

ALTERATIONS IN SERUM GLOBULINS DURING THE FORMATION AND RESORPTION OF AMYLOID IN RABBITS*

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Amyloidosis has long been considered a disorder of protein metabolism because it is characterized by the abnormal deposition of protein complexes in various organs. Several observations have suggested that amyloid deposits are at least partly derived from proteins circulating in the blood under abnormal conditions.

For example, it has been found that the serum protein patterns of patients suffering from amyloidosis are often abnormal (1-4), that abnormal serum globulins and amyloid may both be present in patients with multiple myeloma (5-7), and that the incidence of amyloidosis is high in hyperimmunized horses (8). Yet no single abnormality of the blood proteins has thus far been clearly related to amyloidosis; indeed, the electrophoretic patterns of serum proteins from patients with advanced amyloidosis are variable (1-4). Hence there is reason to question whether abnormalities of the circulating proteins play a part in the pathogenesis of the disease. More evidence on this point has been sought in the present study.

In a previous paper the author reported that amyloidosis uncomplicated by tissue necrosis or infection can be regularly produced in rabbits by means of sodium ribonucleate, and that the amyloid thus produced is resorbed under suitable conditions (9). It has now been found that an abnormal rise in serum beta globulins accompanies the development of this type of amyloidosis, and that proteins with electrophoretic properties of beta globulins are sometimes excreted in relatively high concentrations in the urine of amyloidotic rabbits; furthermore, the circulating beta globulins decrease to normal levels during periods of resorption, while the gamma globulins increase.

Materials and Methods

General.—Sera of rabbits were examined repeatedly by means of electrophoresis during and after a prolonged course of treatment with ribonucleate. Control rabbits were injected with sterile saline instead of ribonucleate. It

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was determined by means of biopsies and postmortem studies whether re-sorption of amyloid had taken place. Formol-gel tests for increased serum globulins were done on all sera to learn whether the formol-gel reaction could be correlated with the development of amyloidosis. Urine from each animal was repeatedly tested for the presence of protein, and positive urine specimens were subjected to electrophoretic analyses. The patterns of urinary proteins were compared with those of serum proteins obtained at the same time.

Rabbits.—Hybrid rabbits of both sexes weighing approximately 2000 gm. were used. They were fed Rockland rabbit pellets, and given water *ad libitum*. Special attention was directed to the problem of coccidiosis, an infectious disease that is caused by *Eimeria stiedae* and is often endemic in colonies of rabbits presumed to be healthy (17). In the author's experience rabbits with manifest infection of the intrahepatic biliary tree have often shown elevations in gamma globulins while animals with infections confined to the gastrointestinal and/or extrahepatic biliary tract have not shown such a change. The rabbits used in the present experiments were obtained when they were quite young and kept under observation for a minimum of 5 weeks prior to being used. Gross and microscopic examinations of the livers of these animals post mortem disclosed no evidence of infection with *Eimeria stiedae*.

Ribonucleate.—Ribose nucleic acid, prepared from yeast, and free from protein, was obtained from Schwarz Laboratories, Inc., of New York. The properties of this preparation have been described previously (10). Five per cent solutions of the material were prepared in 0.5 per cent NaOH, and their pH adjusted to 7.3 by means of HCl and a phosphate buffer (10). These solutions were passed through Seitz filters and yielded no bacteria on agar or in broth.

Bleeding of Animals and Preparation of Sera.—As a routine, the rabbits were bled from ear veins, but at the end of each experiment blood was obtained from the heart. The sera were separated following clot retraction. Whenever possible, analyses were carried out a few hours after bleeding. Since sera obtained from ear veins are commonly unsterile, one drop of a 1:1000 aqueous solution of merthiolate was added to each 3 ml. of serum as a preservative. Following this treatment the sera were stored in a refrigerator at 4°C. This method of preservation did not alter the electrophoretic patterns of the sera during periods of 2 to 3 months.

Collection and Preparation of Urine.—Rabbits were kept in metabolism cages from time to time so that urine could be obtained. The total urinary output during periods of 24 hours was collected and filtered. Small samples were then tested for the presence of protein by means of a 5 per cent solution of sulfosalicylic acid (11) as well as by the application of heat and acetic acid. Urine giving a positive reaction was prepared for electrophoretic analysis by saturating 5 to 10 ml. with crystalline ammonium sulfate. Following vigorous shaking 3 ml. to 5 ml. of diethyl ether were added, and the mixtures shaken again. Then they were centrifuged for 10 minutes at 2000 R.P.M. This resulted in a layering of the precipitated proteins between the ether and urine. The ether was then drawn off by means of a capillary pipette which was later passed into the urine layer near the wall of the test tube. As the urine was pipetted off, the disc of precipitated protein floating on it flipped over and adhered to the wall of the test tube. This material was scooped up with a small spatula and transferred to a watch glass. Small portions were then used for confirmatory ninhydrin and biuret tests according to routine methods (12). The larger remainder was dissolved in a volume of barbiturate buffer (see below) just sufficient to effect solution, and the solution was used for electrophoresis. The pipetted urine was tested with sulfosalicylic acid to make certain that no unprecipitated protein had been left behind in significant amounts.

Electrophoresis.—Electrophoretic analyses of the sera and of the urinary proteins were done on filter paper. A number of sera were also analyzed by the moving boundary method.

For the analyses on filter paper an apparatus similar to that of Flynn and DeMayo (13) was constructed. The analyses were carried out at voltages ranging from 90 to 110 volts, and at temperatures in the neighborhood of 20°C. Strips of Whatman No. 1 filter paper, 4 cm. wide, and 36 cm. long were suspended in the electrophoretic chamber. The electrolyte was the standard barbiturate buffer consisting of sodium diethyl barbiturate and barbituric acid in distilled water, having a pH of 8.6 and an ionic strength of 0.1. In most instances the amount of serum analyzed was 0.02 ml. Occasionally only half this amount was used. When the total protein content proved to be very low, the amounts used were increased proportionately. The same conditions were used in analyses of urinary proteins. Twenty to 22 hours were usually required to achieve the desired degree of separation of the proteins, *i.e.*, a total spread of 15 cm. Following electrophoresis, the strips were dried in an oven at 110°C. for 20 to 30 minutes. They were then stained with naphthalene black 12 BS (Amidoschwarz) according to the method of Grassmann and Hannig (14), and the backgrounds subsequently rendered nearly colorless. The stained strips were dried at room temperature, soaked in a mixture of mineral oil and alpha bromnaphthalene (14), and scanned by direct photometry according to the method of Grassmann and Hannig (14), using a photometer made by Bender and Hobein Co., Munich. The optical densities of the strips were recorded at millimeter intervals on graph paper, and the electrophoretic curves constructed in the usual way. The percentage distributions of the protein components were obtained by means of planimetry. In these measurements boundaries between components were chosen according to the method of Tiselius and Kabat (15). The percentages thus obtained were applied to the total protein concentrations determined by a micro-Kjeldahl method (16).

Electrophoretic analyses by the moving boundary method were carried out in an Aminco-Stern apparatus manufactured by the American Instrument Company, Silver Spring, Maryland. A barbiturate buffer, with pH of 8.52–8.60 was used. The ionic strength of the solutions was 0.1, the temperature generally 0°C. Voltage gradients varied from approximately 5.0 volt/cm. to approximately 8 volt/cm. The final protein concentrations were generally between 2 and 3 per cent.

The accuracy and limitations of the filter paper method used in these experiments are treated in detail in the basic paper of Grassmann and Hannig (14). These investigators found that results obtained by their method for the quantitative evaluation of filter paper strips are superior to those obtained by elution, and are reproducible within limits that are acceptable for studies of a comparative nature, such as the present one, as well as for routine laboratory work. In particular, the alpha globulins can be well developed by the method of Grassmann and Hannig, and relatively small quantities can be rendered visible.

Formol-Gel Tests.—Formol-gel tests were done in order to learn whether the electrophoretic changes that were found in amyloidotic rabbits might be reflected by the results obtainable with this qualitative test. The tests were done at room temperature. One drop of a 40 per cent formaldehyde solution was added to 1 ml. of fresh rabbit serum in a small test tube, and the mixture examined at frequent intervals during the first 2 hours and again after 18 hours. Results were recorded as positive if a gel was formed that adhered partly or wholly to the bottom and sides of the test tube. The time at which this was first seen was also noted. A 5 per cent solution of bovine gamma globulin (Armour) in Ringer's fluid served as material for positive control tests.

Postmortem Examinations and Biopsies.—Detailed postmortem studies were made on all rabbits. The animals were killed by injecting air into ear veins. Duplicate blocks were taken from heart, lungs, spleen, liver, kidneys, adrenals, lymph nodes, and bone marrow, and occasionally from the intestine, urinary bladder, and tongue as well. The blocks were fixed in

Zenker's fluid, and in 10 per cent neutral formalin respectively. They were embedded in paraffin. The hematoxylin and eosin stain was used as routine. When amyloid was found, its presence was confirmed by means of the Congo red stain, and sometimes with the cresyl violet stain also.

To obtain preliminary information, the spleens of a series of rabbits were biopsied as previously described (9). The rabbits subjected to this operative procedure recovered promptly. The resected tissues were prepared as indicated above.

Normal Electrophoretic Patterns of Rabbit Sera

In order to show the natural variations in electrophoretic patterns of healthy laboratory rabbits, the findings on the sera of 38 "normal" animals weighing approximately 1700 to 2350 gm. will first be given. These rabbits had been kept in the laboratory 5 to 6 weeks, and all appeared to be in good health. Most of them were subsequently used in experiments. Sera from these rabbits

TABLE I
*Summary of Serum Protein Concentrations of Thirty-Eight Untreated Rabbits**

Component	Mean		Range		Mean + 2 σ	
	<i>per cent</i>	<i>gm. per 100 ml.</i>	<i>per cent</i>	<i>gm. per 100 ml.</i>	<i>per cent</i>	<i>gm. per 100 ml.</i>
Albumin	71.0	4.3	57-82	2.9-5.5	91	5.5
α -1-globulins	5.0	0.3	2- 7	0.1-0.6	8	0.5
α -2-globulins	6.0	0.4	3- 9	0.3-0.6	9	0.6
β -globulins	9.0	0.6	5-14	0.2-0.9	14	0.9
γ -globulins	9.0	0.6	3-12	0.2-1.0	14	0.9

* As determined by filter paper electrophoresis combined with micro-Kjeldahl analysis.

were studied by filter paper electrophoresis and micro-Kjeldahl analyses. The results, summarized in Table I, show that the percentage distribution of the various protein fractions varies considerably in different animals. In order to indicate the approximate upper limits of normal variation for subsequent comparisons, the means plus two standard deviations are also given in Table I.

Increased Beta Globulins and Amyloidosis in Rabbits Treated with Ribonucleate

As a preliminary step, analyses were made on sera of 6 rabbits that had developed amyloidosis from prolonged treatment with ribonucleate—as later proved by postmortem examinations. These animals had been given subcutaneous injections of a 5 per cent ribonucleate solution in the flanks five times a week during a period of 123 days, 10 ml. being injected each time. At the end of the experiment serum was obtained from each animal, and from seven controls that had received subcutaneous injections of 0.9 per cent NaCl solution instead of ribonucleate. The animals were then killed and autopsied.

Since the anatomical changes have been discussed in a previous paper (9) only a summary is given here. Extensive amyloid deposits were found in the spleens of all six rabbits treated with ribonucleate. These filled the sinusoids, particularly those about the lymphoid follicles. The deposits were stained selectively with Congo red, and proved to be metachromatic upon application of cresyl violet. There were also scattered deposits of amyloid in the glomeruli

TABLE II
Serum Proteins in Rabbits with Amyloidosis and in Controls

Rabbits	Treat-ment	Amyloi-dosis*	Serum proteins‡															
			Filter paper electrophoresis								Moving boundary electrophoresis							
			A		α-1 + α-2		β		γ		A		α-1 + α-2		β		γ	
			per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.	per cent	gm./100 ml.
5-9	RN§	+++	63	3.1	9	0.5	16	0.8	12	0.6	56	2.8	13	0.6	18	0.9	13	0.6
6-0	"	++++	62	3.5	9	0.5	16	0.9	13	0.7	59	3.3	16	0.9	13	0.7	12	0.7
6-1	"	++++	48	2.5	17	0.9	22	1.2	13	0.7	40	2.1	22	1.2	23	1.2	15	0.8
8-4	"	++++	59	3.2	12	0.7	23	1.3	6	0.3								
8-5	"	+++	66	3.2	14	0.7	18	0.9	2	0.1								
8-8	"	++++	62	3.5	10	0.6	22	1.2	6	0.3	58	3.2	9	0.5	25	1.4	8	0.4
Mean..			60	3.2	12	0.7	20	1.1	9	0.4	53	2.9	15	0.8	20	1.1	12	0.6
6-4	Saline	0	75	4.7	10	0.6	7	0.4	8	0.5	See footnote¶							
6-5	"	0	80	5.2	9	0.6	7	0.5	4	0.3								
6-6	"	0	70	5.1	11	0.8	9	0.7	10	0.7								
6-8	"	0	67	3.6	10	0.5	11	0.6	12	0.6								
7-0	"	0	76	4.6	9	0.5	8	0.5	7	0.4								
7-7	"	0	69	4.0	10	0.6	9	0.5	12	0.7								
8-1	"	0	73	4.7	5	0.3	11	0.7	11	0.7								
Mean..			73	4.6	9	0.6	9	0.6	9	0.6								

* As judged from the findings in the spleens (see text).

‡ A: Albumin; α₁ + α₂: α₁ + α₂ globulins; β: β-globulin; γ: γ-globulin.

§ Subcutaneous injections of ribonucleate five times a week during a period of 123 days.

|| Subcutaneous injections of 0.9 per cent NaCl solution five times a week during a period of 123 days.

¶ The mean concentrations of five different rabbits, treated with saline for 92 days, were as follows: A: 70 per cent, 4.3 gm/100 ml., α₁ + α₂: 12 per cent, 0.7 gm/100 ml., β: 9 per cent, 0.5 gm/100 ml., γ: 9 per cent, 0.5 gm/100 ml.

of the kidneys in five of the six rabbits, and two of these animals had small amounts of amyloid in the liver, located between liver cords and sinusoidal lining. No amyloid was found in the organs of the seven control animals.

The results of the electrophoretic analyses on the sera are shown in Table II. It can be seen that the sera of amyloidotic rabbits differed from those of the controls principally in the height of beta globulin and albumin levels. Comparison of the serum protein concentrations of the amyloidotic animals with the normal levels shown in Table I reveals that the amyloidotic rabbits had significant elevations of beta globulins together with depressions of al-

bumin levels. This was particularly true in rabbits 6-1, 8-4, and 8-8. It may also be seen in Table II that a few of the abnormal sera were studied by moving boundary electrophoresis. Although the results were not identical with those obtained by means of the filter paper method, they confirmed the presence of increased quantities of beta globulins and decreased albumin levels. In addition, it may be noted that one rabbit (No. 6-1) also had a high alpha globulin level, a finding that proved to be exceptional. The severity of the amyloidosis was graded roughly on a scale shown in Table II. This grading was based on the amount of amyloid in the spleen, the organ affected earliest and most severely. It appeared that the changes in the serum protein patterns were related to the development of amyloidosis. However, they could not be correlated with the severity of the disease.

The Rise and Fall of Circulating Beta Globulins during the Formation and Resorption of Amyloid

In previous work on hyperglobulinemia the author found that alpha, beta, and gamma globulins of rabbits were often slightly to moderately increased in rabbits given ribonucleate for 1 to 2 months (10). No amyloid was found in the animals after this short period of treatment, and the changes in serum proteins were not considered specific. In the experiment just reported, the sera of the amyloidotic rabbits were examined only once, after the animals had been treated with ribonucleate for 123 days. It remained to be learned what changes occur in the serum proteins during the early stages of amyloidosis produced with ribonucleate.

In another experiment, ten rabbits were treated with ribonucleate as before, but during a period of 164 days, while nine comparable rabbits were concurrently given 0.9 per cent saline. The sera of these animals were examined before the start of treatment, and six times in the course of treatment. Five animals from the former group and four from the latter were killed and autopsied the day the injections were discontinued, while the animals remaining in each group were subjected to biopsy of the spleen. The biopsied rabbits were allowed to survive for 60 to 70 days without receiving any treatment, then sacrificed. The results of this experiment up to the time the injections were discontinued are summarized in Tables III and IV. It was found that the beta globulin concentrations of the rabbits treated with ribonucleate had increased after the 1st month of treatment, and that there was a further increase in subsequent months. The levels were beyond the normal upper limit as may be seen by comparison with the findings on the controls (Table III) and with the data in Table I. A *t* test shows that the differences are significant at *P* values that are much less than 0.001. Nevertheless, there was considerable variation in the height of the beta globulin levels of the animals treated with ribonucleate. Some of these animals also developed moderate,

TABLE III
Serum Beta Globulins in Rabbits Developing Amyloidosis and in Controls

Beta globulin concentrations, gm./100 ml.																						
Treated rabbits*										Control rabbits†												
Days										Days												
	1-5	1-6	1-7	1-8	1-9	2-0	2-1	2-2	2-3		2-4	Mean	4-5	4-6	4-7	4-8	4-9	5-0	5-1	5-3	5-4	Mean
0	0.5	0.4	0.3	0.6	0.4	0.8	0.5	0.4	0.4	0.6	0.5	0	0.8	0.6	0.7	0.4	0.6	0.4	0.7	0.6	0.5	0.6
31	1.1	0.9	0.9	1.0	0.9	1.3	1.2	1.4	0.7	0.9	1.0	33	0.8	0.7	0.7	0.9	0.6	0.4	0.6	0.6	0.9	0.7
74	1.3	1.0	0.9	1.4	0.9	1.6	0.9	1.8	1.0	1.3	1.2	83	0.7	0.5	0.4	0.5	0.5	0.6	0.5	0.7	0.5	0.5
110	1.5	0.8	0.9	1.2	0.9	1.8	1.0	1.2	1.5	0.9	1.2	109	0.7	0.7	0.4	0.6	0.5	0.6	0.6	0.6	0.6	0.6
133	1.6	1.1	1.2	1.6	1.2	1.1	1.1	1.3	1.6	1.2	1.3	180	0.5	0.6	0.9	0.7	0.8	0.7	0.9	0.5	0.6	0.7
164	1.0	1.0	1.3	1.1	1.3	1.1	1.4	1.3	0.9	1.1	1.1											
Max. var.	1.1	0.7	1.0	1.0	0.9	1.0	0.9	1.4	1.2	0.7	1.0	Max. var.	0.3	0.2	0.5	0.5	0.3	0.3	0.4	0.2	0.4	0.3

Difference between means of maximum variation = 0.7 gm./100 ml.

Common variance = 0.036.

S.D. = $\sqrt{0.036}$

$t = 8.03, n = 17$, then $P < 0.001$.

* These rabbits were given subcutaneous injections of 5 per cent ribonucleate solution five times a week

† These rabbits received subcutaneous injections of 0.9 per cent NaCl solution five times a week.

TABLE IV
Distribution of Serum Proteins in Ten Rabbits Developing Amyloidosis

Serum protein fractions	Concentrations, gm./100 ml.					
	Days:					
	0	31	74	110	133	164
<i>Albumin</i>						
Mean	4.1	3.9	3.9	3.9	3.0	3.9
Range	2.9-5.4	3.2-4.5	2.9-4.9	2.6-4.9	2.3-4.1	2.9-4.7
<i>α-1-globulins</i>						
Mean	0.3	0.5	0.3	0.4	0.4	0.6
Range	0.1-0.4	0.1-0.6	0.1-0.5	0.3-0.5	0.2-0.7	0.5-0.8
<i>α-2-globulins</i>						
Mean	0.4	0.3	0.5	0.5	0.4	0.5
Range	0.1-0.5	0.1-0.4	0.1-0.6	0.2-0.6	0.2-0.6	0.4-0.7
<i>β-globulins</i>						
Mean	0.5	1.0	1.2	1.2	1.3	1.1
Range	0.4-0.9	0.7-1.3	0.9-1.8	0.8-1.8	1.1-1.6	0.9-1.4
<i>γ-globulins</i>						
Mean	0.6	0.7	0.5	0.7	0.6	0.6
Range	0.2-0.9	0.3-1.0	0.3-0.8	0.2-1.1	0.3-1.1	0.3-1.2

transitory rises of alpha and gamma globulins during the first 3 months of treatment, with a subsequent return to normal levels, and in some there was a moderate drop in albumin levels. There were no abnormal findings on the sera of the controls that had received injections of saline. Formol-gel tests

performed at the end of the period of treatment with ribonucleate were uniformly negative.

Examination of the splenic tissues obtained by biopsy revealed that the rabbits treated with ribonucleate had extensive amyloidosis of the spleen. The spleens of the controls were normal. Postmortem examinations on the animals that were sacrificed upon cessation of treatment disclosed advanced amyloidosis in the spleens and kidneys of three of the five animals from the ribonucleate group, and moderate amyloidosis in the spleens but not the kidneys of the other two. The tissues of the control rabbits were unremarkable.

TABLE V
Distribution of Serum Proteins during Resorption of Amyloid

Rabbits	Treatment before biopsy	Serum protein concentrations, gm./100 ml.															Amyloid (spleen)		Formol-gel test
		At biopsy					45 days later					60-70 days later					Biopsy	Necropsy	
		A	α -1	α -2	β	γ	A	α -1	α -2	β	γ	A	α -1	α -2	β	γ			
1-6	Ribonucleate	3.5	0.6	0.7	1.0	0.8	4.1	0.7	0.6	0.7	1.9	4.6	0.8	0.6	0.6	1.8	+++	0	+
1-7	"	2.9	0.8	0.5	1.3	0.6	4.4	0.6	0.8	0.6	1.2	4.9	0.2	0.8	0.7	1.2	+++	0	-
1-8	"	4.1	0.5	0.6	1.1	0.7	4.4	0.6	0.6	1.0	1.6	5.8	0.7	0.5	0.8	1.9	+++	0	+
1-9	"	3.4	0.6	0.4	1.3	0.7	4.3	0.5	0.4	0.5	2.0	5.0	0.5	0.6	0.4	1.4	++++	0	+
2-1	"	4.5	0.7	0.5	1.4	0.9	4.3	0.7	0.6	1.1	1.8	5.5	0.2	0.6	1.0	1.4	++++	+	+
4-5	Saline	3.8	0.4	0.5	0.5	0.8	4.0	0.4	0.4	0.9	0.7	4.2	0.2	0.5	0.8	0.8	0	0	-
4-6	"	3.6	0.3	0.2	0.6	0.5	3.8	0.3	0.3	0.6	0.6	3.7	0.5	0.6	0.7	0.6	0	0	-
5-4	"	4.0	0.2	0.4	0.6	0.6	3.9	0.6	0.6	0.5	0.5	4.1	0.4	0.3	0.4	0.7	0	0	-
5-0	"	4.1	0.5	0.3	0.7	0.6	4.3	0.4	0.5	0.7	0.9	4.2	0.6	0.5	0.8	0.7	0	0	-
4-8	"	4.3	0.3	0.3	0.7	0.7	4.5	0.4	0.3	0.6	0.8	4.4	0.5	0.4	0.7	0.8	0	0	-

Difference between means of maximum variation of γ -globulins in the two groups of rabbits: 0.8 gm./100 ml. Common variance = 0.0438; $t = 6.0$, $n = 8$; $P \ll 0.001$ (for γ -globulins).

At this point in the experiment it appeared, therefore, that in rabbits treated with ribonucleate an increase of the beta globulins had regularly accompanied the development of amyloidosis. The principal finding of the earlier experiment was thus confirmed.

To learn whether the beta globulin levels would return to normal if the amyloidosis regressed, no further treatment with ribonucleate was given to the five surviving amyloidotic rabbits, and injections of saline into the five remaining control rabbits were also stopped. Sera from these ten animals were examined 45 days, and 60 to 70 days following cessation of treatment (the date of biopsy). Following the last bleeding the animals were killed and autopsied. The findings are given in Table V. It will be seen that there was

a decline in the beta globulin levels of the amyloidotic rabbits while concurrently the gamma globulin levels rose strikingly by comparison with the levels of the controls and with normal levels (Table I). There were no significant changes in the other fractions.

The levels of beta, gamma, and total globulins of the sera are given in Table VI, together with the results of formol-gel tests. These data indicate that positive formol-gel tests were obtained only in sera with gamma globulin concentrations exceeding 1.2 gm./100 ml. It will be noted that sera with beta globulin concentrations above this value and gamma globulin levels within the normal range gave uniformly negative tests. Moreover, the total globulin

TABLE VI
Results of Formol-Gel Tests in Rabbits with Increased Globulins

Rabbit	Day	Serum globulins, gm./100 ml.			Formol-gel test
		Total	β	γ	
1-6	164	3.1	1.0	0.8	Negative
	223	3.8	0.6	1.8	Positive
1-7	164	3.2	1.3	0.6	Negative
	223	2.9	0.7	1.2	Negative
1-8	164	2.9	1.1	0.7	Negative
	223	3.9	0.8	1.9	Positive
1-9	164	3.0	1.3	0.7	Negative
	223	2.9	0.4	1.4	Positive
2-1	164	3.5	1.4	0.9	Negative
	223	3.2	1.0	1.4	Positive

concentrations *per se* could not be correlated with the results of formol-gel tests. The findings will be considered again in the Discussion.

On the day of the last bleeding, all the remaining rabbits were killed and autopsied. The pertinent anatomical findings in these animals will now be briefly summarized.

The spleens of rabbits previously treated with ribonucleate were now normal in size and gross appearance. In four of the five surviving animals no amyloid was detected on microscopic examination of the spleen, while in the fifth a few, scattered deposits had remained in this organ. The lymphoid follicles had been reconstituted, and were ringed by wide bands of monocytes with large, vesicular nuclei and scanty cytoplasm. Comparison of the post-mortem material with that obtained by biopsy left no doubt that the amyloid had been resorbed from the spleen in every animal, and completely in four out of five rabbits. The kidneys had finely granular cortices with adherent capsules, and on microscopic examination proved to have many amyloidotic glomeruli in most of which there was early fibrosis. Some

of the glomeruli had disappeared, leaving vacant Bowman's spaces. These changes in the glomeruli were interpreted as indicative of resorptive activity. There were areas of interstitial fibrosis, usually associated with atrophy and disappearance of tubules. No amyloid was found in other organs, but there were accumulations of large monocytes and of plasma cells in the axillary and inguinal lymph nodes of three of the rabbits.

The organs of the four control rabbits showed no significant changes with the exception of a slight reaction about the splenic ligatures.

In sum, the electrophoretic and anatomical studies that were carried out following the cessation of treatment with ribonucleate revealed the following: a marked decline of the beta globulin levels, a marked rise of gamma globulin levels, complete or nearly complete resorption of amyloid from the spleen, and probably partial resorption from the kidneys of each amyloidotic animal.

Further Studies of the Increase in Beta Globulins

The findings just presented raised the question whether the increase of beta globulins that accompanied the development of amyloidosis resulted from the appearance of globulin that is not normally present in the rabbit, and moved like a beta globulin. Such a protein might have been produced *de novo* in rabbits receiving injections of ribonucleate. To gain more evidence on this point, attempts were made to subfractionate the beta globulins of appropriate rabbits by means of electrophoresis.

After numerous trial analyses it was found that the beta globulins in the sera of three rabbits that had been treated with ribonucleate (Nos. 1-6, 1-9, and 2-1 in Table III) could be resolved into two components. Optimal results were obtained with the barbital buffer at an initial pH of 7.8, using a potential of 90 volts at a chamber temperature of 15°C. Under these conditions a current of 5 ma. was observed to flow when eight strips were used. Separations took approximately 24 hours. The beta globulins in sera from control rabbits and from eight untreated animals picked at random could not be subfractionated under these conditions although many attempts were made to do so. Fig. 1 (*a* and *b*) shows the separation achieved on serum of one of the three rabbits. As is shown, only one beta globulin peak was separable on serum obtained prior to treatment with ribonucleate, while two distinct peaks were obtained after the animal had been treated with ribonucleate for several months. Analyses on sera from two other amyloidotic rabbits yielded similar results, but sera from five comparable animals that had shown marked rises in total beta globulin concentrations could not be subfractionated by the method described. These findings indicate that qualitative as well as quantitative changes in beta globulins accompanied the development of amyloidosis. It may reasonably be supposed that with more refined methods of analysis qualitative changes in beta globulins could be demonstrated regularly.

Urinary Proteins in Rabbits Developing Amyloidosis

Urine of rabbits receiving ribonucleate, and of controls, was repeatedly collected and tested for the presence of protein by means of 5 per cent sulfosalicylic acid, and by the application of heat and acetic acid. Positive tests were obtained on urine from five treated rabbits on several successive days while the urines of ten controls proved to be negative. The positive tests were

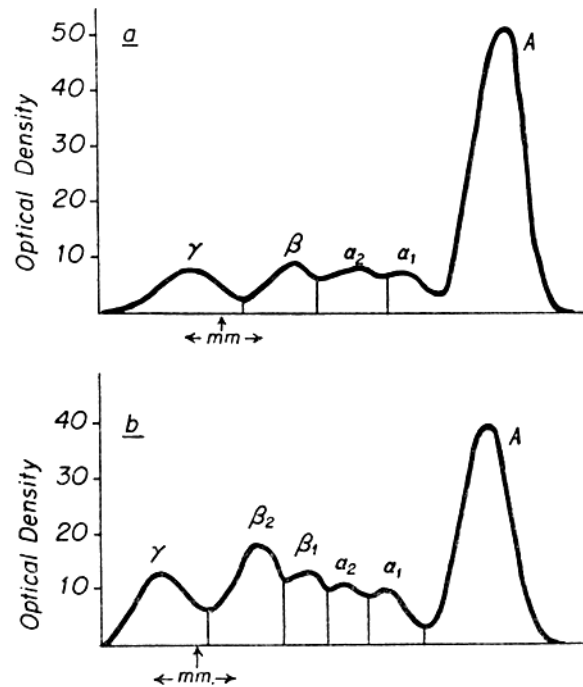


FIG. 1. Serum proteins of a rabbit (a) before and (b) after 133 days of treatment with ribonucleate. Note the appearance of a new peak, marked β_1 , in section b).

obtained during the 4th and 5th months of treatment, at a time when marked changes in the serum proteins of the animals had taken place (Tables III and IV). Three of the rabbits with protein-containing urine were sacrificed approximately 2 weeks after the first positive test (after 164 days of treatment) and were found to have moderate to advanced amyloidosis of the spleen, and moderate to severe amyloidosis of the kidneys. The other two rabbits were in the group receiving no injections of ribonucleate after biopsy. Protein was repeatedly detected in the urine of these two animals until approximately 3 weeks after cessation of treatment. Samples of urine specimens containing

protein were prepared for electrophoresis according to the method already described. Analyses were carried out simultaneously on the urinary proteins and on sera procured from the same animals during the urine collection period. The electrophoretic patterns of urinary and serum proteins were plotted and superimposed for comparison. In one of the five rabbits (sacrificed after 164 days) the excreted protein proved to be almost entirely albumin as judged from electrophoretic analyses. In the remaining four animals this did not prove to be so. Fig. 2 shows the findings on the urine and serum from one of these four. It may be seen that the urinary proteins of this rabbit migrated like its serum proteins during electrophoresis although there was only one alpha globulin peak. Four peaks were separated on each sample, three to one side, and one to the opposite side of the zone of application (origin). The migratory distance of the front component of the urinary protein mixture

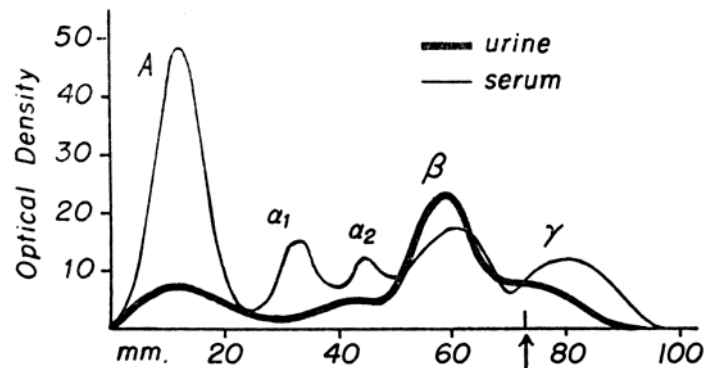


FIG. 2. Serum and urinary proteins in a rabbit treated with ribonucleate for 4 months.

corresponded to that of serum albumin, while the rear component moved as a gamma globulin. However, the findings depicted in Fig. 2 also show that the urinary proteins were present in proportions differing from those of the serum proteins. In particular, there was but a small amount of albumin in the urine. The bulk of the excreted proteins moved as beta globulins. This finding suggests two possibilities. The first is that a protein (globulin?) or protein split product with an abnormally small molecular size was excreted by at least four of the animals treated with ribonucleate, since normal globulins have molecular sizes that are considerably larger than the size of albumin, and hence do not pass the glomerular membrane as readily as albumin. Secondly, both albumin and globulins may have escaped through glomeruli, but the albumin may have been selectively resorbed in the tubules. The second possibility seems unlikely because rabbits readily develop albuminuria under diverse circumstances. However, to substantiate the inference that an abnormal protein was excreted, detailed characterization of the material in question is necessary. Tests for Bence-Jones protein by the usual routine method (11) were negative.

At the subsequent postmortem examinations deposits of amyloid were found in many glomeruli of the four rabbits whose urinary proteins moved predominantly as beta globulins, but there were also many normal appearing glomeruli. By contrast, in the rabbit that had developed predominant albuminuria, there were more extensive deposits of amyloid, involving the majority of the glomeruli. In this relation it may be recalled that extensive renal amyloidosis in man and animals is often associated with massive albuminuria, but not with a preferential excretion of globulins. It seems unlikely, therefore, that anatomical alterations in glomeruli were the principal cause of the unusual type of proteinuria just described.

DISCUSSION

In the experiments here reported the development of an amyloidosis in rabbits was consistently associated with an increase in circulating beta globulins, and, in several instances, with the appearance of a new electrophoretic component that moved as a beta globulin. Moreover, in the urine of some of the animals there appeared a protein or protein derivative that also moved as a beta globulin, and was present in far greater quantity than were the other urinary proteins. These findings lead one to ask whether an abnormal circulating beta globulin, appearing *de novo*, was involved and whether the changes in circulating globulins were related to the deposition of amyloid.

To determine with certainty whether the changes in the beta globulins were merely quantitative or, as seems more likely, qualitative as well, further characterization of the proteins in question is needed. As regards the association of the changes in the beta globulins with the development of amyloidosis, the evidence presented in this and in two previous papers indicates that the electrophoretic changes in the blood proteins precede the deposition of demonstrable amyloid (9, 10). This view is also in accord with the observation that the resorption of amyloid from the tissues is accompanied by a disappearance of the abnormal beta globulin moiety from the blood. Thus, it seems unlikely that the increase in beta globulins results from the liberation of a breakdown product of amyloid into the general circulation, and it is reasonable to assume that this increase is in some way related to the deposition of amyloid in the tissues. That is not to say, however, that all types of amyloidosis—in man or animal—bear a relationship to similar changes in circulating proteins.

The literature on the possible relationship between blood proteins and amyloid is extensive, and also quite contradictory. For this reason only a few pertinent reports will be considered.

In man the classical secondary amyloidosis is generally accompanied by an augmentation of gamma globulins (1-4). It is not known whether distinctive changes in blood proteins precede its development. In cases of multiple myeloma, on the other hand, the development of an amyloidosis may accompany or follow the appearance in the blood of one or more abnormal proteins of differing electrophoretic mobilities

(4, 6, 7, 18). In the author's experience cases of so called primary amyloidosis do not always have electrophoretic changes in the serum proteins. A hypoalbuminemia is commonly found in advanced cases of secondary amyloidosis, and is generally due to severe renal involvement.

A detailed study of the electrophoretic patterns of sera of amyloidotic mice was reported by Bohle, Hartmann, and Pola (19). These workers produced amyloidosis in one strain of mice by means of repeated subcutaneous injections of highly alkaline solutions of ribonucleate according to a method first used by Letterer (21, 22). They noted transient rises of the alpha and beta globulins, followed by more persistent rises of gamma globulins. At the time of the latter, the animals usually had advanced amyloidosis of spleen, liver, and kidneys. Biopsy studies to follow the evolution of the disease were not reported by these authors. However, they stated that the injections produced subcutaneous abscesses of relatively large size. It was their view—in agreement with the earlier work of Letterer—that the production of the amyloidosis depended on the presence of suppuration, and that the injections of alkaline—and unsterile—ribonucleate were the means whereby suppuration was produced. Ott and Schneider (20) have reported an extensive study of plasma proteins of mice in relation to amyloidosis produced by means of subcutaneous injections of alkaline casein solutions. They noted that after five daily injections the animals developed marked increases in alpha-2-globulins, and in a fraction designated as "beta-3-plus fibrinogen," as well as a diminution of albumin and gamma globulin concentrations. Prolonged treatment produced amyloidosis of spleen, liver, and kidneys, moderate elevations of the alpha-2 and "beta-3 plus fibrinogen" fractions, and a marked drop in albumin and gamma globulins, though the latter occasionally rose above the normal level. Latvalahti too, found that the amyloidosis in mice given injections of casein solutions is usually associated with elevations of alpha and beta globulin levels (25, 26). In an analysis of these and other findings in mice, Letterer and Schneider have concluded that the changes in plasma proteins that have been noted are probably non-specific, but that the occurrence of a "dysproteinemia" is nevertheless a constant antecedent of amyloidosis in mice (23).

The fact that amyloidosis develops in hyperimmunized horses (8) has often been cited in support of the hypothesis that the disease is secondary to changes in circulating proteins (21, 22). It has been shown that, in the horse, immunization may result not only in a marked increase of gamma globulins, but also in the appearance of one or more electrophoretic peaks that are not normally present (24). To the author's knowledge there have been no studies in which such alterations were evaluated specifically in relation to amyloidosis.

It has been observed that rabbits receiving cholesterol during periods of several months may develop an amyloidosis (27). Analyses of the sera of such animals revealed, in addition to the usual hypercholesterolemia, considerable elevations of the beta globulins. Presumably, the increases involved lipoproteins, but in view of the presence of amyloidosis in the animals, there may have been beta globulins similar to those detected in the present study. In other work it was found that scorbutic guinea pigs may develop an amyloidosis together with changes in the serum glycoproteins (28, 29). This finding is of interest because of the fact that amyloid consists of protein-carbohydrate complexes.

Two findings in the present study may here be briefly considered, *viz.* the marked

rises of the gamma globulins during the period of resorption, and the results of the formol-gel tests. The former phenomenon may have resulted from autoimmunization. For example, the breakdown of amyloid (by enzymes?) most likely resulted in the liberation of split products, and these may have been antigenic, stimulating the production of gamma globulins. Alternatively, such split products may have had electrophoretic mobilities similar to those of gamma globulins, thus appearing in the same broad electrophoretic peak. In relation to the formol-gel tests it may be recalled that these tests were positive only when the gamma globulin concentrations exceeded 1.2 gm./100 ml., and that sera with beta globulin concentrations above this level, but with gamma globulin concentrations below it, failed to give the reaction. Similar observations have been made on human sera, and the conclusion has been reached that the formol-gel reaction depends upon the quantity of gamma globulins in the serum (18). On the other hand, it has also been stated that the reaction may depend upon the total globulin concentration in the reacting serum (18). Certainly, in the author's experience, the reaction is easily produced with purified gamma globulin fractions of diverse origin when used in sufficiently high concentrations. The fact that rabbit sera with markedly increased beta globulins did not give it, points to a qualitative aspect of this test. Although positive reactions might have been observed, had the concentrations of beta globulins been still higher, it seems more likely that some or all of the beta globulins in the blood of rabbits treated with ribonucleate lacked properties necessary for the formation of a protein gel following the addition of formaldehyde solution.

Thus, studies of the serum proteins of man and animals in relation to amyloidosis have not provided consistent results. This should not be surprising, however, because amyloidosis may develop under widely differing circumstances. Moreover, analyses of sera have generally been carried out when the disease was quite advanced, or while non-specific changes may have masked specific ones. This is particularly true of studies on secondary amyloidosis, the type which is associated with inflammatory and suppurative processes. It is well known that such processes may give rise to alterations in serum protein patterns without leading to amyloidosis. While the findings presented in this report do not warrant generalizations on the pathogenesis of amyloidosis, they indicate that the increases of beta globulins that regularly accompanied the development of the disease in rabbits were in some way involved in its pathogenesis.

It may be recalled that Letterer and others (21, 22, 30) have proposed that amyloidosis, whether produced by ribonucleate or by other means, results from an abnormal antigen-antibody reaction. However, antibodies that are clearly related to the formation of amyloid have not been discovered. The ribonucleate used in the present experiments does not stimulate the production of specific antibodies detectable by precipitin or complement fixation tests (10). It is possible, of course, that the yeast ribonucleate may have stimulated the production of non-specific antibodies. Long ago Fleckseder reported that injections of yeast ribonucleate (of unspecified purity) into volunteers that had previously received typhoid vaccine raised the levels of

antibodies against typhoid antigens (31). However, if a similar anamnestic reaction occurred in the rabbits treated with ribonucleate, it must have been slight for there were no significant changes in the gamma globulin levels of these animals during the period of treatment. Moreover, amyloidosis is not a complication of clearly demonstrable anamnestic reactions of known origin in man or animals. For these reasons it seems unlikely that the amyloidosis in rabbits treated with ribonucleate is a consequence of an anamnestic reaction.

It should be borne in mind that it is not necessary to postulate an antigen-antibody reaction in order to account for what is known about amyloidosis. Amyloid may be a non-specific precipitate resulting from a reaction of circulating globulins with other substances—perhaps sulfur-containing glycosides (32). This hypothesis is based upon observations on serum globulins in amyloidosis and upon chemical analyses of amyloid (33–37). It accounts for the heterogeneity of the various types of amyloid quite as well as the immunologic hypothesis, but like the latter, it cannot account for the peculiar differences in the localization of amyloid without the introduction of further assumptions. In common, and on the basis of inconclusive evidence, both hypotheses postulate that circulating globulins participate directly in the formation of amyloid. The findings here presented provide reasons for further study of this possibility.

SUMMARY

A marked increase of the serum beta globulins was found in rabbits developing amyloidosis as a result of prolonged treatment with ribonucleate administered by subcutaneous injections. Following cessation of treatment the beta globulin levels gradually returned to normal while the gamma globulin levels rose strikingly, the changes being accompanied by a resorption of amyloid from the spleen, and probably also from the kidneys. Electrophoretic studies provided some evidence that the increase in beta globulins which accompanied the development of amyloidosis resulted from the production of a globulin not normally present in rabbit serum. A protein or protein derivative that moved as a beta globulin when subjected to filter paper electrophoresis was excreted in substantial quantities in the urine of several amyloidotic rabbits, along with much smaller quantities of substances moving as albumin, alpha and gamma globulins.

Considered as a whole, the findings indicate a causal relationship between the abnormal production of circulating beta globulins and the deposition of amyloid in rabbits treated with ribonucleate. Hence it appears that a beta globulin may be directly involved in the formation of amyloid under the conditions of the experiments here reported.

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