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Acid-Volatile Selenium Formation Catalyzed by Glutathione Reductase†

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ABSTRACT: The production of acid-volatile selenide (apparently H_2Se) was catalyzed by glutathione reductase in an anaerobic system containing 20 m*M* glutathione, 0.05 m*M* sodium selenite, a TPNH-generating system, and microgram quantities of highly purified yeast glutathione reductase. H_2Se production in this system was proportional to glutathione reductase concentration and was maximal at pH 7. Significant nonenzymic H_2Se production occurred in the system lacking glutathione reductase and TPNH. A concentration of arsenite (0.1 m*M*) which does not inhibit glutathione reductase inhibited selenide volatilization, as did bovine serum albumin (1.67 mg/ml). Both appear to inhibit Se volatilization by reacting with the selenide product(s). The selenotrisulfide derivative of glutathione (GSSeSG) was readily converted to H_2Se by glutathione reductase and TPNH without the addition of glutathione. These results suggest that GSSeSG formed nonenzymically from glutathione and selenite undergoes stepwise reduction by glutathione reductase (or excess GSH) to GSSeH and finally to H_2Se. The same pathway operates when glutathione is used as the reducing agent but to a lesser extent.

Studies on the metabolism of sodium selenite show that selenite undergoes reduction in animals. Selenite is a weak oxidizing agent and reacts with thiol compounds such as cysteine, coenzyme A, 2-mercaptoethanol, glutathione (Ganther, 1968), or reduced pancreatic ribonuclease (Ganther and Corcoran, 1969) as proposed by Painter (1941):

$$H_2SeO_3 + 4RSH \longrightarrow RSSeSR + RSSR + 3H_2O \quad (1)$$

The reaction between selenite and glutathione is particularly interesting because glutathione is the most abundant thiol compound in animal tissues. Moreover, glutathione is specifically required, along with TPNH, for the synthesis of dimethyl selenide from selenite in a cell-free liver system (Ganther, 1966). Selenodiglutathione (GSSeSG[1]) is the first stable intermediate formed in the reaction of selenite and glutathione (Ganther, 1968; Sandholm and Sipponen, 1973). GSSeSG can be further reduced to a labile selenopersulfide (GSSeH) by glutathione reductase (reaction 2) or by excess glutathione (Ganther, 1971):

$$GSSeSG + TPNH + H^+ \xrightarrow{\text{glutathione reductase}} GSSeH + GSH + TPN^+ \quad (2)$$

Diplock et al. (1971) observed that acid-volatile selenium was released by adding strong acid to liver homogenates prepared from rats which had previously received selenite. It was noted that this acid-volatile selenium, believed to be H_2Se, underwent oxidation very easily and was difficult to study. Later these investigators developed a technique for quantitatively trapping H_2Se produced from selenite, zinc dust, and strong acid, in 0.1 *N* $AgNO_3$ (Diplock et al., 1973). Using a similar technique, it was observed in this laboratory that monothiols (glutathione and 2-mercaptoethanol) or dithiols formed H_2Se from selenite. Rhead and Schrauzer (1974) have provided kinetic evidence for such a reaction. This report presents evidence that H_2Se

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[1] Abbreviations used are: GSSeSG, selenodiglutathione; GSSeH, selenopersulfide; GSH, reduced glutathione.

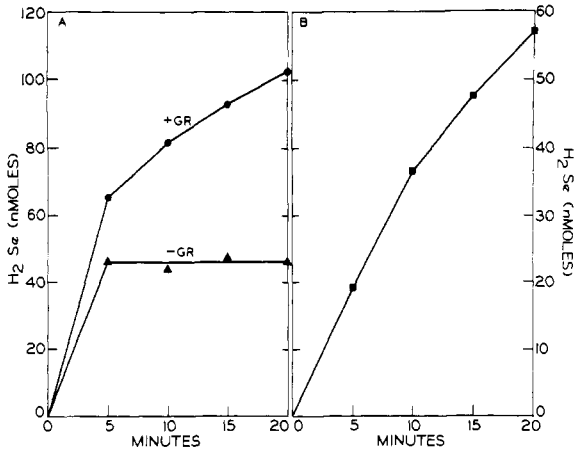


FIGURE 1: Enzymic and nonenzymic formation of H_2Se. The control (-GR) contained selenite (0.05 mM), GSH (20 mM), TPNH-generating system, and phosphate buffer (pH 7.0), while 0.2 unit of glutathione reductase (GR) was added as indicated. (a) Total H_2Se production; (b) the net amount of H_2Se synthesized by glutathione reductase. (H_2Se produced in the presence of GR minus the control.)

can be formed enzymically through the action of glutathione reductase, a previously unknown reaction for this enzyme. The role glutathione reductase plays in the reduction of selenite to selenide is discussed.

Materials and Methods

Nonradioactive sodium selenite was purchased from Alfa Inorganics. The ^{75}Se-labeled H_2SeO_3 was obtained from New England Nuclear and found to consist of 87% selenite by toluene extraction of the piazselenol complex which selenite forms with 2,3-diaminobenzidine (Cheng, 1956). Thin-layer chromatography on cellulose with 1-butanol–acetic acid–water (2:1:1) or dioxane–2 N NH_4OH (1:1) showed in each case that about 85% or more of the ^{75}Se moved with selenite and the remainder with selenate. GSSeSG was prepared as previously described (Ganther, 1971), while glutathione reductase (type III, from yeast) and other chemicals were obtained from Sigma.

Procedures for measuring H_2Se formation were modified from those described by Diplock et al. (1973). The glass apparatus used for collecting dimethyl selenide (Ganther, 1966) was used except 0.1 N $AgNO_3$ replaced 8 N HNO_3 as the trapping solution. Unless indicated otherwise, the complete medium contained the following in a final volume of 3 ml: GSH (20 mM); a TPNH-generating system, which included isocitrate (4 mM), isocitrate dehydrogenase (100 μg), and TPN (0.13 mM); $MgCl_2$ (13 mM); EDTA (1.0 mM); sodium phosphate buffer (pH 7.0, 33 mM) and glutathione reductase (0.2 unit, 1.3 μg) in the main compartment of the Warburg flask and ^{75}Se-labeled sodium selenite (0.05 mM) in the side arm. After flushing the system with prepurified nitrogen gas for 5 min, reactions were started by tipping in selenite. At the end of the incubation period (10 min unless specified), 1.0 ml of 12 N HCl was injected into the medium through the rubber stopper on the side arm and the system was flushed with nitrogen for another 20 min. The radioactivity trapped in 0.1 N $AgNO_3$ was considerably less than that lost from the flask. Presumably H_2Se was deposited in the delivery system, as described by Diplock et al. (1973). Therefore the amount of H_2Se produced was calculated by difference from the radioactivity remaining in the flask.

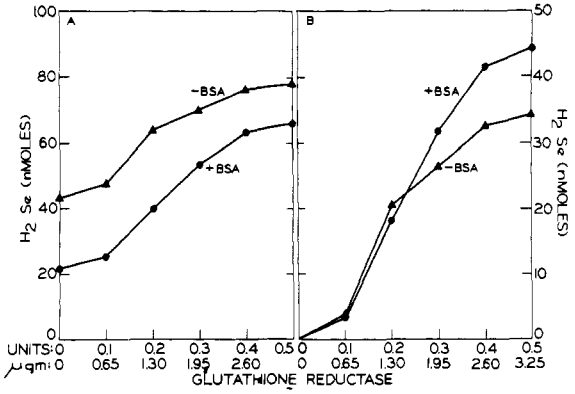


FIGURE 2: H_2Se formation at different levels of glutathione reductase with or without bovine serum albumin. (a) Total amount of H_2Se produced in 10 min; (b) net enzymic synthesis of H_2Se.

Results

The effect of glutathione reductase on H_2Se formation is shown in Figure 1. Although GSH alone could reduce selenite to H_2Se, the presence of glutathione reductase greatly increased H_2Se formation. The amount of H_2Se produced nonenzymically did not increase beyond the initial 5-min time interval (Figure 1a). On the other hand, the addition of glutathione reductase caused H_2Se production to increase linearily with time in the first 10 min (Figure 1b). Evidence was obtained that depletion of Se substrates caused the rate of H_2Se production to decrease with time, since addition of selenite (but not TPNH, glutathione reductase, or TPNH plus glutathione reductase) restored H_2Se production (data not shown).

The rate of H_2Se synthesis was in general proportional to the amount of glutathione reductase added up to approximately 0.3 unit (Figure 2). Bovine serum albumin, which is frequently added as a protective agent to the reaction mixture for glutathione reductase assay (Massey and Williams, 1965), reduced the total volatilization of H_2Se; for example, about 25 nmol of H_2Se was produced nonenzymically in the presence of bovine serum albumin compared to 45 nmol in the absence of bovine serum albumin. The increment in H_2Se production caused by glutathione reductase, however, was not greatly affected by bovine serum albumin (Figure 2b). Indeed, bovine serum albumin seemed to prevent the relative decline in H_2Se production as glutathione reductase (and thus total H_2Se production) was increased.

The effect of pH on H_2Se formation is shown in Figure 3. The nonenzymic reduction of selenite did not show a clear pH optimum. The enzymic reaction, however, showed greatest activity in the neutral pH range, in accord with the pH optimum for this enzyme determined with GSSG as substrate (Massey and Williams, 1965).

The effect of GSH concentration on H_2Se production is shown in Figure 4. A physiological level (2×10^{-3} M) of GSH afforded nearly maximal H_2Se production while a tenfold lower level of GSH (which provided a 4:1 molar ratio of glutathione to selenite) did not. Glutathione reductase stimulated H_2Se production even at a high concentration (2×10^{-2} M) of GSH.

The data in Table I show that 10^{-4} M arsenite completely inhibited H_2Se formation by glutathione reductase in a system containing bovine serum albumin. Since 10^{-4} M arsenite does not inhibit glutathione reductase (Tietze, 1970a,b; also our unpublished data), this inhibition probably is due to a reaction between arsenite and a product of

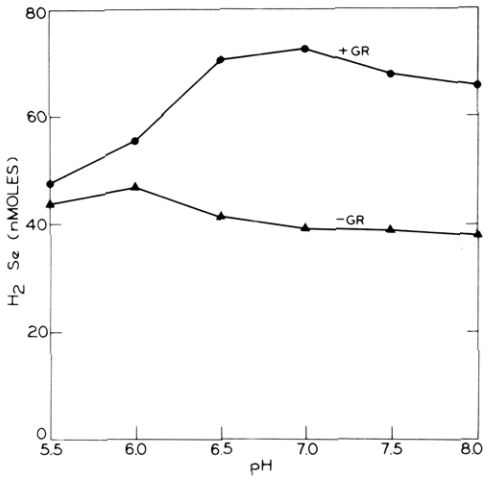


FIGURE 3: Effect of pH on H_2Se formation. Phosphate buffer of indicated pH was used, except for pH 8.0 where phosphate was replaced by Tris buffer.

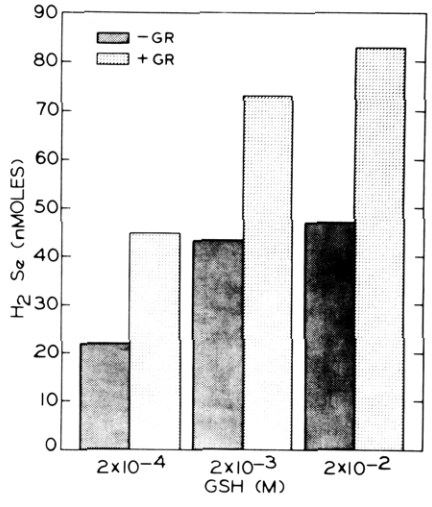


FIGURE 4: H_2Se formation at different levels of GSH. See text for incubation procedures.

selenite reduction. In support of this explanation, inhibition was complete when the arsenite concentration exceeded that of selenium but incomplete when arsenite was less than equimolar with selenium (Table I). In a system not containing bovine serum albumin the effect of arsenite was different (Table II). Arsenite inhibited H_2Se production but inhibition was not complete even at 10^{-3} *M* arsenite, whereas 10^{-4} *M* arsenite gave complete inhibition in systems containing bovine serum albumin (Table I). The stimulatory effect of glutathione reductase on H_2Se production, however, was almost completely abolished by 10^{-4} *M* arsenite (Table II), despite the fact that this level of arsenite is not inhibitory to the enzyme. Perhaps in the absence of bovine serum albumin, arsenite and H_2Se react to form an acid-volatile complex resulting in apparent H_2Se volatilization. Such a compound might be more inhibitory to glutathione reductase than arsenite itself.

GSSeSG, shown to be the first stable product formed by the reaction of GSH and selenite (Ganther, 1968, 1971), could be reduced to H_2Se by glutathione reductase; a submicrogram quantity of enzyme was quite effective with 3.3 $\times$ 10^{-5} *M* GSSeSG (Table III). GSSeSG also was reduced by GSH alone to H_2Se, substantiating the proposal that excess GSH ultimately reduces selenite to H_2Se via GSSeSG and GSSeH.

Table I: Arsenite Inhibition of Hydrogen Selenide Formation in a System Containing Bovine Serum Albumin.[a]

Sodium Arsenite (*M*)	H_2Se[b] (nmol)	% Inhibition
0	50.2	
10^{-6}	49.7	0
10^{-5}	43.3	14
10^{-4}	0	100

[a] 5×10^{-5} *M* selenite; 2×10^{-2} *M* GSH; 2.5 units (16.25 μg) of glutathione reductase plus 5 mg of bovine serum albumin. [b] Total amount produced in 20 min.

Table II: Arsenite Inhibition of Hydrogen Selenide Formation in the Absence of Bovine Serum Albumin.[a]

Sodium Arsenite (*M*)	H_2Se (nmol)[b]		Net Enzymic H_2Se Synthesis (nmol)	% Inhibition
	-GR	+GR		
0	45	81.0	36.0	
10^{-6}	46.5	78.2	31.7	12
10^{-5}	34.5	55.5	21.0	42
10^{-4}	30.0	32.0	2.0	94
10^{-3}	31.0	30.0	0	100

[a] 5×10^{-5} *M* selenite; 2×10^{-2} *M* GSH; 0.2 unit (1.3 μg) of glutathione reductase (GR). [b] Total amount produced in 10 min.

Table III: Reduction of GSSeSG to Hydrogen Selenide by Glutathione Reductase.[a]

Glutathione Reductase		GSH (*M*)	H_2Se (nmol)
μg	units		
0	0	0	0
0.163	0.025	0	22.0
1.63	0.25	0	34.0
16.3	2.5	0	36.3
0	0	2×10^{-3}	33.7

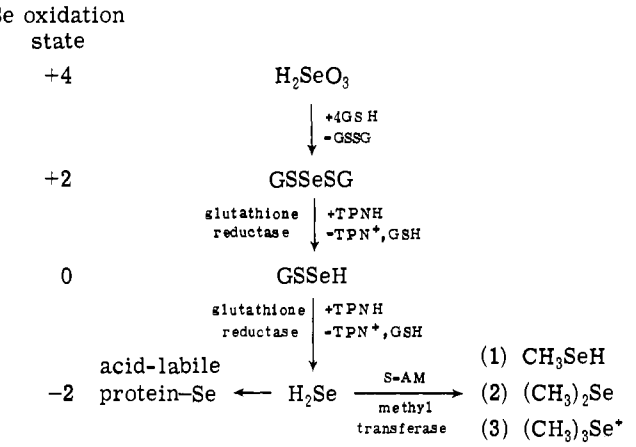
[a] The flasks contained 3.3×10^{-5} *M* ^{75}Se-labeled GSSeSG, a TPNH-generating system (see Materials and Methods), 13 m*M* $MgCl_2$, 1 m*M* EDTA, 33 m*M* sodium phosphate buffer (pH 7), plus glutathione and glutathione reductase as indicated. Incubation was for 20 min.

In other experiments (Hsieh, 1974) it was shown that glutathione reductase could volatilize H_2Se in substantial amounts even without the addition of concentrated HCl; more was volatilized at pH 6.35 than at pH 7. Under the same conditions no volatilization occurred in the absence of glutathione reductase. The addition of acid, however, greatly increased volatilization either in the presence or absence of glutathione reductase, presumably by converting nonvolatile Se^{2-} and HSe^- ions to undissociated H_2Se.

Discussion

The studies reported here plus earlier studies from this laboratory establish a pathway for the reduction of selenite (oxidation state + 4) to H_2Se (−2). This six-electron reduction process in all likelihood involves three sequential two-electron reactions (Scheme I). The first is a nonenzymic reaction between glutathione and selenous acid, producing

Scheme I: Pathway for Biosynthesis of Hydrogen Selenide and Other Selenides from Selenite.[a]



[a] The reductions catalyzed by glutathione reductase can also occur with an excess of GSH but not as rapidly.

GSSeSG as the stable product (reaction 1). Once attached to the glutathione moiety, selenium readily undergoes further reduction by means of TPNH and glutathione reductase (reaction 2), or by an excess of glutathione, to the labile intermediate previously identified as glutathione selenopersulfide, GSSeH (Ganther, 1971). Measurements of the stoichiometry of TPNH oxidation in relation to selenium reduction indicated that further reduction of selenium to H_2Se might be occurring even under aerobic conditions (Ganther, 1971). The results presented here provide evidence that under anaerobic conditions glutathione reductase or excess glutathione effectively reduce GSSeH to the −2 oxidation state. The release of a volatile selenium compound upon acidification is in accord with the formation of H_2Se, as described elsewhere by Diplock et al. (1973). The need for anaerobic conditions to obtain maximum production and release of selenide also fits with the known tendency of H_2Se to undergo rapid oxidation.

If sufficiently high concentrations of glutathione are employed, selenite is reduced to H_2Se under anaerobic conditions. Since earlier studies established that GSSeH was formed by mixing selenite with excess glutathione, the formation of H_2Se can be accounted for by the further reduction of GSSeH (eq 3):

$$GSSeH + GSH \rightleftharpoons H_2Se + GSSG \quad (3)$$

It was of considerable interest to find that highly purified glutathione reductase stimulated H_2Se production from selenite plus glutathione. The effect was proportional to the amount of enzyme added. The optimum pH was similar to that for the reduction of GSSG by glutathione reductase (Massey and Williams, 1965).

Evidence was obtained that bovine serum albumin interfered with H_2Se formation or release, since the amount of H_2Se volatilized in its absence was greater than when it was present. Most of the experiments reported here were performed in the absence of bovine serum albumin, but comparable experiments with bovine serum albumin included are described elsewhere (Hsieh, 1974; Ganther and Hsieh, 1974). Much greater amounts of enzyme must be used to achieve a given amount of H_2Se volatilization when bovine serum albumin is present, presumably because H_2Se reacts with functional groups on bovine serum albumin to prevent the release of Se, even after acidification. It is apparent that investigators studying the production or release of H_2Se from biological systems must be aware of the degree to which these competing reactions can control the release of H_2Se. Thus the apparent H_2Se or acid-labile Se content of a tissue will depend on the nature of other substances present in that system such as proteins or peroxides which may bind to H_2Se or oxidize it to prevent its release upon acidification.

To account for the stimulation by glutathione reductase of H_2Se formation, direct reduction by the enzyme of GSSeH is proposed (reaction 4). Thus TPNH and glutathione reductase, via eq 2 and 4, suffice to generate H_2Se in the absence of GSH if selenium is provided in the form of GSSeSG.

$$GSSeH + TPNH + H^+ \xrightarrow{\text{glutathione reductase}} H_2Se + GSH + TPN^+ \quad (4)$$

The possibility that two molecules of GSSeH might undergo a dismutation reaction leading to H_2Se and GSSeSG is an alternative to reactions 3 and 4 which has not been excluded. However, it has been observed by Hartter and Weber (1974) and also in this laboratory (Ganther and Hsieh, 1974) that the reduction of $GSSCH_3$ to CH_3SH is readily accomplished by glutathione reductase, so it seems reasonable to propose that the enzyme has sufficient versatility to directly reduce GSSeH to H_2Se. Glutathione reductase might shift the equilibrium of reaction 3 by reducing the GSSG product back to GSH. However, this possibility is not believed to explain the stimulatory effect of the enzyme since a tenfold increase in GSH concentration (from 2×10^{-3} to 2×10^{-2} *M*) had little effect on selenide volatilization, but glutathione reductase was stimulatory at all glutathione concentrations (Figure 4).

The demonstration that components found in animal tissues convert inorganic selenite to highly reactive intermediates and ultimately to H_2Se opens many possibilities for considering the biosynthesis of various organoselenium compounds. The importance of such reactions in regard to dimethyl selenide formation has been established, as reviewed elsewhere (Ganther and Hsieh, 1974). Moreover, the biosynthesis of trimethyl selenide (Palmer et al., 1969; Byard, 1969) and the formation of acid-labile selenide (Diplock et al., 1971) might be expected to proceed through such intermediates, and there is the further possibility that they might also be precursors of other forms of selenium having essential biological functions.

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Partial Purification and Properties of Calf Thymus Deoxyribonucleic Acid Dependent RNA Polymerase III†

P A. Weil and S. P. Blatti*

ABSTRACT: DNA-dependent RNA polymerase III (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.-7.6) has been isolated and partially purified from calf thymus tissue. Significant amounts of enzyme III are present in this tissue (up to 15% of the total activity of thymus homogenates). This enzyme has been characterized with respect to its chromatographic properties, broad ammonium sulfate optimum (0.04–0.2 M), template requirements, divalent metal optima, and its unique α-amanitin sensitivity (50% inhibition of activity occurring at an α-amanitin concentration of 10 μg/ml).

The existence of multiple forms of DNA-dependent RNA polymerases in eukaryotic cells is well established (Roeder and Rutter 1969, 1970a). Three forms of RNA polymerase (I, II, and III) were originally described in yeast (Roeder and Rutter, 1969) and later in sea urchin (Roeder and Rutter, 1970a) and in rat liver (Roeder and Rutter, 1970b). RNA polymerase I is localized in the nucleolus (Roeder and Rutter, 1970a) and appears to be responsible for the transcription of rRNA (Blatti et al., 1970; Reeder and Roeder, 1972). Polymerase I is insensitive to inhibition by high levels of the bicyclic fungal toxin, α-amanitin (Lindell et al., 1970; Kedinger et al., 1970). However, the nucleoplasmic enzyme (Roeder and Rutter, 1970a), polymerase II, is inhibited by very low levels of α-amanitin. RNA polymerase II is responsible for the synthesis of heterogeneous nuclear RNA (Blatti et al., 1970; Zylber and Penman, 1971).

The third major class of RNA polymerases, form III, has been described in a number of lower eukaryotes (Roeder and Rutter, 1969; Adman et al., 1972; Ponta et al., 1972, Roeder 1974a,b) but until recently (Sergeant and Krsmanovic, 1973; Weil et al., 1974; Weinmann and Roeder, 1974a,b) RNA polymerase III has never been reproducibly isolated from mammalian cells. We report here the isolation, partial purification, and characterization of RNA polymerase III from calf thymus tissue.

Partially purified calf thymus RNA polymerase III exhibits characteristic elution properties from DEAE-Sephadex columns, ionic strength and divalent cation optima, and template specificities. But most noteworthy is the fact that calf thymus RNA polymerase III exhibits an intermediate sensitivity to α-amanitin, with 50% inhibition of activity occurring at a concentration of 10 μg/ml. This result is in agreement with the data of Weinmann and Roeder (1974a,b). The implications of these data are that the synthesis of any particular mammalian RNA species can be titrated with α-amanitin in vivo or in vitro, and the α-amanitin concentration required for 50% inhibition of its synthesis will be characteristic of the polymerase form responsible (polymerase I, no inhibition at α-amanitin levels >250 μg/ml; polymerase II, 50% inhibition at 0.01 μg/ml; polymerase III, 50% inhibition at 10 μg/ml). This approach has been used to titrate tRNA and 5S RNA synthesis in mouse myeloma nuclei (Weinmann and Roeder, 1974a,b) and HeLa cell nuclei (Weil et al., 1974; P. A. Weil and S. P. Blatti, manuscript submitted for publication) to show that RNA polymerase III is responsible for the transcription of the genes for these low molecular weight RNAs.

Materials and Methods

Biochemicals. All chemicals used were reagent grade. Tritium labeled UTP (specific activity >20 Ci/mmol) was obtained from New England Nuclear. α-Amanitin was purchased from Henley Co., New York, N.Y., calf thymus DNA (Grade I) was from Sigma Chemical Company, and poly[d(A-T)] was from Miles Laboratories. Crystalline bovine serum albumin was purchased from Pentex-Miles.

Calf Thymus. Fresh frozen calf thymus was obtained from Dubuque Pack, Dubuque, Iowa, and maintained at −70° until use.

Solutions. All buffers were prepared from distilled water and, when present, dithiothreitol was added immediately before use.

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