Purification of Gamma Globulin Fragments by Gel Filtration

Abstract. *Treatment with papain of human 7 S gamma globulin which has specific hemagglutinating antibody activity results in 3.5 S univalent fragments contaminated by intact bivalent globulin. The technique described in this report permits separation of univalent antibody fragments from the contaminating undigested molecule.*

In 1959, Porter discovered that rabbit gamma globulin with bivalent antibody activity could be digested by the enzyme papain into smaller molecules whose molecular weight was approximately 50,000, with univalent antibody activity (*1*). This technique provided proteins sufficiently small for the structural basis of antibody specificity to be studied. Treatment of human gamma globulin with papain yields similar fragments. Despite variations in the enzyme digestion procedure and in the chromatographic techniques used for isolating the material, however, the 3.5 S fragments ($s_{20,w}$ = 3.5 S) so obtained are contaminated with intact enzyme-resistant 7 S gamma globulin ($s_{20,w}$ = 7 S) (*2, 3*).

We now present a procedure for separating the 3.5 S fragments from the contaminating 7 S gamma globulin, as judged by ultracentrifugal and immunoelectrophoretic analyses and by serologic analysis based on the hemagglutinating capacity possessed by the bivalent but not by the univalent antibody molecule.

Human 7 S gamma$_2$ globulin isolated from the serum of a patient with chronic thyroiditis and circulating antithyroglobulin antibody was separated by anionic exchange chromatography with diethylaminoethylcellulose (DEAE). Sheep erythrocytes treated with tannic acid and then coated with human thyroglobulin were agglutinated by the isolated gamma$_2$ globulin to a log$_2$-titer of 12; the initial dilution used had an optical density at 280 mμ of 0.025. The isolated gamma globulin was subjected to digestion for 5 hours by mercuripapain under the conditions described by Porter (*1*); the reaction was then stopped by the addition of *p*-chloromercuribenzoate. The digestion product was subjected to chromatography on a carboxymethylcellulose (CM) column and then on a DEAE column by the method of Franklin (*3*); three fractions, designated A, B and C, were recovered. Univalent antibody activity was not present in the B fragment, but was present in fragments A and C.

The present study deals only with the further purification of fraction A. Thirty to forty percent of this fraction consisted of contaminating 7 S gamma globulin, as shown in the first ultracentrifugation pattern in Table 1. We were unable to separate the contaminant completely by DEAE or CM chromatography or starch gel electrophoresis. Immunoelectrophoretic patterns obtained with rabbit antiserum to human gamma$_2$ globulin showed the presence of two lines (Table 1). With dilutions starting at an optical density at 280 mμ of 0.025, the log$_2$-titer of fraction A was 11. This is essentially the same as the titer for the gamma$_2$ globulin before it was subjected to enzymatic digestion.

A newly available preparation of polymerized dextran (Sephadex G-200) (*4*), which has been employed by others for the partial separation of 7 S from 19 S gamma globulin in human serum (*5*), was used in an attempt to separate the components of fraction A. A 2- by

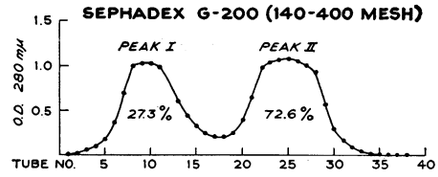

Fig. 1. Gel filtration chromatogram.

55-cm column of this material was equilibrated with 0.1*M* sodium chloride in 0.01*M* phosphate buffer, *p*H 8.0. Before being introduced onto the column, the protein was dialyzed against the phosphate buffer, and elution was carried out with the same buffer. The gel filtration pattern is shown in Fig. 1. On ultracentrifugal analysis the protein of peak I and of peak II (Table 1) sedimented as single peaks with s_{20} values of 6.47 S and 3.5 S, respectively, when centrifuged at concentrations of 3.2 and 5.2 mg/ml, respectively. The hemagglutination log$_2$-titer for thyroglobulin antibody activity of material from peak I was identical with that of the 7 S molecule prior to enzyme digestion, whereas the titer of the material

Table 1. Physical and immunologic characteristics of gamma globulin and of fractions produced by mercuripapain digestion.

Ultracentrifuge pattern	Immunoelectrophoretic pattern	Thyroglobulin hemagglutination log$_2$-titer
Gamma globulin		
		12
Fraction A		
		11
Peak I		
		12
Peak II		
s_{20} = 6.47 3.54		0*

* Same result tested at OD_{280} = 1.0.

from peak II was completely negative even when the protein concentration was 50 times that of the protein from peak I. The presence of univalent antithyroglobulin antibody in the material contained in peak II was established by the capacity of this protein to inhibit specifically the hemagglutination of thyroglobulin coated cells by bivalent 7 S antibody. Based on such studies it is estimated that at the concentration of protein in peak II, no more than 0.5 percent bivalent antibody could be present but not detectable by hemagglutination due to the inhibitory effect of the univalent fragment (*6*).

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Tissue Levels of Norepinephrine and Epinephrine in Hemorrhagic Shock

Abstract. *Severe depletion of endogenous norepinephrine was observed in the brain, heart, liver, and spleen of albino rabbits in which hemorrhagic shock had been induced. On the other hand, the epinephrine content of these tissues was significantly elevated above the levels in tissues of control animals. The norepinephrine and epinephrine levels of skeletal muscle in shocked animals remained unaffected.*

As discussed in an earlier report, the heart in hemorrhagic shock was observed to be markedly depleted of its endogenous norepinephrine (*1*). Although the epinephrine concentration in the myocardium was significantly elevated, the total catecholamine content (norepinephrine plus epinephrine) was reduced to 34 percent of the content for control animals. A study was undertaken (*2*) to determine whether similar alterations of norepinephrine and epinephrine levels occurred in other tissues of animals in hemorrhagic shock.

Table 1. A comparison of norepinephrine (NE) and epinephrine (E) levels in tissue of normal rabbits and of rabbits in which hemorrhagic shock had been induced.

Organ	Levels (μg/g)*				*P* values†	
	Controls		Shocked animals			
	NE	E	NE	E	NE	E
Heart	1.05 ± 0.13 (12)	0.14 ± 0.06 (12)	0.16 ± 0.05 (12)	0.24 ± 0.06 (12)	< .001	< .01
Spleen	0.49 ± 0.10 (10)	.08 ± 0.03 (12)	.00 (4)	.36 ± 0.05 (4)	< .001	< .01
Brain	.32 ± 0.04 (20)	.02 ± 0.009 (20)	.10 ± 0.04 (13)	.12 ± 0.05 (13)	< .01	< .025
Liver	.19 ± 0.05 (12)	.07 ± 0.026 (14)	.06 ± 0.03 (12)	.23 ± 0.07 (12)	< .05	< .025
Muscle	.19 ± 0.05 (12)	.02 ± 0.027 (13)	.10 ± 0.03 (13)	.06 ± 0.04 (13)	> .27	> .30

* Mean; standard error; number of animals (in parentheses). † Calculated by Student's *t*-test.

In albino rabbits ranging in weight from 1 to 3 kg the left femoral artery was cannulated after infiltration of the area with 2-percent procaine. Before the animal was subjected to shock, an initial dose of heparin (5 mg/kg) was administered by vein as an anticoagulant; thereafter, heparin (half of the initial dose) was administered every hour throughout the experiment. Hemorrhagic shock was induced by bleeding from the cannulated artery into a reservoir which was placed at a height equivalent to pressure of 50 mm-Hg (*1*). This level of pressure was maintained for 3 hours, after which the animal was sacrificed by rapid withdrawal of blood from the cannulated artery with a syringe. Samples (approximately 1 g) of brain (diencephalon), heart (left ventricle), liver (left lobe), skeletal muscle (gluteal), and spleen were quickly removed, weighed, and homogenized in a Potter-Elvehjem tissue grinder containing 6 cm^3 of cold 10-percent trichloroacetic acid. The supernatant liquid, after centrifugation under refrigeration, was extracted three times with diethyl ether. The aqueous fraction was diluted with an equal volume of 0.2*N* sodium acetate, and the *p*H was adjusted to 8.2 with 0.5*N* sodium carbonate. The tissue extract was then passed through an alumina column (Fischer) which had previously been washed three times with triple-distilled water. Eluates from the chromatographic columns, together with norepinephrine and epinephrine standards, were analyzed in accordance with a modification of the trihydroxyindole method (*3*). The resulting fluorescence was measured with a Farrand photofluorometer, arbitrarily adjusted to a sensitivity sufficient for detecting 0.01 to 1.5 μg of norepinephrine and epinephrine per gram of tissue. The catecholamine levels determined in the tissue of the shocked animals were compared to levels found in tissue of control rabbits subjected to a cannulation of the femoral artery without bleeding. These animals were killed instantly by a high cervical spinal transection.

In Table 1, the concentrations of norepinephrine and epinephrine in tissue of animals in which hemorrhagic shock had been induced are compared with the concentrations in tissue of control animals. In organs, such as heart and spleen, for which levels of endogenous norepinephrine were found to be relatively high in the controls, levels of the hormone were found to be profoundy reduced in the shocked animals. Levels were reduced in heart and spleen by 85 and 100 percent, respectively. The level of norepinephrine was reduced to a lesser extent in brain (69 percent) and in liver (68 percent), and the concentration in skeletal muscle was not statistically different from that in the controls ($P > .27$). In contrast to the decrease in levels of norepinephrine there was an increase in levels of epinephrine in brain, heart, liver, and spleen in the shocked animals (Table 1). Probably the rise in the concentrations of epinephrine in tissue stems from the high adrenal output of this hormone in hemorrhagic shock (*4*). However, our experimental data do not give any indication of the mechanism of norepinephrine depletion. Experiments for comparing the rate of synthesis with the rate of utilization of norepinephrine would explain the lowering of levels of this catecholamine in tissue of the shocked animal.

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