THE ATPASE ACTIVITY OF AVIAN ERYTHROMYELOBLASTIC LEUKOSIS VIRUS

I. INTRODUCTORY KINETICS*

by

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The particulate etiologic agent of avian erythromyeloblastosis had been shown to possess ATPase** activity[15]. This enzymic capacity is present in the plasmas of infected chicks[16] where its activity occurs coincident with and proportionate to both the virus particle count as determined with the electron microscope and to the infectious titers assayed by inoculation into susceptible chicks[5]. Attempts to separate the particles, ATPase activity and infectious capacity from one another by sedimentation[19] and electrophoresis[19] have failed. Furthermore, it has recently been shown[4] that the particles and ATPase activity are precipitated quantitatively by chicken antiviral serum which also neutralizes completely the infectious capacity.

Since the identity of the particles and ATPase activity has been established and since a thorough survey of the effects of temperature***, pH and ionic environment[3,9] have been completed it seemed a natural consequence that a thorough kinetic and thermodynamic investigation should be undertaken. This communication presents the results obtained in the first of a series of such studies.

The advantages offered by the present system to such a study are practical as well as theoretically provocative. Since the enzyme particles can be seen and counted there is no need for the conventional purity assays, nitrogen analyses or molecular weight determinations. All enzymic rates have been quantitated on the basis of direct observation of enzyme particle number.

EXPERIMENTAL

Materials and methods

The viral enzyme was isolated from the plasmas of chickens with demonstrable signs of erythromyeloblastosis as shown by blood smears[2] and micro ATPase activity[8,14]. These chickens were bled into heparin (Liquaemin, Roche-Organon, 1 ml = 10 mg) in the proportion of 1 ml heparin to a total volume of 10 ml. After removal of clots from the pooled blood by filtration through gauze,

* This work was supported by research grants to Duke University from the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service; from the American Cancer Society, on recommendation of the Committee on Growth; from the Damon Runyon Memorial Fund for Cancer Research, Inc.; and by the Dorothy Beard Research Fund.

** Abbreviations: Adenosinetriphosphatase (ATPase); adenosinetriphosphate (ATP); adenosinediphosphate (ADP); adenosinemonophosphate (AMP); inorganic orthophosphate (P_i).

*** E. A. ECKERT, I. GREEN, D. G. SHARP, D. BEARD AND J. W. BEARD. Manuscript in preparation. I. GREEN. Manuscript in preparation.

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most of the cells were sedimented by low-speed horizontal centrifugation (2,000 *g*) in 50 ml round-bottom glass tubes in the cold room. The plasma was pipetted off and respun at 4,000 *g*. The resulting supernatant was filtered through celite followed by filtration through a Selas 02 porcelain filter candle (Selas Corp. of America, Scientific Equipment Division, Philadelphia, Pa.) to remove residual cells and the enzyme sedimented at 20,000 *g* for 30 minutes. The pellet was resuspended immediately in the minimum volume of cold 0.85% NaCl, the preparation being kept cold throughout the procedure. An aliquot of this or a 10-fold diluted preparation was used to determine both particle count and the rate of ATP hydrolysis.

ATP was obtained from the Sigma Chemical Company as the dipotassium salt. Four hundred mg were dissolved in 25 ml demineralized water, adjusted to pH 8.0 with NaOH and passed over a 3 cm^2 × 10 cm column of Dowex-1 (chloride cycle) at the rate of 3 ml/min. Following the procedure of COHN AND CARTER[1] any adenosine, AMP, ADP or P_i was eluted with 100 ml of 0.01 *N* HCl + 0.02 *M* NaCl, following which the ATP was removed with about 50 ml of 0.01 *N* HCl + 0.2 *M* NaCl. This solution was adjusted to pH 7.0 with NaOH and stored in the cold. Analysis by Dowex column chromatography, Beckman spectrophotometry[11] and P_i determination[6] showed less than 1% P_i or ADP to be present. This procedure was repeated daily before proceeding with the enzymic determinations. The required concentration of ATP was obtained by suitable volumetric dilution after calculating the concentration from the molecular extinction coefficient and the optical density at a wavelength of 260 mμ.

The reaction mixture utilized throughout this study was that which had been shown to yield the greatest activity per unit of enzyme[9]. It contained 1 ml 0.1 *M* tris buffer, pH 7.2 and 3 ml of a salt solution composed of 0.04 *M* Ca^{++}, 0.04 *M* Mg^{++}, 0.05 *M* Na^{+} and 0.05 *M* K^{+}. Ten lambda of the enzyme solution was added, and the reaction mixture was incubated for 10 minutes at 30° C. At this temperature there was no measurable loss of activity either during this period or subsequently during the course of the hydrolysis*. The reaction was initiated by the addition of 1 ml of the ATP solution. A blank containing 1 ml ATP but no enzyme was also included. An enzyme blank was not necessary since it had been shown that no detectable P_i appeared with 10 lambda of virus in the absence of ATP even after incubation for one hour at 40° C. The reactions were terminated by the addition of 2 ml of 25% trichloroacetic acid (TCA), and the 7 ml reaction volume was then transferred to a 10 ml volumetric flask, followed by washings and adjustment to the mark. A 5 ml aliquot was used for the P_i determination[6]. Filtration was unnecessary since no precipitation or turbidity occurred following the TCA addition or after the addition of molybdate during the colorimetric determination of P_i.

Reaction rates were calculated by averaging the results obtained from triplicate enzyme runs. The time during which the reaction was allowed to proceed was chosen so that the maximum quantity of P_i would be produced while the initial linearity of the reaction still obtained. The appropriate time interval was determined from several preliminary runs during which the initial reaction course was determined with the extremes of substrate concentration to be employed.

Determination of enzyme quantity

Usually a statement of specific activity is made in terms of a measured weight of the enzyme, the accuracy of which is related to the purity of the material. With the enzyme of erythromyeloblastic leukosis virus, as with other enzymes, the absolute purity is not definable, consequently it cannot be determined. However, contrary to other enzymes, the particles of erythromyeloblastic leucosis viral enzyme are large enough to be seen and counted[18]. Identification of the enzyme with these particles has been well substantiated[4, 5, 16, 19]. The counting procedure involves sedimentation from an appropriate dilution of the sample directly upon an agar surface. Removal of supernatant fluid and washing of this agar surface does not remove the adhering enzyme particles**. After fixation with osmic acid vapor, 2% collodion in amyl acetate is flooded on the agar surface and allowed to dry in a draining position. When dry, this film is floated off on water. The particles come with it***. After shadowcasting with chromium to increase

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** Supernatant fluids and wash fluids have been resedimented over fresh agar and found to deposit negligible numbers of particles.

*** Second replicas were made in each case. Rarely do they show significant numbers of particles. In those few cases where they did, they were counted and added to make the total figures shown in Table I.

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image contrast, the enzyme particles can then be photographed on this pseudoreplica in the electron microscope. Such a photograph of preparation number 4 is presented in Fig. 1. The number of particles found per unit area is obtained from a known volume of the sample so, from geometry of the cell, magnification of the microscope and dilution of the sample, particle count is calculated.

It is estimated, from counting data on this material analogous to that previously published for influenza virus[17], that the random standard error for these counts is $\pm$ 10%. Uncertainties in magnification in the electron microscope are estimated at $\pm$ 5% in this series. This enters the calculation of particle concentration as a squared term. Overall root-mean-square error calculated from these estimates is $\pm$ 14%.

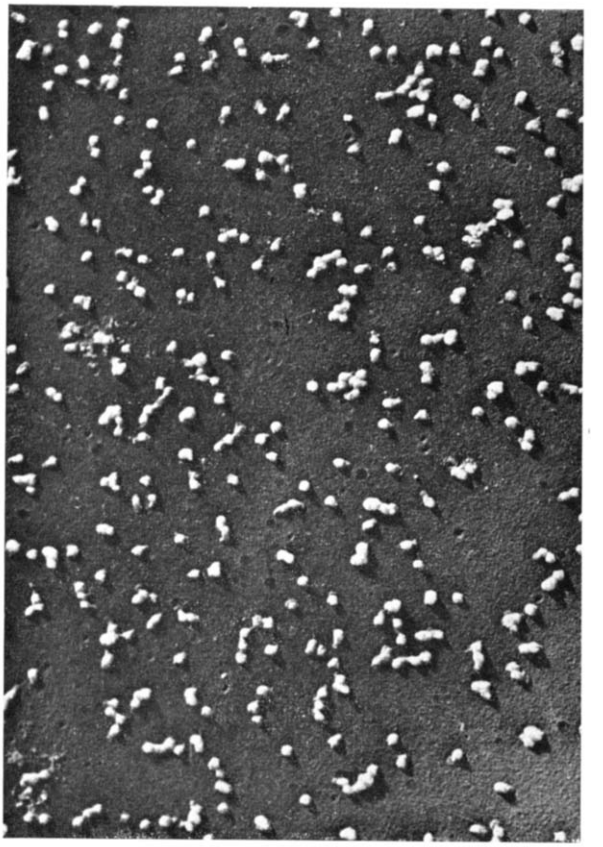

Fig. 1. Avian erythromyeloblastic leukosis virus (enzyme) particles shown in shadowcast pseudoreplica. This electron micrograph is one of those used in establishing particle count in preparation 154. Magnification is 13,500 ×.

From data obtained by ultracentrifugal analysis, these enzyme particles have a mean sedimentation constant of $S_{20,w}$ = 602, hydrated radius of 70 mμ and degree of hydration in physiological saline solution of bovine serum albumin of 80% by volume. The particle density in this medium is 1.059.

Theoretical consideration

The effect of varying substrate concentration was studied by the use of MICHAELIS-MENTEN theory[13] employing LINEWEAVER-BURK plots[12]. The derivations used in this work are based on the following formulation:

$$(1)\ \mathrm{ATP} + V \underset{k_2}{\overset{k_1}{\rightleftharpoons}} V\text{-ATP}$$

$$(2)\ V\text{-ATP} \xrightarrow{k_3^0} V + \mathrm{ADP} + \mathrm{P_i}$$

$$(3)\ K_s = \frac{k_2 + k_3^0}{k_1}$$

where V represents one mole of visible particles of viral enzyme.

It has been assumed that water, the third reactant, is present in constant amount and in such excess as to have no limiting influence in equation (2). It is probably equally

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true that hydroxyl and hydrogen ions do not exert a limiting influence since the optimal pH is close to neutrality.

The use of k_3^0, which has the dimensions of minute^{-1} (when both substrate and enzyme concentrations are expressed in moles per liter and time in minutes), instead of $V_{max.}$ is entirely valid since all experiments were carried out at constant pH and in the constant ionic environment. Hence, the average number of active sites per particle is expected to remain constant. The expediency gained with the use of k_3^0 in future thermodynamic calculations makes it the desirable method of expressing the velocity with which the complex undergoes reaction to yield products and free enzyme.

As has already been pointed out the enzyme particles can be seen and counted. It is therefore possible to express enzyme concentration directly in moles per liter from the electron micrographic counts.

Applicability of the Michaelis-Menten treatment

The simplified MICHAELIS-MENTEN theory assumes that at low molar concentrations of the enzyme, the *free* and the *total* substrate concentrations are equal. Since the present problem is concerned with a relatively large particle which may contain a large number of active sites, it was deemed desirable to investigate this problem with the aid of the STRAUSS-GOLDSTEIN criteria[7,20]. In Fig. 2 is plotted the log of the substrate concentration against the fractional activity, as circles on a theoretical curve $S = K_s\, a/(1-a)$ satisfying the conditions for zone A behavior where substrate is in excess and each active center binds one substrate molecule. The K_s value experimentally determined from the LINEWEAVER-BURK plots (see Fig. 3) was used in deriving the theoretical curve. Although it was not possible to obtain experimental points on the lower half of the plot,

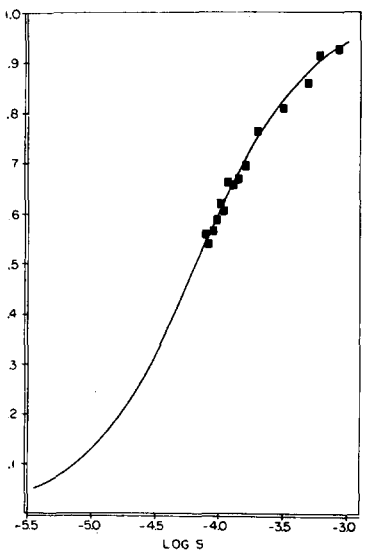

Fig. 2. Dependence of the fractional activity (a) on the logarithm of the ATP concentration. The solid line is plotted using the theoretical equation, $S = K_s\, a/(1-a)$, for zone A behavior where $n = 1$.

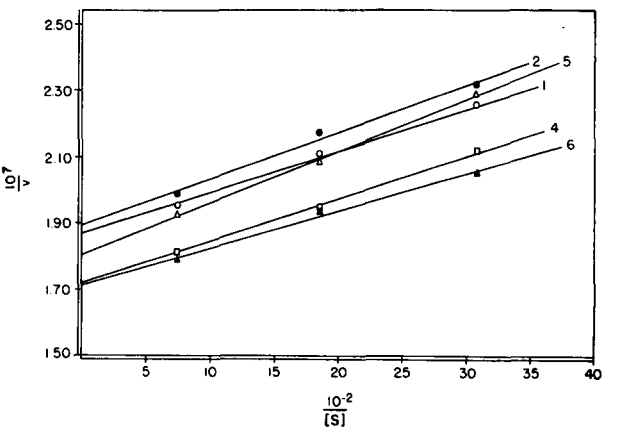

Fig. 3. LINEWEAVER-BURK plot showing the variation of reciprocal initial reaction velocity, expressed in reciprocal moles per liter per minute, with reciprocal initial substrate concentration expressed in reciprocal moles per liter, for the hydrolysis of ATP by viral enzyme at 30° C. All values are reduced to a common enzyme concentration of one mole of enzyme particles per liter. Each line has been obtained by the method of least squares and represents data from a separate preparation, whose number is affixed.

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the agreement on the upper half is sufficiently good to establish the validity of zone A behavior and consequently the applicability of the MICHAELIS-MENTEN treatment used in this paper.

Determination of K_s *and* k_3^0

Five different virus preparations were used for studying the effect of varying substrate concentration on the rate of hydrolysis of ATP. The LINEWEAVER-BURK plots obtained from this study are presented in Fig. 3. The lines have been drawn by the method of least squares and the values of K_s and k_3^0 obtained from them are given in Table I. The mean values for K_s and k_3^0 respectively are $7.34 \pm 0.84 \cdot 10^{-5}$ and $0.556 \pm 0.022 \cdot 10^7$.

TABLE I

(Values found for k_3^0, specific rate of activation of enzyme-substrate complex and K_s, the MICHAELIS-MENTEN constant.)

Preparation No.	*Particles per ml Stock virus* $\times 10^{-12}$	$K_s \cdot 10^5$ *(M/l)*	$k_3^0 \cdot 10^{-7}$ *(M)**
1	6.50	6.68	0.535
2	1.39	7.39	0.527
4	6.17	7.45	0.582
5	7.19	8.65	0.554
6	1.94	6.51	0.583

* Moles per liter per minute per mole enzyme particles per liter (minutes $^{-1}$).

DISCUSSION

The turnover number per particle per minute for the viral enzyme is about 5 million. With the exception of catalase (5 million) this value is much greater than that for any enzyme characterized thus far and is certainly unique among those values obtained for phosphatases. This turnover number must be interpreted, however, with considerable caution. The large size of the enzyme particle suggests the likelihood that there are many more active centers per particle than in the case of other enzymes. Were it possible to express the turnover number per site, the large values reported here might turn out to be more apparent than real. Of course the possibility remains that relatively few sites are available per particle. In this case the viral enzyme activity would be unique among ATPases isolated from normal tissues, not only in its response to Ca^{++} and Mg^{++}[9] and in its ability to dephosphorylate ATP and ITP[10] at equal rates under identical conditions, but also in the ability of the enzyme's active center to turn over substrate.

Thermodynamic studies, under way at present, may allow us to calculate the fraction of the viral surface which is enzymically active. Such information could set a limit to the number of sites available per particle and thereby establish a maximal value for the turnover rate per active center. Since it has been shown that ADP is a competitive inhibitor for the viral ATPase activity*, it should be possible to establish unequivocally the number of sites per particle by the use of equilibrium dialysis with ADP and purified virus. Calculations indicate that under conditions such that relatively few centers are involved, it should be possible to perform such an experiment with the desired precision only by the use of firefly luminescence to assay the change resulting in the free ADP concentration.

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Although we cannot at present make any statement regarding the number of sites per particle we can state with assurance that each site binds only one ATP molecule at a time. This has been determined from the STRAUSS-GOLDSTEIN plot shown in Fig. 2. The solid line represents zone A behavior, where the number of substrate molecules bound per active center equals one. It can be seen that the experimental points obtained fit the curve very well.

Since the discovery of the presence of ATPase in virus preparations[15], it has been a cardinal problem to show that the particles seen in the electron microscope were the enzyme. While the inability to separate the two has afforded negative evidence, the quantitative precipitation of both particles and ATPase activity by chicken antiviral antibody[4], has given direct proof for such a contention. The closeness of the values of K_s and k_3^0 for the five preparations studied in this paper is further direct corroboration.

It must be re-emphasized that only if the particles and the enzyme were identical could the data presented here be possible. In all the work, there have been none of the usual criteria of purity or nitrogen assays or molecular weight determinations. All quantitation of the ATPase activity has been made on the basis of particles which could be seen and counted.

In conclusion it should be pointed out that a kinetic and thermodynamic study based upon direct enzyme particle count is on firmer ground and to be preferred. It is certainly one from which valuable experimental verification of kinetic theory should result.

SUMMARY

An introductory kinetic study of the viral ATPase activity has been based on enzyme particle counts obtained from electron micrographic photographs. This approach has circumvented the necessity of determining enzyme nitrogen, purity and molecular weight.

The values obtained for K_s and k_3^0 are $7.34 \pm 0.84 \cdot 10^{-5}$ and $0.556 \pm 0.022 \cdot 10^7$, respectively.

The close agreement among the values of k_3^0 for five different preparations lends further support for the contention that the virus particle is the enzyme.

A value of 5 million has been calculated for the number of molecules of ATP turned over by each enzyme particle per minute. This large turnover number has been discussed.

RÉSUMÉ

Une étude cinétique préliminaire de l'activité ATPasique virusale, fondée sur le comptage des particules enzymatiques sur des photographies au microscope électronique, a été effectuée. Cette méthode supprime la nécessité de déterminer la teneur en azote de l'enzyme, sa pureté et son poids moléculaire.

Les valeurs obtenues pour K_s et k_3^0 sont respectivement de $7.34 \pm 0.84 \cdot 10^{-5}$ et $0.556 \pm 0.022 \cdot 10^7$.

L'accord étroit entre les valeurs de k_3^0 pour cinq préparations différentes constitue un nouvel argument en faveur de l'identité entre la particule de virus et l'enzyme.

Les auteurs ont calculé que le "turn-over" est de 5 millions de molécules d'ATP par particule enzymatique et par minute. Ce "turn-over" élevé est discuté.

ZUSAMMENFASSUNG

Auf Grund von Enzympartikelzählungen, die an Hand von Elektronenmikrographie-Photographien durchgeführt werden konnten, wurde das einleitende kinetische Studium von Virus-ATPase-Aktivität unternommen. Durch diese Prozedur konnte die Notwendigkeit umgangen werden, Stickstoffgehalt, Reinheit und Molekulargewicht des Enzyms festzustellen.

Die für K_s und k_3^0 erhaltenen Werte sind beziehungsweise $7.34 \pm 0.84 \cdot 10^{-5}$ und $0.556 \pm 0.022 \cdot 10^7$.

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Die enge Übereinstimmung der k_3^0-Werte für 5 verschiedene Präparate unterstützt die Annahme, dass das Viruspartikel mit dem Enzym identisch ist.

Es wurde errechnet, dass jedes Enzympartikel pro Minute 5,000,000 ATP-Moleküle umsetzt. Diese hohe Umsetzungszahl wird erörtert.

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Received February 18th, 1955