IMMOBILIZATION OF RIBONUCLEOTIDE REDUCTASE FROM *Streptomyces aureofaciens*

Peter HALICKÝ[a], Marta KOLLÁROVÁ[a], Pavol KOIŠ[b] and Ján ZELINKA[a]

[a] *Department of Biochemistry, Komenský University, 842 15 Bratislava and*
[b] *Institute of Biochemistry and Biotechnology, Komenský University, 842 15 Bratislava*

Received July 29, 1988
Accepted February 1, 1989

Ribonucleotide reductase from *Streptomyces aureofaciens* (E.C.1.17.4.) has been isolated. The effects of concentration of substrate, effector, coenzyme, reductant, pH, and ionic strength upon the activity of this enzyme have been studied and the data obtained by research have been completed. Based on some properties of this enzyme, we have chosen a proper hydrophobic carrier--Trityl-agarose for immobilization. It has been found out that the properties of the immobilized enzyme are significantly different from those of the free enzyme. Principal differences are in the loss of the effector dependence of the immobilized enzyme and in a significant increase of its stability.

The application of enzymes in practice hinges on the possibilities of a simple and cheap acquisition of the necessary enzyme and on both theoretical and practical knowledge of enzyme kinetics and construction of enzyme reactors. This field evolves from application of free enzymes to the use of immobilized enzymes, and from enzymes with simple kinetics to those that exhibit complex kinetic features.

Ribonucleotide reductase (EC 1.17.4.), a key enzyme in the pathway of DNA biosynthesis in all organisms, is undoubtedly an enzyme with highly complex kinetics. Attempts at immobilizing it are therefore not only practically useful but also theoretically valuable.

Chemically, ribonucleotide reductase catalyzes the reduction of a secondary alcohol by dithiol[1]. All currently known ribonucleotide reductases appear to accomplish this reaction by an essentially identical mechanism which has the chatacter of a complex radical reaction[2-4]. Structurally ribonucleotide reductases are divided into three groups according to the character of the radical of the enzyme[1]. In enzymes belonging to group one and three, the stable tyrosyl radical is generated in the molecule via the action of a non-home Fe^{III} or Mn^{II}. These enzymes have a subunit structure. Enzymes of group two exhibit catalytic activity even in the form of a monomer composed of a single polypeptide chain; here the radical is generated by a homolytic cleavage of a C-Co bond in coenzyme B_{12}. Ribonucleotide reductase

from *Streptomyces aureofaciens* belongs to the second group of ribonucleotide reductases. The fact that it has no subunit structure[5] makes it especially suitable for immobilization experiments.

The kinetic analysis of action of ribonucleotide reductases of this class is based on the fact that the reduction of individual substrates is affected by pH, ionic strength, concentration of divalent cations (especially Mg^{2+} and Ca^{2+}), coenzyme B_{12} and the character and concentration of the reductant, effector and substrate. The reaction is based on a ping-pong mechanism of reduction of the enzyme molecule after each elementary catalytical event[6-13].

Our study of the properties of ribonucleotide reductase from *Streptomyces aureofaciens* was based on our previous results[9] which can be summarized as follows: optimum pH for the activity of the enzyme is between 8·0 and 8·5; optimum temperature is 30°C; Na^+, K^+, NH_4^+ in concentrations of 5 mmol l^{-1} have no effect on enzyme activity, Mg^{2+} and especially Ca^{2+} markedly stimulate the activity; optimum concentration of Ca^{2+} is 2·5 to 7·5 mmol l^{-1}; optimum concentration of dATP functioning as an effector for reduction of CDP and CTP is about 50 μmol l^{-1}; optimum concentration of coenzyme B_{12} is about 10 μmol l^{-1}; ionic strength in the range of 0·04 to 0·17 mol l^{-1} has a progressively inhibitory effect; the best reductant is dithioerythritol with an optimum concentration of 20 mmol l^{-1}, 2-mercaptoethanol is effective only to 20%; the effector for reduction of ADP and ATP is dGTP, for SDP it is dATP, for reduction of CTP both dATP and dCTP act as effectors, the same holds for reduction of GTP, no effector was found for UDP.

Our work aimed at further elaborating and extending these data; it also included an attempt at immobilization of the enzyme and determination of its properties.

EXPERIMENTAL

Solutions: A: 50 mmol l^{-1} Tris-HCl, 28 mmol l^{-1} 2-mercaptoethanol, 20 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} EDTA, pH 8·2. B: as A but with 10 vol.% glycerol. C: 50 mmol l^{-1} Tris-HCl, 28 mmol l^{-1} 2-mercaptoethanol, 20 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} EDTA, 10 vol.% glycerol, pH 8·2. D: 50 mmol l^{-1} Tris-HCl, 28 mmol l^{-1} 2-mercaptoethanol, 20 mmol l^{-1} $MgCl_2$, 2 mmol l^{-1} EDTA, pH 7·4. E: 25 mmol l^{-1} Tris-HCl, 28 mol l^{-1} 2-mercaptoethanol, 10 mmol l^{-1} $CaCl_2$, 2 mmol l^{-1} EDTA, pH 8·2.

Preparation of the enzyme: The enzyme preparation was obtained from an industrial strain of *Streptomyces aureofaciens* BMK. Cultivation was carried out according to previous work[14].

Frozen mycelium was broken to smaller parts. It was then placed in a blender with ice-packed cooling jacket, supplemented with the same weight amount of glass beads No. 8 (Jablonec Glass Works) and a double amount of buffer B, and homogenized 30 times for 30 s with 1 min breaks for cooling. The homogenate was transferred into a vessel in an ice bath and sonicated (20 kHz, 100 W) 5 times for 30 s, with 1 min breaks.

The homogenate was partially clarified by centrifugation and the absorbance of the supernatant (after a suitable dilution) was measured at 260 nm. Buffer B was used to adjust the A_{260} of the homogenate to 60 ml^{-1}. One-fourth volume of chilled distilled water was added and precipitation

was performed with 10 wt.% solution of polyethyleneimine, pH 8, to a final concentration of 0·2 wt.%. The mixture was stirred for 30 min at 0°C, the precipitate was separated by centrifugation 40 min at 1 500 *g* and proteins were precipitated from the supernatant with ammonium sulfate to 80% saturation (0·56 g ml^{-1}). The sediment was again stirred for 30 min at 0°C, centrifuged for 40 min at 1 500 *g*, dissolved in a minimum volume of buffer B and dialyzed overnight against buffer A. The dialyzate was supplemented with glycerol to 10 wt.% concentration and applied on a DEAE cellulose column. The column was washed with buffer C and eluted with a linear gradient of 0·05—0·3 mol l^{-1} KCl in buffer C. Fractions with ribonucleotide reductase activity were pooled and precipitated by dialysis against a saturated ammonium sulfate solution with 28 mmol l^{-1} 2-mercaptoethanol, pH 8·2, overnight. The precipitated proteins were centrifuged, the sediment was dissolved in a minimal volume of buffer D and applied on a column of Sephadex G-150 equilibrated with the same buffer. Fractions with ribonucleotide reductase activity were pooled and loated on to a column of Heparin-Sepharose or Blue-Sepharose equilibrated with buffer D containing 50 mmol l^{-1} KCl. The Heparin-Sepharose column was eluted with a linear gradient of 0·05—0·2 ml l^{-1} KCl in buffer D, Blue-Sepharose was eluted with a linear gradient of 0·05—0·3 mol l^{-1} KCl. Fractions with ribonucleotide reductase activity were pooled and dialyzed overnight against buffer A with 50 mol.% glycerol. The concentrated enzyme preparation was stored at −13°C. Prior to use the enzyme preparation was diluted with the same volume of buffer A and glycerol was removed by chromatography on a Sephadex G-25 column.

This procedure yielded an enzyme preparation purified about 150-fold with a yield of some 25% which had a specific activity of 30—50 pkat mg^{-1} protein.

Protein concentration was determined spectrophotometrically[15]:

$$c_B = 0{\cdot}4\,(A_{235} - A_{280})\ \text{mg ml}^{-1} \qquad (I)$$

Enzyme immobilization: A column of inner dimensions of 0·35 × 10 cm was packed with an appropriate amount of Trityl-Sepharose[16] (40 or 100 µl) with trityl concentration of 75 µmol . . per ml of carrier, and equilibrated with buffer A. The buffer was allowed to seep into the gel and the column outlet was closed. The enzyme preparation in buffer A was placed on the column and resuspended with the carrier. The carrier was left to sediment during the stopped flow and the column was washed with buffer D.

Determination of enzyme activity: Ribonucleotide reductase (RR-ase) activity was assayed according to our previous work[5]. This procedure is a modification of the method described by Gleason[17].

A standard reaction mixture containing in 1 litre: 25 mmol l^{-1} Tris-HCl pH 8·2, 10 mmol l^{-1} $CaCl_2$, 50 mmol l^{-1} dithioerythritol (DTE), 1 mmol l^{-1} EDTA, 50 µmol l^{-1} dATP, 10 µmol l^{-1} coenzyme B_{12}, 20 µmol l^{-1} (^{14}C) or (^{3}H)-CTP with a specific activity of about 0·5 GBq mmol l^{-1}

The final volume of the reaction mixture was 50 µl. Enzyme samples were usually added in a 5 µl volume. The reaction was started by adding coenzyme B_{12} in a faint red light.

Determination of activity of the immobilized enzyme: All measurements with the immobilized enzyme were performed in microcolumns with thermostated water jackets. A volume of 100 µl (or 40 µl) Trityl-Sepharose with immobilized enzyme was overlaid with 90 µl (35 µl) of a reaction mixture thermostated at 30°C. The column outlet was opened, the reaction mixture was allowed to just seep into the column packing and the outlet was again closed. For a certain time the column was incubated in the dark, buffer E was layered on the carrier and the reaction mixture was eluted from the column in a total volume of 250 µl (150 µl). The stationary efficiency factor[19] (relative activity) of the immobilized enzyme was determined as follows. A portion of the enzyme

preparation before immobilization, V_s, was taken for the determination of the initial rate of the enzyme reaction in solution. Another portion, V_e, was immobilized on Trityl-Sepharose and the initial rate was also determined. The stationary efficiency factor was calculated from the formula:

$$\eta_{st} = V_s/V_e \cdot v_{0,i}/v_{0,v} \cdot V_c/V_{rm}\,, \qquad (2)$$

where V_c, V_{rm} is the volume of the carrier in the column and the volume of the reaction mixture.

Analysis of the reaction mixture: The reaction mixture was analyzed by chromatography on Whatman No. 3 paper according to Sato et al.[19]. To speed up the separation, the nucleotides in the reaction mixture were first converted to nucleoside monophosphates by means of potato apyrase. The degree of substrate conversion was calculated from the formula:

$$\eta = [(n_d - n_p)/(n_d + n_r - 2n_p)] - \eta_b\,, \qquad (3)$$

where n_d, n_r, n_p are respective counts per minute in the spot with dNMP, NMP, and the background, η_b is the "conversion" degree determined in a control sample containing buffer instead of the enzyme preparation.

RESULTS AND DISCUSSION

Properties of the free enzyme effect of pH: The effect of the pH of the reaction medium on CTP reduction was studied in some detail in the pH range of 6·4–9·6 in four different buffers: imidazole–HCl, Tris-HCl, triethanolamine–HCl and glycine–NaOH. The concentration of the buffers in the reaction mixture was 80 mmol l^{-1}. The solutions were not adjusted to a constant ionic strength.

The dependences (Fig. 1) show that in the pH range of 7 to 9 the activity of the enzyme does not display substantial changes but depends strongly on the type of the buffer used. The effect of pH on reduction of CDP was not studied because our previous

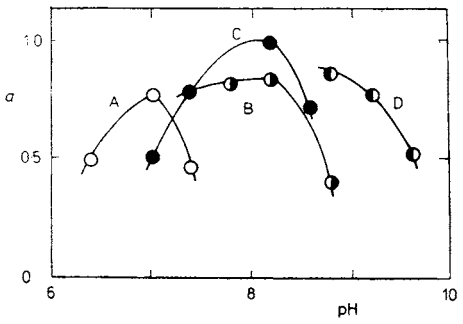

FIG. 1

Effect of pH on CTP reduction. A imidazole–HCl, B Tris-HCl, C triethanolamine–HCl, D glycine–NaOH; *a* relative enzyme activity

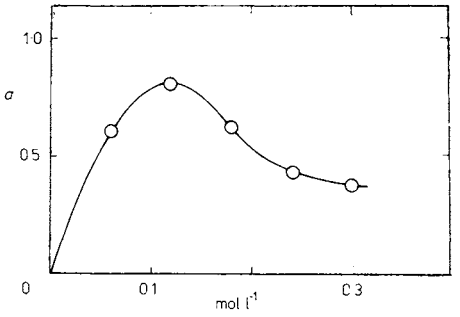

FIG. 2

Dependence of relative activity (*a*) of RR-ase from *S. aureofaciens* on 2-mercaptoethanol concentration

results[9] indicated that the overal dependence should not markedly differ from that for CTP. Likewise, the effect of pH on the reduction of other substrates was not investigated because of insufficient data about the appropriate concentrations of the substrate, effectors and coenzyme B_{12}. Since the pH dependence of the enzyme activity was the least pronounced in Tris-HCl buffer, this buffer was used in all subsequent experiments at pH 8·2.

Effect of 2-mercaptoethanol concentration: Our earlier results[10] indicated that, in contrast to other ribonucleotide reductases, the enzyme from Streptomyces aureofaciens can also use a monothiol, 2-mercaptoethanol, as a reductant. As shown in Fig. 2, even at relatively very high concentrations, about 0·12 mol l^{-1}, 2-mercaptoethanol achieves about 80% afficiency relative to dithioerythritol. At still higher concentrations it has an inhibitory effect.

Effect of effectors: Because of a complete lack of data about suitable concentrations of substrates, we have chosen the following concentrations: CDP, ADP each 10 μmol l^{-1}, CTP and ATP each 20 μmol l^{-1}. At these substrate concentrations a tentative measurement was performed of the dependence of the reduction rate on the concentration of effectors. The selection of suitable effectors was based on our previous findings[9]. The effectors for CDP and CTP was dATP (and also dCTP for CTP), while dGTP was used as effector for ADP and ATP. The dependence of the rate on effector concentration was found to have a saturation character with saturation concentrations of 50 μmol l^{-1} for reduction of cytidine nucleotides and 20 μmol l^{-1} for reduction of adenine nucleotides.

Dependence of the rate of enzyme reaction on substrate concentration: The effect of concentration of coenzyme B_{12} on the rate of the enzyme reaction has been decribed previously[10] and the present study could concentrate on the determination of the dependence of the reaction rate on substrate concentration. The results of measurements in this direction are summarized in Fig. 3.

It is noteworthy that the reduction of CDP and CTP in the presence of dATP has the character of a substrate-inhibited reaction whereas the reduction of CTP in the presence of dCTP, and reduction of ADP and ATP in the presence of dGTP, has an apparently conventional saturation character. When the dependence of the rate of reduction of CDP in the presence of dATP on substrate concentration is approximated by the classical kinetic equation for reaction rate with substrate inhibition[20]

$$v = V \frac{s}{(K_1)_{ap} + s + s^2/(K_2)_{ap}}, \qquad (4)$$

where v is the reaction rate, V is the maximal reaction rate, s is the substrate concentration and $(K_1)_{ap}$, $(K_2)_{ap}$ are apparent kinetic constants and when the other dependences

are approximated by the Michaelis–Menten equation:

$$v = V \frac{s}{(K_m)_{ap} + s}, \tag{5}$$

where $(K_m)_{ap}$ is the apparent Michaelis–Menten constant, the following values of apparent kinetic constants can be determined for individual substrates (Table I).

The dependence of the reduction rate on the concentration of uridine and guanine nucleotides was not determined because the data on the effect of effectors[10] implied that the dependence will be analogous.

TABLE I

Apparent kinetic constants of reduction of cytidine and adenine nucleotides

Substrate	CDP	CTP	CTP	ADP	ATP
Effector	dATP	dATP	dCTP	dGTP	dGTP
$(K_m)_{ap}$ (μmol l^{-1})	20[a]	40[a]	33	7	7
$(K_2)_{ap}$ (μmol l^{-1})	5	12·5	—	—	—

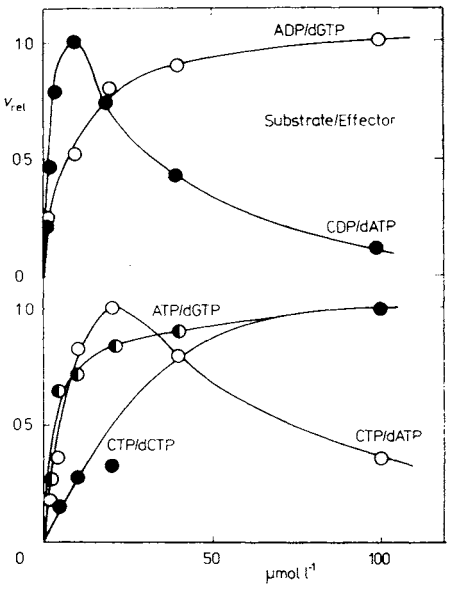

FIG. 3

Dependence of relative reaction rate (v_{rel}) on substrate concentration. The reference value in both graphs is the maximal measured reaction rate for a given substrate

Dependence of reduction rate on coenzyme B_{12} *concentration*: The last factor to be optimized was the concentration of coenzyme B_{12} in the reaction medium. As the rate of reduction of NDP and corresponding NTP depends on substrate concentration in an essentially identical way, and the reduction of NTP to dNTP is more interesting from the practical point of view, we focused our work on studying the dependence of the reduction rate on coenzyme B_{12} concentration for CTP and ATP, with appropriate effectors at 50 μmol l^{-1} concentrations. As seen from the results (Fig. 4 and Table II), optimum concentration of coenzyme B_{12}, i.e. the concentration at which the initial reaction rate is the highest, depends on the type and concentration of both, the substrate and the effector. In all three cases a pronounced inhibition occurs at higher concentrations of the coenzyme.

Properties of the Immobilized Enzyme

Selection of suitable mode of immobilization: Decomposition of coenzyme B_{12} can be taken as a parasitic activity of ribonucleotide reductase, concomitant with the reduction of ribonucleotides. This parasitic reaction has the character of a reaction

TABLE II

Basic characteristics of the effect of coenzyme B_{12} on the reduction of ATP and CTP

Substrate	ATP	CTP	CTP
Concentration (μmol l^{-1})	100	20	100
Effector	dGTP	dATP	dCTP
$(K_I)_{ap}$ (μmol l^{-1})	0·13	0·3	0·16
Optimum concentration (μmol l^{-1})	1	3	5

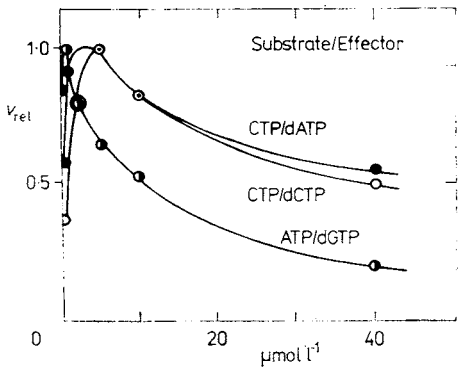

FIG. 4

Dependence of relative rate of reduction (v_{rel}) of ATP and CTP on coenzyme B_{12} concentration

inhibited by its own product. If the enzyme is to be used under these circumstances for the preparative isolation of deoxyribonucleotides, then it has to be immobilized and used in a suitable flow reactor.

Among the types of immobilization under consideration – immobilization in a gel structure, covalent bonding and adsorption – the former two types were excluded for the following reasons: Immoblization in the structure of a polyacrylamide gel was excluded because of the finding made in experiments in which we attempted to perform electrophoresis of the enzyme in polyacrylamide under nondenaturating conditions. Probably owing to the radical mechanism of acrylamide polymerization the enzyme became completely inactivated under these conditions (unpublished data). Immobilization by covalent bonding was rejected on the basis of the folloving considerations: The relatively small molecule of the enzyme has at least three binding sites for relatively bulky molecules of the substrate, the effector and coenzyme B_{12}, and in addition possesses catalycally important dithiol groups. It is thus unlikely that these regions of the enzyme molecule would remain intact after covalent bonding inherent in this type of immobilization. The remaining suitable mode of immobilization was thus adsorption on a suitable carrier. Carriers with electrostatic properties also had to be excluded because both, the enzyme and the substrate, assume the form of anions at pH 8·2. Nonspecific adsorbents such as silica gel, charcoal and others usually exhibit a weak bonding of the enzyme and often inactivate it fairly strongly.

Our previous experience with the strongly hydrophobic carrier, Trityl-Sepharose (ref.[16]), that had been used in our group for successful immobilization of several enzymes (acid and alkaline phosphatase, polynucleotide phosphorylase – unpublished results) induced us to use this carrier.

The presence of triphenylmethoxy groups in the hydrophilic agarose matrix makes this carrier one of the carriers with a hydrophobic character of ligand bonding. The immobilization procedure for these carriers is very simple and fast. The solution of the protein to be immobilized is simply blended in the suspension of the carrier. When the enzyme has become inactivated, the carrier can be easily regenerated by washing with concentrated glycerol solution, a solution of a detergent or a salt with a chaotropic effect (thiocyanates, perchlorates), or their combination. The main advantage of Trityl-Sepharose as compared with carriers for hydrophobic chromatography (Octyl- and Phenyl-Sepharose) is that the large majority of proteins fail to be released from this carrier even at very low ionic strengths.

Binding capacity of trityl-sepharose and release of the enzyme from the carrier: Among the fundamental charactetistics of a carrier used for immobizaton is the binding capacity, i.e. the amount of a protein per volume or weight unit, which the carrier is capable of binding. To determine the binding capacity of our carrier, we mixed the carrier with large amounts of the enzyme preparation, remowed unbound

proteins from the carrier by washing it with a buffer, and determined differentially the adsorbend amount. A similar experiment was performed also with BSA for comparison. The binding capacity for our enzyme was found to be 3·5 mg ml^{-1} carrier, corresponding to some 120 pkat ml^{-1}, while the capacity for BSA was 4·8 mg ml^{-1} carrier.

The immobilized enzyme was not released from the carrier even on treatment with buffer A, or with buffer A supplemented with 0·1% (w/w) Tween-60. A complete desorption can be achieved by washing the carrier with 50 vol. % glycerol in buffer A or 50 vol. % ethylene glycol in the same buffer.

Dependence of the stationary efficiency factor and the rate of reductions on the amount of enzyme in the carrier: The carrier was combined with different amounts of the enzyme preparation and the degree of conversion of 20 μmol l^{-1} CTP was determined after 10-min incubation. The results were used to calculate the stationary efficiency factor (relative activity) of the immobilized enzyme, and for assessment of dependence of this factor on the enzyme concentration in the carrier. These data were also used for constructing a graph of dependence of the enzyme reaction rate on the concentration of the enzyme in the carrier. The stationary efficiency factor was chosen because, with the free enzyme the reaction often stops on attaining a relatively low degree of substrate conversion; this prevented us from using the operational efficiency factor. Moreover, the measurement of the stationary efficiency factor is appreciably simpler.

As seen from Fig. 5, the rate of the enzyme reaction initially increases in proportion to the concentration of the enzyme in the carrier. Above the value of about 4 pkat ml^{-1} of carrier the line begins to curve and from 16 pkat ml^{-1} on the reaction rate no longer depends on the concentration of the immobilized enzyme.

The curve of the stationary efficiency factor has a more complex shape; at an enzyme concentration of about 4 pkat ml^{-1} of carrier it attains maximum and declines at both, lower and higher concentrations. The decrease observed at enzyme concentrations in excess of 4 pkat ml^{-1} of carrier can be satisfactorily explained by limitation of the reaction rate caused by the rate of substrate diffusion while the lower values observed at lower enzyme concentrations are probably due to a non-homogeneous distribution of the enzyme within the carrier volume.

Stability of the immobilized enzyme: An important parameter characterizing the immobilized enzyme is its time stability. In order to ascertain how the stability of the immobilized enzyme changes in dependence of its concentration in the carrier, we performed a series of long-term experiments in which the activity of the immobilized enzyme was investigated at two concentrations of the enzyme in the carrier – 4 pkat ml^{-1} and 24 pkat ml^{-1}. The lower concentration of the enzyme corresponds to the maximal value of the stationary efficiency while the higher is the saturation concentration (in the sense that the rate of the enzyme reaction at this

concentration is no longer a function of the concentration of the enzyme in the carrier). As seen from Fig. 6, higher amounts of the enzyme in the carrier appear to be more stable than lower amounts.

Immobilization stabilizes the enzyme substantially. As compared with the free enzyme, which has an inactivation half-life of less than one day[9], on immobilization the incativation half-life is about 15 days in the case of an optimum concentration of the enzyme in the carrier and about 30 days at higher enzyme concentrations.

Effect of pH: The pH-dependence of the activity of the immobilized enzyme was determined in a Tris-HCl buffer at pH 7·8–8·6. The ionic strength was adjusted by adding KCl to 0·033 mol l^{-1}. In this buffer system both, the free and the immobilized enzyme were found to have an essentially identical pH-dependence of activity.

Effect of ionic strength: The effect of ionic strength on CTP reduction was studied in the Tris-HCl buffer in which the ionic strength was adjusted to (0·018–0·218) mol l^{-1} by the addition of KCl. The results of the measurements showed that the dependence of enzyme activity on the ionic strenght is about same as that found previously for the free enzyme[10].

Effect of substrate concentration on reduction rate: In view of the finding that the basic characteristics of the free and immobilized enzyme essentially the same, we next determined the dependence of the reaction rate on the concentration of the substrate in the presence of 50 μmol l^{-1} of effector and 10 μmol l^{-1} of coenzyme B_{12}.

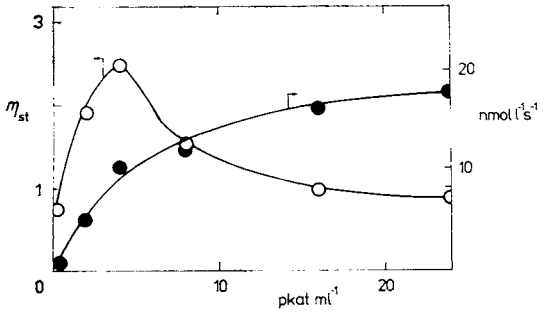

FIG. 5

Dependence of the stationary efficiency factor (η_{st}) and the rate of the enzyme reaction on the amount of the immobilized enzyme in the carrier

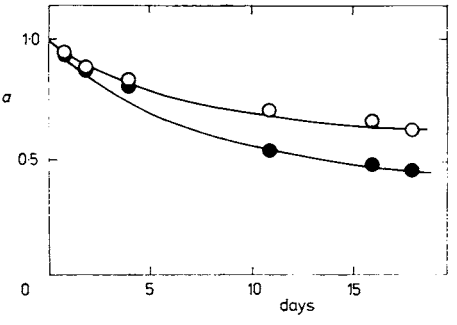

FIG. 6

Dependence of relative activity (*a*) on the length of storage (days) for two different concentrations of the enzyme in the carrier. ○ 24 pkat ml^{-1}, ● 4 pkat ml^{-1}

As shown in Fig. 7, in contrast to the free enzyme no inhibition by substrate occurs with CDP and CTP at concentrations up to 100 μmol l^{-1}.

Evaluation of the experimental data yielded the following values of apparent K_m (Table III). Comparison of these data with those for free enzyme points to a 2·5 – 10-fold increase in the value of this constant.

Effect of concentration of effectors on the activity of the immobilized enzyme: The measurement of the reaction rate at different substrate concentrations showed a substantial difference in the properties of the immobilized and the free enzyme. The next step was therefore the optimization of allosteric effectors. Results in Fig. 8 show that all dNTP, which in a free enzyme are nearly essential for reduction of some substrates, act as inhibitors of the immobilized enzyme in the whole concentration range under study.

TABLE III

$(K_m)_{ap}$ values for some substrates in the presence of effectors

Substrate	ADP	ATP	CDP	CTP
Effector	dGTP	dGTP	dATP	dATP
$(K_m)_{ap}$ (μmol l^{-1})	80	70	110	85

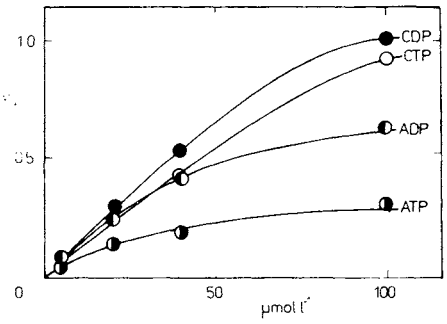

FIG. 7

Effect of substrate concentration on relative reaction rate (v_{rel}) in the presence of effectors

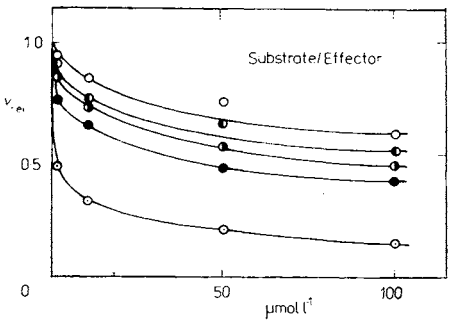

FIG. 8

Inhibitory effect of dNTP on reduction of NTP by the immobilized enzyme (NTP concentration 100 μmol l^{-1}). v_{rel}. Relative reduction rate; ○ CTP/dATP (20 μmol l^{-1}), ◐ GTP/dTTP, ◑ UTP/dTTP, ● CTP/dATP, ⊙ ATP/dGTP

Dependence of reduction rate on substrate concentration after omission of dNTP *from reaction mixture*: The finding that reduction of NTP by the immobilized enzymes does not require the presence of effectors compelled us to test again the dependence of the rate of the enzyme reaction on substrate concentration, using all four NTP. The resulting curves (Fig. 9) served for evaluating the values of $(K_m)_{ap}$. Comparison of data in Tables I and IV documents that the properties of the immobilized enzyme in the absence of dNTP in the reaction mixture resemble those on the free enzyme in the presence of dNTP.

Measurements of the rate of CTP reduction, at an initial concentration of 100 μmol l^{-1}, as a function of the concentration of the reductants dithioerythritol and 2-mercaptoethanol (DTE and 2-ME) (Fig. 10) show that, in comparison with the free enzyme, the optimum concentrations of these agents decreased for the immobilized enzyme. Like in the free enzyme, 2-ME at an optimum concentrations

TABLE IV

Values of $(K_m)_{ap}$ for NTP reduction by the immobilized enzyme in the absence of dNTP

Substrate	ATP	CTP	GTP	UTP
$(K_m)_{ap}$ (μmol l^{-1})	15	10[a]	90	10
$(K_2)_{ap}$ (μmol l^{-1})	—	250	—	—

[a] $(K_1)_{ap}$.

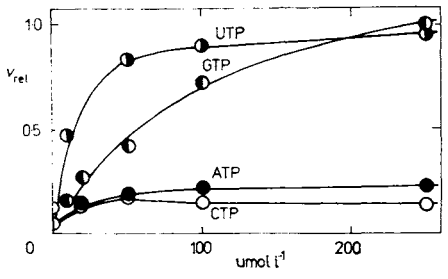

FIG. 9

Dependence of relative reduction rate (v_{re}) on substrate concentration after omission of dNTP from the reaction mixture

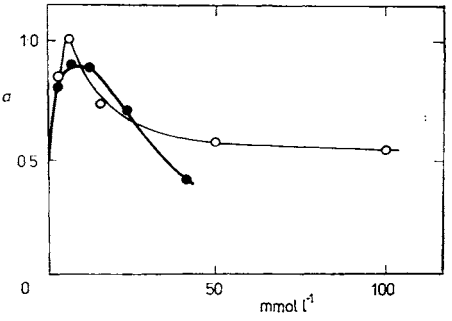

FIG. 10

Dependence of relative enzyme activity (*a*) on the concentration of the reductants dithioerythritol (○) and 2-mercaptoethanol (●) for the immobilized enzyme

reaches a mere 80% of efficiency of DTE. Optimum concentration of DTE is 5 mmol l^{-1}, that of 2-ME is 10 mmol l^{-1}. Comparison with the effect of DTE on the activity of the free enzyme[9] shows that at superoptimal value the inhibitory effect of DTE on the immobilized enzyme is much more pronounced that on the free enzyme. Optimum concentration of 2-ME for the immobilized enzyme is 10 times lower than the optimum concentration for its free counterpart.

CONCLUSION

Our study of properties of the free enzyme indicates that a kinetic analysis of the ribonucleotide reductase reaction is a highly complex problem. In our opinion this problem cannot be solved in its entirety by current methods of enzyme kinetics. The results appear to provide a sufficiently comprehensive picture of the complexity of processes of DNA synthesis.

Our results obtained in the study of the immobilized enzyme are surprising in many respects, especially concerning the loss of effector dependence. Other results that deserve attention concern the increased stability and changes in optimum concentrations of reductants. These results indicate that under in vivo conditions, when the enzyme in the cell is probably in some way "immobilized" its properties may appreciably differ from those determined on an isolated enzyme. On the other hand, the results appear to be promising in view of a possible preparative use of the enzyme.

LIST OF ABBREVIATIONS

A_{235}, A_{280}	absorbance at 235 and 280 nm respectively
BSA	bovine serum albumin
DTE	dithioerythritol
c_B	protein concentration
2-ME	2-mercaptoethanol
RR-ase	ribonucleotide reductase
dNTP	deoxyribonucleoside triphosphate
NTP	ribonucleoside triphosphate

REFERENCES

1. Follman H.: Angew. Chem. *17*, 624 (1974).
2. Stubbe J. A.: Mol. Cell. Biochem. *50*, 25 (1983).
3. Reichard P., Ehrenberg A.: Science *221*, 514 (1983).
4. Sjöberg B. M., Gräslund A., Eckstein F.: J. Biol. Chem. *258*, 8060 (1983).
5. Kollárová M., Perečko D., Zelinka J.: Biológia *35*, 907 (1980).
6. Vitols E., Brownson C., Gardiner W., Blakley R. L.: J. Biol. Chem. *242*, 3035 (1967).
7. Gleason F. K., Frick T. D.: J. Biol. Chem. *255*, 7728, (1980).
8. Ludwig W., Follman H.: Eur. J. Biochem. *82*, 393 (1978).
9. Kollárová M., Halický P., Perečko D., Bukovská G., Zelinka J.: Biológia *37*, 777 (1982).
10. Kollárová M., Halický P., Bukovská G., Zelinka J.: Biológia *38*, 1189 (1983).

11. Halický P., Kollárová M., Koiš P., Szutorová K., Kormanec J., Zelinka J. in: *5- International Symposium on Metabolism and Enzymology of Nucleic Acids Including Gene Manipulations 5* (J. Zelinka and J. Balan, Eds), p. 145. Veda – Publishing House of the Slovak Academy of Sciences, Bratislava 1984.
12. Tsai P. K., Hogenkamp H. P. C.: Biol. Chem. *255*, 1273 (1980).
13. Hogenkamp H. P. C. in: *Vitamin* B_{12} (B. Zagalak and W. Friedrich, Eds), p. 489. Walter de Gruiter, Berlin, New York 1979.
14. Kollárová M.: *Thesis*. Komenský University, Bratislava 1982.
15. Whitaker J. R., Grunun P. E.: Anal. Biochem. *109*, 156 (1980).
16. Cashion P., Javed A., Harrison D., Seley J., Lentini V., Sathe G.: Biotechnol. Bioenerg. *24*, 403 (1982).
17. Gleason F. K., Wood J. M.: J. Bacteriol. *128*, 673 (1976).
18. Kasche V.: Enzyme Microb. Technol. *5*, 1 (1983).
19. Sato K., Seki T., Inukai S., Shimizu S.: Anal. Biochem. *116*, 185 (1980).
20. Kotyk A., Horák J.: *Enzýmová kinetika*. Academia, Praha 1977.

Translated by the author (P. H.).