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Partial Purification and Characterization of the Ribonucleotide Reductase Induced by Herpes Simplex Virus Infection of Mammalian Cells

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In this report we confirm and further characterize the induction of a novel ribonucleotide reductase after herpes simplex virus infection of mammalian cells. Induction of the enzyme was observed at a multiplicity of infection of 1 PFU/cell or greater and was found to be maximal (three- to sixfold the activity in mock-infected controls) at 6 to 8 h postinfection at a multiplicity of infection of 10 PFU/cell. Partial purification and subsequent characterization of the reductase activity from infected cells demonstrated the existence of two enzymes which could be separated by precipitation with ammonium sulfate. One of the activities precipitated at between 35 and 55% salt saturation, as did the enzyme from control cells, whereas the novel activity precipitated at 0 to 35% saturation. This latter enzyme was similar to the herpes simplex virus-induced reductase described by others in its lack of requirement for Mg^{2+} and its resistance to inhibition by dTTP and dATP; in addition, we found that it was inhibited by ATP, whereas the enzyme from control cells displayed an absolute requirement for the nucleotide. Both enzymes were equally inhibited by pyridoxal phosphate and showed similar cold and heat stability. The enzyme induced by herpes simplex virus infection, however, was much more labile than the control enzyme upon purification.

Infection of mammalian cells with herpes simplex virus (HSV) results in the induction of several enzymes involved in DNA synthesis (29). This phenomenon may play an important role in viral infection and perhaps also in the establishment of latency by the virus. Since the activity of DNA-synthesizing enzymes is low or absent in stationary-phase and nonreplicating cells (15, 24), virally induced enzymes could serve to free viral replication from dependence on the cell cycle. Furthermore, some of the induced enzymes have regulatory properties which differ from those of the cellular isozymes (18, 19, 26), and thus they might allow viral replication to continue under conditions which are inhibitory to cellular DNA synthesis.

Of the many enzymes whose induction has been observed after HSV infection, only two are known to be coded for by the virus: thymidine kinase (TK) and DNA polymerase (27, 34; for a review, see reference 29). The current unavailability of mutants (viral as well as cellular) or of assays for biochemical transformation for the other induced enzymes has rendered it difficult to attribute their synthesis to the transcription of a viral gene. Moreover, in some cases either the induction of the enzymes awaits further confirmation or the characterization of the enzymes is still at a preliminary stage.

Induction of an altered ribonucleotide reductase has been observed after HSV infections (19, 26), as well as after Epstein-Barr virus and equine herpesvirus infections (2, 8). The enzyme, which is found in all procaryotic and eucaryotic cells (except erythrocytes and other cells not synthesizing DNA) catalyzes the synthesis of deoxyribonucleotides by direct reduction of the four ribonucleotides, and its activity is closely correlated to the regulation of DNA synthesis (33). The reductase is subject to a complex system of allosteric control, best understood in bacteria (33), which involves nucleotides as both positive and negative effectors. The ribonucleotide reductase induced by HSV has previously been partially purified and found to coprecipitate with the endogenous cellular enzyme at 0 to 40% salt saturation (26). The induced enzyme differs from the control enzyme in its lack of absolute requirement for Mg^{2+} and in its sensitivity to inhibition by dTTP and dATP (19, 26).

In this paper we confirm the induction of an altered ribonucleotide reductase in HSV-infected cells and describe the further characterization of this enzyme. In our system, partial purification of crude extracts from infected cells effected the physical separation of the induced and control activities, with the former precipitating at 0 to 35% salt saturation and the latter

at 35 to 55% saturation. The two enzymes were further found to differ with regard to susceptibility to allosteric inhibition and requirement for Mg^{2+} and ATP.

MATERIALS AND METHODS

Cells. BHK-21 clone 13 (cl.13) and Vero cells (Flow Laboratories) were grown in monolayer in Dulbecco-modified minimum essential medium and in minimum essential medium-F15, respectively; BHK-21 cl.13 cells modified for suspension culture (1) were grown in Joklik-modified minimum essential medium. All media were supplemented with 5% heat-inactivated calf serum and antibiotics.

Virus. For preparation of virus stocks, HSV type 2 (HSV-2) strain 219 (31), HSV-1 cl.101 and the B2006 TK^- mutant derived from it (10) were propagated in Vero cells. The cells were infected at a multiplicity of infection (MOI) of 0.2 to 0.5 PFU/cell, harvested 24 h later, pelleted, and resuspended in medium. Virus was released from the cells by freezing and thawing followed by sonication of the cell suspension. After removal of cell debris by centrifugation, the clarified supernatant was aliquoted and stored at −70°C. Virus titers were determined by plaque assays on Vero cells. For experimental purposes, BHK-21 cl.13 cells growing in monolayers or suspension cultures were infected at an MOI of 7 to 12 PFU/cell, unless otherwise specified. All infections were carried out in medium with 1% calf serum.

Enzyme assays. Ribonucleotide reductase was assayed by monitoring the conversion of CDP to dCDP, using a modified version of the assay described by Moore (22). The standard reaction mixture (in a total volume of 400 μl for crude extracts or 160 μl for partially purified extracts) contained: 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.2), 0.06 mM $FeCl_3$, 2.7 mM magnesium acetate, 8.3 mM NaF, 6.2 mM dithioerythritol (DTE), 4.4 mM ATP, 0.1 mM CDP, and 2.5 μCi of [^{3}H]CDP (specific activity, 21 to 26 Ci/mmol). As assay conditions for partially purified enzymes were optimized in the course of the study, the concentrations of specific cofactors was altered as indicated in Results. The amount of crude extract protein used in the assay is also indicated in Results; unless otherwise specified, partially purified enzymes were assayed with 300 μg of protein per reaction, after ensuring that this amount corresponded to the linear region of the enzyme concentration curve. After incubation for 30 min at 37°C, the reaction was terminated by addition of $HClO_4$ to a final concentration of 1 N. Unlabeled CMP and dCMP were added to final concentrations of 1 mM each as carriers and markers. The nucleotides were then converted to the monophosphate form by boiling for 15 min, after which the samples were cooled on ice and brought to a pH of between 4 and 8 with KOH. The resulting precipitate was pelleted, and aliquots of the supernatant were spotted on Whatmann 3 MM paper and analyzed by descending chromatography with EDTA (250 mM)-ammonium acetate (5 M)-sodium tetraborate (saturated)-ethanol (1:20:80:220, by volume) as solvent (28) for 40 h. Analysis of the chromatograms, using cold nucleotides as markers, demonstrated that after enzymatic reaction and acid hydrolysis the substrate was converted only to dCMP. Moreover, the chromatographic system used resulted in good separation of dCMP from CMP and from contaminants present at low levels in the label.

TK activity was assayed according to Munyon et al. (23).

Preparation and partial purification of crude extracts. Infected or control cells were washed twice with phosphate-buffered saline and usually frozen as a dry pellet at −20°C. All subsequent steps were carried out at 4°C. The cells were thawed or resuspended, or both, in buffer A (20 mM HEPES, 1 mM dithiothreitol (DTT), pH 7.2), sonicated (2 × 30 s, on ice, at the maximum setting in a Bronwill Biosonik cup sonicator), and centrifuged at 70,000 × *g* for 30 min. The supernatant was used as the crude extract. In initial experiments the extracts were passed through a column (of volume equal to that of the extract) of the cation-exchange resin AG1X8 (Bio-Rad Laboratories) to remove deoxynucleotides (25); this step was omitted in later experiments as it gave little or no increase in reductase activity. Partial purification of the crude extracts was carried out as follows. A 5% solution of streptomycin sulfate in buffer A was added to the extracts (dropwise and under continuous stirring) to a final concentration of 1%. The suspension was stirred for 20 min more and then centrifuged at 11,000 × *g* for 15 min. The pellet was discarded, and the supernatant was brought to 35% saturation by the slow addition, under constant stirring, of a saturated solution of $(NH_4)_2SO_4$ in buffer A. After 20 min of further stirring and centrifugation as above, the saturation level was raised to 55% and then again to nearly complete saturation. The pellets obtained from each centrifugation were redissolved in 0.5 to 1 ml of buffer A and filtered through a Sephadex G-25 column (0.8 by 23 cm) in buffer A. Finally, the eluate was brought to a protein concentration of 9 to 12 mg/ml by filtration through Minicon B15 concentrators (Amicon Corp.) and stored at −70°C. Protein concentration was determined by the method of Lowry et al. (21).

Sedimentation on glycerol gradients. Glycerol gradients (5 to 25%) were prepared in buffer A containing magnesium acetate at the concentrations indicated in Results and were centrifuged for 15 h at 39,000 rpm (130,000 × *g*) and 4°C in an IEC swinging-bucket rotor, model SB405. The gradients were fractionated and assayed for reductase activity immediately thereafter. The conditions of the assay for different enzyme preparations are also specified in Results.

Heat stability. Aliquots of induced and control enzymes in buffer A, containing equal protein concentrations, were incubated at the indicated temperatures in a water bath. At the times given in Results, the samples were removed from the bath, supplemented with the complete reaction mixture, and further incubated at 37°C for 30 min. The reaction was terminated and processed as described above.

Chemicals and radiochemicals. [^{3}H]CDP and [^{3}H]thymidine were purchased from New England Nuclear Corp. and used as received. Streptomycin sulfate, ATP, DTT, CDP, and pyridoxal phosphate were obtained from Sigma Chemical Co.; DTE, dTTP, dATP, and the markers for chromatography (CMP,

dCMP, dCDP, dC, dUMP, cytidine, and cytosine) were obtained from Boehringer Mannheim Corp. Ultra-pure ammonium sulfate was obtained from Schwarz/Mann.

RESULTS

Induction of ribonucleotide reductase. Unless otherwise indicated, the results presented in this report describe the induction of ribonucleotide reductase after infection of BHK-21 cl.13 cells with HSV-2 strain 219; however, both HSV-1 and HSV-2 were found to be capable of inducing this enzyme in a variety of mammalian cell types (hamster, human, monkey, mouse). After infection of confluent monolayers of BHK-21 cl.13 with strain 219, an increase in reductase activity could be detected as early as 2 to 4 h postinfection (p.i.); maximal activity was attained at 6 to 8 h p.i. and was followed by a steady decline such that at 10 h p.i. approximately 30 to 60% of the maximum activity remained. This time course is essentially the same as the one illustrated in Fig. 1B, which refers to cells infected with HSV-1. A similar time course was observed for the induction of the well-characterized viral TK. Infection of cells in suspension (not shown) yielded essentially the same pattern except that the time course was much abbreviated. Both reductase and TK activities increased rapidly from 1 h p.i. to a maximum at 3 to 4 h p.i. and then gradually declined. In all subsequent experiments, unless otherwise noted, infected monolayers were harvested at 7 h p.i. and infected suspension cultures were harvested at 4 h p.i.

The induction of the reductase, as well as that of TK, was dependent on the MOI. An increase in the activity of both enzymes could be detected upon infection at an MOI of 1, and the activity was proportional to the input of virus up to an MOI of 10, after which the response persisted at a plateau level until at least an MOI of 50 (Fig. 1A). Under similar experimental conditions, no increase in the activity of deoxycytidine or deoxycytidylate deaminase (6, 30) was observed in a time period ranging from 1 to 10 h p.i. (data not shown). All subsequent experiments on reductase induction were carried out at an MOI of 7 to 12. The degree of induction at a given MOI and at the time of maximum activity p.i. varied between experiments from three- to sixfold the enzyme level in mock-infected controls. This value, however, is likely an underestimate, since in the course of this study it became apparent that the conditions used in the assay of crude extracts were suboptimal for the assay of the induced enzyme. To rule out the possibility that the induction of the reductase resulted from nonspecific elevation of cellular enzymes by the high input of virus, confluent monolayers were infected with the B2006 TK$^-$ mutant of HSV-1 cl.101 at an MOI of 12 and assayed for both ribonucleotide reductase and TK activity (Fig. 1B). If high input of virus results in nonspecific enhancement of cellular enzymes, both reductase and TK should be induced by the TK$^-$ mutant. However, only the induction of ribonucleotide reductase was observed; TK activity did not increase (see Fig. 1B). As expected, wild-type HSV-1 cl.101, included as a control, induced both enzymes (data not shown).

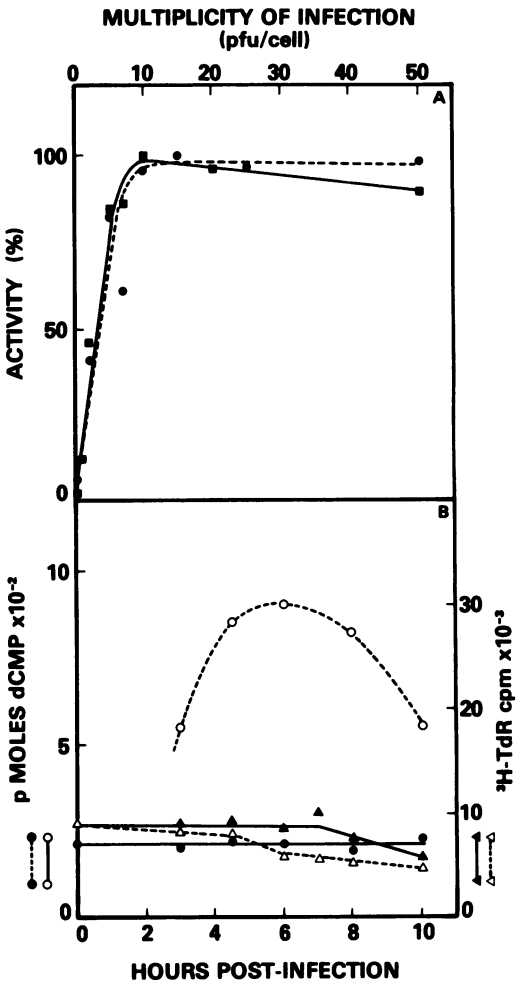

FIG. 1. *(A) Dependence of reductase and TK induction on the MOI. Crude extracts of infected (7 h p.i.) and uninfected cells were assayed for ribonucleotide reductase (●) and TK activity (■) under standard conditions as described in the text. (B) Reductase and TK activity after infection with the B2006 TK$^-$ mutant of HSV-1 at an MOI of 12. Symbols: Reductase activity of infected (○) and uninfected (●) cells; TK activity of infected (△) and uninfected (▲) cells.*

Figure 2 shows the enzyme concentration curve for the reductase from infected and uninfected cells. In both cases, at protein concentrations below approximately 750 μg per reaction, the enzyme activity is not proportional to the protein concentration, whereas a linear relationship exists from approximately 0.75 to 3 mg per reaction. Similar kinetics have been reported by others (11) and seem to be characteristic of ribonucleotide reductase from a variety of cell types. All assays of crude extracts were carried out at 1 mg per reaction.

Partial purification of ribonucleotide reductase. To further characterize the induced reductase, infected and uninfected BHK-21 cl.13 cells growing in suspension were used as source of enzyme. Generally, 2×10^9 to 3×10^9 cells were used for each purification to yield 200 to 300 mg of protein in the 70,000 × *g* supernatant. After streptomycin sulfate precipitation and ammonium sulfate fractionation, approximately 50% of the protein was recovered from both infected and uninfected cells; however, whereas the recovery of reductase activity from control cells was as high as 70%, only 35% of the activity of infected cells was recovered (Table 1). The distribution of the enzyme activity among the ammonium sulfate fractions was also markedly different in the two extracts. Whereas virtually all (85 to 95%) of the activity recovered from uninfected cells precipitated at 35 to 55% salt saturation (fraction 2), up to 55% of the activity recovered from infected cells could be precipitated separately at a lower salt concentration (0 to 35% saturation, fraction 1), with the remainder precipitating at the same salt concentration as the enzyme from uninfected controls. The partial purification thus seemed to effect the physical separation of two enzyme activities from infected cells; this separation, however, was not complete. As described below, the enzyme which precipitated in fraction 1 from infected cells had properties different from those of the enzyme from control cells. On the other hand, the properties of the reductase activity from fraction 2 of infected cells were intermediate between those of the induced and control enzymes, suggesting that this fraction contained a mixture of the two activities. Attempts to maximize the degree of separation by raising the salt saturation level of fraction 1 to 40% resulted in contamination of this fraction with the cellular enzyme from fraction 2. For these reasons, in the data to follow, "control enzyme" refers to fraction 2 of uninfected cells and "induced enzyme" refers to fraction 1 of infected cells.

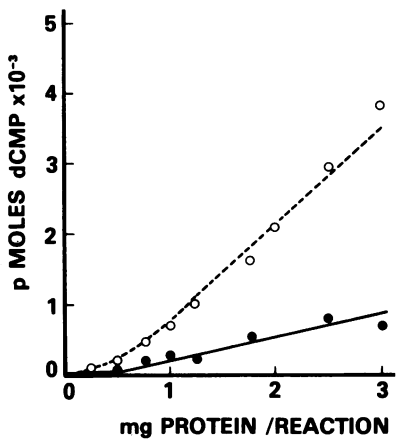

FIG. 2. *Relationship between reductase activity and protein concentrations. Crude extracts were assayed at the indicated protein concentrations under standard assay conditions as described in the text. Symbols:* (○) *infected cells;* (●) *uninfected cells.*

The partial purification resulted in an increase in specific activity of four- to fivefold for the control enzyme and of at least two- to threefold for the induced enzyme (Table 1). This latter value, however, is likely an underestimate since crude extracts of infected cells contain a mixture of control and induced enzyme in relative proportions varying with the degree of induction.

TABLE 1. *Partial purification of ribonucleotide reductase*[a]

Fraction	Fraction no.	Infected cells				Control cells			
		U (total)	Protein (mg)	Sp act (U/mg)	Recovery (%)	U (total)	Protein (mg)	Sp act (U/mg)	Recovery (%)
70,000 × *g* supernatant		120,000	200	600	100	90,000	300	300	100
Streptomycin sulfate supernatant		—[b]	—	—	—	—	—	—	—
$(NH_4)_2SO_4$ (0–35%)	1	23,250	17	1,370	19	800	16	50	1
$(NH_4)_2SO_4$ (35–55%)	2	22,000	24	917	18	58,500	45	1,300	65
$(NH_4)_2SO_4$ (55–>95%)	3	2,400	55	44	2	6,000	80	75	7

[a] Enzyme activity was assayed under standard conditions, using 1 mg of protein per reaction. One unit of ribonucleotide reductase activity is defined as the amount of enzyme which catalyzes the formation of 1 pmol of dCMP in 30 min at 37°C.

[b] —, Not done.

After partial purification, the induced and control enzymes were characterized with regard to the following parameters.

Susceptibility to inhibition by dTTP or dATP. As already mentioned, it has been reported that the HSV-induced ribonucleotide reductase is refractory to inhibition by dTTP and dATP (19, 26). To ascertain whether the activity in fraction 1 of infected cells actually represented the altered HSV-induced activity, this fraction and the control enzyme were tested for susceptibility to both allosteric inhibitors (Fig. 3A). Whereas the control enzyme was inhibited by 50% at a 0.05 mM concentration and by 80 to 95% at a 1 mM concentration of either deoxynucleotide, the induced enzyme behaved as the reported HSV-induced activity and was only inhibited up to a maximum of 20% by 1 mM dTTP or dATP. Fraction 2 of infected cells was inhibited by 60% at 1 mM dTTP (data not shown), suggesting that this fraction contained some of the induced enzyme as well as the control enzyme.

Effect of ATP. ATP is a positive effector which regulates the overall activity of the mammalian reductase (33). The control enzyme had maximal activity at 3 mM ATP and was inhibited by 95% in the absence of ATP (Fig. 3B). The induced enzyme, on the other hand, showed maximal activity only in the absence of exogenous ATP and was inhibited by 65% at 3 mM ATP. As described in Materials and Methods, the fractions resulting from $(NH_4)_2SO_4$ precipitation were filtered through a Sephadex G-25 column as the last step in partial purification and thus should have been relatively free of endogenous ATP. The possibility remained, however, that removal of the nucleotide might have been incomplete or unequal in different samples, especially if endogenous levels of ATP were higher in infected than in control cells. To test this possibility, aliquots of the induced and control enzymes were extensively dialyzed against buffer A at 4°C and again assayed at different ATP concentrations. The results obtained for both enzymes were the same as those shown in Fig. 3B for the undialyzed fractions, indicating no interference by residual ATP in the preparations.

As described previously, crude extracts had been assayed not only without removal of endogenous ATP but also in the presence of 4.4 mM exogenous ATP. On the basis of the results of Fig. 3B, therefore, we can conclude that the total amount of reductase activity detected in extracts from infected cells, as well as the observed degree of induction, were likely underestimated.

Effect of pyridoxal phosphate. In light of the differences thus far observed in the regulatory properties of the induced and control enzymes, the effect of pyridoxal phosphate was tested. Pyridoxal phosphate inhibits a wide range of enzymes by reacting with the ϵ-NH_2 groups of lysyl residues to form a Schiff base. In particular, the compound has been shown to reversibly inhibit mammalian ribonucleotide reductase, possibly by interacting with the allosteric site of the enzyme (9). Despite the altered regulatory properties of the HSV-induced reduc-

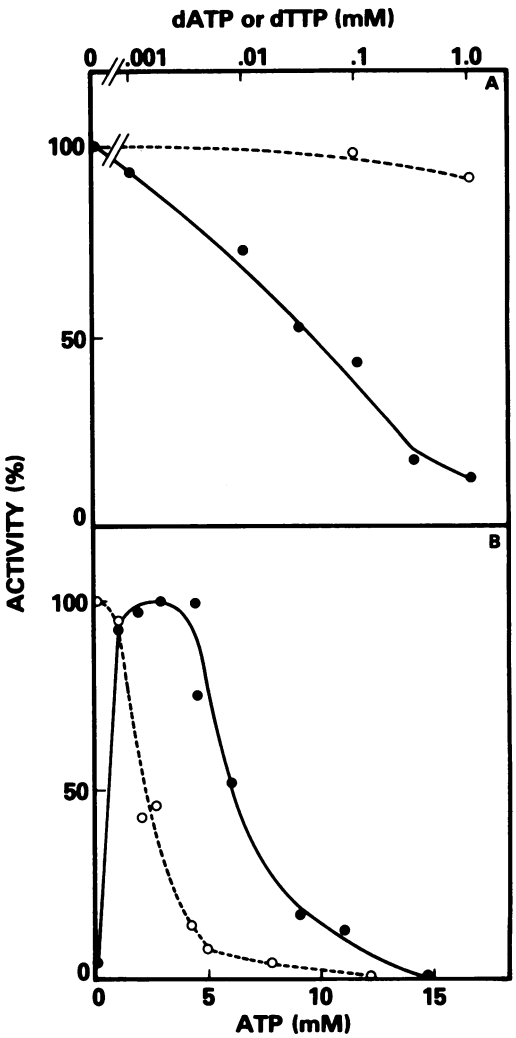

FIG. 3. *Response of the induced* (○) *and control* (●) *enzymes to the allosteric effectors dATP, dTTP, and ATP. (A) Effect of dTTP and dATP. The enzymes were assayed under standard conditions as described in the text. (B) Effect of ATP. The control enzyme was assayed at 5 mM magnesium acetate, and the induced enzyme was assayed in the absence of magnesium acetate (see text).*

tase, however, both induced and control enzymes proved to be equally susceptible to inhibition by this compound; in either case enzyme activity was reduced by 65% at 1 mM and by approximately 90% at 3 mM pyridoxal phosphate (data not shown).

Requirement for Mg^{2+}. Mg^{2+} is required by ribonucleotide reductase for the binding of the two subunits of the enzyme, which is in turn necessary for enzymatic activity. In our system, the control enzyme displayed a broad peak of maximum activity between 5 and 10 mM Mg^{2+} and was inhibited by over 85% in the absence of the ion. The induced enzyme, on the other hand, did not require exogenous Mg^{2+} and indeed retained between 70 and 90% of its activity in the absence of the ion. Similar observations have been reported by others (26). When exogenous Mg^{2+} was added, the following response was obtained for the induced enzyme: an initial decrease in activity between 1 and 3 mM Mg^{2+} was followed by an increase to maximum levels at about 6 mM, which persisted until at least 20 mM Mg^{2+}. We have thus far been unsuccessful in further clarifying the Mg^{2+} requirement of the induced enzyme. The presence or absence of Mg^{2+} could nevertheless be used as a criterion for distinguishing between the induced and control enzymes, and thus we routinely assayed the induced enzyme in the absence of the ion and the control enzyme in the presence of 5 mM magnesium acetate.

Enzyme stability. In view of the low recovery obtained during purification of the enzyme from infected cells (35%), compared with that from control cells (70%), experiments were carried out to determine whether this resulted from a difference in the stability of the two enzymes. Both enzymes, however, were found to be equally stable upon incubation at 4°C for at least 24 h or upon storage at −70°C for up to 8 months: no loss of activity was detected in either case. In addition, both enzymes were inactivated at approximately the same rate and to the same extent by preincubation at 50°C (Fig. 4). Similar results were also obtained at 46 and 42°C (data not shown).

Sedimentation properties of the enzymes. In an attempt to distinguish, and possibly further purify, the induced and control enzymes on the basis of their sedimentation properties, crude or partially purified extracts from HSV-2- or mock-infected cells were centrifuged through glycerol gradients as described in Materials and Methods. In all cases, equal amounts of enzymes (in terms of units and protein concentration) were loaded on each gradient. The gradient fractions were assayed under the following conditions: the control enzyme was assayed by raising the Mg^{2+} concentration in the standard reaction mixture to 5 mM, whereas the induced enzyme was assayed in the absence of ATP and either at 5 mM Mg^{2+} or without addition of Mg^{2+}. (Due to the presence of magnesium acetate in the gradients, the actual final concentration of the ion in this latter case was approximately 1 mM.) Crude extracts from infected cells, containing unfractionated induced and control enzymes, were assayed both in the presence of 3 mM ATP and 5 mM Mg^{2+} and in the absence of the cofactors to optimize either one of the two activities or to inhibit the other.

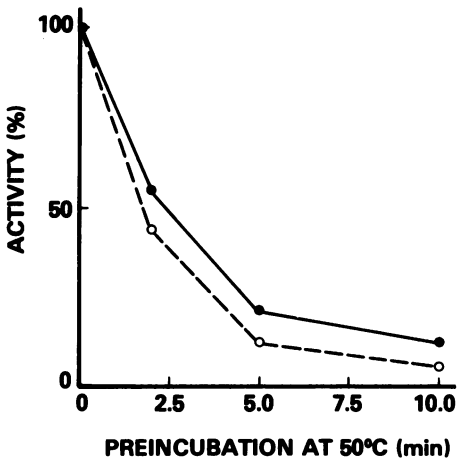

FIG. 4. *Effect of preincubation of the enzymes at 50°C. Aliquots of both enzymes were preincubated at 50°C for the indicated periods of time and assayed immediately thereafter. The assays were carried out at the optimal Mg^{2+} and ATP concentrations described in the text for the induced* (○) *and control* (●) *enzymes.*

Recovery of the control enzyme was strictly dependent upon the presence of Mg^{2+} in the gradients (Fig. 5); approximately 50% of the input activity was recovered when 6 mM Mg^{2+} was present, whereas only 20% could be recovered at 4 mM. No recovery at all was obtained when the gradients were prepared without magnesium acetate. On the other hand, sedimentation of the induced enzyme, whether from partially purified or crude extracts, always resulted in complete loss of activity (data not shown). This was the case even when the glycerol gradients were prepared in the complete reaction mixture except for the labeled substrate. Since, as described earlier, both control and induced enzyme exhibited the same stability at 4°C and upon dialysis, we examined the possibility that the loss of the induced activity resulted from its being separated from an accessory (and essential) protein moiety with different sedimentation properties. If this was the

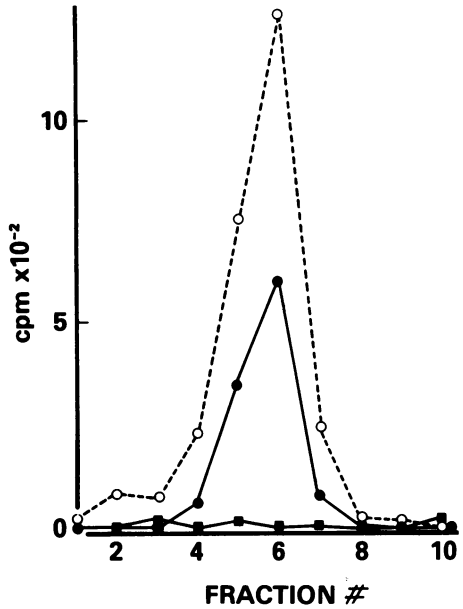

FIG. 5. *Sedimentation of the control enzyme in glycerol gradients: dependence on magnesium acetate. Aliquots of the enzyme preparation corresponding to 1 mg of protein and 1,500 enzyme units were loaded on 5 to 25% glycerol gradients containing 6 mM* (○), *4 mM* (●), *or no* (■) *magnesium acetate. After centrifugation and fractionation as described in the text, fractions were assayed under standard conditions except that the concentration of magnesium acetate was raised to 5 mM. Sedimentation is from left to right.*

case, however, the separation may be irreversible since we were unsuccessful in attempts to reconstitute enzymatic activity by pooling fractions of the gradient.

DISCUSSION

HSV-infected mammalian cells exhibit a novel ribonucleotide reductase, in addition to the endogenous cellular enzyme. The two activities can be differentiated on the basis of their susceptibility to inhibition by dTTP and dATP, requirement for Mg^{2+}, and response to ATP; these observations have been described in part by other authors in cells infected with HSV (19, 26) or equine herpesvirus (8). In addition, as described in this paper, they can be separated by precipitation of infected cell extracts with ammonium sulfate, such that the induced enzyme is obtained essentially free of the endogenous one.

These observations could be interpreted as indicating that viral infection results in modification of part (but not all) of the cellular enzymatic activity or induces a normally nonexpressed cellular isozyme. These effects, however, would have to be directed specifically at the reductase, since no alteration in the activity of other enzymes, such as deoxycytidine or deoxycytidylate deaminase, has been observed by us or by others (19). Our results are also compatible with the hypothesis that the novel reductase is coded for by the virus. In support of this hypothesis is the observed similarity in the kinetics of induction of reductase and TK, a similarity which further suggests that, as observed for enzymes involved in viral DNA synthesis, the reductase might belong to the β class of viral polypeptides (14). Indeed, that HSV codes for at least two enzymes for DNA replication (TK and DNA polymerase [27, 34]) suggests that the virus may be fairly self-sufficient in this process.

Although an understanding of the function of the induced reductase in HSV infections awaits further characterization of this system, there exists a variety of situations where a role for this enzyme (and for TK and DNA polymerase as well) could be envisaged. For example, induction of a reductase could be required for viral replication in resting cells, in which the levels of the cellular reductase and the pool of DNA precursors are very low (15, 24, 33). Moreover, as suggested by the inability of mutant TK^- virus to persist in neural ganglion cells (12, 32), enzymes such as the induced reductase could also be involved in the establishment of viral latency. Lastly, given that infection with HSV reportedly results in elevated intracellular levels of dTTP (7, 15), likely due mostly to the viral TK (16), a reductase insensitive to allosteric inhibition by the deoxynucleotide may be essential for viral DNA synthesis to continue.

An interesting parallel exists between the induced enzyme described in this report and the one induced after infection of *Escherichia coli* with bacteriophage T4. T4 also codes for a ribonucleotide reductase which precipitates at a lower salt saturation than the bacterial enzyme, is resistant to dATP, and does not require exogenous Mg^{2+} (3–5). The bacterial enzyme, on the other hand, is inhibited by the deoxynucleotide as well as by the absence of Mg^{2+} (33). The relevance of this comparison to the HSV system lies in the observation that T4 codes also for its own thioredoxin, a small protein which acts as hydrogen donor during ribonucleotide reduction. The T4 reductase, moreover, shows specificity for this thioredoxin and is unable to use the cellular analog (3, 5). It is tempting to speculate that the similarity between the T4 and HSV systems might extend to the synthesis of a specific thioredoxin preferentially used by the HSV-induced reductase over other hydrogen donors, such as the cellular thioredoxin or the

dithioerythritol added to the reaction. If this were indeed the case, the relative instability of the virally induced enzyme during purification could result from its partial or complete separation from the viral thioredoxin.

Direct evidence for the viral origin of the reductase could derive from correlating viral mutants with the absence or alteration of the induced enzyme. Convincing evidence could also be obtained by a number of other approaches, such as the purification of antibodies directed against the enzyme or the development of a cell transformation assay. In the case of TK, the existence of TK^- viral and cell mutants (10, 17, 20), of the HAT selection system for TK^+ cells (20) and of a method for gene transfer (13) were all essential tools for establishing the viral origin of the enzyme. Neither viral nor cellular mutants deficient in reductase are available; however, this requirement might be bypassed by devising a selection system based on the differential properties of the induced and cellular reductases. Thus, high concentrations of exogenous deoxynucleosides, resulting in elevated intracellular pools of triphosphates, might allow survival only of cells expressing the induced enzyme. Under these conditions, transformation of cells with restricted viral DNA fragments might ultimately result in the mapping of a viral reductase gene.

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