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Affinity Chromatography of Viral DNA Polymerases on Pyran-Sepharose

(RNA tumor viruses/RNA-dependent DNA polymerase)

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ABSTRACT **Pyran covalently linked to cyanogen bromide-activated Sepharose has been shown to be an effective affinity matrix for several viral DNA polymerases. Differential salt elution of viral compared with cellular polymerases, as well as substrate elution, suggests the affinity nature for the matrix. Unlike some other affinity systems described, pyran-Sepharose is totally resistant to nuclease digestion and is stable at 4° for several months. DNA polymerases isolated from several viruses by detergent treatment were recovered in good yield. Analysis of iodinated proteins by sodium dodecyl sulfate-gel electrophoresis revealed that the DNA polymerase of avian myeloblastosis virus found in crude preparations of the virus could be purified nearly to homogeneity by a single passage through the column. These results suggest that pyran-Sepharose is an effective affinity column that is potentially adaptable as part of a general purification procedure for viral DNA polymerases.**

The discovery of DNA polymerase activities associated with RNA retraviruses initiated various studies dealing with the search for specific inhibitors of these enzymes (1, 2). To date there are several reports dealing with inhibition of viral DNA polymerases (3–6). The general classification of such inhibitors varies from large synthetic polynucleotides (7) to small molecules such as alkaloids (8) and inorganic cations (9). In most cases these compounds inhibited by interfering with the nucleic acid templates (10, 11). There are, however, several inhibitors that bind to the enzyme at either the catalytic site (7, 12, 13) or other sites (14). One such compound, which binds in a noncompetitive fashion, is the pyran copolymer. The copolymer is a divinyl ether of maleic anhydride (Fig. 1) which, in aqueous solution, hydrolyzes to its corresponding polycarboxylic acid. The highly negatively charged pyran molecule is thought to simulate the nucleic acid phosphodiester backbone and has an approximate molecular weight of 17,000 (15).

Pyran inhibits viral DNA polymerases from several sources, yet has little inhibitory effect on cellular DNA polymerase (14). This observation, as well as the ability of pyran to bind to the enzyme, prompted us to exploit these properties as an affinity ligand for fractionation of viral enzymes from crude extracts. An additional property is its biostability to nucleases which are prevalent in crude viral and cellular extracts.

In this paper we report chemical coupling of pyran to Sepharose and its affinity properties for viral DNA polymerases. We demonstrate a viral DNA polymerase affinity fractionation that uses as the affinity ligand a non-nucleic acid inhibitor.

Abbreviations: AMV, avian myeloblastosis virus; RLV, Rauscher leukemia virus; RSV, Rous sarcoma virus, FeLV, Feline leukemia virus; MTV, murine mammary tumor virus.

MATERIALS AND METHODS

Reagents. Unlabeled and labeled deoxynucleoside triphosphates were obtained from Schwarz/Mann Bioresearch; specific activities (Ci/mmol) were: dTTP, 17.3; dATP, 20; dCTP, 30; dGTP, 20. Calf thymus DNA was obtained from Worthington Biochemicals, and "activated" calf thymus DNA was prepared by limited DNase I digestion as described (16). All synthetic polynucleotides used were obtained from Collaborative Research.

Pyran Source. The pyran copolymer was synthesized by Hercules Research Center, Wilmington, Del.

Viruses. Avian myeloblastosis virus (AMV) from the plasma of infected chicks was obtained through the Virus Cancer Program of the National Cancer Institute. The virus was concentrated and purified as described (17).

RIII murine mammary tumor virus (MTV) was prepared from mouse milk obtained from Meloy Laboratories. A sample of 2.5 ml of milk was added to an equal volume of 10 mM Tris·HCl (pH 8.2) buffer containing 15 mM EDTA and 0.15 M NaCl and centrifuged at 5000 × *g* at 4° for 20 min. The sample was then layered onto a 10–40% (w/w) sucrose gradient and centrifuged for 2.5 hr at 100,000 × *g*. The visible band (with a density of 1.16 g/cm^3) was removed and pelleted through cold buffer containing 10 mM Tris·HCl (pH 8.2), 2 mM EDTA, 0.15 M NaCl for 1 hr at 100,000 × *g*. The resulting pellet was resuspended in 10 mM Tris·HCl (pH 8.2) and stored at −90° until further use.

Rauscher leukemia virus (RLV), Rous sarcoma virus (RSV), feline leukemia virus (FeLV), and JLSV9 cells were obtained from the office of Program Resources and Logistics of Virus Cancer Program, NCI.

Preparation of Crude Extracts from JLSV9 Cells Producing RLV Virus. Approximately 2 × 10^8 cells were suspended at 10 times their pelleted cell volume containing the following: 50 mM Tris·HCl (pH 7.8), 500 mM KCl, 2 mM dithiothreitol, and 10% glycerol. Cells were homogenized by sonication for 15 min. The extracts were then made 0.5% with NP40. After successive centrifugation at 4000 × *g* and 10,000 × *g*, the supernatant was finally centrifuged at 100,000 × *g* (S-100). The S-100 fraction was passed through a DEAE-cellulose column to remove the nucleic acid components. The eluate was dialyzed and fractionated on the pyran-Sepharose column.

Preparation of 70S AMV RNA. High-molecular-weight 70S RNA from AMV was extracted (18) and layered onto linear sucrose gradients of 5–30% (w/w) in 0.1 M NaCl, 10 mM Tris·HCl (pH 7.3), and 1 mM EDTA. Centrifugation was performed for 3 hr at 105,000 × *g*. Fractions were collected

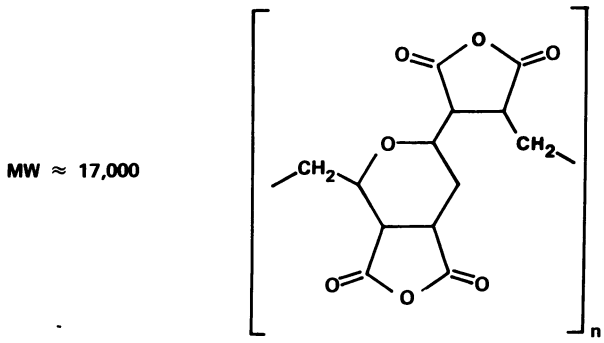


FIG. 1. Structure of the pyran copolymer.

and precipitated with ethanol. External markers used were 28S and 18S [^{3}H]RNA from NC-37 cells, a generous gift from Steve Turner, Schwarz/Mann.

Assay Conditions for DNA Polymerases. Viral DNA polymerases were assayed essentially as described (12). *Escherichia coli* and *Micrococcus luteus* DNA polymerases were assayed by established procedures (19, 20).

Preparations of Viral Extracts. Samples of different viruses were disrupted as described (12).

Electrophoresis of Denatured and ^{125}I-Labeled Proteins on Sodium Dodecyl Sulfate-Polyacrylamide Gels. Protein samples from crude AMV viral extracts and AMV DNA polymerase fractionated on pyran-Sepharose were labeled with ^{125}I using chloramine T (21). Protein samples were denatured and subjected to electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel for 5 hr. The protein bands were fixed in a solution containing 7.5% methanol and 5% acetic acid. The gel was dried and exposed to Kodak safety film.

RESULTS

Preparation of Pyran-Sepharose. Hydrazine-Sepharose was prepared and washed as described (22). The gel was suspended in an equal volume of water and adjusted to pH 11 with dilute KOH. The pyran copolymer (3 mg/ml), dissolved in the same manner, was added to Sepharose at a final concentration of 5 mg/ml of gel. The coupling reaction was carried out for 16 hr at 50° and terminated by removing unbound pyran with several washes with salt and distilled water. The pyran-Sepharose was then packed in 0.3 × 5 cm pipettes. The total volume of the column was 2.0 ml. Columns were equilibrated with 50 mM phosphate (K^+) buffer (pH 7.2) containing 20% glycerol and 1 mM dithiothreitol. The flow rate was adjusted to 10 ml/hr.

Elution Patterns of Viral DNA Polymerases from Pyran-Sepharose Column. DNA polymerase activities from several oncogenic viral sources showed affinity for pyran-Sepharose (Fig. 2). The two murine polymerases (RLV and MTV) appear to bind to the pyran column less tightly than the avian enzymes and were eluted at a lower salt concentration (0.22–0.25 M). Feline leukemic viral polymerase elute at an intermediate salt concentration of 0.3 M. By contrast to viral polymerases, the two bacterial enzymes eluted at a much lower salt concentration. Table 1 summarizes the recovery of activities from the pyran-Sepharose column and the salt concentration required for elution of the different viral polymerases.

To examine whether the binding of viral polymerases was due to the pyran ligand or to binding with the activated hydrazo-Sepharose matrix, we tested affinity of AMV DNA polymerase for the latter matrix. In the absence of pyran, AMV polymerase eluted at 0.08 M KCl in contrast to 0.28 M when the ligand was present (Fig. 2).

Substrate Affinity Elution of AMV DNA Polymerase. In order to further examine the affinity nature of the pyran-Sepharose to DNA polymerases, we attempted to elute the enzyme using its natural 70S RNA and synthetic substrates. Substrate elution has also the added advantage of amplifying the purification (23). In both experiments (Fig. 3), the affinity column was first saturated with AMV DNA polymerase and the excess unbound enzyme was washed with the column buffer. When poly(rA)·$(dT)_{12-18}$ was included in the elution buffer (which was free of salt), all the bound enzyme was recovered in two column volumes. Subsequent elution with high salt (0.45 M KCl) initiated at fraction 23 did not elute any additional activity. A similar experiment was done with oligo(dT)-primed AMV 70S RNA as the eluting substrate. Due to the unavailability of 70S RNA in substrate quantities, limiting amounts of the primed 70S RNA were used as the eluting substrate. Correspondingly, a small amount of polymerase was eluted and the remaining activity was recovered in the high salt fraction.

To assure that the described elutions were not due to salt effects or small variations in pH, all fractions were monitored for conductivity and pH. The conductivity and pH for all fractions showed no change compared to the eluting buffer. In control experiments where the pH was varied between 7.2 and 7.8, essentially no polymerase activity was eluted, further verifying the affinity nature of the pyran matrix. When total unfractionated yeast RNA or tRNA was used to elute the polymerase activity, only a small amount of activity was recovered.

Fractionation of RLV DNA Polymerase from JLSV9 Cell Extracts. In order to determine the selectivity of the affinity matrix for viral DNA polymerases in the presence of extracts containing cellular polymerases, we examined the elution profile of RLV viral polymerase in extracts from JLSV9 cells. The affinity of the major peak of the viral DNA polymerase (Fig. 4) remained the same as that of the enzyme from purified viral extracts eluting at 0.25 M KCl (Fig. 2).

DISCUSSION

Affinity chromatography is a widely used method for the fractionation of biological compounds. This method, unlike all other classical modes of fractionation, takes advantage of specific interactions between biological macromolecules (24). A predominant use of this technique has been in enzyme purification. The several advantages include rapid and selective fractionation as well as protection of the enzyme against denaturation, which may be due to inhibitors or to physical alterations of the native protein conformation. The affinity ligands used for enzyme fractionation may be a substrate, a cofactor, or an inhibitor. The proper choice of the ligand is based on the degree and reversibility of its affinity to the enzyme. In addition, the ligand should be covalently bound to the supporting matrix without any damage to its biological properties. In all cases, elution with specific substrates is an important test for the affinity nature of the ligand-matrix (23).

There are very few reports describing the application of

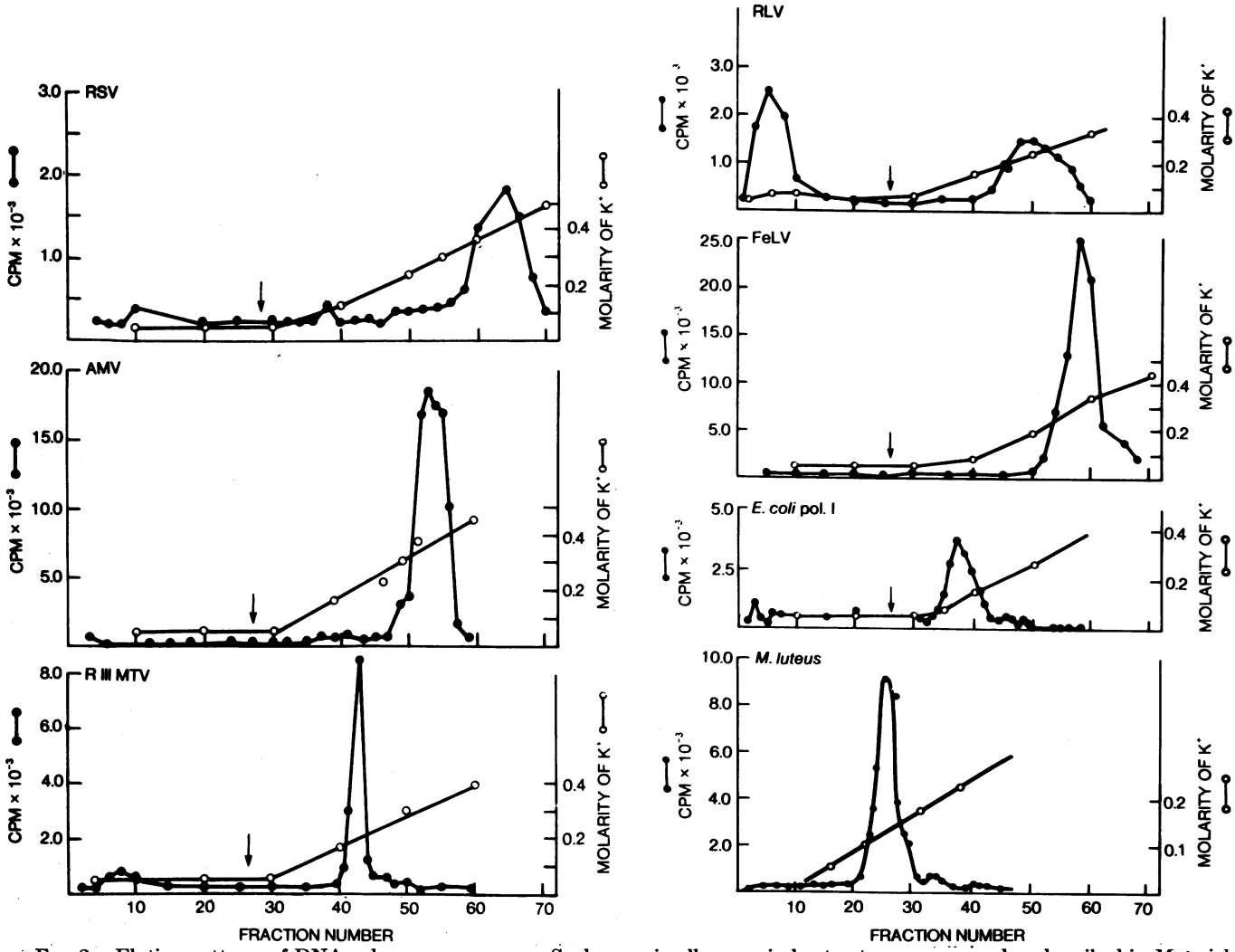


FIG. 2. Elution patterns of DNA polymerases on pyran-Sepharose: in all cases viral extracts were prepared as described in *Materials and Methods*. The samples were diluted to a final concentration of 20 mM (K^+) in a total volume of 2 ml. The column was washed with three column volumes of equilibrating buffer. A linear salt gradient (0–0.4 M KCl) started at the fractions indicated by the solid arrow, and 0.1-ml fractions were collected. All viral polymerases were assayed as described in *Materials and Methods*, with poly(rC) oligo$(dG)_{12-18}$. The template used for the bacterial polymerases was d(AT). [^{3}H]dTMP incorporations in acid-precipitable counts were measured.

affinity chromatography to viral DNA polymerase. Livingston *et al.* developed a selective immunoadsorbent for the DNA polymerase from murine leukemia virus (25). This affinity column, although effective for the system, could not be used for other viral polymerases due to its restricted specificity. Gerwin and Milstein (26) reported an oligo-$(dT)_{12-18}$-cellulose to purify DNA polymerase from Rauscher leukemia virus and RD-114 infected cells; however, the specificity of this matrix has been recently questioned (27). Marcus *et al.* (28) described a polycytidylate-agarose matrix which was effectively used for the purification of AMV DNA polymerase. The latter system, as well as that of the oligo-(dT)-cellulose column, suffers from the disadvantage of being nuclease-sensitive, making it difficult to purify viral enzymes in crude cellular extracts.

TABLE 1. *Recovery of activities from the pyran-Sepharose column*

Viral polymerase*	cpm in starting sample ($\times 10^{-6}$)	cpm recovered in high salt ($\times 10^{-6}$)	% activity recovered	K^+ concentration of peak fraction (M)
AMV	3.5	2.6	75	0.38
MTV	0.54	0.5	99	0.22
RLV	1.2	0.87	72	0.25
RSV	0.6	0.05	8–10	0.40
FeLV	12.8	7.6	60	0.30

* Assayed with [^{3}H]dGTP, 3.0 × 10^3 cpm/pmol.

In this paper we report an affinity matrix for viral DNA polymerases using the pyran copolymer. Although the details of the covalent linkage between pyran and the hydrazine residues are not yet established, we favor the mechanism shown in Scheme I. Since heating was involved during the preparation, it is assumed that the cyclic derivative (II) is the major form of the ligand, in addition to the half-acylated product (I). Since the matrix can be reused without loss of effectiveness, it is also unlikely that the DNA polymerases are being bound by virtue of the unhydrolyzed anhydride residue acylating free amino groups in the polymerases. The intermediate half-acylated matrix (I) has free carboxyl residues, and it can be argued that it is functioning as a cation exchanger.

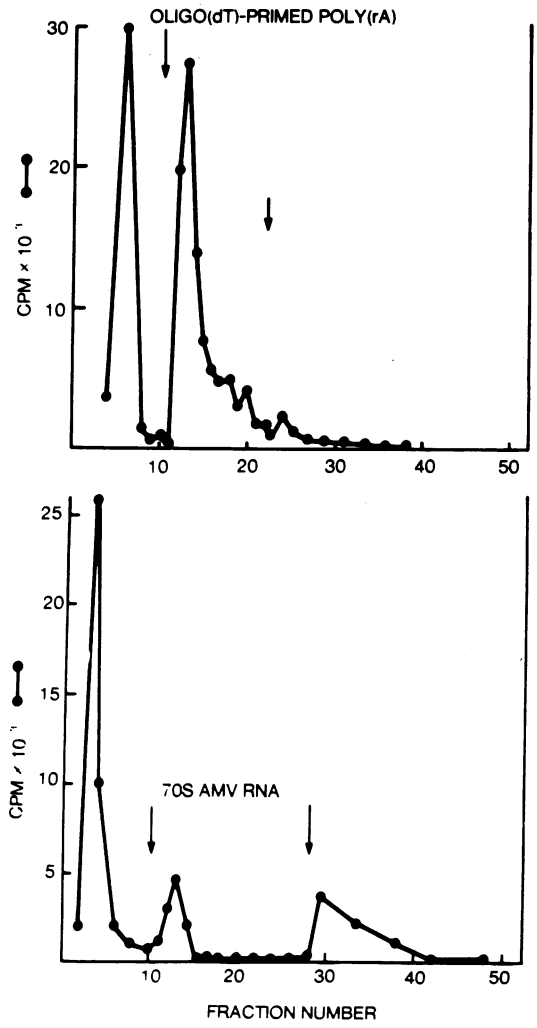


FIG. 3. Affinity elution of AMV DNA polymerase by natural and synthetic templates. The concentration of the templates were 20 A_{260} units/ml in the eluting buffer [50 mM phosphate (K^+) pH 7.2 containing the usual amounts of glycerol and dithiothreitol]. AMV 70S RNA was primed with oligo(dT)$_{12-18}$ in the ratio of 1:5 by A_{260}. The concentration of the primed 70S RNA was 2.0 A_{260} units/ml in the elution buffer described.

This possibility has been eliminated on the basis of the experiment where AMV DNA polymerase was eluted with substrates (Fig. 3), verifying the affinity nature of the column. Secondly, in control experiments (not shown) using a cation exchanger (IRC-50), AMV DNA polymerase showed little affinity for carboxyl residues eluting at a very low salt concentration. Additional evidence for the affinity nature of the binding was provided by experiments where pyran copolymer was not linked to Sepharose, in which case no binding of the AMV viral polymerase was observed. All the viral polymerases showed great affinity for this column and reproducibly eluted at a salt concentration ranging between 0.2 and 0.4 M KCl. There appears to be a correlation between the molecular weights and the salt elution profiles. The two avian enzymes (AMV and RSV), which have molecular weights of about 160,000, were eluted between 0.38 and 0.40 M KCl. On the other hand, the murine enzymes (MTV and RLV), having molecular weights of about half of those of the avian enzymes, eluted at a relatively lower salt concentration of (0.22–0.25

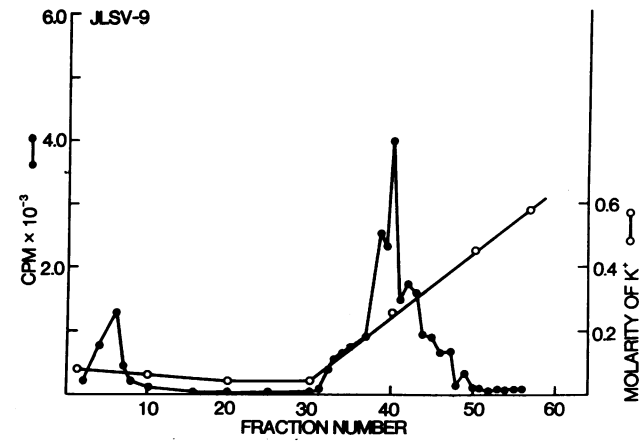
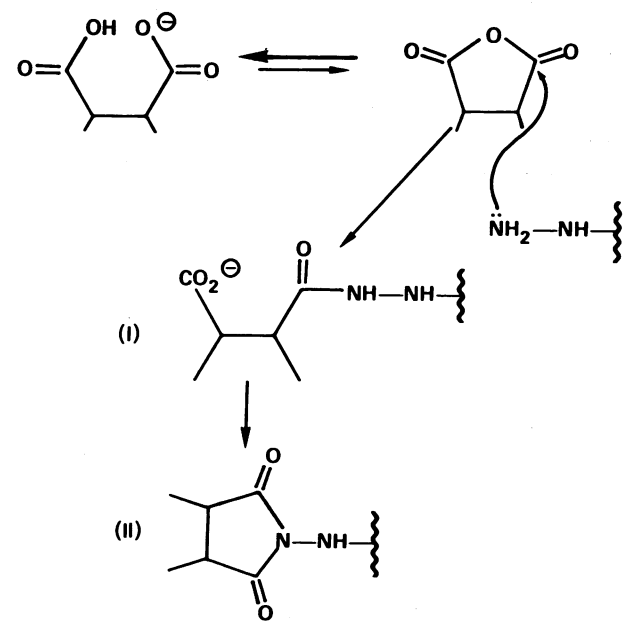


FIG. 4. Detection of RLV DNA polymerase in JLSV9 cell extracts. All fractions were assayed with poly(rC)·oligo(dG)$_{12-18}$ as described in *Materials and Methods*. The peak eluted in the initial wash was not further investigated; however, it could be due either to overloading or to the detection of a small amount of RLV DNA polymerase subunit. Upon assaying again with poly(rA)·oligo(dT)$_{12-18}$ (not shown), several other DNA polymerase peaks could be detected, which have been assigned to be of cellular origin based on assay with poly(rA)·oligo(dT), while the peak shown was reduced to a relatively insignificant amount.

M KCl). With the exception of the RSV polymerase, all recoveries were better than 60% of the original activity (Table 1). Conceivably, by optimizing the conditions for the elution of these enzymes, better yields may be obtained. The purification was assessed by iodination of proteins from crude AMV extracts and AMV DNA polymerase fractions purified on pyran-Sepharose. The iodinated AMV DNA polymerase purified by the pyran-Sepharose column showed essentially two bands (Fig. 5). Since AMV DNA polymerase is composed of two nonidentical subunits (28), it is reasonable to assume that the two bands observed correspond to those of the subunits of the polymerase. Pyran-Sepharose displays several



SCHEME I. Proposed mechanism of linkage between pyran and the hydrazine residues.

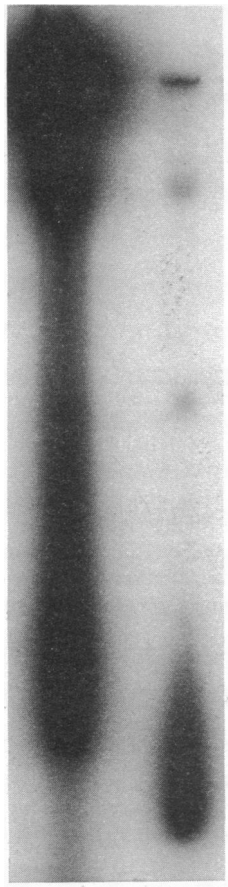


FIG. 5. Autoradiography of iodinated proteins from AMV showing the result of purification on pyran-Sepharose. Protein samples were iodinated and denatured in 1% sodium dodecyl sulfate + 1% mercaptoethanol. The sample on the left corresponds to the proteins from crude AMV extracts, while the sample on the right corresponds to the purified DNA polymerase.

advantages essential for a good affinity matrix. These include (*a*) stable attachment to activated Sepharose, (*b*) total insensitivity to nuclease activities found in crude extracts, and (*c*) specificity for viral DNA polymerase from several sources (14).

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