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Kinetics of the Photochemical Addition of [^{35}S]Cysteine to Polynucleotides and Nucleic Acids*

Kendric C. Smith† and Dieter H. C. Meun

ABSTRACT: [^{35}S]Cysteine was ultraviolet irradiated in the presence of poly rU, poly rU:rA, poly rA, poly rC, poly dC, poly dC:dG, poly dT, poly dA:dT, poly dAT:dAT, ribonucleic acid (RNA), or deoxyribonucleic acid (DNA), and the rate constants for the uptake of cysteine per dose of ultraviolet light were tabulated. Whether the polymers were single or double stranded (and/or protonated) had a profound effect upon the reaction rate. Preliminary experiments indicate that tyrosine and serine also add photochemically to DNA but threonine and methionine appear unreactive under the conditions used.

These photochemical reactions may provide the mechanism by which DNA and protein are cross-linked *in vivo* when cells are irradiated with ultraviolet light.

The biological importance of the ultraviolet-induced cross-linking of DNA and protein has been demonstrated (Smith, *et al.*, 1966; Smith and O'Leary, 1967) but the nature of the chemical linkage involved is still unknown. The technical complexities of using a bacterial system caused us to search for a suitable *in vitro* system with which to study the chemical nature of the linkage group(s). We had shown that purified *Escherichia coli* DNA would cross-link readily with bovine serum albumin (Smith, 1964a,b), and that a mixed photoproduct of cysteine and uracil (5-*S*-cysteine-6-hydrouracil) (Figure 1) could be formed when uracil and cysteine were irradiated together in solution (Smith and Aplin, 1966). This mixed photoproduct of cysteine and uracil was offered as a possible model for the cross-linking of DNA and protein (Smith and Aplin, 1966), but since uracil is not present in DNA, the question arose as to whether cytosine or thymine (or both) would undergo this type of addition with cysteine. We therefore investigated the photochemical reactivity of [^{35}S]cysteine with poly rU,[1] poly rU:rA, poly rA, poly rC, poly dC, poly dC:dG, poly dT, poly dA:dT, poly dAT:dAT, RNA, and DNA. This paper deals with the kinetic aspects of this interaction and demonstrates that cysteine reacts readily with cytosine, uracil, and thymine polymers. Whether the polymers are single or double stranded, however, has a profound effect upon the reaction rates.

Materials and Methods

Chemicals. Poly rU and poly rC were purchased from Miles Chemical Co. and poly rA from Sigma Chemical Co. DNA (calf thymus) and RNA (yeast) were purchased from Worthington Biochemical Corp. Poly dC, poly dC:dG, poly dT, and poly dA:dT were a generous gift of Dr. Fred J. Bollum (1966). The sample of poly dAT:dAT was a generous gift of Dr. Arthur Kornberg. DL- and L-[^{35}S]cysteine were purchased from the Radiochemical Centre (Amersham, England). Nonradioactive L-cysteine hydrochloride was purchased from Eastman Kodak Co.

Preparation of Mixture for Irradiation. Each sample to be irradiated contained 1.5 ml of cysteine hydrochloride (0.02 M) freshly made up, 0.075 ml of [^{35}S]cysteine hydrochloride (0.02 M, 1 mCi/ml), 1.5 ml of polynucleotide solution (0.2 mg/ml in 0.075 M NaCl), and approximately 0.2 ml of 0.1 M NaOH. The pH of the cysteine solution was adjusted to ~4.5 before the polynucleotide solution was added. After the addition of the polynucleotide the pH was adjusted to 5.0 (unless otherwise stated). The final salt concentration should be ~0.04 M.

Irradiation Conditions. The reaction mixture (~3 ml) was irradiated in Beckman quartz cuvets placed 2.15 cm from the filter of a low-pressure mercury lamp (Mineralight, Model SL 2537, Ultraviolet Products, Inc.). The output of the lamp at this distance was 8000 ergs/mm^2 as measured by uranyl oxalate actinometry (Bowen, 1946).

Recovery of the Irradiated Polynucleotides. The irradiated sample was transferred to a conical centrifuge tube, made to approximately 0.1 M NaCl by the addition of 0.12 ml of saturated NaCl, and precipitated with two

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† To whom to address inquiries.

[1] Abbreviations used: poly rU, poly rA, and poly rC refer to the polyribonucleotides of uracil, adenine, and cytosine, respectively. Poly rU:rA is the two-stranded hydrogen-bonded polymer composed of a mixture of poly rU and poly rA. A similar terminology exists for the corresponding polydeoxyribonucleotides dC, dT, dC:dG, and dA:dT, where G and T stand for guanine and thymine, respectively. Poly dAT:dAT is composed of two hydrogen-bonded strands of the alternating single-chain polymer dAT.

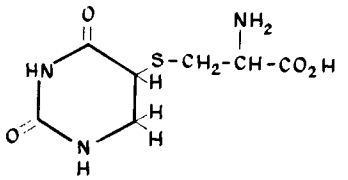

FIGURE 1: 5-*S*-cysteine-6-hydrouracil.

volumes of cold, 95% ethanol. After standing at 4° overnight, the precipitated polynucleotide was recovered by centrifugation at 2000 rpm (4°) for 60 min. The supernatant was decanted and the precipitate was washed with 10 ml of 0.1 M NaCl in 70% EtOH and again centrifuged. The supernatant was decanted and the tube was drained. The excess alcohol was evaporated and the polynucleotide was redissolved in 0.5 ml of H_2O (required several hours for DNA). Insoluble cystine crystals (present in varying amounts but absent from the RNA samples) were sedimented before an aliquot was taken for gel filtration.

Gel Filtration. The gel (Bio-Gel P-2, Bio-Rad Laboratories, Richmond, Calif., 200–400 mesh (wet) (exclusion limit mol wt 1600) was washed several times with 0.15 M NaCl and allowed to come to equilibrium with 0.15 M NaCl for 24 hr before use. Glass columns which had been treated with silicone (Dri-Film, SC-87, General Electric Co.) were packed with gel (0.8 × 35 cm). The top of the gel column was protected by a disk of Whatman 3MM filter paper. A 0.20-ml aliquot of the redissolved polynucleotide was put on the column. The column was eluted with 0.15 M NaCl at the rate of 0.5 ml every 2 min. The column fractions were assayed for absorbancy (A_{260}) and for radioactivity (10 μl on a Whatman 3MM filter paper disk in toluene liquid scintillation counting solution; Mans and Novelli, 1960) to determine the location of the polynucleotide peak and whether a good separation from the [^{35}S]cysteine peak had been achieved (Figure 2). The two peak tubes of the polynucleotide fraction were pooled and 0.1 ml was counted on a disk along with a blank and a[^{35}S]cysteine standard. Another aliquot was then used for the determination of phosphorus by our modification (K. C. Smith and D. H. C. Meun, submitted for publication) of the method of Griswold *et al.* (1951).[2] The micromoles of cysteine per micromole of phosphorus per dose of ultraviolet light could then be calculated.

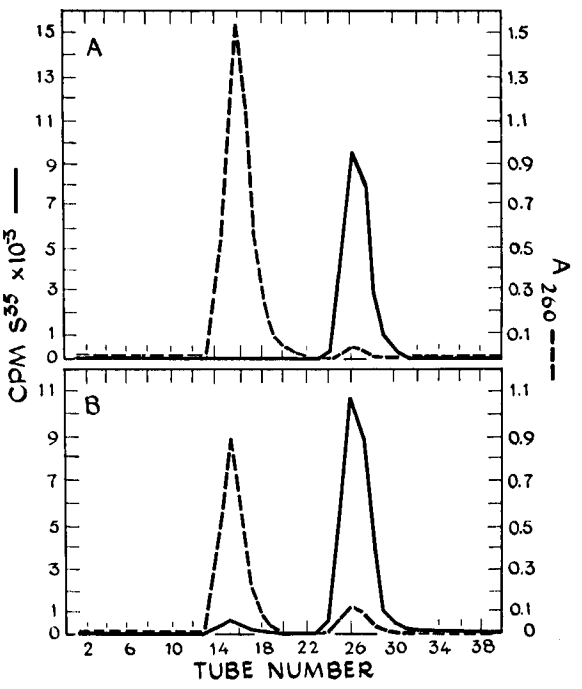

FIGURE 2: Separation of polyuridylic acid from [^{35}S]-cysteine on Bio-Gel P-2. Columns (0.8 × 35 cm) packed with 200–400 mesh gel (exclusion limit mol wt 1600) were eluted with 0.15 M NaCl at the rate of 0.5 ml every 2 min. (a) Before ultraviolet irradiation of the [^{35}S]cysteine–poly rU mixture. (b) After 60-min ultraviolet irradiation.

TABLE I: Rate Constants for the Photochemical Addition of [^{35}S]Cysteine to Polynucleotides.

Polynucleotide	K^a Exptl	K^a Calcd
Poly rU	21.8 (13.3)[d]	
Poly rU:rA	0.7[d] (U only)	
Poly rA	0.6	
Poly rC	8.1 (0.6)[d]	
RNA (yeast)[b]	21.8[e]	
	4.8[e]	
Poly dC	2.6	
Poly dC:dG	2.6 (C only)	
Poly dT	5.4	
Poly dT:dA	4.2 (T only)	
(heated)[e]		
Poly dT:dA	2.6 (T only)	
Poly dAT:dAT	1.1 (T only)	
DNA (calf thymus)	2.6[f]	2.6[g]
DNA (heated)[e]	4.2[f]	4.2[h]

[a] K = (μmoles of cysteine/μmole of PO_4 involved)/ergs/mm^2 × 10^8 (at pH 5). [b] RNA shows a biphasic uptake of cysteine (see Figure 3). [c] For 15 min at 100° in 0.075 M NaCl. Quick cooled. [d] At pH 6.5. [e] For (20% C + 27% U). [f] For (21% C + 29% T). [g] For (dC:dG + dA:dT). [h] For (dC + dT).

Results and Discussion

Polymers Containing rU. Of all the polymers tested, poly rU (single stranded at room temperature; Brown, 1966) showed the greatest photochemical reactivity with cysteine (Figures 3 and 4, Table I). This is consistent

[2] Reagents: ammonium molybdate ($10H_2O$), 0.28%; 7.2 N H_2SO_4; and aminonaphtholsulfonic acid (Griswold *et al.*, 1951); standard solution of KH_2PO_4. Place sample in a 30-ml micro-Kjeldahl flask along with 1 ml of H_2SO_4. Digest for 30 min after constant-boiling sulfuric acid is achieved. Cool, add 4.5 ml of ammonium molybdate and 0.5 ml of aminonaphtholsulfonic acid. Heat in a boiling-water bath for 10 min, cool, read the absorbancy at 830 nm.

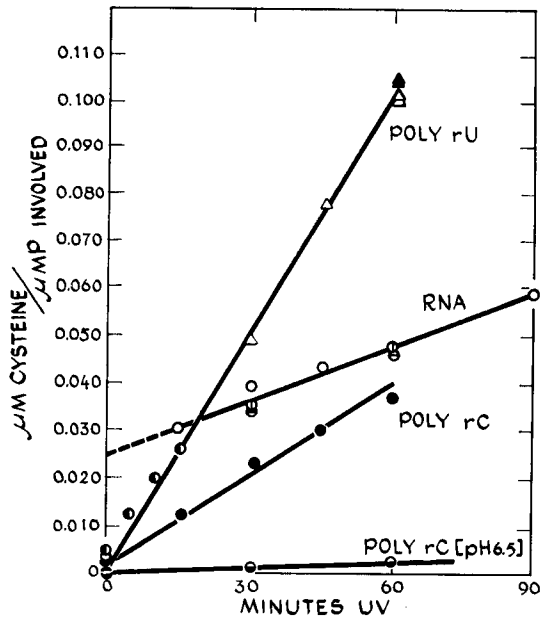

FIGURE 3: Photochemical addition of [^{35}S]cysteine to RNA, poly rU, and poly rC.

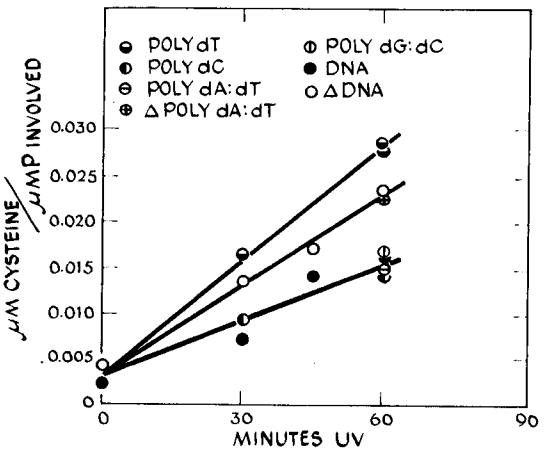

FIGURE 4: Photochemical addition of [^{35}S]cysteine to DNA, poly dT, poly dC, poly dC:dG, and poly dA:dT. Δ = heated for 15 min at 100° and quick cooled before irradiation.

with the previous observation of the ready uptake of cysteine by monomeric uracil (Smith and Aplin, 1966). On the other hand, poly rU:rA (double stranded above pH 5.2; Felsenfeld and Rich, 1957) took up almost no cysteine (Table I). It is therefore of interest that the rate of hydration in poly rU:rA is suppressed by a factor of 10 and the rate of dimer formation a factor of 5 relative to poly rU (Pearson and Johns, 1966). As these authors state: "These results provide direct proof of the importance of secondary structure in determining the photochemical behavior of U in polynucleotides."

That the uptake by poly rU is decreased significantly by raising the pH from 5 to 6.5 suggests that the photochemical reaction may involve an ionic species of either uracil or cysteine. The pK values for cysteine are 1.96 (COOH), 8.18 (NH_3^+), and 10.28 (SH) and the pI is 5.07 (Cohn and Edsall, 1943). Changing the pH from 5 to 6.5 would therefore not be expected to have a large effect upon the ionization of individual groups of cysteine but might have an important effect upon the pI. The pK for uracil is ~0.5 (Cohn, 1955) so one would also not expect a significant change in the ionization of uracil in going from pH 5 to 6.5. The involvement of the ionic form of uracil (probably in the excited state) in the photohydration reaction, however, has been reported (Burr and Park, 1967), and there are some similarities between the photohydration reaction and the addition of a molecule of cysteine to the 5–6 double bond of uracil (Smith and Aplin, 1966).

Although 5-*S*-cysteine-6-hydrouracil had been shown to be slowly converted to other products when heated at high temperatures in strong acid under conditions used to hydrolyze nucleic acids (Smith and Aplin, 1966) we felt that partial hydrolysis of the poly rU–cysteine photoproduct might liberate enough of the uracil–cysteine photoproduct for identification purposes. To this end the poly rU–[^{35}S]cysteine photoproduct was heated in formic acid at 175° for various times and chromatographed in butanol–acetic acid–water (80:12:30). Material appearing at the R_F of authentic 5-*S*-cysteine-6-hydrouracil increased up to 30-min hydrolysis (to a maximum of 35% of the total radioactivity present) and then decreased with longer times. The material at R_F 0.1 was recovered and rechromatographed in three additional solvents (Smith and Aplin, 1966). In each case it behaved identically with authentic 5-*S*-cysteine-6-hydrouracil. As a control, it was determined that only 45% of a sample of authentic 5-*S*-cysteine-6-hydrouracil remained unaltered after 30-min hydrolysis in formic acid. Considering the chromatographic results, the acid hydrolysis kinetics, and the stability to heat at neutral pH (see below) of the poly rU–cysteine photoproduct and of 5-*S*-cysteine-6-hydrouracil we may infer that essentially all of the cysteine adds to poly rU in the same manner as it does to monomeric uracil, that is, to form 5-*S*-cysteine-6-hydrouracil.

Polymers Containing rC and dC. The rate of uptake of cysteine by poly rC was less than half of that observed under similar conditions for poly rU (Table I). At pH 5, poly rC should be mainly (but not completely) in a two-stranded form (Hartman and Rich, 1965). When poly rC was assayed at pH 6.5, where it should be single stranded, there was almost no photochemical uptake of cysteine. The protonated form of cytosine therefore seems to be required both for the formation of double-stranded molecules of poly rC and for the efficient uptake of cysteine.

At pH 5 there is a greater uptake of cysteine by poly rC than by poly dC (Table I). This may possibly be due to the lesser degree of double strandedness in poly rC at pH 5. Strandedness depends upon protonation and the pK value for C in poly dC is 7.5 (Inman, 1964) and in poly rC it is 5.7 (Hartman and Rich, 1965).

Polymers Containing dT. The uptake of cysteine by poly dT was completely unexpected, inasmuch as thymine is not reported to undergo hydration-type reactions (Smith, 1966). When we try to explain the result for the uptake of cysteine by two-stranded polymers containing dT, we are faced with several apparent inconsistencies.

In agreement with the results for the uridylic acid polymers but in contrast with those for the cytidylic acid polymers, the rate of uptake of cysteine by double-stranded poly dA:dT was only about half that for single-stranded poly dT (Table I). A heated and quick-cooled sample of poly dA:dT took up more cysteine than an unheated sample. However, our sample of poly dAT:dAT took up almost no cysteine at all. These results would suggest that poly dAT:dAT was more tightly hydrogen bonded than poly dA:dT and would predict a higher T_m for poly dAT:dAT. Unfortunately, the T_m for poly dAT:dAT has been reported to be 7.5° lower than that for poly dA:dT (Riley *et al.*, 1966). The T_m for poly dA:dT is 12° higher than that for poly rA:rU (Riley *et al.*, 1966), yet poly dA:dT shows a significant uptake of cysteine whereas poly rA:rU shows almost none. The tightness of the helical structure would appear then not to be the only factor involved in determining the rate of the uptake of cysteine by polymers containing dT.

Localized melting is caused by the formation of hydrates or dimers in a double-stranded polynucleotide (Pearson and Johns, 1966); however, poly dAT:dAT does not form cyclobutane-type thymine dimers (Smith, 1964c; Deering and Setlow, 1963). One might therefore expect less photochemically induced denaturation in poly dAT:dAT than for poly dA:dT, and therefore more reaction of cysteine with poly dA:dT. One would also expect a lag in the production of cysteine addition photoproducts at the lower doses of ultraviolet light until sufficient denaturation had been produced. Our data are not sufficient to detect a lag.

A thymine radical is formed when thymine or DNA is irradiated with ultraviolet light (Pershan *et al.*, 1964). Recently, Yamane *et al.* (1967) identified dihydrothymine as an ultraviolet-irradiation product of thymine in DNA and observed that its production appeared to follow the thymine radical yield. One may speculate that the addition of cysteine to thymine in DNA might follow a similar reaction scheme to yield 5-*S*-cysteine-6-hydrothymine. We have recently isolated a mixed photoproduct of thymine and cysteine and are in the process of determining its structure.

Deoxyribonucleic Acid and Ribonucleic Acid. Denaturated DNA took up cysteine at almost twice the rate as native DNA (Figure 4 and Table I). Furthermore, using the rate constants for the appropriate single- and double-stranded deoxypolymers the rate constants for heat-denaturated and native DNA were accurately predicted (Table I). It has been pointed out, however, that "the environment of the AT pair in DNA appears to be intermediate between that of the homopolymer pair and that of the strictly alternating helix" (Riley *et al.*, 1966). This conclusion would not appear to hold for the photochemical reactivity of cysteine with the different AT pairs. The AT pair in DNA would appear to have the same photochemical reactivity toward cysteine as does the AT pair in poly dA:dT but differs markedly from the AT pair in poly dAT:dAT.

Evidence that the photochemical linkage of cysteine to DNA is covalent is as follows. (1) Cysteine combines with DNA as a function of the dose of ultraviolet irradiation; (2) when cysteine and DNA were irradiated separately (60 min of ultraviolet light) and then mixed and assayed by the gel filtration method very little interaction was observed (K value of 0.4 *vs.* 2.6 when irradiated together; see Table I); (3) when samples of the DNA-[^{35}S]cysteine photoproduct were dialyzed against water for 24 hr and then assayed they gave the same results for cysteine attachment as samples that had not been dialyzed; and (4) when the [^{35}S]cysteine adduct of DNA was chromatographed on paper in isobutyric acid–0.5 N ammonia (10:6, v/v) essentially all of the radioactivity remained at the origin but after treatment with DNase and snake venom phosphodiesterase the radioactivity moved out from the origin in several bands.

In contrast to all the other polymers studied, RNA showed a biphasic uptake of cysteine *vs.* dose of ultraviolet light. Approximately 3% of the pyrimidines took up cysteine at about the rate exhibited by poly rU, while the second slope of the curve represented a rate only slightly more than half that of poly rC (Figure 3 and Table I). Since RNA (in contrast to DNA) also contains single-stranded regions, the biphasic response curve may represent this fact and the position of the break in the curve may give an estimate of the extent of single-strandedness present.

Heat Stability of the Cysteine–Polymer Photoproduct. In order to obtain some preliminary information relevant to the nature of the linkage between cysteine and the several polymers, we have studied the heat stability of the various cysteine–polymer photoproducts. The peak tubes containing the photoproduct were pooled from several analytical experiments, alcohol precipitated, and redissolved in a small amount of water. One aliquot was run through the gel filtration column, the peak was again recovered, and the micromoles of cysteine per micromole of phosphorus was determined. Other aliquots were heated at 65° (the temperature currently used during the extraction of DNA from ultraviolet-irradiated bacterial cells; Smith and O'Leary, 1967) for various times before being put on the gel filtration column. The DNA–cysteine photoproduct showed a linear loss of radioactivity with time of heating up to 30 min and then no further change even after 90-min heating. A maximum of 36% of the cysteine was removable from the DNA by heating at 65°. No difference in results were observed if the solutions were heated at pH 5 or 7.

When the poly dT–cysteine and poly dC–cysteine photoproduct peaks were similarly processed, heated for 60 min at 65°, and assayed, there was only a 15.6% loss of cysteine from poly dT and 49.3% loss from poly dC. Using these percentage lability figures and correcting for the relative percentage of T and C in the DNA used (21% C and 29% T) we calculate that 30% of the cysteine in the DNA–cysteine photoproduct should be heat labile which is in reasonable agreement with the value of 36% observed experimentally.

When poly rU–cysteine photoproduct peaks were similarly processed, heated for 60 min at 65°, and assayed, there was only a 1% loss of cysteine from the poly rU attesting to the unique stability of its photochemical attachment. The photoproduct, 5-*S*-cysteine-6-hy-

drouracil, is stable at 100° for 15 min at neutral pH (Smith and Aplin, 1966).

Now that we have an indication that cysteine can add to DNA by at least two different types of chemical linkages (one heat stable and one heat labile) we can profitably discuss the possible reasons why we have been essentially unsuccessful in isolating the cross-linked DNA–protein complex in purified form from ultraviolet-irradiated bacteria. We have tried to purify this complex by solubilizing it in various detergents at 60–65° and by gentle shearing so that this complex would not sediment to the bottom but would appear somewhere in the middle of a sucrose gradient. However, after these treatments we find that the protein is no longer bound to the DNA. This failure may be related to the present observation that certain of the modes of attachment of cysteine to DNA is through heat-labile linkages.

The lability of certain amino acid adducts to the nucleic acids may also explain the results of Goddard *et al.* (1966) who found for ultraviolet-irradiated tobacco mosaic virus that one molecule of protein was cross-linked per molecule of RNA per lethal hit. The cross-linked protein–RNA complex withstood sodium lauryl sulfate at 50° for 5 min but was split by subsequent treatment with phenol, 5 M guanidine hydrochloride, or 66% acetic acid.

Photochemical Reactivity of Other Amino Acids with DNA and RNA. Under conditions similar to those used for cysteine we have tried preliminary experiments with uniformly ^{14}C-labeled L-tyrosine, L-serine, and L-threonine and with L-[2-^{14}C]methionine. Because of the insolubility of tyrosine, it was used at a final concentration of 0.0015 M rather than at 0.01 M as used for the other amino acids. Using only one ultraviolet dose point (4.8×10^5 ergs/mm^2) we have calculated the K values (see Table I for definition) to be 5.5 and 3.2 for tyrosine with DNA and RNA, respectively. The results for serine were 0.7 and 0.8 for DNA and RNA, respectively. Threonine gave a value of 0.2 for both DNA and RNA. Methionine gave a value of 0.2 for RNA.

Thus, tyrosine and serine add photochemically to DNA and to RNA and can also be considered as models for the mechanism by which DNA and protein are cross-linked *in vivo* and RNA and protein are cross-linked in ultraviolet-irradiated tobacco mosaic virus (Goddard *et al.*, 1966). The fact that there may be other mechanisms for the cross-linking of DNA and protein besides those involving SH groups had been suggested by the observation that gelatin (which contains no cysteine) also cross-links with DNA *in vitro*, although at a much reduced efficiency relative to bovine serum albumin (Smith, 1967).

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