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CHROMATOGRAPHY OF NATIVE AND γ-IRRADIATED LYSOZYMES ON DENSE GELS

V. I. MAKSIMOV AND V. A. MOSIN
N.D. Zelinsky Institute of Organic Chemistry, U.S.S.R. Academy of Sciences, Moscow (U.S.S.R.)
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SUMMARY

A chromatographic method has been developed for the isolation of the most heavily damaged part (fraction I) of γ-irradiated lysozyme. This method cuts secondary changes of protein structure occurring after irradiation to a minimum. The chromatography of native and γ-irradiated lysozymes on gels of high density (Sephadex G-25, Bio-Gels P-6 and P-10) was investigated and the characteristics of chromatography established, *viz.* 2 types of adsorption, relative large capacity of adsorption, concentration dependence for elution volumes, zonal asymmetry and the existence of Johnson–Ogston effect. It is suggested that the chromatographic anomalies of lysozyme are due to the osmotic pressure of its solutions which could reach a large value owing to the Donnan effect. Chromatography of irradiated and thermally denatured lysozymes on Sephadex G-75 as compared to that on Sephadex G-25 shows sharp differences.

INTRODUCTION

A number of proteins, particularly the lysozymes, are known to be adsorbed irreversibly on high density gels (*e.g.*, on Sephadex G-25 and G-50) from aqueous medium[1,2]. Such adsorption phenomena have only been studied briefly. They usually interfere with the chromatographic process and result in incomplete recovery of the protein. However, in some cases the protein adsorption on Sephadex G-25 can help the fractionation[1]. It was thought that adsorption chromatography could be used for the fractionation of γ-irradiated lysozyme. Our former fractionation method was based on the different solubilities of the most and least heavily damaged fractions (fractions I and II) in acetic acid containing NaCl (ref. 3). This method was not sufficiently mild as the lysozyme, made unstable by irradiation, underwent further changes during fractionation. In this investigation a simple and rapid method for the isolation of fraction I from γ-irradiated lysozyme was developed. It is based on chromatography on Sephadex G-25 under very mild conditions. It could be demonstrated that fraction I retained in general outline the structure of native protein. As a preliminary, we also investigated the chromatographic behaviour of native and irradiated lysozymes on Sephadex G-25 and G-75 and Bio-Gels P-6 and P-10.

MATERIALS AND METHODS

Native lysozyme

This was obtained by direct crystallisation from hen's egg white, which was then recrystallised 4 times.

Lysozyme irradiation

^{60}Co γ-rays were used to irradiate the lysozyme *in vacuo* (15 min, less than 10^{-2} mm Hg) in sealed ampules at 30–40°; the dose was ~ 36 mrad. Fractions I and II were isolated, their separation with NaCl in acetic acid was performed as described by MAKSIMOV AND OSIPOV[3].

Denatured lysozyme

This was obtained as follows: 100 mg of native protein in 10 ml of 0.5 *M* CH_3COOH were warmed in a boiling water bath for 4 h 20 min, then cooled and lyophylised.

Enzymatic activity

This was determined by the turbidimetric method involving the lysis of *Micrococcus lysodeikticus* suspension in $^1/_{15}$ *M* phosphate buffer, pH 6.2. The activity of γ-irradiated and denatured protein was 56 and 6% respectively.

Chromatography

Gels were allowed to swell more than a day before packing the columns. Such columns have been used over a period of 2–3 or more months and showed good reproducibility. Experiments corresponding with Figs. 1b–e, 2a–e, 4a, 4b and 5a–c were all made on the same column, in each case. The presence of protein in the effluent was registered by a flow-through optical density recorder (AMN CKB of U.S.S.R.). Elution volume (V_e) was determined from the maximum in the chromatographic pattern. When V_e depended on concentration the data for the lowest were used in Table I.

Tryptophan

The tryptophan content was determined according to the method of OPIENSKA-BLAUTH *et al.*[4].

Optical rotation dispersion

This was measured on a Cary instrument (U.S.A.) in the range of 350–550 nm (see Table II) Solvent: 0.1 *M* acetic acid. Protein concentration: 2 mg/ml.

RESULTS AND DISCUSSION

Chromatography in water

The first questions investigated were: how much native lysozyme could be irreversibly adsorbed on the gels in water, and how the excess non-adsorbed part of the lysozyme behaved during gel chromatography under these conditions. The data are given in Table I. The capacity of gels for irreversibly adsorbed protein was not a constant value; it increased with the amount of substance applied.

TABLE I

CHROMATOGRAPHY OF NATIVE LYSOZYME ON DIFFERENT GELS

Gel and column dimensions	*Irreversible adsorption in water (mg)*		*Values of V_e (ml) in water*		*Values of V_e (ml) in 0.1 M CH_3COOH for lysozyme*	*Approximate fractionation range for proteins*[5]
	Applied	*Adsorbed*	*Lysozyme*	*Glucose*		
Bio-Gel P-6 (1.2 × 45.5)	5	~ 0.5	14	38	14	1000–5000
Sephadex G-25 (1.2 × 43)	5	~ 5	24	30	14	1000–5000
	30	18				
	86	30				
Bio-Gel P-10 (1.3 × 38)	5	~ 5	45	45	18	5000–17000
	100	12				
Sephadex G-75 (1.2 × 42.5)	10	~ 10	32–33	35–37	32	3000–70000
	30	15				

It was surprising that the excess lysozyme (with respect to that irreversibly bound) was not eluted in the water at void volume, but much later. Such observations were made for both Sephadex G-25 and Bio-Gel P-10 (Table I). In the latter case the excess of protein was eluted at the same volume as glucose and the elution diagrams were nearly symmetrical (Fig. 1b). An increase in the amount of protein used for the experiment by five times (from 20 to 100 mg, see Fig. 1b) did not appreciably change the position of the maximum, but the zone became broader. When chromatography in water was repeated several times on the same column of P-10 without removal of the irreversibly adsorbed protein the same elution volume was practically always obtained. This means that the adsorbed protein did not influence the chromatography of the excess.

A significant difference was observed when we used Bio-Gel P-6. Irreversible adsorption of native lysozyme was slight in this case and the protein was eluted practically at void volume (Table I, Fig. 1a).

The large amounts of lysozyme irreversibly adsorbed on Sephadex G-25 and Bio-Gel P-10 make these gels unsuitable for chromatography in water. The irradiated protein was also strongly adsorbed on these gels. Chromatography of excess irradiated lysozyme on P-10 (0.9 × 45 cm) in water did not give a satisfactory separation. The elution patterns for native and irradiated lysozyme in this case showed only small differences. Our task was to eliminate the irreversible adsorption properties of the gel but retain the reversible ones. The adsorption is thought to be due principally to the carboxyl groups of the gels[5]. The most advisable procedure seemed to be the use of a dilute acid as an eluent, because under such conditions the ion-exchange interactions could be suppressed.

Chromatography in dilute solutions of acetic acid

Tentative experiments showed that under appropriate conditions the irreversibly adsorbed part could be small and the protein was almost quantitatively eluted

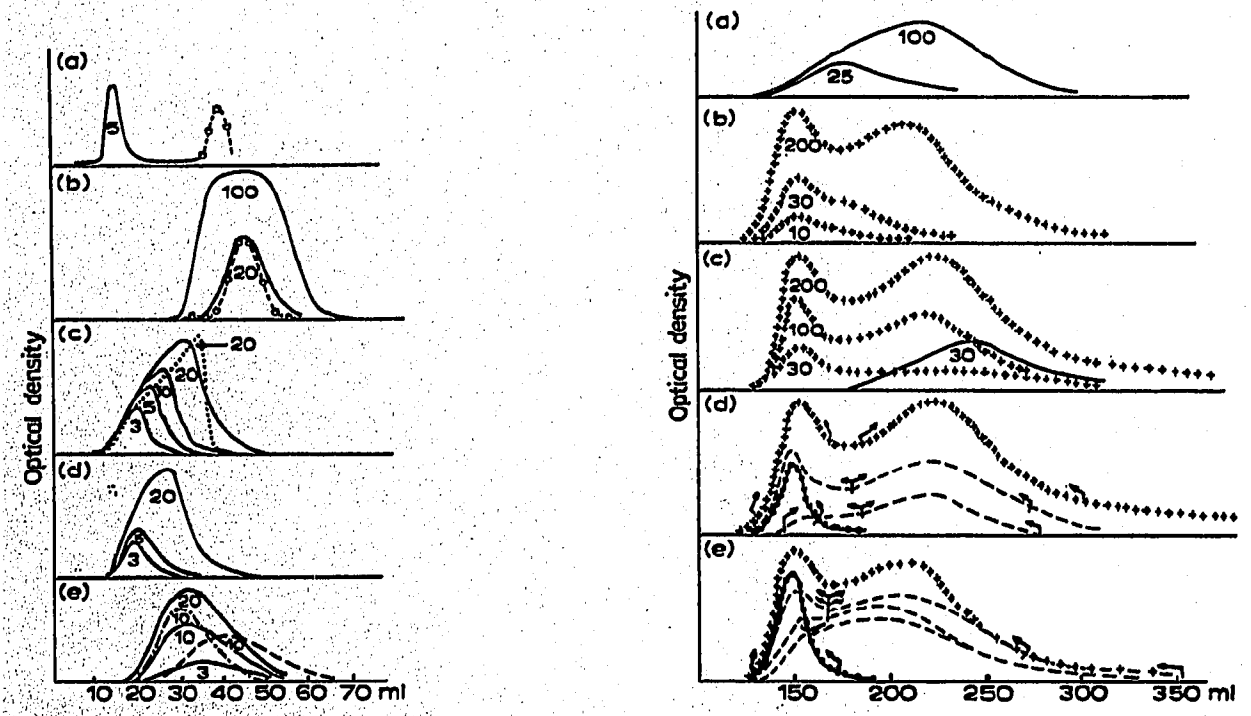

Fig. 1a. Chromatography of native lysozyme and glucose on Bio-Gel P-6 (1.2 × 45.5 cm) in water. The other chromatograms are the same on Bio-Gel P-10 (1.3 × 38 cm) in: (b) water; (c) 0.005 *M* CH_3COOH; (d) 0.1 *M* CH_3COOH and (e) NaCl solutions. Definitions: --O--O-- for glucose on (a) and (b), others for lysozyme. The curves in (e) correspond to the following concentrations of NaCl: (– – – –) 0.02 *M*, (————) 0.1 *M* and (–·–·–) 0.2 *M*. Flow rate: 1.5 ml/min in all cases with one exception : 0.1 ml/min (shown as the dotted line on (c). Numerals under the curves show the amount of protein (mg) in 1 ml applied to the column.

Fig. 2. Chromatography of native (————), γ-irradiated (+ + +) lysozymes and fractions (–·–·–) I and (– – – –) II on Sephadex G-25 (3.4 × 46 cm), coarse, in 0.05 *M* (a, b, e) and 0.02 *M* (c, d) CH_3COOH. Numerals under the curves show the amount of protein (mg); concentration of sample was 15–25 mg/ml. In (d) and (e) chromatograms are shown of γ-irradiated lysozyme and a series of chromatograms after rechromatography of both fractions. Boundary volumes of the solutions of the collected fractions are marked. Flow rate: 3–4 ml/min.

from the column. However, where some irreversible adsorption remained it seemed to depend on the column dimensions. Practically, it would appear that the larger the column, the higher the acid concentration needed for elution.

Chromatography of native lysozyme in acetic acid was found to be non-linear as shown by both the asymmetry of the curves and the position of the maxima (Figs. 1c, d, 2a and 3). The more highly concentrated zones migrated along the column more slowly. The zonal asymmetry of the chromatographic patterns and the concentration dependence for the rate of migration are very clearly shown in the case of Bio-Gel P-10. The dependence of V_e, defined as the position of the peak maxima, on the initial protein concentration in 0.005 *M* CH_3COOH is plotted in Fig. 3. The dotted line designates the extrapolation of the plot to zero protein concentration. This was possible owing to the complete concurrence of the values in the ascending part of all chromatographic patterns given in Fig. 1c. The elution volume changed in our case from ~ 12 to 38 ml, *i.e.* within the range of almost the total gel volume available. In the experiments depicted in Figs. 1c, d and 2a irreversible adsorption was practically

absent. This was verified by subsequently washing the column with 0.1 M CH_3COOH. It is characteristic that in all the experiments performed under different conditions (see for example Figs. 1b, c, d, e) the value of V_e for lysozyme did not exceed that of glucose. This observation is of importance in the understanding of the retention mechanism of substances on the column.

Zonal asymmetry in gel chromatography is a rather rare phenomenon, nevertheless a number of cases are known[6,7]. Zonal asymmetry has been proved to be associated not with the heterogeneity of a substance, but with the nonlinear character of the chromatography[6].

The chromatographic properties of native lysozyme on dense gels predetermine the chromatographic behaviour of γ-irradiated lysozyme. We observed in the first place that the shape of the chromatographic diagrams depended upon the amount of substance applied to a column. From Fig. 2b it is seen that irradiated lysozyme gave two peaks and the quality of separation depended upon the amount of the sample. The total yield of irradiated protein after lyophilisation was 75 and 90% for 0.02 M and 0.05 M CH_3COOH, respectively (for experiments on Figs. 2d, e).

Both peaks of irradiated lysozyme were identified by chromatography on a small analytical column of Sephadex G-25 (1.2 × 43.5 cm) in 0.01 M CH_3COOH (Fig. 4a). In this case a significant amount of irradiated protein was eluted with void volume. Fractions I and II obtained earlier from irradiated lysozyme by salt fractionation[3] were used for comparison. Fraction I corresponds to the most heavily damaged part of the irradiated protein and fraction II—to the least[3]. It can be seen that there is a fraction I that elutes with the void volume. The position of fraction II in the elution pattern, however, corresponds more nearly to that of native lysozyme. On rechromatography, fraction I always gave one peak, but fraction II repeatedly separated into two peaks (see Figs. 2d, e). An attempt was made to obtain complete separation of the two fractions by changing the conditions (column dimensions, eluent, flow rate, etc.). This, however, has not yet been achieved. We suggest that this is

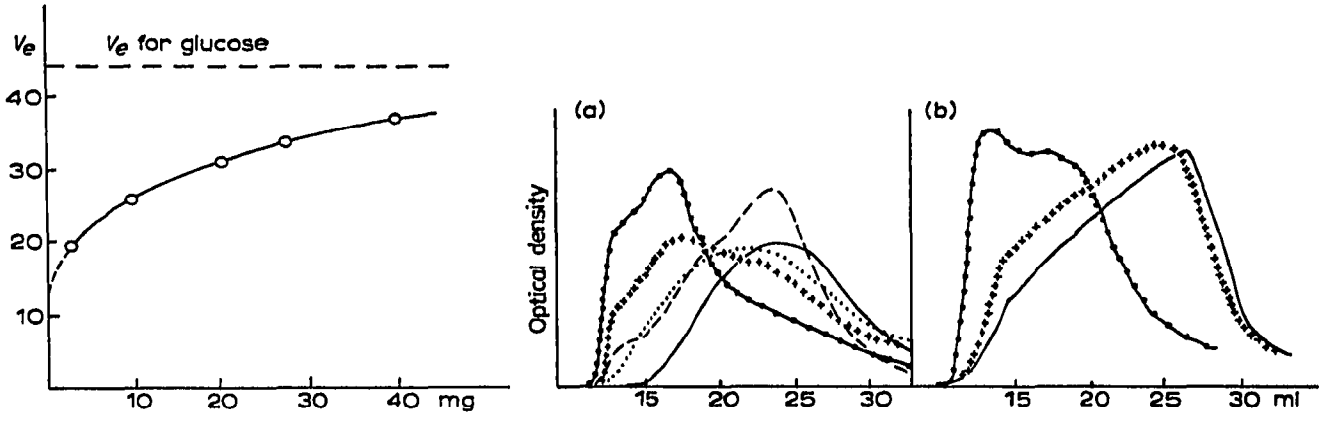

Fig. 3. Dependence of the position of the maximum (V_e) on the amount of sample (mg) applied. Chromatography of native lysozyme on Bio-Gel P-10 (1.3 × 38 cm) in 0.005 M CH_3COOH. Flow rate: 1.5 ml/min.

Fig. 4. Chromatography of different lysozyme derivatives on Sephadex G-25 (1.2 × 43 cm), medium, in 0.01 M CH_3COOH (a) and Bio-Gel P-10 (1.3 × 38 cm) in 0.005 M CH_3COOH (b). Definition for native, γ-irradiated lysozymes and fractions I and II are the same as in Fig. 2; for thermally denatured lysozyme ·····.

primarily because of the Johnson–Ogston effect which should be apparent from the concentration dependence of the elution pattern. It also results in part of the faster component moving along the column at the same rate as the retarded one. This effect has already been discussed in literature[6] with respect to the gel chromatography of other substances. The difficulties arising for the isolation of fraction II are principally bound up with the nature of this chromatographic method. It is fortunate that the most labile and most difficultly obtainable part of irradiated lysozyme, fraction I, can be isolated without admixture with fraction II in such a simple way. Fraction II can be purified by other methods (*e.g.* by ion-exchange chromatography) since its behaviour, in particular its solubility, differs very little from that of native protein.

Bio-Gel P-10 seems to be less suitable for such a fractionation of irradiated protein. Typical elution diagrams are given in Fig. 4b. The lack of separation in this case may also be caused by the Johnson–Ogston effect.

Chromatography on Sephadex G-75

It is known that Sephadex G-75 only partly excludes lysozyme from its inner phase (see Table I). It was interesting to compare the data on gels of high density (*e.g.* Sephadex G-25) with those on Sephadex G-75. Marked differences in the chromatograms of γ-irradiated lysozyme on Sephadex G-25 and G-75 were observed. Increase in acetic acid concentration on a Sephadex G-25 column resulted in the convergence of both peaks. For example, on an analytical column γ-irradiated lysozyme was eluted in 0.1 *M* CH_3COOH as a single peak near void volume. On the other hand, chromatography on Sephadex G-75 only gave a single peak in the dilute acetic acid (0.02 *M*). A separation of γ-irradiated lysozyme on G-75 could only be obtained in more concentrated acetic acid (*cf.* Figs. 5a, b). Similar changes in the elution diagrams were

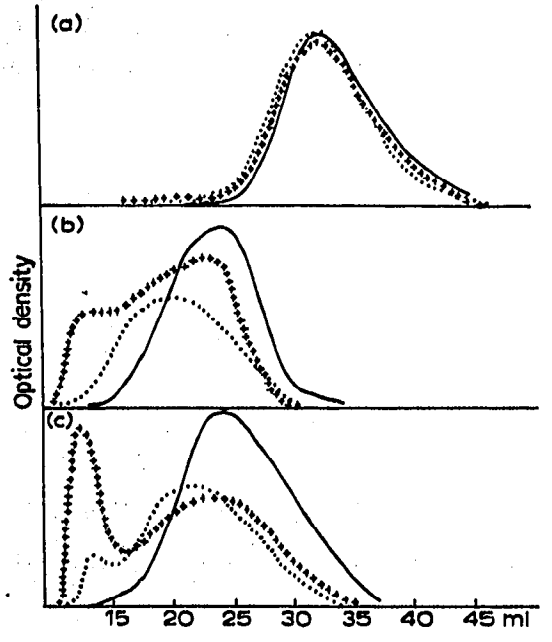

Fig. 5. Chromatography of different lysozyme derivatives on Sephadex G-75 (1.2 × 42.5 cm) in: (a) 0.02 *M* CH_3COOH; (b) 0.5 *M* CH_3COOH; and (c) 0.2 *M* CH_3COONa. Flow rate: 0.3 ml/min. In each case 5 mg of sample in 1 ml was applied to the column. Definitions are the same as in Figs. 2 and 4.

also observed in sodium acetate solution (Fig. 5c). These data for irradiated protein are compatible with those obtained on Sephadex G-100[3]. The protein eluted in the first peak refers to an aggregate of molecular weights. In both acetic acid and sodium acetate, the height of this peak depended on the eluent concentration and this dependence was of S-shaped character. It is the difference in molecular weights that causes the fractionation in these cases. Such conditions could, in principle, be used for the fractionation of γ-irradiated lysozyme. However, increasing the acid concentration would probably result in further denaturation changes in fraction I, and chromatography in sodium acetate also demands an additional step for desalting.

The mechanism of lysozyme retardation on high density gels

Three types of adsorption interactions between a substance and gel matrix are in general known[5]: Coulomb or ion exchange, van der Waals and adsorption interaction (adsorption of aromatic molecules by unknown mechanism). In addition, *Ve* displacement may also be achieved by osmotic pressure on the gel[8]. All these four explanations of abnormal gel chromatography are possible for lysozyme, since its molecules contain hydrophobic and aromatic residues on the surface and carry a positive charge over a wide range of pH. The decrease of adsorption capacity of lysozyme in CH_3COOH solutions could be explained by the suppression of the ion-exchange interaction mechanism.

Furthermore, in order to explain the observed facts the possibility that the lysozyme molecules could penetrate the grains of such gels as Sephadex G-25 and Bio-Gel P-10 should also be considered on account of the following: (1) The high degree of irreversible adsorption in water. The capacity of the gel for reversible adsorption of the excess part was particularly large, this comes about from the linear character of the chromatography. (2) The sharp difference in adsorption for Bio-Gels P-6 and P-10. (3) The identical elution volumes for the excess part of the lysozyme and glucose on Bio-Gel P-10. The ability of lysozyme to penetrate such gels as G-25 and P-10 may be caused by osmotic pressure as noted above. This assumption seems very likely if we take into consideration the Donnan correction to osmotic pressure of lysozyme solutions. The high charge of the molecules and the low ionic power of the solutions are known to result in the osmotic pressure of proteins rising sharply[9]. Recently it was shown that the Donnan effect could become apparent in chromatography on Sephadex G-25[10]. Such an assumption may explain the observed concentration dependence. At the same time it excludes any chemical interaction between a substance and gel matrix. The elution volume therefore can not exceed the sum of the void and inner volumes ($V_o + V_i$). This indeed was a fact.

It is not possible at present to go into the details of the mechanism, particularly for the separation of γ-irradiated lysozyme, but some possibilities may be excluded. The separation of fraction I from fraction II cannot be explained either by denaturation or by aggregation. Thermally denatured lysozyme is chromatographically closer to native protein (and fraction II, Fig. 3a), and when it was also irradiated, the same "fraction I" appeared in the chromatographic pattern. Aggregates can be observed only in solutions with higher concentration of acetic acid, as was shown on Sephadex G-75.

The mechanism of lysozyme adsorption on Bio-Gel P-10 in NaCl solutions (Fig. 1e) still remains unsolved.

ISOLATION OF FRACTION I

200 mg of γ-irradiated lysozyme were dissolved in 15–25 ml of 0.05 *M* CH_3COOH. A sample was applied on a Sephadex G-25 column (3.4 × 46 cm, coarse) and was eluted as usual with 0.05 *M* CH_3COOH. After eluting 120 ml a detector was connected to the column in order to record the UV-absorption of the effluent (UV flow-through densitometer). Fraction I was collected between 130–165 ml, as shown by the arrows on Fig. 2e. Fraction II is also marked by the arrows on Fig. 2e. Collected fractions were lyophilized. Yields of fractions I and II were 20 and 70 %. The yield of fraction I may be increased to 40 % by rechromatography of fraction II. Rechromatography of fraction I gave only one peak (Fig. 2e). The flow rate was 3–4 ml/min and the isolation procedure took about an hour.

TABLE II

A COMPARISON OF THE CHARACTERISTICS OF FRACTION I PREPARED BY SALT FRACTIONATION[3] AND BY THE CHROMATOGRAPHIC METHOD IN THIS PAPER

Proteins	*Optical rotation dispersion parameters*			*Tryptophan content* (%)	*Activity on Micrococcus lysodeikticus* (%)
	$-a_0$	$-b_0$	λc (*nm*)		
Native lysozyme[a]	267	145	251	100	100
Fraction I[a] (by salt fractionation method)	346	88	239	54	5–7
Fraction I (by chromatographic method)	335	159	247	86	3–5

[a] Unpublished data of E. D. KAVERSNEVA, V. I. MAKSIMOV AND V. I. OSIPOV.

Two samples of fraction I prepared by two different methods, and showing identical chromatographic diagrams on Sephadex G-25 in dilute acetic acid are compared in Table II. However, the fraction I obtained chromatographically has less damaged tryptophan, and the parameters of optical rotation dispersion b_0 and λ_c show the retention of a general characteristic structure. In fraction I, prepared by salt fractionation, greater damage of tryptophan and significant denaturation changes in protein structure are apparent. Therefore, the chromatographic method of isolation of fraction I avoids to some extent secondary structure alteration occurring in the irradiated protein during fractionation.

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REFERENCES

1 F. MIRANDA, H. ROCHAT AND S. LISSITZKY, *J. Chromatog.*, 7 (1962) 142.
2 A. N. GLAZER AND D. WELLNER, *Nature*, 194 (1962) 862.
3 V. I. MAKSIMOV AND V. I. OSIPOV, *Biochimia*, 32 (1967) 835.
4 J. OPIENSKA-BLAUTH, M. CHAREZINSKI AND H. BERBEC, *Anal. Biochem.*, 6 (1963) 69.
5 H. DETERMANN, *Gel Chromatography*, 2nd ed., Springer-Verlag, Berlin, Heidelberg, New York, 1969, pp. 26, 29, 27, 81.
6 D. J. WINZOR AND L. W. NICHOL, *Biochim. Biophys. Acta*, 104 (1965) 1.
7 M. V. TRACEY, *Australian J. Biol. Sci.*, 17 (1964) 792.
8 E. EDMOND, S. FARQUHAR, I. R. DUNSTONE AND A. G. OGSTON, *Biochem. J.*, 108 (1968) 755.
9 R. B. MARTIN, *Introduction to Biophysical Chemistry*, 1964; Russian translation, Moscow, 1966, p. 140.
10 L. W. NICHOL, W. H. SAWYER AND D. I. WINZOR, *Biochem. J.*, 112 (1969) 259.