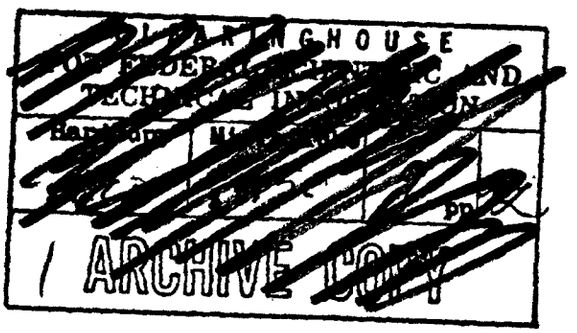
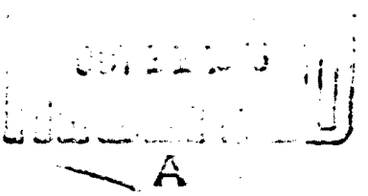
PURIFICATION OF FLUORESCENT CONJUGATES FROM FREE FLUOROCHROME WITH THE AID OF ION-EXCHANGE RESINS

Translation No. 1608



July 1965



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PURIFICATION OF FLUORESCENT CONJUGATES FROM FREE FLUOROCHROME WITH THE AID OF ION-EXCHANGE RESINS

Report II

Comparative Results of Fluorescent Antibody Purification by the Methods of Ion Exchange and Filtration Through Gel

/Following is the translation of an article by I. O. Dashkevich, G. M. Mayboroda and R. B. Goldin, Military Medical Order of Lenin Academy imeni S. M. Kirova, published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiology) No. 2, 1965, pages 116-120. It was submitted on 28 Oct 1963. Translation performed by Sp/7 Charles T. Ostertag Jr.7

In the practice of the preparation of fluorescent antibodies numerous methods were used for the purification of labeled proteins from the unreacted fluorochrome (reprecipitation with ammonium sulfate, organic solvents, sorption with activated carbon and other substances, extraction by ethyl-acetone, etc.). Recently there has been widespread use of methods based on ion exchange (Mayboroda and Dashkevich, 1963) or gel filtration (Goldstein et al., 1961; Lipp, 1961). In the present report materials are presented which make it possible to compare the merits and deficiencies of these two virtually different methods of purifying proteins.

For the purification of labeled proteins by the ion exchange method we used ion-exchange resins of Soviet origin, AV-17, AV-18 and AV-27 (containing from 2 up to 6% divinylbenzene). For the purification of proteins by the gel filtration method we used preparation of "Sephadex" (Swedish origin) brands Zh-25 and Zh-50, which represent dextrans, easily swelling in water and forming gels, and inhibiting substances with a molecular weight lower than a specific value. Thus sephadex Zh-25 inhibits particles with a molecular weight less than 3500-4000, and sephadex Zh-50 - less than 8000-10,000. Molecules of substances with a greater molecular weight do not enter the granule of gel and pass freely through a column filled with sephadex.

In the tests we used freshly prepared, fluorescein isothiocyanate labeled, antibacterial, antirickettsial, antiviral, and also antiprotein antibodies (the globulin fractions of the corresponding immune rabbit, human and pigeon sera). Labeling of antibodies was conducted with a 1-2% concentration of protein; the amount of fluorochrome introduced into the reaction mixtures comprised 2% of the weight of the protein. Conjugation was performed by mixing in an alkaline medium (pH 9.0) for 18-20 hours at 4°; then each conjugate was divided into four equal parts, each of which was purified by one of the types of anion-exchange resins or sephadex.

As it was shown in our previous investigations, the most complete purification with anion-exchange resins is possible following dialysis of the conjugates for 1 - 2 days against an 0.15 M solution of sodium chloride, containing an 0.01 M phosphate buffer with a pH of 7.2-7.3 (FB-1). After such a preliminary purification we diluted the conjugates with a buffer of the stated composition (up to 0.5% concentration of protein) and passed them through a column, 4--5 mm in diameter, filled with ion-exchange resin. For the purification of 15-20 ml of conjugate, 200-250 mg of anion-exchange resin was required. The latter was preliminarily wetted in a buffer solution of sodium chloride (FB-1) and placed into the column in the form of a suspension. The column, charged with the ion-exchange resin, was washed with a buffer unti its pH was equal with the initial one. Then the conjugate was passed through the column with a speed of 50 ml/cm^2/hour. For the purpose of removing suspended particles the purified conjugate was subjected to centrifuging and in order to avoid intergrowth was preserved with sodium merthiolate or by means of lyophilic drying.

The removal of free fluorochrome with the help of sephadex was done without the preliminary dialysis of the conjugates. The sephadex was preliminarily wetted up to swelling in the FB-1 and in the form of a gel was transferred to a column with a diameter of 15 mm and brought into equilibrium with a buffer. After this the conjugate was passed through the column. As a measure of its passage there appeared a clearer dividing zone between the inhibited free fluorochrome and the purified conjugate. When the conjugate had completely entered the layer of sephadex, we eluted the purified protein, adding a phosphate buffer of the mentioned composition to the column. As a result we obtained a solution of protein, diluted by approximately 2 times in comparison with the initial. For the purification of 6 ml of conjugate, 5 g of sephadex was required.

When evaluating the quality of the purification of the fluorescent antibodies, we recorded the completeness of removal of the free fluorochrome with the help of a chromatographic method which we developed (Mayboroda and Dashkevich, 1963). The content of protein in the conjugates was determined by the nitrogen (microkjeldahl method); the amount of fluorochrome connected to the protein was determined spectrophotometrically (SF-4) with a wave length equal to 495 millimicrons. A solution of fluorescein served as the standard. The serological properties of the conjugates were judged according to their staining titers, that is, based on the maximum dilution which ensured the distinct specific fluorescent staining of the homologous organisms or antigens As an additional feature characterizing the quality of purification of the conjugates, we considered the degree of nonspecific luminescence of the cellul or protein background of the preparations in relation to the luminosity of the specific fluorescence of the homologous microorganisms (especially in the identification of rickettsiae and viruses).

Investigations in ultraviolet rays of the chromatograms of all the conjugates prepared by us, taken before and after purification, showed that both the ion-exchange resins and the sephadex almost completely removed the free fluorochrome from the solutions of labeled proteins. Consequently, in this respect the methods of purification must be considered equal.

Since any method of purification is connected with certain losses of protein, we made a comparison of these losses during purification with ion-exchange resins and with sephadex.

It turned out (table 1) that the losses were comparatively small.

Investigations of the serological properties of the conjugates showed that the purification did not reflect on the activity of the immune proteins in their reactions with antigens.

During the purification of conjugates with sephadex the losses of protein depended on the volume of the eluate selected (table 2). During the elution of purified conjugate with the help of a buffer solution of sodium chloride, in the first batch of the solution of protein, which is equal in volume to that which was initially taken for purification, the main mass of protein is included, in the second batch -- almost all the remaining amount. If another, third batch, is collected, as this is recommended by several authors, then it is practically possible to collect all the protein. However, along with this a strong dilution of the entire conjugate takes place. Therefore it is better to be limited to elutions of the first two batches of purified protein, disregarding the losses. In a comparison of the results of purification of conjugates by sephadex Zh-25 and Zh-50, it can be seen that when using the first of these the losses of protein were less, though the difference turned out to be comparatively small.

In comparing the results of purification by the stated methods it can be seen that the total losses of protein during purification by anion-exchange resins and sephadex differed insignificantly.

The serological properties of the proteins (titers of antibodies, calculated per 1 ml of protein, amount of fluorochrome connected per unit weight of protein, etc.) in the conjugates which were purified by the different methods also differed little (table 3). The relationships between the brightness of luminescence of the antigens and the background of the stained preparations (cells of tissues in smear imprints from organs, the protein layer in smears from microbial suspensions, containing serumal globulin, allantois fluid, etc.) turned out to be the same. In all cases the luminescence of the background was extremely weak. It should be noted, however, that in a number of cases, with all other conditions equal, the staining titers of the antibodies which were purified with sephadex turned out to be 1--2 dilutions lower than in the corresponding preparations which were purified with anion-exchange resins. The reason for this phenomenon has not been investigated by us and requires further study.

Conclusions

1. Purification of luminescent antibodies with the help of anion-exchange resin and sephadex produces practically the same results.

2. A deficiency in purification with anion-exchange resins is

connected with the necessity of the preliminary dialysis of the conjugates, which somewhat prolongs the time for purification; a deficiency of sephadex is its great expense and the considerable dilution of the solutions of purified antibodies.

3. The extreme low price and availability of ion-exchange resins, and also their insignificant expenditure in work make it possible to recommend the latter, especially when it is necessary to purify significant amounts of luminescent antibodies, and this may have special importance under industrial conditions.

Literature

a. Mayboroda, G. M., Dashkevich, I. O., Zh. mikrobiol., 1963, No 3, page 55.

b. Goldstein G., Slizys, I. S., Chase, M. W., J. exp. Med., 1961, v. 114, p. 89.

c. Lipp, W., J. Histochem. Cytochem., 1961, v 9, p. 458.

Table 1

Losses of protein in fluorescent conjugates during purification with ion-exchange resins.

No. of conjugate	Concentration of protein in conjugates(%)		Amount of protein in relation to initial (%)	Losses of protein (in %)
	Before purification	After purification		
212	0.52	0.51	98	2
212_1	0.5	0.47	94	6
427	0.6	0.55	92	8
231	0.61	0.47	92	8
217	0.53	0.53	100	0

Notes: Conjugate No 212 -- gamma globulin of rabbit species specific antiplague serum; No 472 -- gamma globulin of rabbit normal serum; No 231 -- gamma globulin of rabbit antitularemia serum; No 217 -- gamma globulin of rabbit antiduck serum.

Table 2

Content of protein in various volumes of eluates following purification of the conjugates with sephadex.

No. of conjugate	Type of sephadex	Initial conjugate		Conjugate following purification									
		Concentration of protein (in %)	Amount of protein in 5 ml of conjugate(mg)	Content of protein in separate batches, equal to the initial volume in 5 ml (in mg)				Amount of protein in 2 batches (in mg)	In % to the initial amount	Concentration of solution	Amount of protein		
				I	II	III	IV				In 3 batches	In % to the initial amount	Concentration of solution
218		0.94	47	37.5	10.0	--	--	47	100	0.47			
230	Zh-25	1.03	51.5	35.5	15.0	1.5	--	50	97	0.5			
RM		1.93	96.5	74.0	14.5	5.5	--	88.5	91.7	0.88	94	97	0.62
RM		1.93	96.5	61.5	21.5	6	--	83	86.4	0.83	89	92	0.59
230	Zh-50	1.03	51.5	21.5	18.5	10	--	40	77.6	0.4	50	97	0.33

Note: Conjugate No 218 -- gamma globulin antiornithosis serum, conjugate No 230 -- gamma globulin rabbit antitularemia serum, conjugate RM -- gamma globulin rabbit antirabic serum.

Table 3

Immunological and staining properties of conjugates, purified with anion-exchange resins and sephadex.

Serological reaction	No. of conjugate *	Brand of anion-exchange resin and sephadex	Titers of antibodies for 1 mg of protein before and after purification**	Amount of fluorochrome (in mg) per 1 mg of protein	Staining titers for 1% solutions of conjugates	Brilliance of luminescence of homologous antigens*** and background
Neutralization reaction	21	AV-17	2.11/2.11	8.0	1:8	++++/+
		AV-18	2.11/2.11	8.2	1:10	++++/+
		AV-27	2.11/2.11	8.1	1:10	++++/+
		Zh-25	2.11/2.11	7.5	1:6	++++/+
	22	AV-17	2.87/2.87	9.0	1:8	+++/-
		AV-18	2.87/2.87	8.0	1:8	++++/+
		AV-27	2.87/2.87	8.3	1:6	++++/+
		Zh-25	2.87/2.87	7.0	1:4	++++/+
	23	AV-17	2.37/2.37	5.9	1:6	++++/+
		AV-18	2.37/2.37	6.1	1:6	++++/+
		AV-27	2.37/2.37	5.8	1:4	++++/+
		Zh-25	2.37/2.37	5.3	1:3	++++/-
Precipitation reaction	210	AV-17	1:100/1:100	9.8	1:8	++++/
		AV-18	1:100/1:100	8.7	1:6	++++/+
		AV-27	1:100/1:100	9.0	1:6	+++/-
		Zh-25	1:100/1;100	8.3	1:4	+++/-
	212	AV-17	1:10/1:10	7.2	1:10	++++/-
		AV-18	1:10/1:00	6.3	1:6	++++/-
		AV-27	1:10/1:10	6,6	1:10	++++/-
		Zh-25	1:10/1:10	6.6	1:10	++++/-
	213	AV-17	1:8/1:8	7.1	1:8	+++/-
		AV-18	1:10/1:10	5.7	1:15	+++/-
		AV-27	1:10/1:10	7.0	1:10	++++/+
		Zh-25	1:10/1:10	7.0	1:6	++++/-
	213_2	AV-17	1:10/1:10	7.2	1:10	++++/-
		Zh-25	1:20/1:20	7.0	1:6	+++/-
Complement fixation reaction	975	AV-17	1:40/1:40	7.0	1:4	+++/-
		AV-27	1;40/1:40	6.4	--	---
		AV-27	1:40/1:40	7.4	--	---
		Zh-25	1:40/1:40	7.0	1:2	---

* Conjugates No 21 and 22 -- gamma globulin of specific rabbit serum of Japanese encephalitis, No 23 -- gamma globulin of specific rabbit serum of tick-borne encephalitis, No 975 -- gamma globulin of specific rabbit serum of murine typhus , No 210 -- gamma globulin of rabbit antipigeon serum, No 212, 213 -- gamma globulin of rabbit type specific antiplague serum.

** Numerator -- titers of antibodies before purification, denominator - after.

*** Numerator -- brilliance of specific luminescence of homologous antigen, denominator -- degree of dying out of background of the preparation.

Note: In the event the denominator is $\pm$ the $-$ appears to belong to the slash in the expression below it.