pressures recorded. The remainder of the circulation was left intact. Following injection of GTN into the pump outflow, essentially the main pulmonary artery, no rise above baseline pulmonary artery pressure was seen. In fact the pulmonary artery pressure, left atrial pressure and pressure fall across the lungs decreased. This indicates that the increased pulmonary artery pressure seen with the intact right ventricle is due to increased blood flow. At this time, after injection of the drug, the pulmonary vascular resistance is in fact decreased. The increased myocardial contractile force and tachycardia seen coincident with the increase in pulmonary artery pressure also are consistent with an associated increased output from the right ventricle.

Summary. The effect of GTN on the pulmonary vasculature was tested in animals with an intact circulation, with a mechanical pump substituted for the left ventricle and with the pump by-passing the right ventricle. The results of the experiments indicate that in anesthetized dogs the direct effect of GTN in the pulmonary circuit is to decrease pulmonary vascular resistance. The observed increase in pulmonary artery pressure in intact dogs following the injection of GTN is due to increased pulmonary blood flow.

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Contribution of Allelic Genes A_b^4 and A_b^5 To Formation of Rabbit 7S γ-Globulins.* (28264)

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The 7S γ-globulins of individual rabbits are antigenic for other rabbits and yield precipitating antibodies(1-3). These isoantibodies identify antigenic specificities on the γ-globulin molecules in the sera of some but not all rabbits. Such antigenic specificities are called allotypic specificities and distinguish among 7S γ-globulins thus far not separable by chemical criteria. The allotypic specificities A1, A2 and A3 appear to be controlled by 3 allelic genes at one locus, *a*; the allotypic specificities A4, A5 and A6 by 3 allelic genes at a second locus, *b*(4-9). These 2 loci, *a* and *b*, are not closely linked(9,10). Distinct molecules carrying different allotypic specificities have systematically been found together in sera, as though controlled by the same allele or by closely linked genes(4,6). In the case of such families of allotypic specificities, the family is designated by a number and the different members of the family by the same number with the superscript "prime" or "double prime," for example, Ab4, Ab4′, Ab4″(7). Agar gel tube analysis of sera from individual rabbits indicates that the non-allelic controlled Aa1 and Ab6 are found on the same molecule whereas the allelic controlled Aa1 and Aa3 are on different molecules(11).

This paper presents a study of the contribution of allelic genes to formation of 7S γ-globulin molecules using a method of quantitative analysis based on successive precipitations of specific allotype from I^{131}-labeled 7S γ-globulins with anti-allotype sera. The contributions of allelic genes A_b^4 and A_b^5 were evaluated by determining the quantity of γ-globulin with the Ab4 and Ab5 specificities among 7S γ-globulin molecules prepared from

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genetically defined rabbits. These rabbits were homozygous or heterozygous for allelic genes A_b^4 and A_b^5. The amount of γ-globulin I^{131} precipitable by antisera revealing specificities Ab5 and Ab5′ (by agar gel studies) was compared with antisera revealing only Ab5.

Preparation of immunological reagents. The γ-globulin preparations were made from the serum of each of 3 pedigreed rabbits with different genotypes. Rabbit ZX-24 was obtained by mating genotypes A_b^4/A_b^4 and A_b^4/A_b^4; rabbit 31FZ-2 by mating A_b^4/A_b^5 and A_b^4/A_b^5; rabbit 20FZ-2 by mating A_b^5/A_b^5 and A_b^4/A_b^4. The genotypes of the three rabbits are as follows: ZX-24 = A_b^4/A_b^4; 31FZ-2 = A_b^5/A_b^5; 20FZ-2 = A_b^4/A_b^5. Gamma-globulin preparations were made by precipitating the serum 3 times with decreasing concentrations of sodium sulfate(12), followed by passage through a column of DEAE-cellulose(13) in 0.0175 M phosphate buffer, pH 6.9. The proteins (10 mg/ml) migrated almost entirely as a single peak in the ultracentrifuge ($s_{20,w} = 6.2 \pm 0.1$ S). The γ-globulin preparations were tested by immunoelectrophoresis with sheep anti-rabbit serum and one precipitin arc was formed, characteristic of γ-globulin.

Gamma-globulin preparations were iodinated with I^{131} by the method of McFarlane (14). The amount of iodine coupled to protein was 0.8 to 1.1 atoms/mole, and the coupling efficiency was 44 to 60%. Subsequent to iodination the samples were dialyzed in the cold for 3 days against 3 portions of saline-borate buffer, pH 8.0, containing 0.02 M potassium iodide, and then for one day against saline-borate buffer, pH 8.0, ionic strength 0.16. In each case, more than 99% of the radioactivity in the dialyzed product was precipitated by trichloroacetic acid, added to a final concentration of 5%; 96.7% to 98.8% was precipitable by a sheep antiserum which was monospecific for rabbit γ-globulin as shown by analysis in agar gel tubes and by immunoelectrophoresis.

The anti-allotype sera were prepared by selecting donor and recipient rabbits of known genotypes at the *a* and *b* loci so that the recipient would lack only one allotypic specificity present in the donor rabbit. Rabbits were immunized with 7S γ-globulin incorporated in Freund's complete adjuvant. The antisera were characterized by agar gel studies (15). Two kinds of anti-Ab5 sera were used: 1) serum 38V3 which reacted with all sera from rabbits with the A_b^5 gene to give one precipitin line; and 2) serum TDU-21 which reacted with all of the same sera to give 2 precipitin lines in agar gel tubes or in immunoelectrophoresis(6). The 2 precipitin lines represent reactions of antibodies to specificities Ab5 and Ab5′.

Quantitative analysis of allotypes. Amounts of labeled allotype were estimated by precipitation with antiserum. To insure that all of the precipitable allotype of a given variety was being removed, repeated precipitations were carried out in which unlabeled carrier globulin of the allotype being precipitated, together with additional antibody, were added to the supernatant at each stage.

Radiolabeled allotypic γ-globulin and antiserum were mixed and allowed to stand for 1 to 2 days in the refrigerator. Reactions were carried out in saline-borate buffer of pH 8.0 and ionic strength 0.16 in a total volume of less than 1 ml. An amount of antibody in excess of equivalence, as determined by preliminary experiments, was used. After standing for the desired period of time, the mixture was centrifuged and the supernatant removed. The precipitate was washed twice with saline-borate buffer, pH 8.0, and twice with 0.16 M saline, and was dissolved in 0.3 to 0.5 ml of 0.02 M NaOH. Radioactivities of the dissolved precipitate and of the supernatant were determined in a well-type γ-scintillation spectrometer. The sodium iodide crystal of the latter was heavily shielded with lead; the background count was 75-90 cpm throughout these experiments. A minimum of 6000 counts was recorded for each sample. Measurements of radioactivity on the supernatant and precipitate were carried out in succession. Since the removal of supernatant from the precipitate was not quantitative, its total radioactivity was calculated by correcting for the volume loss. The volume of supernatant collected was determined from its weight, as measured with an analytical balance, and spe-

cific gravity. The latter was taken as that of whole rabbit serum, 1.02. Since the precipitate weighed less than a milligram its contribution to the total volume was negligible. After counting the dissolved precipitate, it was diluted to 1.0 ml and the absorbancy at 280 mμ was measured to obtain an estimate of the amount of precipitate that had formed. The extinction coefficient used was 1.5 optical density units per mg per ml.

It was considered desirable to maintain conditions as nearly constant as possible during the successive precipitations; a marked decrease in the percentage of radioactivity that was precipitated would then necessarily indicate approaching exhaustion of the allotype. This was accomplished by using only a portion of a supernatant (no more than one-third in most cases) in the succeeding precipitation step, together with unlabeled carrier γ-globulin. (In 2 cases a larger fraction of the supernatant was used because insufficient radioactivity remained. In each instance, the data for the preceding steps already indicated approaching exhaustion of the allotype being precipitated.)

Using a small portion of the supernatant in the following precipitation served 2 purposes. First, there was little difference in total volume in the successive precipitations. Secondly, the amount of antigen present at each stage was known with a considerable degree of accuracy, since it consisted largely of carrier globulin, with only a small contribution by the antigen brought over from the preceding step. Also, it was possible to estimate fairly accurately, after preliminary experiments, the relatively small amount of antigen being carried over into the next stage.

Another advantage of the method is that it was possible to exhaust a given allotype by successive precipitations, and then to perform additional precipitations with anti-allotype sera of a different specificity. Thus, for the Ab4-Ab5 heterozygote, the amount of labeled Ab4 γ-globulin present was determined by successive precipitations, and aliquots of the supernatant solution were then tested with anti-Ab5. In this way the amounts of 2 allotypes were estimated on a single sample. In the example cited above, the precipitating antiserum (anti-Ab4) contained γ-globulin of the Ab5 variety. Therefore, to precipitate with anti-Ab5 after removal of labeled Ab4, it was necessary to use a very small aliquot of the supernatant solution. One microliter of the antiserum was estimated to contain about 10 μg of Ab5 γ-globulin; thus 5 μl represented a substantial amount of antigen of the Ab5 variety for a precipitin test. Since it was necessary to use such a small fraction of the supernatant, a radioactive preparation with sufficiently high initial specific radioactivity was required.

The fraction of molecules in the original radioactive preparation having a given allotypic specificity was calculated from the values for the fraction of radioactivity precipitated in the successive stages. As an example of the method of calculation, assume that 60% of the radioactivity in the first mixture was found in the precipitate and 40%, corrected for volume loss, was in the supernatant; and that unlabeled carrier of the same allotype, together with additional antibody, was then added to a portion of the supernatant. This time, 30% of the radioactivity was found in the precipitate and 70% in the supernatant. Then the total percentage of the original material precipitated was $60\% + 30\% \times 40\% = 72\%$. Similar calculations apply to subsequent precipitations. Molecules with a given allotypic specificity were considered to be essentially exhausted if the increment in the amount precipitated, expressed as percentage of total number of molecules originally present, was less than 3%. It should be noted that conditions in the successive precipitations were very similar so that a sharp decrease in percentage of radioactivity precipitated was considered to be highly significant.

To determine the extent of non-specific occlusion in precipitates, an experiment was carried out in which unlabeled Ab4 γ-globulin was precipitated with excess antibody in the presence of labeled Ab5 γ-globulin; in a second experiment unlabeled Ab5 γ-globulin was precipitated with excess antibody in the presence of labeled Ab4 γ-globulin. Forty μg quantities of each antigen were used in each experiment and total volume was 0.5 or 0.6

TABLE I. Precipitation of γ-Globulin-I^{131} from a Rabbit of Genotype A_a^1/A_a^3, A_b^4/A_b^5, by Anti-Ab4 Followed by Anti-Ab5.†

Precipitating mixture	(a) 70 μg γ-glob.-I^{131} + .25 ml anti-Ab4	(b) .11 ml super. of (a) + 65 μg unlabeled γ-glob.* + .25 ml anti-Ab4	(c) .14 ml super. of (b) + 65 μg unlabeled γ-glob.* + .25 ml anti-Ab4	(d) .005 ml super. of (b) + .3 ml anti-Ab5	(e) .15 ml super. of (d) + 90 μg unlabeled γ-glob.* + .3 ml anti-Ab5	(f) Entire super. of (e) + 90 μg unlabeled γ-glob.* + .3 ml anti-Ab5
Total volume (ml)	.32	.43	.46	.31	.54	.89
μg precipitate formed‡	220	241	266	199	126	144
Counts/min of supernatant (corrected for volume loss)	288,500§	76,800	23,490	312	120	95
Counts/min of dissolved precipitate	379,800§	13,270	296	551	27	4
% of labeled protein precipitated	56.8	14.7	1.2	63.8	18.3	4.0
Cumulative % precipitated	56.8	63.2	63.7	86.7‖	89.1	89.5

* Rabbit 20FZ-2; Aa1-Aa3, Ab4-Ab5 double heterozygote.
† Anti-Ab4, Rabbit BZ-446-2; Aa2, Ab5 double homozygote.
Anti-Ab5, Rabbit TDU-21; Aa2, Ab4 double homozygote.
‡ Determined by measurement of optical density at 280 mμ of a solution of the precipitate in 1.0 ml .02 M NaOH. Extinction coefficient used, 1.50 optical density units per mg per ml.
§ To reduce coincidence error a small aliquot was counted and total radioactivity was calculated.
‖ The value, 86.7 = 63.2 + .638 (100-63.2).

ml. In the first experiment 0.9% of the radioactive antigen was co-precipitated and in the second, 1.6%.

The calculation of precipitable γ-globulin-I^{131} from the radioactivity in the precipitates requires a determination of the rates of labeling with I^{131} of γ-globulins with different allotypic specificities, *e.g.*, Ab4 and Ab5. The γ-globulins used for this experiment were from an Aa1, Ab4, double homozygote (ZX-24) and an Aa1, Ab5 double homozygote (35FZ-3). When trace-labeled and tested singly, 91% of the I^{131} label on the γ-globulin from ZX-24 was precipitable by anti-Ab4 and 74% of the I^{131} label on the γ-globulin of 35FZ-3 was precipitable by anti-Ab5. (In each experiment, the allotypic γ-globulin was exhausted by successive precipitations, with unlabeled carrier added at each stage.) When equal quantities of the two γ-globulins were mixed and then labeled, 42% of the I^{131} was precipitable by anti-Ab4 and 36% by anti-Ab5; these values agree closely with the values 45.5% (91/2) and 37% (74/2) calculated on the basis of equal rates of labeling. In view of the close chemical similarity of γ-globulins with different allotypic specificities, this result was not unexpected.

Experimental results. The methodology and results of a representative experiment are presented in Table I. Radiolabeled γ-globulin from a rabbit of genotype A_b^4/A_b^5 was precipitated successively with anti-Ab4, followed by anti-Ab5. An aliquot of the supernatant in step (b), Table I, was used for the successive precipitations with anti-Ab5. Table I was included to illustrate the method. These and other data similarly obtained are shown graphically in Fig. 1, and this figure will be used as a basis for the discussion. The figure presents the results of quantitative analysis for Ab4 and Ab5 in 7S γ-globulin preparations from an Ab4 homozygote, an Ab5 homozygote, and an Ab4-Ab5 heterozygote.

Three precipitations with anti-Ab4 removed 91% of γ-globulin-I^{131} from the Ab4 homozygote ZX-24. Three precipitations with anti-Ab5 removed 82% of the γ-globulin-I^{131} from the Ab5 homozygote 31FZ-2.

In the Ab4-Ab5 heterozygote, 20FZ-2, 64% of the γ-globulin-I^{131} was removed by anti-Ab4 (solid line), 26% of the γ-globulin-I^{131} originally present was then precipitable

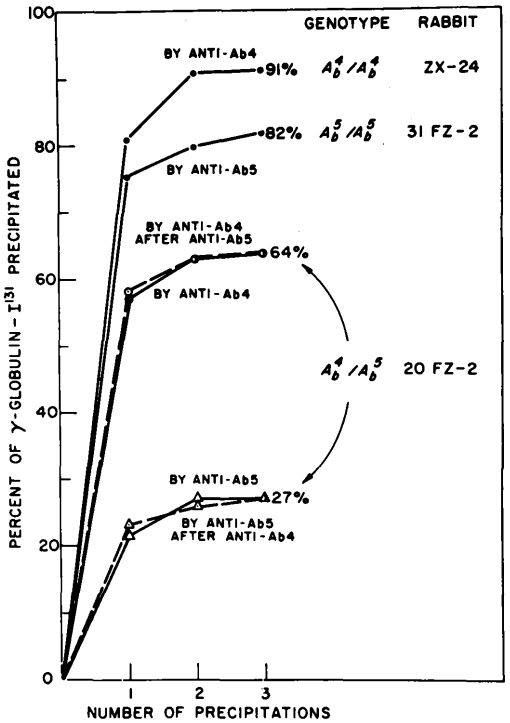

FIG. 1. Quantitative analysis of γ-globulin allotypes based on successive precipitations of specific allotype from I^{131}-labeled 7S γ-globulins with anti-allotype sera.

from the supernatant by anti-Ab5 (broken line). Alternatively, initial precipitation by anti-Ab5 removed 27% of the γ-globulin-I^{131} (solid line); 64% of the total γ-globulin-I^{131} was then precipitable from the supernatant by anti-Ab4 (broken line). This very close agreement is probably somewhat fortuitous since co-precipitation should give a slightly higher result when a given allotype is precipitated first, as compared to its removal subsequent to the other allotypic globulin.

These results in Fig. 1 show that: 1) in the 2 homozygotes or the heterozygote, 80-90% of the γ-globulin molecules have Ab4 or Ab5; 10-20% of the γ-globulin molecules have neither Ab4 nor Ab5 by the criterion of precipitation used; 2) in the heterozygote, the quantities of molecules precipitated by anti-Ab4 or anti-Ab5 are independent of the order of precipitation, which indicates that (within the experimental error) Ab4 and Ab5 are not found on the same γ-globulin molecules but only on separate molecules.

In other experiments, the results obtained with the 2 antisera presented in Fig. 1 were checked by using antisera prepared in other rabbits. Thus another anti-Ab5 (38V3) precipitated 80% of the γ-globulin-I^{131} from the Ab5 homozygote (31FZ-2). Similarly, another anti-Ab4 (12LF-3) removed 92% of the γ-globulin-I^{131} from the Ab4 homozygote (ZX-24).

Additional analyses were also made of the γ-globulin preparations from other rabbits. Thus, 90% of the γ-globulin-I^{131} was precipitated from another Ab4 homozygote (8651); 90% from another Ab5 homozygote (13LF-5). Finally, 24% and 66% of the γ-globulin-I^{131} from another Ab4-Ab5 heterozygote were precipitated by anti-Ab5 and anti-Ab4, respectively, in close agreement with the results shown for the heterozygote (20FZ-2) in Fig. 1.

Discussion. Although agar gel studies suggested that the allotypic specificities were represented on the bulk of the γ-globulin molecules, the absolute quantities of γ-globulin bearing a given specificity had not yet been determined. This paper describes a method of quantitative analysis based on successive precipitations of specific allotype from I^{131}-labeled 7S γ-globulin with anti-allotype sera. Thus, at the *b* locus (Fig. 1), 80-90% of the γ-globulin-I^{131} have Ab4 or Ab5; 10-20% of the γ-globulin-I^{131} is not precipitable by anti-Ab4 or anti-Ab5. The shapes of the curves in Fig. 1 indicate that the results obtained are very close to the maximum precipitable. The 10-20% of γ-globulin-I^{131} not precipitable by anti-Ab4 or anti-Ab5 probably has allotypic specificities defined at one or more different genetic loci. Thus far, the quantitative analysis of allotypes was obtained for the γ-globulin preparations of only a few normal rabbits. For example, three Ab5 homozygous rabbits were tested and values of 74% (35FZ-3), 80-82% (31FZ-2), and 90% (13LF-5) were obtained for the amount of γ-globulin-I^{131} precipitable. This variation in 3 rabbit γ-globulin preparations indicates that further studies are needed to evaluate the extent of and factors influencing such variation(4).

On the basis of agar gel tube analysis, Oudin has shown that the allelic controlled Aa1 and Aa3 were not found on the same molecule (11). The data presented in Fig. 1 establish the same conclusion for the *b* locus and also set the limit of overlap quantitatively at less than 5%. A quantitative limit is not readily determined by agar gel methods.

Since 80-90% of the γ-globulin has Ab4 or Ab5 specificity in the Ab4 or Ab5 homozygote, the allotypic specificity should serve as an efficient marker for cell transfer studies (16).

It is of interest that 38V3, an anti-Ab5 giving one precipitin band, precipitates virtually the same quantity of γ-globulin-I^{131} as TDU-21 (80% *vs* 82%) from the same γ-globulin preparation. Thus the antiserum exhibiting one band is capable of precipitating as much γ-globulin as the antiserum exhibiting 2 bands. The simplest explanation for this result is that both antisera, TDU-21 and 38V3, have anti-Ab5 and anti-Ab5′ but in different concentrations and that Ab5 molecules with and without Ab5′ exist in rabbits with the A_b^5 allele. That these are "cross reacting" systems is also indicated by agar gel studies(4,6,10).

In the Ab4-Ab5 heterozygote (Fig. 1), 64% of the γ-globulin-I^{131} was precipitable by anti-Ab4, 27% by anti-Ab5. If Ab4 and Ab5 molecules were synthesized in different cells, the greater number of Ab4 molecules might be accounted for by a greater number of Ab4-synthesizing cells. However, present evidence, based on studies with fluorescent antibodies(17), indicates that the two types of molecules are made in the same cells. This would suggest that the A_b^4 gene is more efficient than the A_b^5 gene.

Of considerable interest for studies of γ-globulin synthesis is the question as to why allelic genes do not contribute to the same molecule(11) although both alleles appear to be operating in the synthesis of γ-globulin within the same cell(17). A similar question applies to hemoglobin synthesis(18). The half-molecules of hemoglobin consist of dimers of polypeptide chains produced by a single gene; mixed dimers are not observed even when allelic polypeptide chains are being produced in the same cell(18). However, it is of interest to note that the findings for human blood groups differ; A and B specificities determined by allelic genes do appear on the same mucopolysaccharide molecules(19).

Porter(20) has proposed a 4-chain structure for γ-globulin, consisting of 2 "A chains" each of molecular weight 55,000, and 2 "B chains" of molecular weight 20,000. These correspond, respectively, to the "H and L chains" of Edelman and collaborators(21,22). Edelman and Gally(23) have shown that the L chains readily form dimers, and Porter's work indicates that the A chains tend to associate with one another. We suggest the following explanation for the failure of allelic genes to contribute to the same molecule; namely, that polypeptide chains carrying the allotypic specificity Ab4 form pairs immediately after formation; and that the Ab4 pairs do not combine with similarly formed Ab5 pairs but do combine with pairs of polypeptide chains produced by genes at other loci. This interaction of polypeptide pairs to form the γ-globulin molecules presumably takes place in another location within the cell and is not the result of direct gene action. This proposal is directly analogous to that of Singer and Itano(24) for hemoglobin biosynthesis.

To continue the analogy with hemoglobin, one might expect that the dimers would remain intact after interaction. Indeed, there is evidence that Porter's A chains are linked in the γ-globulin molecule; fragment III appears to consist of parts of both A chains linked by disulfide and noncovalent bonds (20). However, there is no evidence for association of B (or L) chains in the native molecule; in fact, the ease with which one can separate univalent fragments, each containing a B (or L) chain, by treatment with papain or pepsin and a reducing agent could be interpreted as evidence that the B chains are not associated. Nevertheless, this evidence does not exclude the possibility that Porter's 2-dimensional model is folded so that the 2 B chains are contiguous with each other as well as with the A chains, and that the

enzymatic digestion causes their separation. This modification of Porter's model is consistent with chemical and genetic studies of γ-globulin. Obviously, additional experiments are necessary to test the ideas presented here in discussion.

It will be of considerable interest to relate the allotypic specificities to the polypeptide chains of γ-globulin. Finally, quantitative studies of the amounts of γ-globulin carrying allotypic specificities defined at other genetic loci are under investigation(25).

Summary. 1. A method for quantitative analysis of allotypes is presented, based on successive precipitations of specific allotype from I^{131}-labeled 7S γ-globulin with anti-allotype sera. 2. In the homozygotes or heterozygotes, 80-90% of the γ-globulin molecules have Ab4 or Ab5; 10-20% of the γ-globulin molecules have neither Ab4 nor Ab5. 3. An anti-Ab5 serum giving 2 precipitin bands with Ab5 sera was compared with an anti-Ab5 serum giving one precipitin band and was found to precipitate virtually the same quantity of γ-globulin-I^{131} (82% *vs* 80%) from the same γ-globulin preparation. This indicates that the 2 precipitin bands represent cross-reacting antigen-antibody systems. 4. In the heterozygote, 64% of the molecules have Ab4, 27% have Ab5. These quantities are independent of the order of precipitation, indicating that Ab4 and Ab5 are not found on the same γ-globuin molecules but only on separate molecules. The limit of overlap is less than 5%. 5. The question as to why allelic genes do not contribute to the same molecule, although both alleles appear to be operating in the synthesis of γ-globulin within the same cell, is discussed. It is suggested that polypeptide chains carrying the allotypic specificity Ab4 form pairs immediately after synthesis; and that the Ab4 pairs do not combine with similarly formed Ab5 pairs.

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