproducible (Table 1). The control rate of lactate production was again increased (about 80–100%) upon transformation, and the ratio control rate/dinitrophenol rate was again greater for the transformed phenotype than for normal, but oligomycin did not change the glycolytic rate in the transformed cells as it had in the set A experiments. However, oligomycin, reproducibly increased the glycolytic rate by about 20% with cells exhibiting the normal phenotype. The elimination of this increase was thus found to be a characteristic of the transformed state in set B experiments. When these cells were grown at the permissive temperature in the presence of abrin, a return to the characteristics of the normal phenotype was observed as in the set A experiments. These changes toward normal were already noticeable within 2 hr after initiation of the abrin treatment (Fig. 2), and the normal characteristics were fully recovered by 6–8 hr.

In the set B experiments, we looked for other metabolic changes that might be correlated with transformation, so that we could test these also for their reversibility after cycloheximide treatment. Glucose transport, hexokinase activity, and phosphofructokinase activity were investigated. With the same conditions under which LA23-infected cells grown at 33° or 39° exhibited the differences in rates of aerobic glycolysis presented in Table 1, their uptake parameters for the glucose analogues 2-deoxyglucose (V_{max} = 36 nmol/min per mg of protein, K_m = 8 mM); and 3-*O*-methylglucose (V_{max} = 28 nmol/min per mg of protein; K_M = 9 mM) were indistinguishable. These parameters were also essentially the same for uninfected NRK cells grown under the same conditions. The hexokinase and phosphofructokinase activities of extracts of the LA23-infected cells (65 and 135 nmol/min per mg of protein, respectively) were likewise unaffected by the temperature shift.

DISCUSSION

The first objective of these experiments was to find one or more metabolic parameters in the temperature-sensitive LA23-infected NRK cells that were closely correlated with the interconversion between the normal and transformed phenotypes. To this end we focussed initially on aerobic glycolysis. The overall rate of aerobic glycolysis is known to vary with (*i*) the degree of confluency of the cell culture (17); (*ii*) serum stimulation (18); and (*iii*) the glucose concentration used in the lactate production assay (10). We attempted to minimize these variables by (*i*) confining our experiments to subconfluent populations, (*ii*) refeeding the cells with fresh medium containing 10% fetal calf serum within 24 hr of any experiment, and (*iii*) using the saturating concentration of 25 mM glucose for all lactate production assays, thus always measuring effective V_{max} values for glycolysis.

Under these circumstances, we found that the rate of aerobic glycolysis was increased in the LA23-infected NRK cells exhibiting the transformed phenotype compared to the infected cells exhibiting the normal phenotype or to uninfected cells. This result is consistent with earlier findings that chick fibroblasts infected with and transformed by wild-type Rous sarcoma virus exhibit enhanced rates of aerobic glycolysis (10). Furthermore, despite quantitative differences between two separate sets of experiments, significant differences between the transformed and normal phenotypes in LA23-infected NRK cells were also observed in the effects of dinitrophenol and oligomycin, but not of ouabain, on the rates of aerobic glycolysis.

An enhanced rate of aerobic glycolysis is one of the most general characteristics of tumor cells (5–8). The fact that a shift from nonpermissive to permissive growth temperature of the LA23-infected NRK cells induces the changes to the glycolytic properties of the transformed phenotype (Fig. 1) as rapidly as any other phenotypic characteristics we have observed is consistent with an important role for the enhanced aerobic glycolysis in the process of transformation. On the other hand, a general molecular explanation for this enhancement upon transformation is still a matter of controversy (13, 18–23). In one important sense, however, that controversy is irrelevant to our purposes because we have used these glycolytic changes only as markers to differentiate the normal and transformed phenotypes in the LA23-infected cells.* Similarly, the findings that the rates of glucose transport,† hexokinase activity, and phosphofructokinase activity did not differ between the two phenotypes in these cells may be of interest for the mechanism of the enhanced aerobic glycolysis,* but for our present purposes these results were negative—that is, they provided us with no additional markers to distinguish the two phenotypes.

The main result of this study is that the addition of the protein synthesis inhibitors cycloheximide or abrin to the LA23-infected NRK cells *at the permissive temperature* induced the glycolytic properties of the transformed phenotype to return toward normal. The resultant decrease in the rate of aerobic glycolysis (Fig. 2; Table 1) cannot simply be attributed to glycolytic enzyme degradations in the presence of the protein synthesis inhibitors, for several reasons: (*i*) the effects of dinitrophenol and oligomycin on the glycolytic rates also reverted toward the normal phenotype, which would not be expected simply from a generalized decay of glycolytic capacity of the cell; (*ii*) the changes in glycolytic rate, and in the effects of dinitrophenol and oligomycin on that rate, were rapidly and coordinately expressed (Fig. 2) after protein synthesis was arrested; and (*iii*) three other independent protein-mediated processes—glucose transport, hexokinase activity, and phosphofructokinase activity—showed no decreases over a much longer period than was required for the glycolytic changes to be observed. These results are therefore consistent with the conclusion that the aerobic glycolysis characteristics of the transformed phenotype reverted to those of the normal phenotype in these cycloheximide- and abrin-treated cells.

In our previous report (1), we showed in similar cycloheximide and abrin experiments that reversion from transformed to normal phenotype occurred at the permissive temperature, with respect to cell morphology, the structure of intracellular myosin filaments, and the mobilities of surface concanavalin A receptors. We infer, therefore, that the cycloheximide-induced reversion from transformed to normal phenotype is pleiotypic, involving many of the structural and metabolic parameters of the LA23-infected NRK cells.‡ The reversion to the normal phenotype induced by the inhibitors of protein synthesis leads to two conclusions: (*i*) protein synthesis is required to maintain the transformed state in LA23-infected cells at the permissive temperature, presumably because the *src* gene product of the LA23 mutant virus is labile even at 33°; and (*ii*) protein synthesis is *not* required to revert the cells from transformed to normal phenotype (i.e., normal cellular components are not irreversibly altered upon transformation). The latter conclusion is consistent with the hypothesis (1) "that the product of the viral *src* gene is directly or indirectly involved in a reversible chemical modification" of many components in the cell whose functions were "controlled metabolically by a reversible enzymatic modification (e.g., by phosphorylation-dephosphorylation reactions)."

* The investigations by Racker and his co-workers (8–10, 24) provide evidence that the overall glycolytic rates of normal and transformed cells are determined by the steady-state concentrations of ADP and P_i, compared to ATP, that are accessible to the enzymes of the glycolytic pathway. Any cellular activities that increase ADP or P_i at the expense of ATP may accelerate the glycolytic rate. These include, among others, Na^+,K^+-ATPase (involved in Na^+ and K^+ active transport, and inhibitable by ouabain) and mitochondrial ATPase (which is activated to hydrolyze ATP in the presence of the uncoupler dinitrophenol and is specifically inhibited by oligomycin). Thus, our results with ouabain may be interpreted to mean that the glycolytic rates of both normal and transformed LA23-infected NRK cells were, on a percentage basis, equally dependent on the Na^+,K^+-ATPase-catalyzed hydrolysis of ATP. Our dinitrophenol and oligomycin results may signify that, in the transformed phenotype, the mitochondrial ATPase was more uncoupled from electron transport than in the normal phenotype (9, 10)—i.e., generated more ADP and P_i in the transformed than in the normal state. This would explain why dinitrophenol stimulated glycolysis less in the transformed state than in the normal, and why oligomycin inhibited glycolysis more (set A experiments) or stimulated glycolysis less (set B experiments) in the transformed state than in the normal. Our glucose transport results indicate that the enhanced rate of glycolysis in the transformed state cannot be accounted for by an enhanced rate of glucose transport (18, 23) in these cells. Our hexokinase and phosphofructokinase results imply that neither of these kinase activities was responsible for the enhanced glycolytic rate of the transformed phenotype, although it is possible that, during cell rupture for their assay, any differences between these enzyme activities in the intact cells were lost.

† The results obtained on glucose uptake differ from previous observations on this cell line (2). It should be noted that the assay methods differ considerably and the cells used in the present study are serum stimulated. Serum stimulation has been reported (25) to eliminate differences in glucose transport between the normal and transformed phenotypes of chicken embryo fibroblasts transformed by a temperature-sensitive Rous sarcoma virus mutant.

‡ We have obtained some preliminary indirect evidence that mitochondrial structural stability is also affected reversibly by the temperature-sensitive transformation. Because of our inference* that the mitochondrial ATPase becomes more uncoupled from electron transport as an early event in the transformation process, we investigated the mitochondria in these intact cells by immunofluorescent procedures. Cells were lightly fixed with formaldehyde and then stained by indirect immunofluorescence with antibodies specific for cytochrome *c* oxidase (26). After fixation but prior to staining, cells were rendered permeable by brief exposure to Triton X-100. Under these circumstances, we were surprised to find a reproducible reduction in the number (one-third to one-fifth) of stained intact mitochondria per cell in the LA23-infected NRK cells (set B) grown at 33° compared to 39°. Upon growth of these cells at 33° in the presence of cycloheximide, the number of mitochondria apparently reverted to normal. It was then found that, if these cells, after the same light fixation, were rendered permeable by freezing and thawing or by −70° acetone treatment instead of by Triton X-100 treatment, there were no such changes in numbers of stained intact mitochondria with change in phenotype. The explanation we suggest is that, in the transformed phenotype, the mitochondria are for some unknown reason more sensitive, compared to the normal phenotype, to disruption by Triton X-100 after mild formaldehyde fixation and that this sensitivity also reverts to normal after cycloheximide or abrin treatment of the cells at 33°.

As this work was being prepared for publication, it was reported (27) that the product of the viral *src* gene is itself, or is closely associated with, a protein kinase. Our conclusions clearly conform with this finding. The nature of this protein kinase activity is thus of great interest. If it is relatively nonspecific in its protein substrates, the extra phosphorylation it might induce in a wide variety of cellular proteins could itself be the pleiotypic reversible modification reaction that we have proposed.[§] On the other hand, if the protein kinase were highly specific, its action might be to stimulate some other host cell enzyme which in turn produced a nonspecific pleiotypic modification of cellular proteins (which might or might not involve reversible phosphorylation reactions).

One may think of the transformed cell as having been placed into a new steady state, different from that of the normal cell. The steady state of a normal cell is maintained by a complex network of biochemical regulatory mechanisms, an important feature of which is the allosteric control of enzyme activities (cf. 28). It is remarkable that the introduction into the cell of a single protein, the product of the *src* gene, can induce a new steady state. In this connection, it is of great interest that the genome of normal chicken cells contains a gene that is substantially, but apparently not completely, homologous to the DNA that is complementary to the *src* gene of the viral RNA (29–31). This suggests that, if the *src* gene product is indeed a protein kinase, it is closely related to a normal cell protein kinase. The escape from the steady state of the normal cell into the new steady state of the transformed cell could then be simply a dosage effect, in which the *src* gene protein kinase might be functionally the same as the normal cell kinase but present in much larger amounts in the transformed cell. The normal concentrations of its allosteric effectors might be unable to regulate its activity effectively. Alternatively, the *src* gene product might be a mutant form of the normal cell protein kinase, which was normal in its catalytic site but was altered in one or more of its regulatory sites and, because of such alteration, it partially escaped allosteric regulation.

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[§] The ADP liberated by such kinase activity might be a positive modulator contributing to the enhanced aerobic glycolysis in the transformed cells (9, 10).*

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