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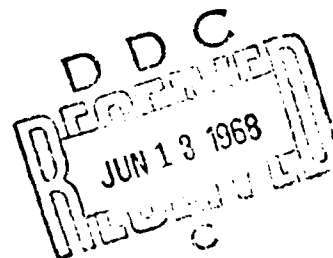
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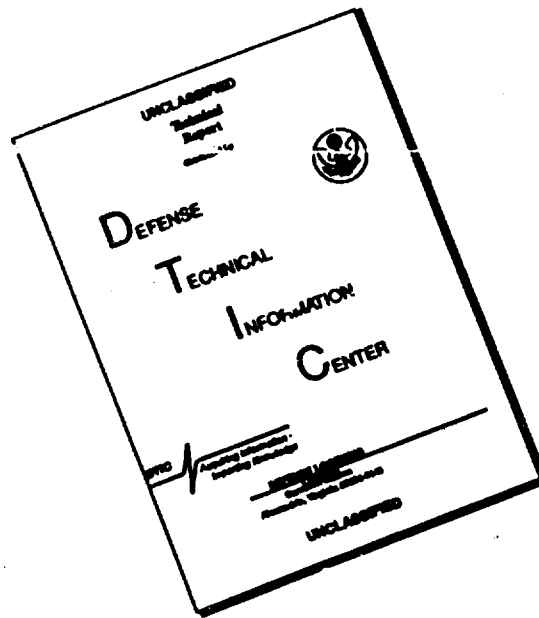
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**FRACTIONATION OF BOVINE GAMMA-GLOBULIN ON COLUMNS
OF CARBOXYMETHYL- AND DIETHYLAMINETHYL-CELLULOSE**

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**FRACTIONATION OF BOVINE GAMMA-GLOBULIN ON COLUMNS
OF CARBOXYMETHYL- AND DIETHYLAMINETHYL-CELLULOSE**

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A.S. Tsyporovich and I.P. Galich

One of the most complex protein systems is the γ -globulin of the blood serum. The chromatographic properties of the γ -globulins have been extensively studied and are also being studied at the present time. The subject investigated is usually human γ -globulin, which is investigated particularly in connection with various pathological states. Less research has been devoted to bovine γ -globulin, although it is rather valuable in the production of diverse medical preparations.

This paper studies bovine γ -globulin by chromatography on ion-exchanger-cellulose derivative columns.

The ion-exchanging adsorbents introduced by Peterson and Sober [1, 2] uncovered great possibilities for studying individual proteins and complex protein systems. For the characteristics of normal bovine γ -globulin we used two adsorbents: the cationite carboxymethyl cellulose (CM-cellulose) and the anionite diethylaminethyl cellulose (DEAE-cellulose).

The literature contains practically no works studying bovine γ -globulin on cellulose ion-exchangers. There has also been little fractionation of bovine γ -globulin on other adsorbents. Thus, Porter and Press [3], studying this protein by separation chromatography on celite 545, in essence obtained only a single wide peak which could only tentatively be separated into components. As for chromatography on CM-cellulose [4], the most painstaking experiments may be considered to be those of Sober, Peterson, and Martin [5] on human γ -globulin. The protein, it was found, was combined with a number of components which were rather clearly separated. Human γ -globulin was eluted as a single wide peak in the investigations of Khase and Putnam [6] [names transliterated from the Ukrainian].

A few more data are known about fractionation of γ -globulin on DEAE-cellulose [2, 5-10]. For the most part, however, whole human blood serum, normal and pathological, has been investigated on this adsorbent, and γ -globulin only in composition with this serum [2, 5, 7]. Columns with DEAE were used by Albert and Johnson [10] to separate macroglobulins. There is only one paper [11] on direct investigation of γ -globulin microfractions, but there, too, it was human γ -globulin separated by preparative electrophoresis which was studied. The authors showed that DEAE-cellulose can be used to separate human γ -globulin into five components, four of which have a sedimentation factor of 6.6 S, while the fifth has 18 S. We set ourselves the task of separating bovine γ -globulin more completely into its individual fractions. We had to work out the conditions for this separation on CM- and DEAE-cellulose.

Materials and Methods

Gamma-globulin was obtained from normal bovine blood serum or plasma by the rivanol method after Horejsl and Smetana [12-13]. We used a method of supplementary purification with salting out by ammonium sulfate. (How γ -globulin was obtained is described below.) The rivanol was used in the form of the usual medical preparation. Protein homogeneity was checked by electrophoresis on paper.

We prepared the CM-cellulose from the Ellis-Simpson prescription [14]. The initial material used was enriched sulfite pulp which is produced in the form of cardboard sheets. Instead of the usually recommended [14] cellulose of the Arkhangel' Paper Combine we used that of the Svyatogorsk Combine. The adsorbent obtained was quite satisfactory in quality and the number of milliequivalents per gram of protein was 0.47-0.52. The monochloroacetic acid and other reagents needed to prepare CM were the ordinary commercial preparations.

The other adsorbent used -- DEAE-cellulose -- was prepared from the prescription of Peterson and Sober [1].*

To fill the columns, whose dimensions as a rule were 130-160 mm by 9-10 mm, it took 2.5-3.0 grams of CM-cellulose or 1.5 grams of DEAE-cellulose. Into the CM column was poured 20-25 mg of γ -globulin previously dissolved in 2 ml of M/1000 potassium-sodium-phosphate buffer of pH 6.6, and into the DEAE columns in all experiments 30 mg of protein also dissolved in 2 ml of M/1000 or M/5000 phosphate buffer of pH 8.0. Elution by degrees was used to wash out the γ -globulin. In the CM experiments the protein was eluted by NaCl solutions of various concentrations in M/1000 sodium-potassium-phosphate buffer of pH 6.6. Two series of experiments were conducted with DEAE-cellulose. In one of them the γ -globulin was eluted with a phosphate buffer of pH values which were decreased by degrees from 8.0 to 5.3. In the other series elution by degrees was

*We take this opportunity to express our thanks to E.L. Khodorova, who gave us some of the preparation which she had derived.

conducted in the same way as in the CM-cellulose experiments by changing the NaCl concentration in $M/1000$ or $M/5000$ phosphate buffer while the medium (pH 8.0) is continuously reacting.

The fraction during elution of approximately 5 ml is taken off by automatic fraction collectors of our design [15]. The amount of protein in the samplings was determined from optical density of the solutions ($\lambda = 280$ millimicrons). The chromatographic experiments were conducted at a temperature of $5-6^{\circ}\text{C}$ [1-5].

Deriving γ -globulin from Bovine Blood

There are several methods of deriving γ -globulin [5, 12, 13, 16-21]. The familiar method is salt fractionation by means of $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 , KH_2PO_4 , ZnSO_4 , and other salts. Heavy metal salts (especially of zinc), organic compounds and solutions, and preparative electrophoresis are extensively used to separate blood serum proteins. The alcohol method developed by Kon [transliterated from the Ukrainian] and his collaborators was formerly very extensively used in producing γ -globulin. This method has many advantages, but certain drawbacks cannot be eliminated -- the complexity and time-consuming nature of the process, great losses of alcohol, γ -globulin losses because of the many steps in the process, and the need to conduct operations at a low temperature (-5 to 6°C).

We fixed on the rivanol method of obtaining γ -globulin developed by Gorzheyshi and Smetana [12, 13]. Its principle is that the action of rivanol solutions on the blood serum precipitates all proteins but γ -globulin, which remains in solution. The rivanol is removed from the latter by adsorbing it on active carbon and then lyophilizing the transparent and colorless γ -globulin solution. The yield of γ -globulin obtained by this method is 95-96%. Its properties in a whole series of biological, chemical, and physicochemical characteristics are in no wise different from the γ -globulin obtained by the alcohol method.

The inconvenience of the rivanol method is that as a result of all the operations the separated protein is in a very weak solution (the serum diluted 4 to 4.5 times). This complicates the drying process. Some authors have introduced extra operations to separate protein from this sort of solution. Thus, Gurvich, Guberniyeva, and Myasoyedova [19] precipitated rabbit γ -globulin by alkalizing the medium to pH 9.5. Gubenko [20] has developed a so-called rivanol-alcohol method for production purposes: here the γ -globulin is separated from the dilute solution by alcohol precipitation at a temperature of -5 to 6°C . It is interesting that this work salts out the rivanol with sodium chloride in an 0.85% concentration instead of removing it by adsorption on active carbon.

In our work we also used a supplementary operation by working out a procedure which by analogy with the rivanol-alcohol method may be called the rivanol-salt method. The purpose of the additional operation was to

remove the hemoglobin which was always present in bovine serum, was not precipitated by rivanol, and contaminated the protein obtained.

Usually, according to the directions of Horejsi and Smetana, we added to one volume of blood serum 3.5 volumes of 0.4% rivanol solution, dropping it in in small amounts. The proteins which settled were filtered out through a double filter and complete transparency of the solution was required. To adsorb the rivanol we chose the most suitable grade of carbon (AR-3). Since it is of granular manufacture in the form of small cylinders we carefully ground it in a mortar before using it. Sixty grams of ground carbon per liter of solution obtained was needed for complete separation of the rivanol. The carbon was filtered out. We were careful that the final solution (after removal of the carbon) containing the purified γ -globulin was always slightly pink. It was found that it contained a certain amount of hemoglobin which had not been precipitated by the rivanol. Bovine blood serum always contained a certain amount of this protein. Even completely colorless ("yellow") serums contained dissolved hemoglobin which stayed in the γ -globulin fraction and contaminated it. In order to remove the hemoglobin impurities we introduced an extra salting out with ammonium sulfate, which was conducted at a concentration of the salt where the γ -globulin was completely precipitated, while the hemoglobin remained in solution (final degree of salt saturation was 0.33-0.38%). In addition to salting out the hemoglobin this also concentrated the protein before lyophilization. As already mentioned, the usually obtained γ -globulin solution was greatly diluted. More than 1800 cc of solution had to be dried to give 2.5-3.0 grams of dry protein. The protein had to be separated in advance, but we tried to avoid alcohol precipitation because of the relatively great danger of denaturation and the inconvenience of working at a temperature of -5 to 6°C.

Salting-out and dialysis seemed the more convenient operations. The additional purification introduced made it possible completely to remove the hemoglobin. When the solution was saturated with $(\text{NH}_4)_2\text{SO}_4$ to 0.33-0.38% there then fell out a white precipitate of γ -globulin which was isolated by centrifuging; the colored hemoglobin remained in the supernatant liquid. The precipitate was washed with a solution of ammonium sulfate and dissolved in the minimum; the solution was dialyzed against water to almost complete separation of the sulfate ions, after which the protein was dried by lyophilization.

The protein obtained was electrophoretically studied. Electrophoresis on paper was conducted in the usual way [21] in a veronal-medinal buffer of pH 8.6 and in a sodium-potassium-phosphate buffer of pH 7.1 and 7.7 at room temperature. Leningrad chromatographic paper No. 2 was used: size of the electrophoretic strips was 42 x 4 cm. The experiment was conducted at 260-300 volts and 6-8 ma. The electrophoregrams were colored by means of murexide black. On the strips was always applied 0.01 cc of γ -globulin solution in the appropriate buffer; the solution

contains 0.5 mg of protein. Duration of electrophoresis in the veronal-medinal buffer was 16 and 40 hr; in the phosphate buffer it was 16 hr.

In all cases the γ -globulin obtained acted like an electrophoretically homogeneous protein (Fig. 1).

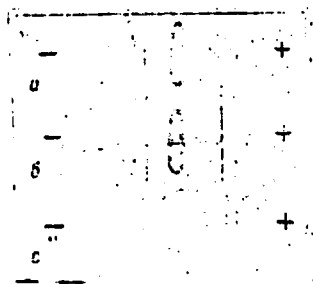


Fig. 1. Electrophoregrams of γ -globulin: a -- electrophoresis in phosphate buffer of pH 7.1 for 16 hr; b -- in phosphate buffer of pH 7.7 for 16 hr; c -- in veronal-medinal buffer of pH 8.6 for 40 hr.

Studying γ -globulin on CM-cellulose

The electrophoretically homogeneous preparation of γ -globulin derived by the above-described method was chromatographically studied on a cation-exchanging adsorbent (CM-cellulose). The experiments were conducted as follows. The column was washed with 10-20 volumes of M/1000 phosphate buffer of pH 6.6 and then 20-25 mg of γ -globulin dissolved in the same buffer was applied to it. When checking completeness of protein adsorption in the column we began to elute the protein by degrees, adding washing solutions which contained 0.05-0.15-0.20-0.30-0.40-0.50-0.6 M of sodium chloride in an M/1000 phosphate buffer of pH 6.6. The column was regenerated in the usual way after the conclusion of each experiment [14].

Figure 2 shows the characteristic fractionation curve of bovine γ -globulin on CM-cellulose.

The experimental results permit us to proceed to rather definite conclusions. First of all, attention is drawn to the presence in γ -globulin of no less than seven microfractions. The relative amounts thereof in our experiments vary somewhat, but it was always noted that the components which were eluted by M/500 and M/300 sodium chloride were the greatest in quantity, the first containing 50-60% protein, the second, 20-35%. In the other protein fractions the distribution was: fraction eluted by M/10 NaCl solution always contained a very insignificant amount of it, 0.5-1%; the fractions following it contained 1-3% (M/150), 4-10% (M/20), 6-9% (M/40), 7-11% (M/50). Finally, the last

fraction when eluted by M/60 of NaCl did not always appear. When it did it contained 1-2% of γ -globulin.

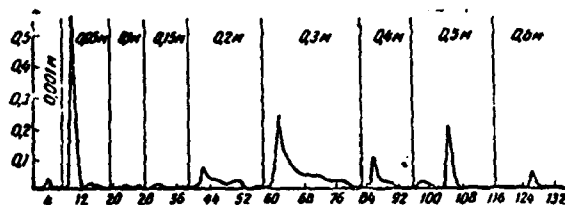


Fig. 2. Fractionation of γ -globulin on CM-cellulose: vertically -- amount of protein in samples eluted from column, measured in degrees of extinction; horizontally -- number of tests; horizontally above -- molar concentration of eluent.

Investigation of bovine γ -globulin on CM-cellulose thus showed that this protein, like human γ -globulin, is composed of a number of components (seven or eight). The experimental conditions chosen enable us to distinguish microfractions with adequate clarity. In this respect the method of elution by degrees obviously has advantages over the gradient method of elution, for it permits each fraction to be completely washed out.

Study of γ -globulin on DEAE-Cellulose

In order to derive more detailed characteristics of bovine γ -globulin the second part of the work was devoted to studying this protein on an ion-exchanger -- DEAE-cellulose. The experiments were conducted in sodium-potassium-phosphate buffer. The following conditions for regeneration and preparation of the column for the experiment were chosen: it was treated with 50-60 cc of 1% NaCl, washed with water until reaction was neutral (to phenolphthalein), and then with 200-230 cc of M/1000 or M/5000 phosphate buffer of pH 8.0. After this amount of buffer has been passed through the column the pH of the effluent solution is 0.0-9.2; column capacity was meanwhile rather high and when 30 mg of γ -globulin was applied it was completely adsorbed. It is to be noted that even in the first samplings chosen the required pH (8.0-8.2) was found.

It was decided to conduct the first series of experiments by fractionating proteins by changing the pH of the eluent solution. The first eluent buffer was a M/10 solution of phosphate buffer of pH 8.0, then buffers of the same concentration but different pH ranging from 7.85 to 5.3 were successively added, i.e., 7.85, 7.7, 7.52, 7.38, 7.17, 6.98, 6.8, 6.6, 6.4, 6.2, 5.9, 5.6, and 5.3. Then further to acidify the medium were added solutions of KH_2PO_4 alone, first in the same M/100

concentration and then with successive increases to M/50, M/1, and M/20. The protein remaining in the column was washed out with a 1% solution of NaOH. Figure 3 gives the characteristic curve of this series of experiments.

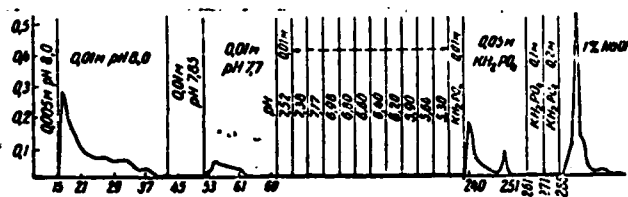


Fig. 3. Fractionation of γ -globulin on DEAE-Cellulose: horizontally above -- pH values of eluent; other notation as in Fig. 2.

In this method of fractionation two γ -globulin microfractions were always detected at the very beginning of the chromatogram. The first of them appeared when buffer (pH 8.0) concentration alone increased from M/5000 to M/100 and contained about 50% of all the protein; the second followed the first when eluting with M/100 buffer of pH 7.85 or 7.7 and contained a small amount of γ -globulin, about 7%. Then, as is evident from Fig. 3, successive decrease of buffer pH from 7.7 to 5.3 and even to 4.5 (KH_2PO_4 solution) did not result in eluting the protein from the column. The new third fraction containing 13-15% protein appeared only after concentration of the KH_2PO_4 solution was increased. The remaining protein was washed out with 1% NaOH as the fourth fraction containing 20-25% of the material used. The data of these experiments therefore already have shown that even a considerable change in pH of the medium does not make it possible to fractionate γ -globulin. At the same time a slight increase in eluent concentration immediately washes out the protein.

In some experiments we also increased the phosphate buffer concentration from M/100 to M/10 at pH 5.9, after which the protein immediately began to be eluted. In other experiments there was an increase in concentration of the first eluent buffer from M/100 to M/200 at pH 8.0. This led to an increase in the first fraction to 80%. In the experiments where, however, the first eluent buffer was an M/500 phosphate one of pH 8.0 almost all the protein (up to 90%) was washed out in a single peak.

It therefore proved to be that γ -globulin may be separated into a greater number of microfractions by changing salt concentration in the eluent. It was therefore decided to conduct a second series of experiments by increasing the concentration of NaCl ions in the M/5000 phosphate buffer of pH 8.0. First were prepared the same NaCl solutions as in the experiments with CM-cellulose: molar values of 0.05, 0.01, 0.15, 0.2, 0.3,

0.4, 0.5, and 0.6. By their means we separated the γ -globulin into seven microfractions. The first was always a very sharp and high peak and contained 58-60% of all the protein applied; this fraction was eluted by the M/500 NaCl solution. The second fraction was eluted by the next M/10 and contained about 21% of the protein. Following this came the third, fourth, fifth, and sixth fractions which were eluted by the M/150, M/20, M/30, and M/40 solutions of NaCl; all of these were small and each contained from 0.6 to 5% of the protein. The seventh fraction was taken off by the 1% NaCl and contained 12-14% of the γ -globulin.

It was found to be of interest to divide the first and largest component and thus to achieve more careful fractionation of all the protein. To do this we decreased the first eluent concentration (M/500) and used the following molar values of NaCl: 0.01 -- 0.02 -- 0.03 -- 0.04 -- 0.05. After them fractionation was conducted with the usual solutions: M/700, M/10, M/150, M/20, M/30, M/40, and M/60. In so doing the first and largest component was successfully separated into its component parts.

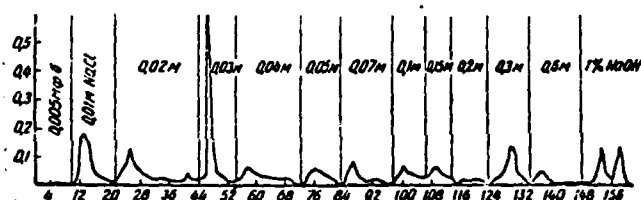


Fig. 4. Fractionation of γ -globulin on DEAE-Cellulose (notation as in Fig. 2).

Figure 4 gives the results of a representative experiment. As we see, under the conditions worked out γ -globulin may be fractionated into 11 or 12 microfractions. It is interesting that the first five fractions contained 58-65% of all the protein, i.e., the same amount as in the large component in the preceding series of experiments. By selecting the proper conditions we have thus separated the first fraction obtained in the preceding experiments into five individual microfractions. The protein in them is distributed as follows: first microfraction, 10-15%; second, 14-18%; third, 14-19%; fourth, 10-16%; fifth, 5-8% (percentages of all the protein). The rest of the components eluted by each successive buffer contained lesser amounts of protein: sixth microfraction, 4-9%; seventh, 3-5%; eighth, 2-4.4%; ninth, 1-2.5%; tenth, 1-5%; eleventh (washed out by M/40 or M/60 NaCl), up to 2% of the γ -globulin; and finally in the last fraction which was eluted by a 1% solution of NaCl there was always 10-14% of the γ -globulin.

By choosing the proper conditions we have therefore been able to separate bovine γ -globulin into 11 or 12 microfractions. This is twice as many as were found by Fahey and Horbett [11] in their clearly executed work on human γ -globulin.

Conclusions

Chromatographic fractionation of bovine γ -globulin was carried out in columns of ion-exchangers -- cellulose derivatives.

The γ -globulin preparation was obtained by the rivanol method. It is shown that when bovine serum or plasma is precipitated by rivanol the hemoglobin admixture which is always present remains in solution and contaminates the γ -globulin. A method of supplementary purification is proposed which permits separation of the hemoglobin by salting off with ammonium sulfate. The resultant γ -globulin preparation is found to be electrophoretically homogeneous in phosphate and veronal buffers of pH 7.1, 7.7, and 8.6.

The protein is chromatographed on a cation-exchange adsorbent -- carboxymethyl cellulose (CM-cellulose), and on an anion-exchange adsorbent -- diethylaminethyl cellulose (DEAE-cellulose). In all cases the method of elution by degrees was employed.

Conditions have been worked out under which bovine γ -globulin on CM-cellulose can be separated into seven microfractions by sodium chloride solutions of increasing concentration.

In chromatography on DEAE-cellulose it is demonstrated that a change in pH of the eluting solutions from 8.0 to 4.5 makes it impossible to fractionate the protein. Conditions have been worked out under which bovine γ -globulin may be separated into 11 or 12 microfractions also by changing the sodium chloride concentration in the eluent.

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