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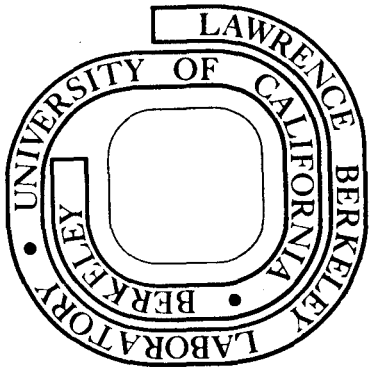

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ISOELECTRIC FOCUSING OF PURIFIED MURINE LEUKEMIA AND SARCOMA VIRUS DNA POLYMERASE. DETECTION OF TEMPLATE SPECIFIC STIMULATION

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Summary

RNA dependent DNA polymerase from Mo-MSV(MLV) was purified by DEAE-cellulose, phospho-cellulose, poly rC-agarose and G-100 Sephadex chromatography. Further purification and analysis by polyacrylamide gel and glycerol density gradient isoelectric focusing revealed a heterogeneous mixture of enzyme activities including an acid focusing activity that utilized only $(rC)_n$-$(dG)_{12-18}$ as template-primer. The uniqueness of the exclusive $(rC)_n$-$(dG)_{12-18}$ activity, which is descriptive of viral DNA polymerase, may be a result of the interaction of endogenous protein kinase and "DNA binding" proteins with the DNA polymerase. These interactions most likely contribute to the heterogenity of the isoelectric focusing profile. During purification, the $(rC)_n$-$(dG)_{12-18}$ activity of the DNA polymerase was diminished compared to $(rA)_n$-$(dT)_{12-18}$ from an original ratio of 0.2 to less than 0.05 in some cases. Addition of fractions that did not bind to phospho-cellulose and low molecular weight fractions from G-100 Sephadex chromatography partially restored the decreased $(rC)_n$-$(dG)_{12-18}$ activity. "DNA binding" proteins isolated from the virus on single-stranded DNA-cellulose stimulated the purified enzyme activity for both templates.

The enzyme most likely exists as a complex with protein kinase, nucleic acid binding proteins, nucleic acids and possibly other factors which have a considerable effect on enzyme stability and the template specificity of the enzyme.

Introduction

RNA tumor virus RNA-dependent DNA polymerases have been shown to copy the synthetic templates $(rA)_n$-$(dT)_{12-18}$ and $(rC)_n$-$(dG)_{12-18}$ preferentially over DNA templates or 70S viral RNA. The ability of viral DNA polymerase to copy $(rC)_n$-$(dG)_{12-18}$ and methylated-$(rC)_n$-$(dG)_{12-18}$ is regarded as descriptive for distinguishing viral DNA polymerase from polymerases of cellular origin[2]. Furthermore, after purification, mouse viral DNA polymerase does not copy its own 70S viral RNA very efficiently and it has been postulated that factors present in crude extracts to promote endogenous synthesis are missing in purified preparations of the enzyme[3,4]. Also a role for cellular transfer-RNA has been proposed for the interaction with virus polymerase[5,13,14].

Recent reports show that Rous sarcoma virus (RSV) reverse transcriptase is stimulated by protein kinase and by cytoplasmic proteins with DNA-binding activities[1,6]. We would like to confirm and extend these reports by showing that viral DNA polymerasé from Mo-MSV(MLV) can be affected by ATP dependent protein kinase present in crude extracts of mouse virus and by protein which binds to single-stranded DNA-cellulose. In addition, we report that mouse viral DNA polymerase preparations contain information to copy $(rC)_n$-$(dG)_{12-18}$ exclusively as evidenced by an acidic factor detected by isoelectric focusing in polyacrylamide gels and glycerol density gradients. When Mo-MSV(MLV) DNA polymerase was purified away from ATP dependent protein kinase and DNA-binding proteins, much of its activity was lost and the template specificity that describes $(rC)_n$-$(dG)_{12-18}$ was severely diminished with respect to $(rA)_n$-$(dT)_{12-18}$ synthesis. The indication is that viral DNA polymerase activity and perhaps its <u>in vivo</u> function is determined by cellular and viral factors which interact with viral DNA polymerase and perhaps play

a regulatory role in imparting viral-like character to the DNA polymerase found in RNA tumor viruses.

Materials and Methods

Rauscher leukemia virus grown in JLS-V9-cells and supplied as a sucrose density gradient centrifugation purified suspension at 1.5×10^{11} particles/ml was obtained as a generous gift of Jack Gruber from the Office of Program Resources and Logistics, National Cancer Institute, Bethesda, Maryland.

Moloney murine sarcoma and leukemia virus, Mo-MSV(MLV), was obtained from the medium of Balb 3T3 A31 cells grown in culture. Medium was centrifuged at 2000 x g for 10 min to remove cells and then centrifuged at 141,000 x g for 1 h in a Ti-14 zonal rotor. The virus containing material was scraped from the wall of the rotor into 5 ml of virus disruption buffer (VDB; 50 mM Tris-HCl pH 8.0, 500 mM KCl, 50 mM NaCl, 0.1 mM EDTA, 0.25% Triton X-100, 10 mM dithiothreitol, and 10% glycerol). The virus extract was allowed to stand for 2 h before centrifugation at 39,000 x g for 1/2 h in a SW 50.1 rotor. The supernate was stored at $4^{o}C$ until use. On some occasions crude virus was further purified by sucrose density gradient centrifugation and particles banding at 1.6-1.17 g/cm^3 were used as the enzyme source. However, results of the enzyme purification were substantially the same whether the sucrose gradient centrifugation step was included or not, except that purifying the virus greatly reduced the amount of enzyme activity in the starting material.

Virus extract was put through DEAE-cellulose and phospho-cellulose according to methods used by Verma[7]. Virus extract (10 ml, 0.5-1.0 mg/ml) was dialyzed against Buffer A (0.05 M Tris-HCl pH 8.0, 10% glycerol, 1 mM 2-mercaptoethanol, 0.05% Triton X-100) overnight and passed through a 4 ml DEAE-cellulose column. The column was washed with 10 ml of 0.2 M KCl

in Buffer A. The flow through and 0.2 M KCl wash were combined and loaded onto a 5 ml phospho-cellulose column and washed with 5 ml of 0.1 M KCl in Buffer A. The enzyme was eluted with 50 ml of a 0.1-0.5 M KCl gradient in Buffer A and 1.7 ml fractions were collected at 0.85 ml/min. The peak of enzyme activity eluted from phospho-cellulose at 0.24 M KCl. The peak fractions were pooled and dialyzed against Buffer A before applying to a 2 ml poly rC-agarose column[21]. Poly rC-agarose chromatography was performed at a flow rate of 0.5 ml/min and 1.5 ml fractions were collected. The column was washed with 2.5 ml of Buffer A and then eluted with a 25 ml 0-0.5 M KCl gradient in Buffer A. The major peak of $(rC)_n$-$(dG)_{12-18}$ activity eluted at 0.25 M KCl. Pooled fractions were dialyzed (volume 8 ml) and 2 ml was put on a 2 x 25 cm Sephadex G-100 column and eluted with Buffer A. One ml fractions were collected at a flow rate of 0.2 ml/min. Peak fractions were pooled and 2.5 ml was subjected to glycerol density gradient isoelectric focusing. Enzyme activity focusing near pH 5.8 was collected and stored at -70°C.

Glycerol Density Gradient Isoelectric Focusing. A 5 ml 5-55% glycerol density gradient was made in a 0.7 x 15 cm glass tube that composed one arm of a U-tube. The other arm of the U-tube and the tygon tubing that formed the apex which connected the two arms was filled with 60% glycerol in 0.74 M H_3PO_4. The U-tube was fitted in a Hoefer Scientific Model EF-301 electrophoresis apparatus (San Francisco, CA) with the gradient arm fitted in the rubber o-rings and the dense arm projecting up through the center well to achieve hydrostatic equilibrium. The U-tube was clamped at the apex before the 60% glycerol-H_3PO_4 solution was put in the center well arm and apex, and the density gradient pumped into the other arm. The 24 fractions (0.23 ml) that composed the 5 ml stepwise density gradient were made by mixing decreasing amounts

of dense solution (75 µl of 40% carrier ampholytes pH 3-10 (LKB), 1.375 ml glycerol, 250 µl protein sample and 0.8 H_2O) with increasing amounts of light solution (50 µl ampholytes, 125 µl glycerol, 250 µl sample and 2.075 ml H_2O) in separate holes of a plastic mini-well culture dish. The 24 fractions were pumped in the order of decreasing density onto the 60% glycerol-H_3PO_4 solution. The clamp was transferred to a piece of tubing above the center well arm and 0.08 M NaOH was placed in the upper reservoir over the top of the gradient and 0.74 M H_3PO_4 placed in the lower reservoir and center well to the same level as the upper reservoir solution. The upper clamp was removed and focusing was performed at 4°C at 250 V and 1 mA for 16 h. After focusing, the tubing was clamped at the apex and the 60% glycerol-H_3PO_4 solution was replaced with 70% glycerol. The gradient arm was stoppered with a serum cap punctured with a #16 gauge needle attached to a pump and fraction collector. The clamp was removed and the gradient pumped out the top at 0.25 ml/min; 125 µl fractions were collected. During fractionation, 70% glycerol was continuously added to the dense arm to prevent inversion of the gradient.

RDP Assay. Assay of RNA-dependent DNA polymerase (RDP) activity was done by the usual methods[9,10]. Reaction mixture components (0.1 ml) included 50 mM Tris-HCl pH 8.0, 50 mM KCl, 10 mM 2-mercaptoethanol, 0.02% Triton X-100, 0.02 mg/ml of $(rA)_n$-$(dT)_{12-18}$ or $(rC)_n$-$(dG)_{12-18}$ or 125 µg/ml "activated" salmon sperm DNA, 1 mM $MnCl_2$, $MgCl_2$(1mM with rA-dT, 5mM with rC-dG and DNA as template), and 0.02 mM [^{3}H] TTP or [^{3}H] dGTP (500 cpm/pmole). The complementary deoxynucleoside triphosphate (0.1 mM each) were also present when DNA served as template. Activity is expressed as pmoles of [^{3}H] dXMP incorporated per h for 10 µl enzyme extract.

Polyacrylamide Gel Isoelectric Focusing. A variation of the procedure used by Catsimpoolis was used to focus proteins[8]. The gel was polymerized in the presence of 3.4 µl of n,n,n',n'-tetramethylenediamine (TEMED), 1.4 mg of ammonium persulfate, 50 µl of 40% carrier ampholytes (pH 3-10), 0.66 ml of 22.5% acrylamide solution (22.2 g acrylamide and 0.3 g methylene-bis-acrylamide in 100 ml H_2O) and 1.30 ml of protein solution. Each sample (2.0 ml) was added to 5 mm i.d. x 130 mm glass tubes designed to fit a Hoefer Scientific gel electrophoresis apparatus. After polymerization (45 min), 0.08 M NaOH was used to fill the lower reservoir and 0.74 M H_3PO_4 filled the upper reservoir.

Focusing was performed at a constant current of 2.5 mA per tube until the voltage reached 250 V, whereupon the voltage was allowed to remain constant and the current was allowed to decrease. Total time of focusing was 16 h at 4°C.

After focusing, gels were removed from the tubes by syringe-forced water extraction and either stained for protein in a trichloroacetic acid-methanol-acetic acid solution (10% : 20% ; 7% : 0.01% Coomassie blue) or sliced into 2 mm sections for assay of RDP activity or pH measurement.

The 2 mm sections of the gel (vol. 40 µl) were each incubated in 100 µl of standard reaction mixture for 3 h at 37°C. Aliquots (60 µl) of each assay mix were then added to 10% trichloroacetic acid solutions and the precipitable radioactivity measured as for the usual assay.

Determination of the pH profile was done after overnight incubation of each slice in 0.2 ml of distilled H_2O at room temperature.

Isolation of Polymerase Stimulator on DNA-cellulose. Binding protein activity was isolated by a similar method to that employed by Alberts

et al.[11]. The virus preparation (12 mg in 10 ml of VDB) was made 1.7 M NaCl and 10% polyethylene glycol (6-7000 Mr) by addition of 30% polyethylene glycol in VDB. After mixing, the solution was allowed to stand for 30 min before centrifugation. The supernate was dialyzed against Buffer A overnight to reduce the salt and glycol before applying it to a 2 ml single-stranded DNA-cellulose column (P-L Biochemicals).

The column was washed successively with 5 ml of 0.2 M KCl, 5 ml of 0.5 M KCl and 5 ml of 2 M KCl in Buffer A. The fractions eluting at the different salt concentrations were dialyzed in Buffer A to remove the salt.

Protein Kinase Assay. Phosphorylation of substrate protein by an endogeneous protein kinase present in the crude virus preparation was done as described by Lee et al.[1]. Protein kinase activity was measured for 15 min at 37°C in crude virus preparation with and without added H_1 histone (0.2 mg/ml, Sigma Chemical Co., St. Louis MO). The total reaction mixture (1.0 ml) contained 50 mM sodium glycerol phosphate (pH 7.0), 5 mM NaF, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 2 mM theophylline and 2.4 μM [γ-^{32}P] ATP (9.26 Ci/mmole). Aliquots of fractions from glycerol density gradient isoelectric focusing (20 μl) were assayed by the above procedure for 30 min at 37°C. Precipitable radioactivity was collected on Millipore filters after the reaction was terminated by the addition of 10% trichoroacetic acid. After phosphorylation of endogenous protein by the above procedure without H_1 histone, a 0.2 ml sample was polymerized in a 2 ml gel and focused as usual. The gel was extracted from the tube, sliced into 2 mm sections and the ^{32}P was counted in a toluene based liquid scintillation fluid.

Estimation of Protein. Estimations of purity were made by gel focusing and determining relative amounts of protein by Coomassie blue staining procedures. The limits of detectability of stained bands visually after focusing were approximately 0.5 ug of protein in a focused band[8].

Since the amount of virus used as starting material was small, conventional methods of protein estimation could not be made and the amounts of enzyme present in the purified extracts could not be accurately determined. Considering a protein of 70,000 daltons is responsible for catalysis and using estimates of activities for the purified enzyme obtained by other workers[7,15,18] for the standard conditions for the $(rA)_n$-$(dT)_{12-18}$ assay, the amount of protein responsible for this activity can be estimated. Estimates indicate that 2.2 µg/ml of enzyme was present in the G-100 Sephadex preparation assuming a pure enzyme. Other workers have shown that the enzyme was greater than 90% pure after the phospho-cellulose stage[15] and it is assumed that even greater purification was achieved after G-100 Sephadex (See Table 1).

The effects of stimulators and the appearance of particular modifiers including the acid focusing activity were variable from one preparation to another. It should be noted that the variations of experimental results experienced from repeated runs were similar to those encountered by other investigators.

Results

Focusing of Purified Enzyme and Detection of Template-Specific Focusing Activity. After purification of DNA polymerase from murine leukemia virus by DEAE-cellulose, phospho-cellulose, poly rC-agarose and Sephadex G-100 chromatography, a considerable loss of $(rC)_n$-$(dG)_{12-18}$ template activity relative to $(rA)_n$-$(dT)_{12-18}$ was observed (Table 1). In some cases $(rC)_n$-$(dG)_{12-18}$ activity relative to $(rA)_n$-$(dT)_{12-18}$ decreased from an original value of 0.2 down to 0.05.

The loss of template activity for $(rC)_n$-$(dG)_{12-18}$, the template regarded as descriptive for DNA polymerase of viral origin, has led us to investigate what factors may be affecting the activity of the

enzyme in the crude and the purified state. The known complex interaction of nucleic acids, protein kinases and phosphatases, and the recently described DNA binding proteins has prompted us to consider their effect on enzyme activity[1,5,13]. Other workers have experienced variations during purification and have suggested that soluble fractions obtained during purification which did not contain the enzyme were capable of stimulating the polymerase[4]. In addition most reports had neglected to include a rigorous analysis of $(rC)_n$-$(dG)_{12-18}$ activity of the purified enzyme and may have overlooked the diminished transcribing ability of the pure enzyme for this template.

Figure 1 shows the glycerol density gradient isoelectric focusing profile of crude detergent disrupted RLV. DNA polymerase activity focused at several pHs with the main peaks of activity at pH 5.5 and 6.6. Unexpectedly, additional peaks of activity that were expressed with only $(rC)_n$-$(dG)_{12-18}$ occurred in the acid region of the gradient. Similar results were obtained by subjecting the enzyme to focusing after purification through the poly rC-agarose chromatography step (Fig. 2). The results presented are for Mo-MSV(MLV) as the enzyme source but are essentially the same as for RLV. Focusing after purification reflected the reduced $(rC)_n$-$(dG)_{12-18}$ activity of the purified enzyme, as is evidenced by the decreased rC/rA ratio. Polyacrylamide gel isoelectric focusing offered better resolution than glycerol density focusing and an almost complete separation of the major peaks was achieved (Fig. 3). The acid focusing activity elicited with only $(rC)_n$-$(dG)_{12-18}$ was very labile and variable from different preparations but was expressed in varying amounts in 19 out of 24 trials on gels. The total activity in the acid region ranged from amounts barely detectable over background to in some cases almost all the $(rC)_n$-$(dG)_{12-18}$ directed activity observed[23]. The

reason for this variability is not clear but the variability was also seen in 12 out of 18 trials in density gradients. E. coli and M. luteus DNA polymerase I produced only single, sharp peaks of activity and no activity was produced in the extreme acid region with the above gel focusing techniques. Other methods and experiments also showed the curious $(rC)_n$-$(dG)_{12-18}$ specific template activity (see below and Fig. 7).

Protein Kinase Effect on DNA Polymerase. Since the variability of expression of the acid focusing activity and the differences in the main peaks might be a result of the action of phosphatases and protein kinases present in the virus preparation[1,12], endogenous protein kinase activity and the endogenous products of the kinase reaction were examined by the focusing techniques. Using H_1 histone as a receptor and [γ^{32}P]ATP as substrate, we found that protein kinase activity was present in our extracts and focused on glycerol density gradients mostly at pH 5.8 (Fig. 4). The product of the kinase reaction when endogenous material acted as receptor focused predominately at pH 5.5 with some ^{32}P detectable at pH 6.6 (Fig. 5). The endogenous kinase in crude extracts incorporated 10 pmoles of phosphate per mg of protein in 20 min at 37°C. Since this would amount to approximately 21,000 dpm of phosphorylated products present in the 0.2 ml sample of crude extract that was put on gel focusing and the amount of radioactivity detected in the pH 5.5 and 6.6 peaks did not reflect this amount, it is possible that the phosporylated species were largely eliminated by a phosphatase known to be present in crude extracts.[1] Alternatively, the major phosphate receptor might be the acid focusing species detected in the gel slice assay for DNA polymerase activity (Fig. 3). However, this species probably could not be detected over the high background of radioactivity due to the unreacted [^{32}p] ATP that migrated to the

acid region. Attempts to remove the ATP and phosphatase before analysis have so far been successful. Further analysis of more purified preparations and, in particular, the pH 5.5 and 6.6 peaks has been hampered by small amounts of starting material and limited stability of the activities.

Protein kinase was largely removed from the enzyme preparation after phospho-cellulose since the majority of the kinase activity was found in the phospho-cellulose flow through. Addition of the phospho-cellulose flow through extracts back to purified DNA polymerase resulted in the stimulation of the $(rC)_n$-$(dG)_{12-18}$ activity (Table II). After subjecting phospho-cellulose flow through extracts to glycerol density focusing, additions of fractions to purified enzyme showed that a major fraction of stimulator focused at pH 5.6. This corresponded closely with the protein kinase focusing position (Fig. 4). In addition, another stimulator species was present in the acid region that acted only on $(rC)_n$-$(dG)_{12-18}$ template activity (data not shown).

Since DNA synthesis activity of crude extracts was stimulated by ATP while purified enzyme was not (Table III), it was desirable to see what effect ATP produced on the focusing profile of preparations that contained the protein kinase, the kinase receptor and the DNA polymerase. Therefore, crude virus preparations were treated with cold ATP for the endogenous kinase reaction as described in Materials and Methods and subjected to gel focusing. An increase in the amount of enzyme activity that focused at pH 5.5 in the ATP treated sample occurred with a concommitant reduction in the neutral pH region (Fig. 6). E. coli alkaline phosphatase treatment of the preparation resulted in an increase in the activity in the neutral region accompanied

by a decrease in activity at pH 5.5 suggesting that the usual profile of the enzyme consists of a mixture of partially phosphorylated species.

It is uncertain whether the DNA polymerase itself or another associated protein that interacts with the enzyme and regulates enzyme activity is phosphorylated[1]. It has been shown above that phosphorylated proteins focused at pH 5.5 and 6.6 in gel focusing. These coincide with the enzyme activity peaks, and is an indication that the enzyme might be the phosphate receptor and the multiple peaks are the result of different states of phosphorylation of the enzyme. However, additional experiments suggest that a protein in intimate association with the enzyme may be the receptor and that the relative amounts of binding of this protein with the enzyme are responsible for the different focusing points of the activity.

Detection of DNA Binding Protein in Virus Preparations. Disrupted virus extract was subjected to high NaCl concentrations and polyethylene glycol treatment (to disrupt complex interactions) before chromatography on a single-stranded DNA-cellulose column. Most of the enzyme was eluted from the column at 0.5 M KCl or less. However, a fraction that eluted from the column with high salt (2 M KCl) stimulated purified DNA polymerase slightly after the 2M KCl eluate was dialyzed (Table III). Although high degrees of stimulation were never achieved by addition of this preparation to purified enzyme, this factor was reminiscient of the DNA binding or unwinding protein[11]. The stimulation could not be explained by other well-known effects such as salt, divalent metal ion or detergent effects[10,24]. The stimulation was non-specific in that it increased synthesis with all the templates

tested and the 2M KCl eluate stimulator was not stable to storage. The 2M KCl eluate served as a receptor for ^{32}P in the protein kinase assay since 0.86 pmoles of phosphate were incorporated into acid precipitable material in 20 min/ml of 2M KCl eluate with the protein kinase assay described in Materials and Methods. The protein concentration in the 2M KCl eluate was extremely low, much less than 2.5 µg/5 ml of eluate according to gel focusing and subsequent staining procedures and around 10 ng/ml as determined by the ^{32}P assay above.

Considering the limited complexity of the virus and since the only detectable phosphorylated low molecular weight polypeptide from murine leukemia virus has been shown to be the p12 protein (Mr 12,000)[20] which Sen et al. have described as having RNA binding properties[19], our isolated 2M KCl eluate from DNA-cellulose is most likely a similar species. Also, in referring to the possibility of the enzyme being the phosphorylated species instead of the binding protein, the amount of DNA polymerase present in this virus is small and a phosphorylated polymerase would be difficult to detect with even the most sensitive labeling techniques. Therefore, a phosphorylated DNA polymerase probably would not have been detected by Pal et al.[20] using his methods and this same conclusion can be drawn concerning our work since even less starting material was used. A detectable phosphorylated species therefore probably is not the polymerase but is a species that most likely exists in higher proportional amounts than the enzyme such as the p12 protein. It has not been possible yet to directly compare phosphorylated species from the 2M KCl eluate with the pH 5.5 and 6.6 peaks seen in phosphorylation experiments of crude extracts (Fig. 5).

The interaction of the binding proteins with enzyme and nucleic acids in other systems is a strong and specific one and would most

likely withstand the strong electrophoretic separation effects of isoelectric focusing. The existence of a binding protein therefore is the most likely explanation for both the ATP dependent stimulation of DNA polymerase activity and the cause of the heterogeneity of enzyme activity observed in isoelectric focusing.

An interaction of viral RNA with the polymerase must also be considered since tritiated uridine that was incorporated into viral RNA de novo by the method of Fan et al.[17] was detected at pH 5.5 when crude labeled virus was density focused. Other labeled peaks occurred in the more acid region where one would expect uncomplexed RNA to focus (data not shown).

Other Experiments Indicating the Existence of Template-specific Activity. After treating the crude virus extract with Ribonuclease A and T_1 and then performing gel isoelectric focusing, the $(rC)_n$-$(dG)_{12-18}$ activity profile of the treated sample increased compared to the control. The ribonuclease treated sample also produced more acid focusing activity specific for $(rC)_n$-$(dG)_{12-18}$. The $(rA)_n$-$(dT)_{12-18}$ activity profile was almost unchanged compared to the control (manuscript in press)[23]. This result could be explained by assuming that a complex of RNA, binding protein, and DNA polymerase was degraded by the ribonuclease and the liberated binding protein including ones specific for $(rC)_n$-$(dG)_{12-18}$ were made available for the exogeneously added template after focusing.

Other experiments not related to focusing techniques also produced the template specific activity. As observed several times during Sephadex G-100 chromatography runs, a low molecular weight species expressed small amounts of only $(rC)_n$-$(dG)_{12-18}$ activity (Fig. 7). When this fraction was added back to the main peak of activity that eluted close

to the void volume, only the $(rC)_n$-$(dG)_{12-18}$ activity was enhanced (Table III). Experiments are in progress to determine if the low molecular weight species from G-100 Sephadex and the acid focusing activity, both of which are specific for only $(rC)_n$-$(dG)_{12-18}$ synthesis, have some properties in common and if they contain a class of p12 binding proteins that regulate a specific function for DNA synthesis. Characteristics common to both phenomena were that the activity was low, unstable and existed in varying amounts from one preparation to the other.

The importance of such a factor is emphasized by the fact that viral DNA polymerase utilize $(rC)_n$-$(dG)_{12-18}$ as template characteristically and that rC-rich regions have been proposed for specific initiation sites for other DNA polymerases from bacterial systems[16]. The loss of this factor during purification may be the explanation for the decreased $(rC)_n$-$(dG)_{12-18}$ activity in purified samples and perhaps for the inability of the purified enzyme to utilize its own 70S RNA efficiency as template to support the endogenous reaction[4,18].

Discussion

According to the results presented above from isoelectric focusing experiments, the viral DNA polymerase from murine tumor viruses interacts with other viral components which cause a change in its electrophoretic behavior and the specificity of the template-directed DNA synthesis. The results suggest that a complex interaction might exist *in vivo* which is necessary for full expression of enzyme activity and perhaps for the ability of the enzyme to copy endogenous viral RNA. This selectivity might provide a species specific method of expressing virus function. The exact mechanism of initiation of copying viral sequences is unknown but the revelation of a template-specific synthesis as evidenced by the unique $(rC)_n$-$(dG)_{12-18}$ directed synthesis implies

that a specific site for tumor virus initiation of replication might exist. This increases the probability that a specific inhibitor could be found to interfere with the initial stages of viral replication.

Since this template-specific function was associated with the enzyme throughout the purification (albeit in lesser amounts in the final stages of purification as evidenced by the reduced ability of the enzyme to copy $(rC)_n$-$(dG)_{12-18}$), it is assumed that a stable, undissociated enzyme complex is necessary for optimal activity and that the complex can be dissociated by dilution or by removal of stabilizing proteins. The initial concentration of factors present or the state of activation of the modifiers may be responsible for the variability observed in different preparations of starting material. These factors could be either of cellular or viral origin[13] and include cellular transfer RNA[22]. Isoelectric focusing also showed that the protein kinase activity might be intimately attached to the enzyme since the kinase activity coincided with the polymerase activity.

The stimulation observed can be summarized into two categories: a) a general stimulator that was found in phospho-cellulose flow through (focused at pH 5.5), that was present in the 2M KCl eluate from DNA-cellulose, and that was activated by the addition of ATP to crude extracts; and b) an $(rC)_n$-$(dG)_{12-18}$ specific stimulator that was detected in the acid focused fractions after gel focusing of crude extracts and whose effect was enhanced by ribonuclease treatment, was detected in the phospho-cellulose flow through as an acid focused species, and was still associated with enzyme after rC-agarose since it was detected as a low molecular weight species after the G-100 Sephadex step. Since two kinds of stimulation were observed, the proposal of Sen et al.[19] for the existence of at least two sub-

populations of p12 binding proteins would be an attractive hypothesis. In our system, maximum stimulation was achieved when ATP, protein kinase from phospho-cellulose flow through and the 2M KCl eluate were added to the purified enzyme. Optimal activity was produced for $(rC)_n$-$(dG)_{12-18}$ when fraction 33 from G-100 Sephadex was added to the above mixture.

Even though the catalytic function of the enzyme is carried out by a polypeptide of 70,000 daltons, an integrated replication complex consisting of the ancillary proteins for binding to the template-primer, the kinase that regulates the specific activity of the enzyme, and perhaps other factors not yet known is necessary for optimal enzyme activity[4]. These factors can conceivably determine the specificity of the reaction[4]. It was therefore necessary to look at the behavior of the enzyme more closely in the crude, unpurified state. The evidence presented suggests that one of the major virus constituents, the p12 phosphoprotein, is responsible for modifying the enzyme activity. This promotes the idea that other virus constituents, which previously were thought to have only a structural function, may yet turn out to have important regulatory functions in the replication complex.

The intriguing result of the isoelectric focusing profile is the appearance of enzyme activity that utilized only $(rC)_n$-$(dG)_{12-18}$. This raises the possibility that an rC-specific initiation site exists. It has been reported that a rC-rich region forming a hairpin double-stranded loop is synthesized and used as a primer for DNA synthesis initiation in some bacterial system[16]. If the ability of viral DNA polymerase to copy $(rC)_n$-$(dG)_{12-18}$ while other cellular DNA polymerases cannot readily copy this template is an indication of the existence of a specific recognition signal for initiation of DNA synthesis by viral DNA polymerase, a site would be available for directing

inhibitors for regulating viral replication in mammalian systems.

In analyzing and interpreting our data for the viral DNA polymerase and the template-specific activity, we have drawn largely on the considerable information accumulated for bacterial DNA polymerases and the large number of factors found in those DNA replication systems, including binding protein fractions, specific initiation sites and other factors needed during replication. Since we were unable to work with sufficient quantities of material to carry out the depth of research that bacterial DNA polymerase has suggested is needed, we are using the inferences gained from our research and information about other systems to develop methods of inhibition and regulation of virus function. One of the approaches is to look for an inhibitor of initiation of viral replication much like the rifampicin inhibition of E. coli RNA polymerase.

It is hoped that in the future the reason for the variability of the acid focusing activity and its function might be more completely understood and provide a specific handle for the control of virus replication. This might allow a specific inhibition of viral DNA polymerase without inhibition of normal cellular DNA replication.

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Table I Purification of Viral DNA Polymerase

step	vol(ml)	pmole dXMP[1] rA	rC	Total enzyme rA	rC	% recovery[2] rA	rC	rC/rA
DEAE-cell	20	720	190	14400	3800	115	53	0.26
Phospho-cell	15	830	480	12500	7200	100	100	0.59
rC-agarose	8	1040	520	8320	4160	66	57	0.5
G-100 Sephadex	4	318	48	5120[2]	760[2]	41	10.5	0.167
density isoelectric focus	0.4	268	33	2080[2]	330[2]	16	4.2	0.12

[1] Enzyme activity expressed as pmoles ^{3}H-dXMP polymerized/10 µl/h at 37°C. The template-primers used for assay are $(rA)_n$-$(dT)_{12-18}$ and $(rC)_n$-$(dG)_{12-18}$ designated respectively, rA and rC.

[2] Percent recovery expressed as the total amount of enzyme recovered in pooled peak fractions, assuming that all the recoverable enzyme was used for the following step. The purification procedure used was described in Materials and Methods. The protein concentrations of the fractions are as follows: 2 mg/ml before applying 10 ml to DEAE-cellulose, approximately 5 µg/ml after rC-agarose. This represents at least a 720-fold purification through poly rC- agarose step. After G-100 Sephadex, the protein concentration was undetectable by conventional means and the limited quantities of material precluded accurate determinations of purity.

Table II Stimulation of Purified DNA Polymerase by Phospho-cellulose Flow Through Extracts

	cmp dXMP incorporated using:	
	$(rA)_n$-$(dT)_{12-18}$	$(rC)_n$-$(dG)_{12-18}$
G-100 Sephadex purified enzyme[1]	88,182	5,087
P-cell flow through extract[2]	58,712	9,081
Both of the above reacted together	102,584	29,529

[1] The G-100 Sephadex preparation was obtained after purification of enzyme using the purification procedure outlined in Materials and Methods. The enzyme was purified from the main peak of material that was bound to the phospho-cellulose.

[2] The phospho-cellulose flow through contained some residual DNA polymerase as a result of overloading but represented only a small fraction (5%) of the total polymerase put on the column.

Table III Stimulation of DNA Polymerase Activity in Crude and Purified Enzyme Preparation

Additions	Crude Enzyme[1] pmoles of ^{3}H-dXMP incorporated with		Purified enzyme[2] pmoles of ^{3}H-dXMP incorporated with	
	$(rA)_n$-$(dT)_{12-18}$	$(rC)_n$-$(dG)_{12-18}$	$(rA)_n$-$(dT)_{12-18}$	$(rC)_n$-$(dG)_{12-18}$
none	196	55	180	34
2M KCl eluate DNA-cellulose	196	55	232	44
G-100 fract 33	196	55	172	52
2M KCl eluate and G-100 fract 33	196	55	214	52
ATP	240	83	180	34
ATP and 2M KCl eluate	244	90	230	45

[1]The crude preparation was detergent disrupted and dialyzed Mo-MSV(MLV).

[2]The purified preparation was obtained after the purification scheme outlined in Materials and Methods was followed through the poly rC-agarose step. Subsequent purification through Sephadex G-100 produced fraction 33 which when added to the main peak of enzyme from G-100 (fraction 22, see Fig. 7) or poly rC-agarose purified enzyme produced similar results. Enzyme activity is expressed as pmoles of acid precipitable radioactivity. Additions were 10 µl of the dialyzed 2M KCl eluate from the DNA-cellulose chromatography of 10 ml of detergent disrupted and polyethylene glycol treated crude Mo-MSV(MLV) as described in Materials and Methods; 10 µl of fraction 33 of G-100 Sephadex chromatographed enzyme; and 10 µl of 0.2 mM ATP to complete the reaction mix (0.1 ml). Adjustments were made to account for the small amounts of DNA polymerase activity present in the 2M KCl eluate and fraction 33 samples. Crude enzyme protein concentration was determined to be 0.60 mg/ml. Poly rC-agarose protein concentration was estimated to be 5 µg/ml by staining procedures after

Table III (cont'd)

gel focusing. The protein concentration of the 2M KCl eluate was estimated to be below 0.5 µg/ml by the same method.

Figure Legends

Figure 1. Glycerol density gradient isoelectric focusing of dialyzed detergent disrupted RLV. Focusing was performed with a 0.5 ml sample applied in a 5 ml gradient according to the procedure outlined in Materials and Methods. After focusing, 10 µl aliquots of each 125 µl fraction were assayed as usual for $(rA)_n$-$(dT)_{12-18}$ (O----O) and $(rC)_n$-$(dG)_{12-18}$ (●——●) template activity. An acid focusing material that expressed DNA polymerase activity with only $(rC)_n$-$(dG)_{12-18}$ was repeatedly seen in the acid region of the gradient to various extents in 12 out of 18 attempts.

Figure 2. Glycerol density gradient focusing of purified Mo-MSV(MLV) after rC-agarose chromatography. Conditions were the same as for Fig. 1 except that 2.5 ml of the peak fraction from rC-agarose were added to the 5 ml gradient. The assay conditions and symbols used were the same as for Fig. 1.

Figure 3. Polyacrylamide gel isoelectric focusing of Mo- MSV(MLV) obtained after phospho-cellulose chromatography. A dialyzed 0.2 ml sample was added to the 2 ml gel solution before polymerization. Focusing was performed according to the procedure in Materials and Methods. The symbols for enzyme activity are the same as for Fig. 1. The pH gradient is shown by the thin line and the characteristic $(rC)_n$-$(dG)_{12-18}$ peak in the acid region is shown at

the lower left hand corner. It should be noted that the acid focusing activity was extremely variable and was not expressed in all the experiments. The amount of acid focusing activity detected ranged from 90% of all the $(rC)_n$-$(dG)_{12-18}$ activity seen to amounts that were barely detectable above background. However, in 24 experiments on gels the acid peak was detected 19 times in varying degrees.

Figure 4. Protein kinase activity profile after glycerol density focusing of dialized crude Mo-MSV(MLV). After focusing 0.5 ml of crude Mo-MSV(MLV), aliquots (20 µl) were assayed for protein kinase activity as described in Materials and Methods with H_1 histone as substrate. The major peak of kinase activity focused at pH 5.8 while a small peak of activity appeared in the neutral region.

Figure 5. The gel focusing profile of the ^{32}P labelled products of the endogenous viral protein kinase present in crude Mo-MSV(MLV). Crude dialyzed virus was incubated with [$\gamma^{32}P$] ATP as described in Materials and Methods without added H_1 histone. After incubation, aliquots of the reaction mix (0.1 ml, ●——●, and 0.2 ml, 0----0) were added to gel solution and focused as usual. After focusing and slicing into 2 mm sections, the slices were put into toluene based liquid scintillation solution and counted. The unreacted [^{32}P]ATP was not removed from the preparation before focusing and therefore the ATP migrated to the extreme

acid region of the pH gradient which accounted for the large amounts of radioactivity in the gel length up to about 30 mm. The pH profile is represented by the thin solid line. The products of the kinase reaction focused at pH 5.5 and 6.6.

Figure 6. Gel focusing of crude, dialyzed disrupted Mo-MSV(MLV) after incubation with ATP or E. coli alkaline phosphatase. For ATP preincubation (O----O), 0.2 ml of virus extract in virus disruption buffer was heated to 37°C for 15 min in the presence of 0.2 mM ATP. For phosphatase reaction (●——●), 0.2 ml reaction mixture contained 45 µg phosphatase (Sigma), and 5 mM $MgCl_2$. The samples were then run in identical fashion as described in Materials and Methods. Enzyme activity was measured with $(rA)_n$-$(dT)_{12-18}$. The stippled area (pH 5.5 peak) represents activity enhanced by ATP treatment. The area represented by the horizontal bars (pH 7.5) is activity enhanced by the phosphatase treatment.

Figure 7. Sephadex G-100 chromatography of Mo-MSV(MLV) polymerase after poly rC-agarose. Enzyme activity was measured with $(rA)_n$-$(dT)_{12-18}$ (O----O) and $(rC)_n$-$(dG)_{12-18}$ (O——O). Fraction 33 exhibited activity with only $(rC)_n$-$(dG)_{12-18}$ but not with other templates. When aliquots of tube 33 were added to the main activity

peak, the $(rC)_n$-$(dG)_{12-18}$ activity was stimulated specifically. Again the $(rC)_n$-$(dG)_{12-18}$ activity expressed in tube 33 was variable but was seen in 5 out of 7 runs with enzyme in various stages of purity. The activity in tube 33 was very unstable and was not amenable to further characterization.

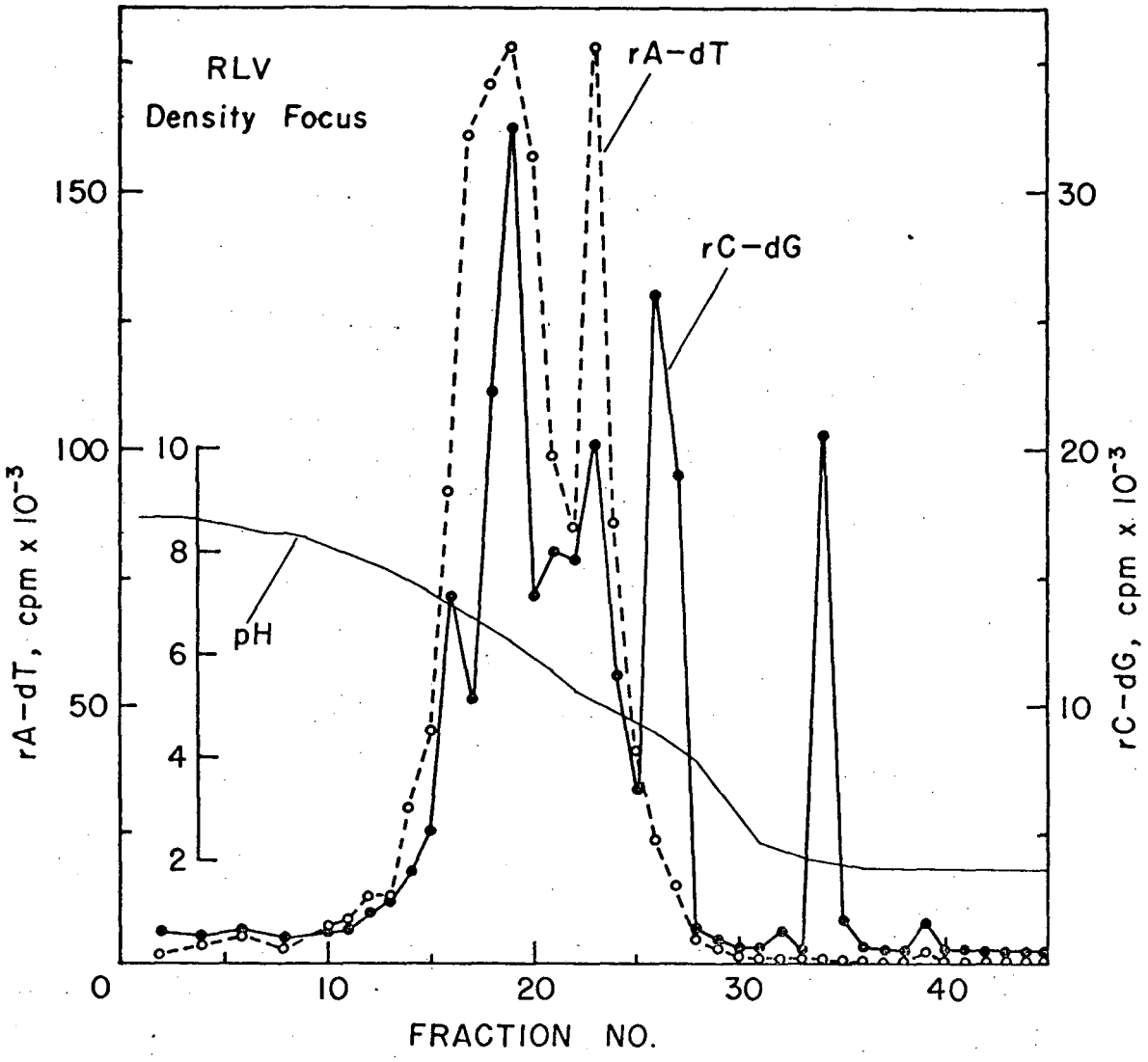

FIGURE 1
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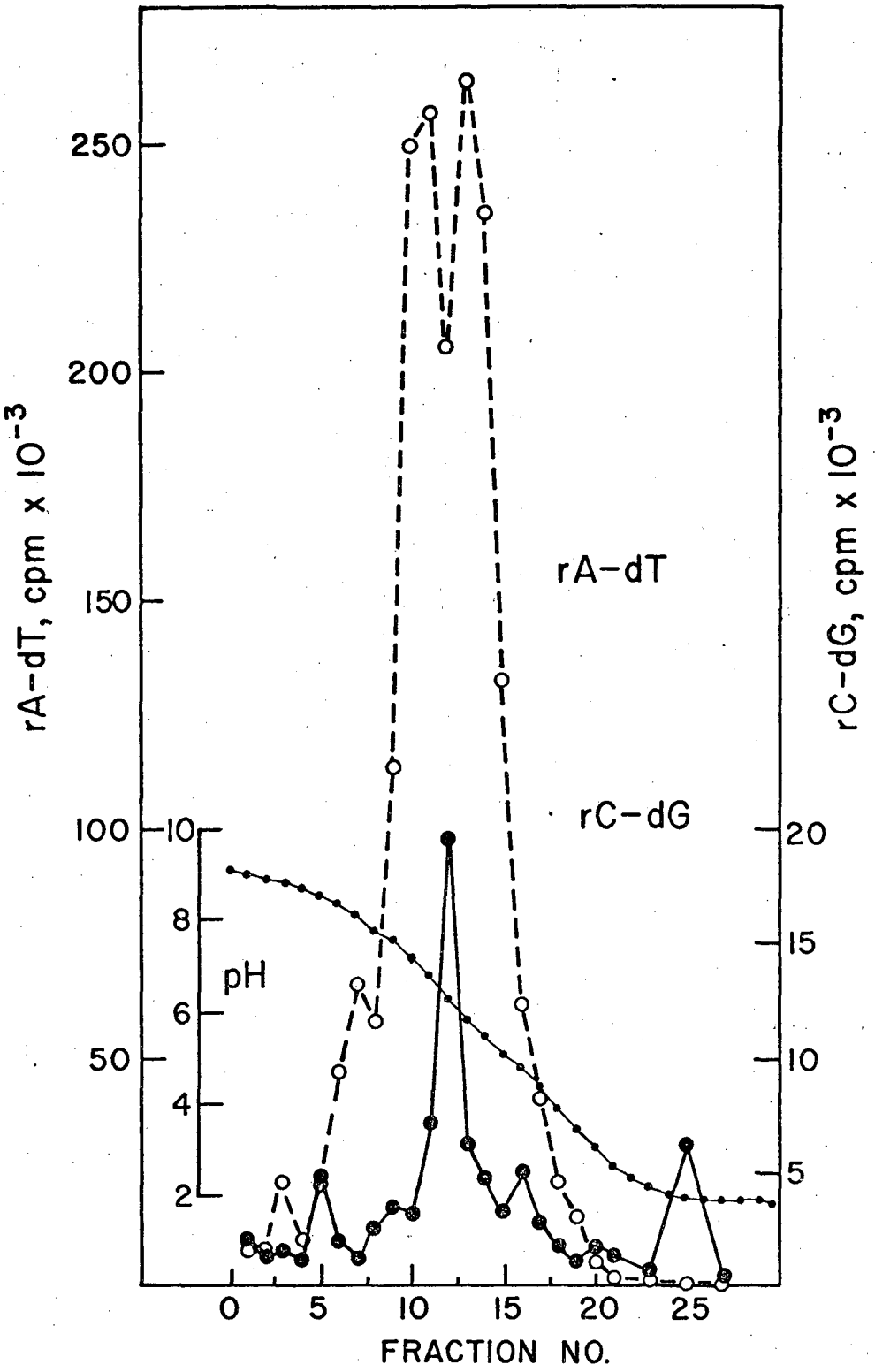

FIGURE 2

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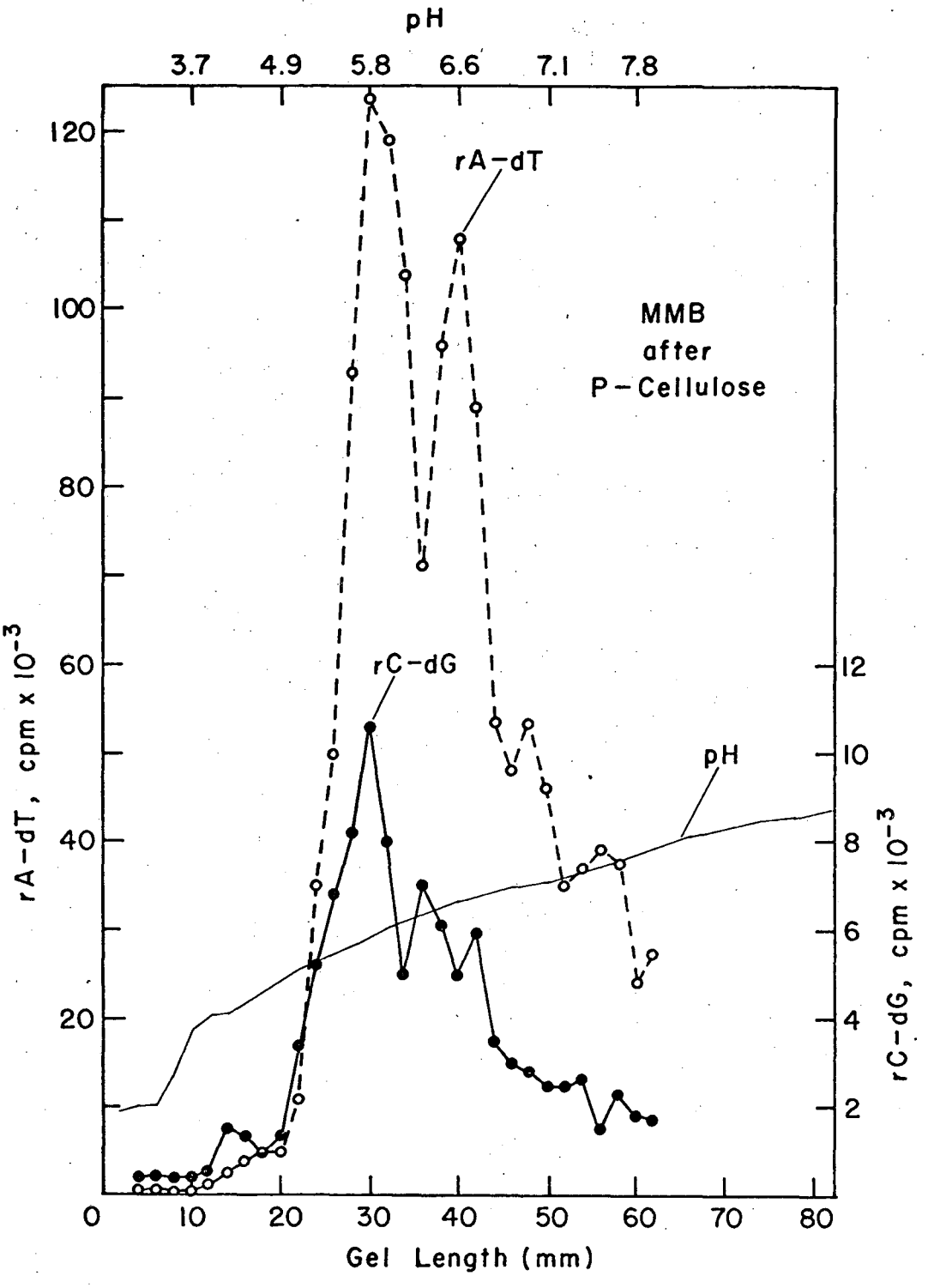

FIGURE 3

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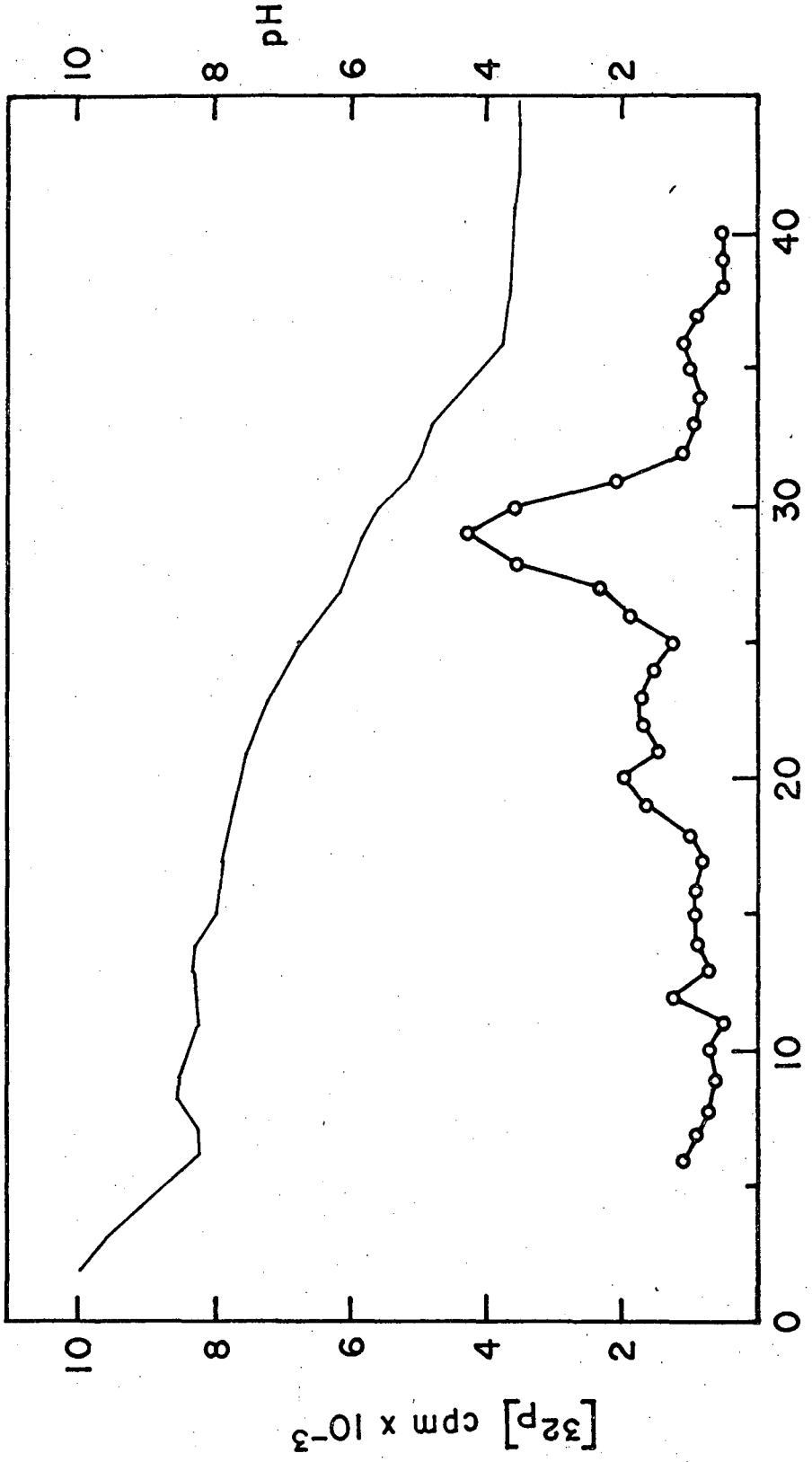
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4
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30
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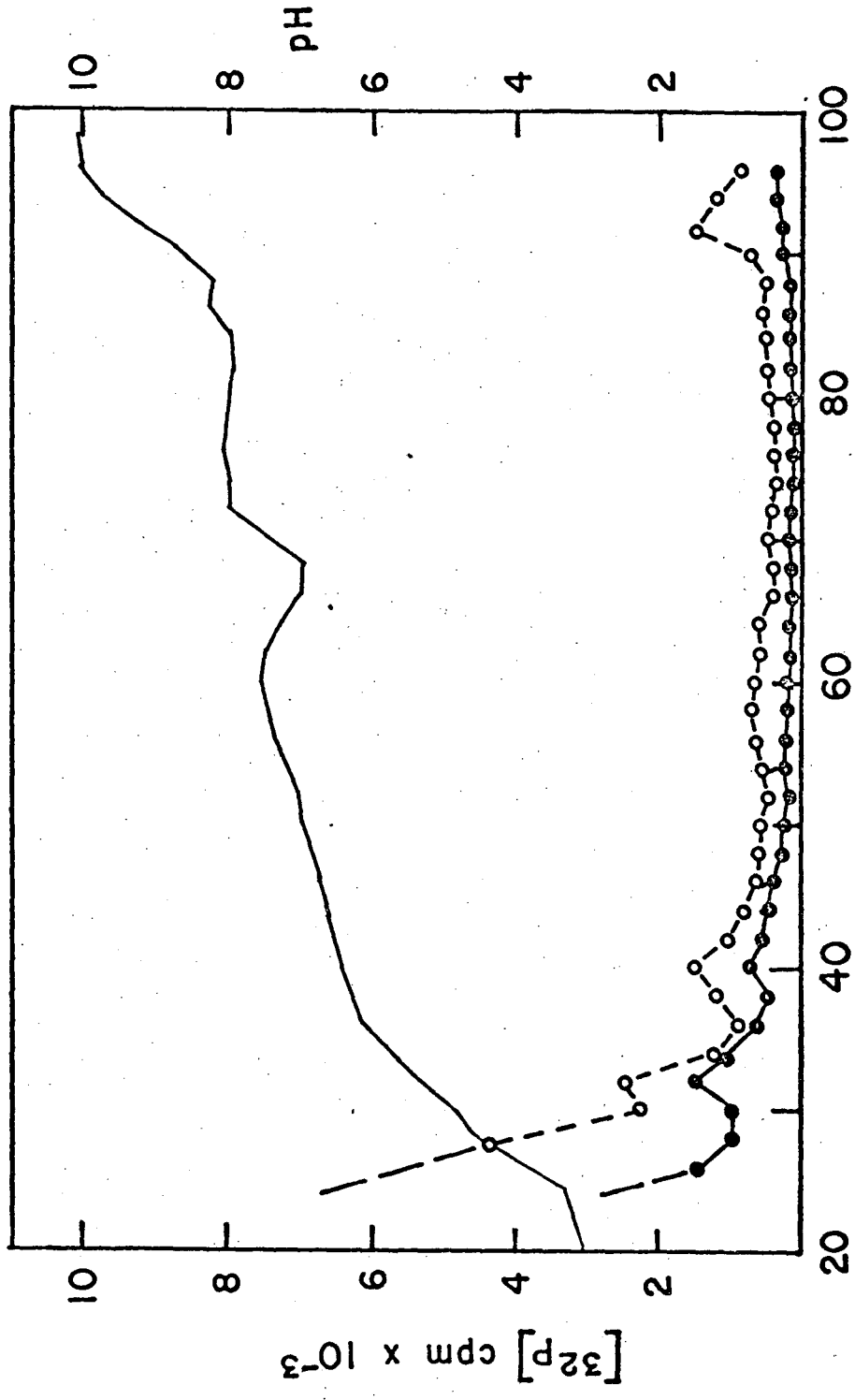
FRACTION NO.
XBL769-9549

Fig. 4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GEL LENGTH (mm)
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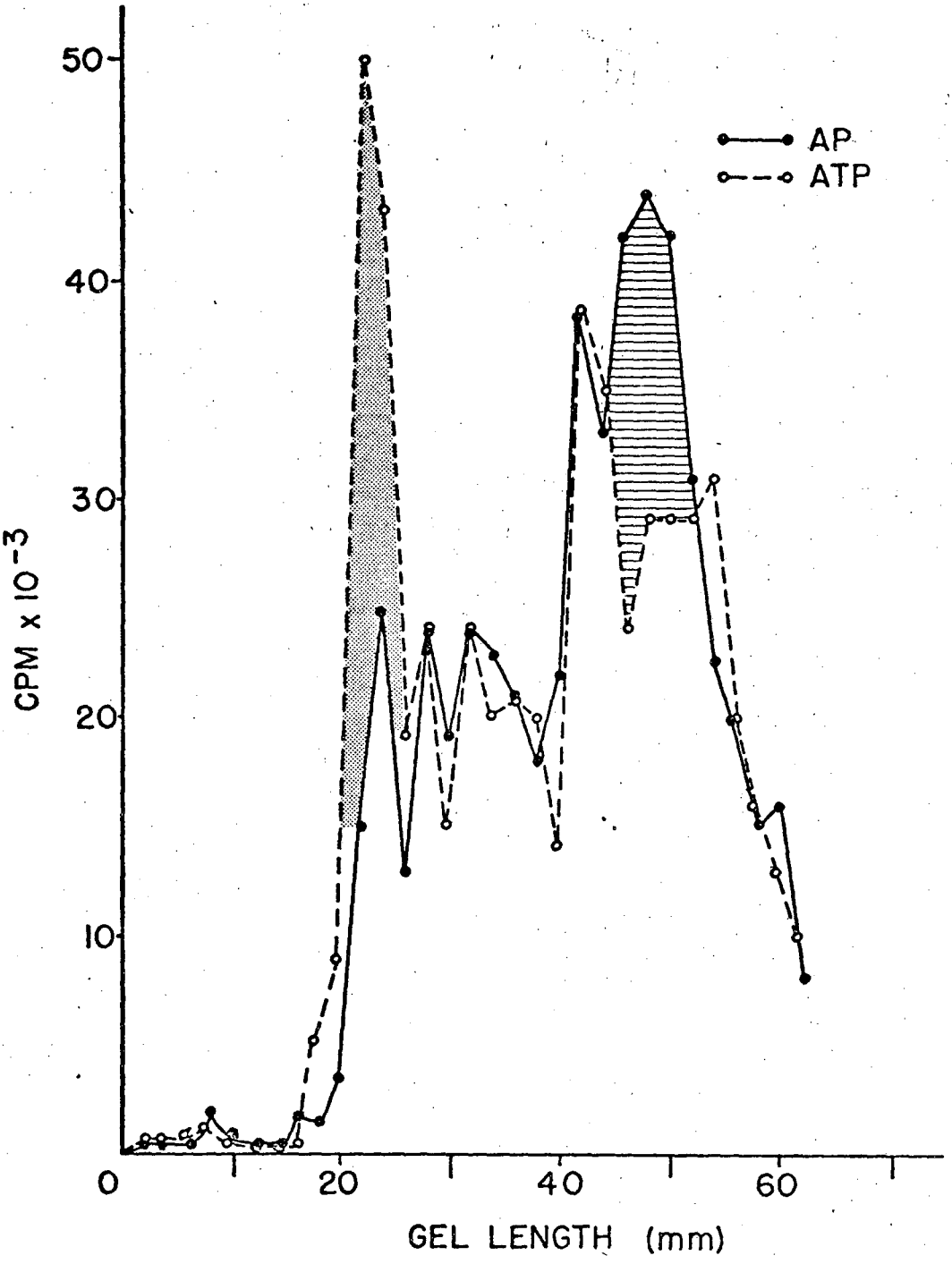
Fig. 5



XBL 767-6011

FIGURE 6

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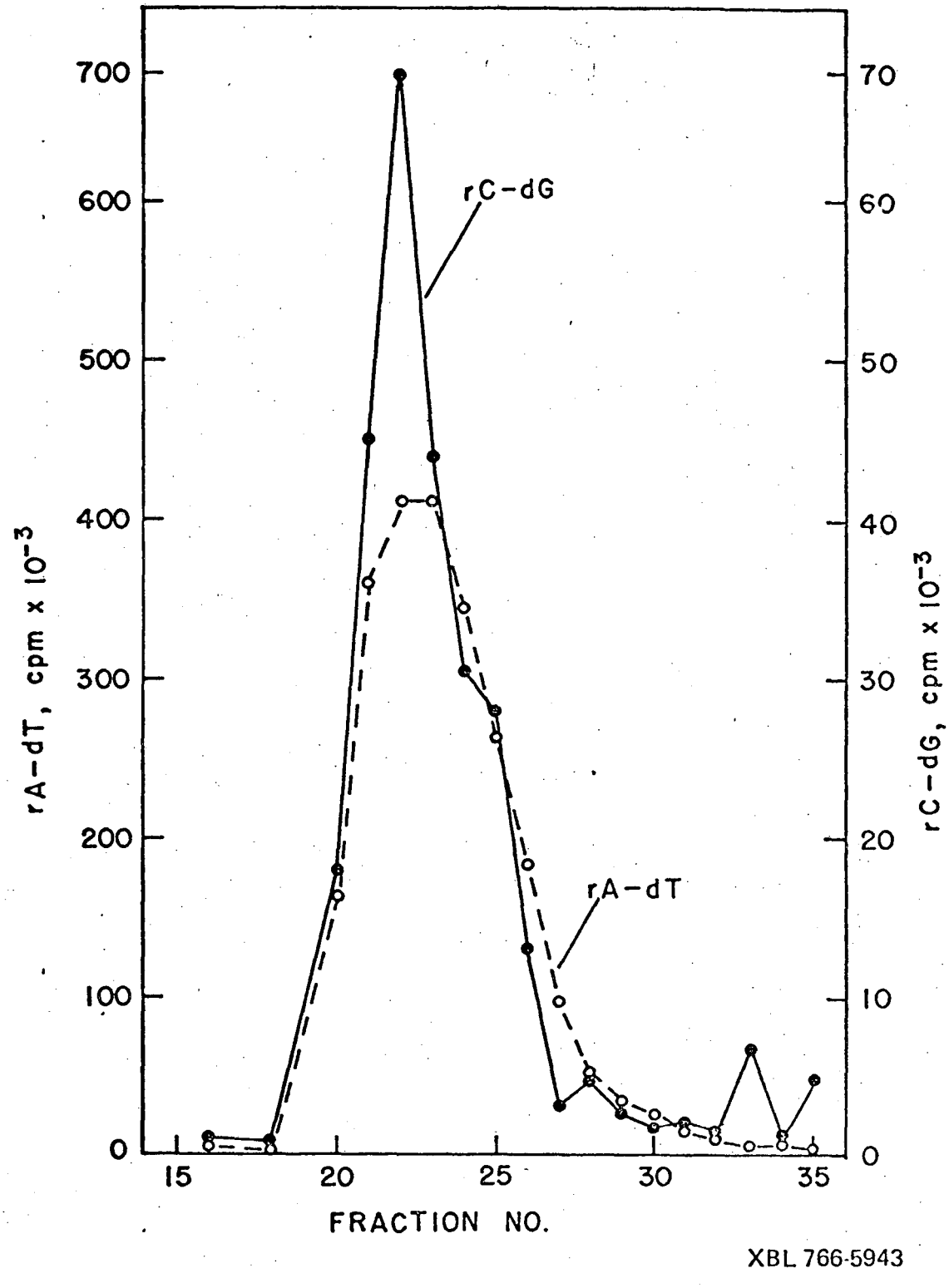

FIGURE 7

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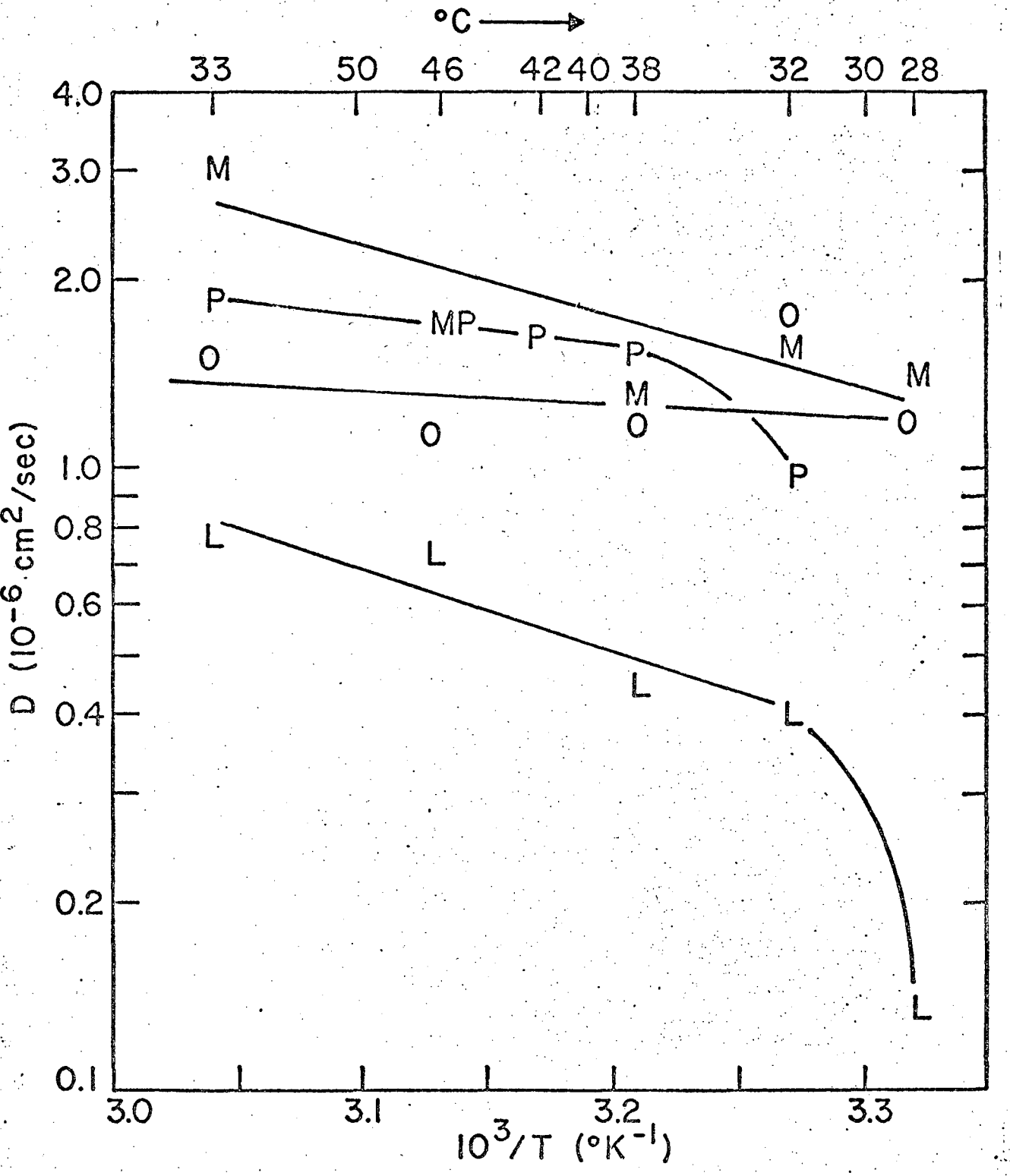

XBL 7611-10775

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