Proc. Nat. Acad. Sci. USA
Vol. 69, No. 1, pp. 15–19, January 1972

Adenosine Triphosphatase Associated with Adenosine Triphosphate-Dependent Deoxyribonuclease

(*recB-recC* enzyme/*E. coli*/ATP to phosphodiester hydrolysis ratio/DNA-dependent ATPase activity)

F. G. NOBREGA*, F. H. ROLA†, M. PASETTO-NOBREGA*, AND M. OISHI

The Public Health Research Institute of the City of New York, Inc., New York, N.Y. 10016

Communicated by George K. Hirst, October 14, 1971

ABSTRACT **An ATPase activity that is completely dependent on DNA is associated with the ATP-dependent DNase (*recB-recC* enzyme) purified from *Escherichia coli*. There is a strong correlation between the ATPase and the DNase activities under various assay conditions. With *E. coli* DNA as substrate, 8–9 molecules of ATP are hydrolyzed to ADP and inorganic phosphate for every phosphodiester bond hydrolyzed by the DNase. The possible functional relationship of the ATPase and DNase activities is discussed.**

We reported the partial purification and characterization of an ATP-dependent DNase from *Escherichia coli* (1). Since this enzyme activity is absent in extracts of certain types of recombination-deficient mutant strains (*recB* and *recC*) of *E. coli* (1–4), it is reasonable to speculate that this enzyme is responsible for a certain step in the recombination process. The ATP-dependent DNase is an enzyme of large molecular weight (300,000–350,000), and appears to be determined by at least two genes (*recB* and *recC*), suggesting that it is composed of subunits. As a first step in elucidating the gene-product (protein) relationship and the molecular mechanism of the enzyme action, we have searched for an additional enzyme activity that may be associated with this enzyme.

We now report the presence of a DNA-dependent ATPase activity associated with this DNase, and some of the basic characteristics of the ATPase. A preliminary report of this study has appeared.‡

MATERIALS AND METHODS

Medium for Bacterial Growth. G medium contains the following in 1 liter: 3 g beef extract, 6 g yeast extract, 10 g peptone, 0.5 g K_2HPO_4, 0.1 g $MgSO_4$, 5 g NaCl, and 2 g glucose (autoclaved separately). Antifoam A (0.1 ml) was added to the fresh medium after inoculation with an overnight culture.

Preparation of DNA. [^{32}P]DNA from *E. coli* (K12, KL16) was prepared as reported (1). Bacteriophage T4 and T7 [^{32}P]DNA were purified by the method of Grossman *et al.* (5). PM2 [^{32}P]DNA was purified from sodium dodecyl sulfate-treated PM2 phage (kindly provided by Drs. R. M. Franklin and S. Braunstein) by a neutral sucrose-gradient centrifugation procedure (6). Poly(dA-dT) and poly(dG·dC) were purchased from Miles Laboratories. DNA for the ATPase assay was prepared by the procedure used for DNA labeled with ^{32}P except that it was unlabeled.

Enzyme assays

ATP-Dependent DNase. The DNase was assayed by measurement of the acid-soluble radioactivity released from ^{32}P-labeled DNA (1). The reaction mixture (0.5 ml) contained 12.5 μmol of Tris–maleic acid–NaOH buffer (pH 7.5), 5 μmol of $MgCl_2$, 250 μmol of glycerol, 0.5 μmol of dithiothreitol (DTT), 0.1 μmol of EDTA, 125 nmol of ATP, 15 nmol of ^{32}P-labeled DNA (nucleotide equivalent), 100 μg of bovine serum albumin, and 0.5–5.0 units of enzyme. In order to obtain reproducible results, [^{32}P]DNA was sheared by a Sorvall mechanical mixer to a mean molecular weight of 8×10^6 before use. For the standard assay, *E. coli* DNA was used as a substrate for the DNase. The reaction mixture was incubated for 20 min at 37°C unless otherwise specified, and the reaction was terminated by the addition of 0.1 ml of a carrier solution (5 mg/ml of DNA and 10 mg/ml of bovine serum albumin) and 0.5 ml of cold 3.5% perchloric acid. After 5 min at 0°C followed by centrifugation ($2000 \times g$ for 5 min), all the supernatant fluid was transferred to a planchet and, after addition of one drop of 5 N KOH, the sample was evaporated to dryness. The radioactivity was measured by a gas-flow counter (Nuclear-Chicago, model 4342). 1 unit of enzyme is defined as the amount catalyzing the conversion of 1 nmol (nucleotide equivalent) of *E. coli* DNA to an acid-soluble product in 20 min at 37°C.

DNA-Dependent ATPase. The ATPase was assayed by measurement of the amount (measured as radioactivity) of inorganic phosphate released from [γ-^{32}P]ATP. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (7). The reaction mixture was the same as for the ATP-dependent DNase, except that (*a*) [γ-^{32}P]labeled ATP was used instead of unlabeled ATP, and that (*b*) unlabeled DNA was used instead of [^{32}P]DNA, unless otherwise stated. (Since there is no detectable phosphatase activity in the purified enzyme preparation, ^{32}P-labeled DNA can be used for the assay of ATPase activity instead of unlabeled DNA). After 20 min of incubation at 37°C, the reaction was terminated with 4 ml of 1.25 N perchloric acid containing 1 μmol/ml of monosodium phosphate, followed by 4 ml of isobutanol–benzene (1:1) and 0.5 ml of 10% ammonium molybdate solution. After the whole solution was mixed with a vortex mixer, 1 ml of the

* Present address: Departmento de Bioquimica, Instituto de Quimica, Caixa Postal 20780, São Paulo, Brazil.
† Present address: Instituto de Biologia, Fortaleza, Ceara, Brazil.
‡ Oishi M., and Rola, F. H. (1970) *Fed. Proc.* **29**, 405.

TABLE 1. *Purification of the enzyme*

	Fraction	Protein		Total activity	Specific activity	
		Total mg	mg/ml	(units)	(units/mg protein)	
I	Crude extract	14,080	61	87,630	6	(1.0)
II	82,000 × *g*	9,200	50	75,570	8	(1.3)
III	Streptomycin	330	3	26,320	80	(13)
IV	Phase separation	132	0.85	30,840	233	(38)
V	DEAE-cellulose	10	0.07	15,300	1,530	(247)
VI	DEAE-Sephadex	0.29	0.009	6,270	21,630	(3480)

isobutanol–benzene phase was transferred to a planchet and dried, and the radioactivity was measured with a gas-flow counter.

Purification of the enzyme

All manipulations were at 0–2°C unless otherwise stated.

Preparation of Crude Extract. E. coli K12 strain AB 1157 (*rec*$^+$) was grown in G medium at 37°C with vigorous aeration. The culture was maintained at pH 7 by the addition of 2 N NaOH. The cells were harvested during the late-log phase of cell growth (3×10^9 cells/ml). The medium was chilled with crushed ice and the cells were collected by centrifugation in a Sharples centrifuge. After the cells were washed with TMSK buffer [0.01 M Tris·HCl (pH 7.8)–0.01 M $MgCl_2$–0.05 M KCl–5 mM 2-mercaptoethanol–0.2 mM EDTA], they (120 g) were mixed with 10 ml of TMS buffer [0.01 M Tris·HCl (pH 7.8)–0.01 M $MgCl_2$–5 mM 2-mercaptoethanol–0.2 mM EDTA] and 300 g of acid-washed glass beads (Superbrite, type 100, 3M Co.) and were disrupted by shaking in a cell mill (Edmund Bühler Co., Tübingen, Germany) equipped with a cooling jacket for 30 min at maximum speed. The disrupted cell–glass bead mixture was extracted repeatedly with TMS buffer. The combined cell extract (260 ml) was centrifuged at 27,000 × *g* for 10 min and the supernate (Fraction I, 230 ml) was collected.

Ultracentrifugation. Fraction I was centrifuged at 82,000 × *g* (25,000 rpm) for 120 min in a Beckman SW 27.1 rotor and the supernate (Fraction II, 184 ml) was collected.

Streptomycin Precipitation. Fraction II was diluted with TMS buffer to give 200 A_{260} units/ml and then 57 ml of 5% streptomycin sulfate (Eli Lilly Co.) solution was added, with constant stirring, over a 10-min period. (The amount of streptomycin sulfate to be added was calculated as 30 μg of streptomycin sulfate per A_{260} unit of Fraction II.) After centrifugation at 12,000 × *g* for 10 min, the tightly packed precipitate was homogenized by blending in 80 ml of TS buffer [0.02 M Tris·HCl (pH 7.8)–5 mM 2-mercaptoethanol–1 mM EDTA–1 M glycerol]. Solid $(NH_4)_2SO_4$ was then added to give a concentration of 0.6 M, and the resulting viscous solution was dialyzed once against 30 volumes of TS buffer containing 0.2 M $(NH_4)_2SO_4$, and twice against 30 volumes of TS buffer (Fraction III, 107 ml).

Phase Separation. To 104 ml of Fraction III, 31.2 ml of polyethylene glycol 6000 (Union Carbide) solution (30% w/w), 10.4 ml of dextran T500 (Pharmacia) solution (20% w/w), and 16.8 g of NaCl were added and, after it was mixed for 5 min, the solution was centrifuged (10,000 × *g* for 5 min) and the upper phase was dialyzed twice against 30 volumes of TS buffer for a total of 7 hr. (Fraction IV, 150 ml).

DEAE-Cellulose Chromatography. Immediately after dialysis, Fraction IV, (150 ml, NaCl concentration 0.05–0.10 M) was applied to a DEAE-cellulose column (2.2 × 20 cm) that had been equilibrated with TS buffer (Tris·HCl, 0.05 M) and chromatography was performed with a linear gradient of Tris·HCl (pH 7.8) from 0.1 to 0.7 M in TS buffer (total 600 ml) for 24 hr. Each 11-ml fraction was collected, and the peak fractions were pooled and dialyzed against TS buffer (Tris·HCl concentration 0.1 M) overnight. (Fraction V, 143 ml).

DEAE-Sephadex Chromatography. Fraction V was then applied to a DEAE-Sephadex A50 column (2.2 × 20 cm), that had been equilibrated with TS buffer (Tris·HCl concentration 0.2 M) and chromatography was performed with a linear Tris·HCl gradient from 0.2 to 0.7 M in TS buffer (total 650 ml) for 48 hr. Each 11-ml fraction was collected and the peak fractions were pooled (Fraction VI, 32 ml). The pooled fractions were diluted with TS buffer (Tris·HCl concentration 0.01 M) to about 150 ml, and applied to a DEAE-cellulose column (0.5 × 5 cm). The enzyme was eluted from the column with TS buffer (Tris·HCl concentration 0.7 M) in a 2-ml volume and dialyzed against 30 volumes of TS II buffer [0.02 M Tris·HCl(pH 7.8)–1 mM EDTA–1 mM dithiothreitol–1 M glycerol] for 10 hr. Fraction VI was stored frozen at −80°C. The purification is summarized in Table 1.

RESULTS AND DISCUSSION

Association of ATPase with the DNase

During the purification of the ATP-dependent DNase, we observed that a DNA-dependent ATPase activity accompanies the DNase activity throughout the purification process. This ATPase, observed in the purified ATP-dependent DNase preparation, is completely dependent upon the presence of DNA. Double-stranded linear DNA is more effective in promoting the ATPase activity than is single-stranded DNA. Before characterizing the enzymatic properties of the ATPase, we tried to establish whether the ATPase activity is associated with the ATP-dependent DNase molecule. As is shown in Fig. 1, when the purified DNase enzyme preparation is subjected to polyacrylamide gel electrophoresis, the DNA-dependent ATPase activity is found in the same fraction as the ATP-dependent DNase, suggesting that the ATPase and the DNase activities are, in

fact, associated with the same enzyme molecule. This conclusion was further supported by comparison of the heat-inactivation profiles of both the DNase and ATPase activities of the purified enzyme. The results of Fig. 2 show a parallel loss of ATPase and DNase activity when the enzyme is incubated at 45°C. This observation suggests that the ATPase not only is physically associated with DNase, but also has some essential role in the DNase action.

Products of ATPase

The products of the ATPase action on ATP in the presence of DNA were identified as ADP and inorganic phosphate. As is shown in Table 2, essentially equimolar amounts of ADP and inorganic phosphate were produced during the reaction, while an equimolar amount of ATP had disappeared. No appreciable amount of AMP production was detected. These results indicate that the ATPase action consists of a hydrolysis of ATP into ADP and inorganic phosphate.

The role of ATPase in the DNase reaction

As mentioned above, the DNA-dependent ATPase activity associated with the ATP-dependent DNase apparently participates in the degradation of DNA. To study this assumption further, the activity of ATPase (as measured by the release of phosphate from ATP) was compared with the DNase activity by time-course studies, as well as by the use of various assay conditions. As shown in Fig. 3, the release of inorganic phosphate from ATP is paralleled by the release of acid-soluble material from DNA during the course of the reaction. The ratio of phosphate released from ATP to

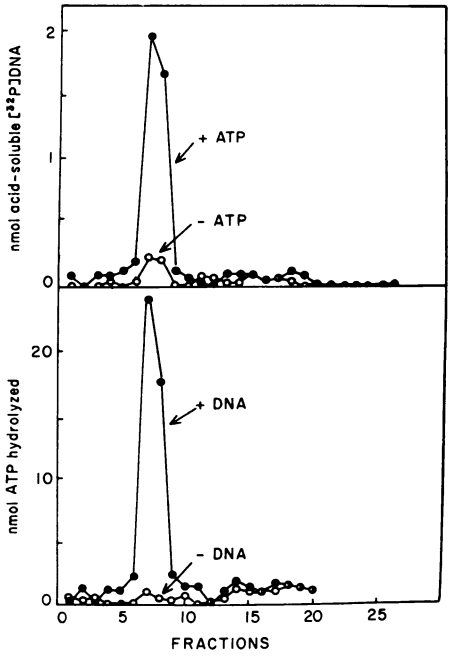

FIG. 1. Polyacrylamide gel electrophoresis of the purified ATP-dependent DNase: Gels (0.6 × 7 cm) were prepared by mixing solutions of acrylamide (32% acrylamide, 0.6% bis-acrylamide), dimethylaminopropionitrile (3%), buffer [0.05 M Tris–borate (pH 7.8)–1 mM EDTA–2 mM 2-mercaptoethanol–10% glycerol], and ammonium persulfate (0.5%) in a ratio of 2:1:2.2:5.2. After the gel was left overnight at 3°C, it was run for 1 hr before sample application. The reservoir buffer was 25 mM Tris–borate (pH 7.8), that contained 1 mM EDTA, 2 mM 2-mercaptoethanol, 10% glycerol, 0.1 mM ATP, and 5 mM $MgCl_2$. After the enzyme preparation (88 units) was applied on the top of the column, electrophoresis was run at 3 mA/tube for 150 min at 3°C. Gels were then sliced into 1-mm discs; each two slices were placed in 0.5 ml of the 0.02 M Tris·HCl (pH 7.7) that contained 1 mM EDTA, 0.7 M glycerol, 5 mM 2-mercaptoethanol, 0.02% sodium azide, and 1 mM aminocaproic acid. After standing overnight at 4°C, portions of each fraction were assayed for DNase (*top*) and ATPase (*bottom*) activity.

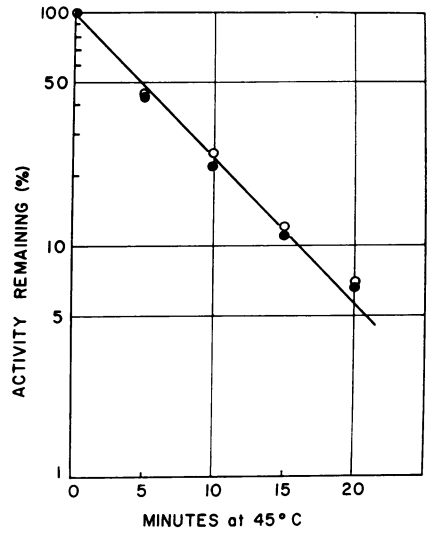

FIG. 2. Heat inactivation of the DNase and ATPase activities of the enzyme. The enzyme preparation (8 ml, 34 units/ml) was incubated at 45°C in Tris–maleic acid–NaOH buffer (20 mM, pH 7.5) that contained 100 μmol/ml glycerol, 1 μmol/ml DTT, 1 μmol/ml EDTA, and 200 μg/ml of bovine serum albumin. At the time indicated, 0.1-ml samples were withdrawn and chilled. Without heat treatment (at 0 min) 3.6 nmol of [^{32}P]DNA became acid soluble and 9.1 nmol of [γ-^{32}P]ATP were hydrolyzed.

TABLE 2. *Product analysis of the ATPase reaction*

Minutes	nmoles of ATP	ADP	AMP	Pi
0	124.6	12.8	1.7	2.4
30	54.8	78.2	2.4	67.2
Difference	−69.8	+65.4	+0.7	+64.8

Analysis of the products of the ATPase reaction. Products of the ATPase reaction were identified by column chromatography on Dowex I(Cl^-) (8). The composition of the reaction mixture (0.5 ml, 15 units of enzyme) was the same as for the DNase assay, except for the presence of 140 nmol of a mixture of [γ-^{32}P]-ATP (0.1 μCi) and [8-C^{14}]ATP (New England Nuclear, 0.2 μCi), and 45 nmol of sheared *E. coli* DNA. After incubation of the reaction mixture for 30 min at 37°C and chilling it in ice-water, 0.5 ml of the carrier solution (mixture of ATP, ADP, AMP, and inorganic phosphate, 500 nmol each) was added to the solution. It was then heated at 70°C for 3 min and applied to Dowex I(Cl^-) column. AMP (and inorganic phosphate), ADP, and ATP were eluted from the column with 10-ml (5 × 2 ml) each of 0.01 N, 0.04 N, and 0.50 N HCl, respectively. Small portions (0.2 ml) of each fraction (2.0 ml) were applied to paper filters (Schleicher and Schuell, 470C, 2 × 2 cm), and radioactivities were measured by a Beckman Scintillation counter.

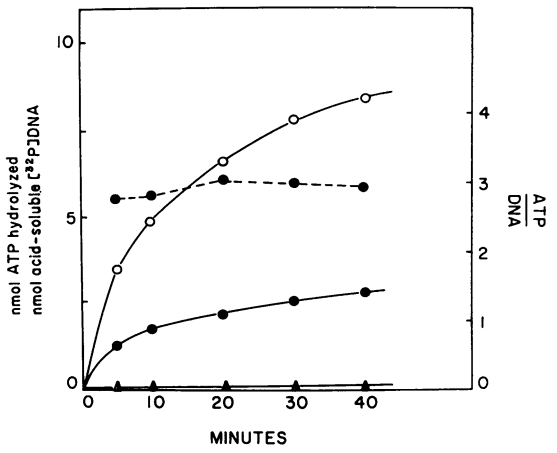

FIG. 3. Comparison of DNase and ATPase activities as a function of time of incubation. Labeled and unlabeled *E. coli* DNA were used for DNase and ATPase assays, respectively. O——O, ATPase (+DNA); ●——●, DNase (+ATP); ▲——▲, ATPase or DNase (−DNA and −ATP, respectively); ●- - -●, ATP/DNA ratio.

nucleotides released from DNA (about 3) remains relatively constant throughout the incubation period, which indicates that when *E. coli* DNA is used as substrate, about three ATP molecules are hydrolyzed to ADP and inorganic phosphate by the ATPase action, while one nucleotide is released from DNA by the DNase action. However, recent experiments in this laboratory (unpublished data) show that the product of DNase action on double- (as well as single-) stranded DNA is a mixture of 5'-oligodeoxyribonucleotides, ranging from mono- to penta-deoxyribonucleotides (average chain length, 2.8). Therefore, the consumption of ATP that is required for the hydrolysis of one phosphodiester bond of *E. coli* DNA should be about 8-9, and much greater than the value obtained by simply calculating the amount of acid-soluble radioactive material released from DNA during the reaction.

Table 3 compares the DNase and ATPase activities with various species of DNA. It is apparent that the DNase activity of the purified enzyme is completely dependent upon the presence of ATP, regardless of whether single- or double-stranded DNA is used as substrate. The activities, however, vary considerably depending upon the source of the DNA. In any case, as we have reported (1), double-stranded DNA is a better substrate than is the corresponding denatured DNA. The ATPase activities, as shown in the same table, also vary depending upon the species of DNA used, but correlate with the DNase activities. Thus, the ratio of the activity of ATPase to DNase for each DNA tested was relatively constant (about 2-3). There is essentially no difference in the ATPase/DNase ratio for double-stranded DNA and the corresponding denatured single-stranded DNA. On the other hand, no ATPase activity is detected in the presence of RNA or superhelical. double-stranded, circular(PM2)DNA, neither of which serves as a substrate for the DNase. The time-course studies and the results with different substrates all indicate that there is a strong correlation between the extent of the hydrolysis of DNA and that of ATP.

We also tested a few other DNA species, such as poly-(dA-dT), poly(dG·dC), and calf-thymus DNA for their effect on the ATPase activity (Table 3). From the data obtained, and the presumed constant ATPase/DNase ratio, one can predict the efficiency of these materials as substrates for the DNase activity, which has been difficult to assay because of unavailability of labeled material. The prediction made in this way for poly(dA-dT) was recently confirmed with labeled polymer.

Although there is a strong correlation between ATP consumption and DNA hydrolysis, the exact role of the ATPase activity in the hydrolysis of DNA still remains to be determined. The results presented in this paper favor the idea that ATP is directly involved in the process of hydrolysis of phosphodiester bonds of DNA. However, an alternate possibility is that the ATP is used in the conversion of the DNA structure (whether double- or single-stranded) into a specific transition state that is essential for the DNase action. We are attempting to detect the intermediate complex(es) of the enzyme reaction, such as enzyme-ADP(Pi) or DNA-ADP(Pi) in order to examine the precise role of ATP in the DNase reaction.

The number of ATP molecules consumed for the hydrolysis of one phosphodiester bond of DNA by the enzyme (calculated as about 8-9 in the case of *E. coli* DNA) seems to be unusually high; the total consumption of ATP molecules would be enormous if the enzyme functions *in vivo* as it does *in vitro*, where it displays uncontrolled digestion of DNA. Extensive digestion of DNA, as observed *in vitro*, is not observed *in vivo*, except under very unusual conditions such as when a *recA*$^-$ strain is irradiated (9). Under normal conditions, *in vivo*, there must be some mechanism to regulate or

TABLE 3. *Comparison of DNase and ATPase activities for various DNA substrates*

DNA	nmoles hydrolyzed: DNase −ATP	DNase +ATP	ATPase	ATPase/DNase
E. coli	<0.02	2.40	6.84	2.85
E. coli (denatured)	<0.02	0.50	1.61	3.26
Phage T4	<0.02	6.92	11.74	1.70
Phage T4 (denatured)	<0.02	0.58	1.26	2.12
Phage T7	<0.02	4.32	9.34	2.20
Phage T7 (denatured)	<0.02	1.00	2.07	2.07
Phage PM2	<0.02	<0.02	0.20	—
poly(dA-dT)	—	—	8.17	—
poly(dG·dC)	—	—	1.70	—
Calf-thymus	—	—	8.04	—
Ribosomal RNA	<0.02	<0.02	<0.20	—

DNase and ATPase activities with various DNA substrates. For the DNase assay, 15 nmol of ^{32}P-labeled *E. coli*, T4, T7, and PM2 DNA, as well as *E. coli* ribosomal RNA (a mixture of 16S and 23S ribosomal RNA) were incubated with 2.4 units of the enzyme. For the ATPase assay, 15 nmol of unlabeled DNA (except for PM2 DNA, where ^{32}P-labeled DNA was used) were incubated with 2.4 units of enzyme. *E. coli* and T4 DNA were sheared to a mean molecular weight of 8×10^6 before use. Denatured DNA was prepared by heating the DNA (300 nmol/ml in 15 mM NaCl-1.5 mM sodium citrate) at 100°C for 10 min.

limit the ATP-dependent DNase action on DNA, conceivably mediated by the *recA* product, so that the digestion of DNA and the total consumption of ATP is kept at a minimum. It is also possible that *in vivo* the structure of DNA on which the enzyme acts is very much different from that of the substrates that we provide *in vitro*. Thus, the whole process of ATPase action, as well as DNase action, *in vivo* may be quite different from those observed *in vitro*.

The enzymatic characteristics of the ATPase described in this paper are similar to those of the ATPase that is associated with ATP-dependent DNase from *Micrococcus luteus* (10), although the biological role of the *Micrococcus* enzyme still remains to be determined.

We thank Miss G. Y. Slywka and A. Oishi (D.D.S.) for skillful technical assistance, Drs. P. Margolin and A. Tzagoloff for their help during the preparation of this manuscript, and Miss J. Tamayo for her expert secretarial assistance. This work is supported by grants from the National Science Foundation (GB 14313) awarded to M. O.

1. Oishi, M. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 1292–1299.
2. Wright, M., & Buttin, G. (1969) *Bull. Soc. Chim. Biol.* **51**, 1073.
3. Barbour, S. D., & Clark, A. J. (1970) *Proc. Nat. Acad. Sci. USA* **65**, 955–961.
4. Goldmark, J., & Linn, S. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 434–441.
5. Grossman, L., Levine, S. S. & Allison, W. S. (1961) *J. Mol. Biol.* **3**, 47.
6. Espejo, R. T., & Canelo, E. S. (1969) *Virology* **37**, 495.
7. Glynn, I. M., & Chappell, J. B. (1964) *Biochem. J.* **90**, 147.
8. Pullman, M. E. (1967) *Methods Enzymol.*, **X**, 57
9. Howard-Flanders, P., & Boyce, R. P. (1966) *Radiat. Res.* (Suppl) **6**, 156.
10. Anai, M., Hirahashi, T., Yamanaka, M. & Takagi, T. (1970) *J. Biol. Chem.* **245**, 775