Contribution of Allelic Genes to the Formation of Individual α_2-Macroglobulin Molecules*

Katherine L. Knight and Sheldon Dray

ABSTRACT: The contribution of allelic genes to the formation of individual α_2-macroglobulin molecules was examined by precipitation of α_2-macroglobulin from rabbits of known genotypes with antiallotype antisera. The α_2-macroglobulin was isolated from z^1z^1 and z^2z^2 homozygous rabbits and z^1z^2 heterozygous rabbits. The α_2-macroglobulin was iodinated with ^{125}I and subsequently precipitated either with goat anti-α_2-macroglobulin or with the antiallotype antisera, anti-Mtz-1 and anti-Mtz-2. Nearly all the α_2-macroglobulin molecules have the allotypic specificity of the *Mtz* locus. Approximately 60–80% of the α_2-macroglobulin molecules in a z^1z^2 heterozygous rabbit have both allotypic specificities in the same molecule indicating that the allelic genes Mtz^1 and Mtz^2 are contributing to the formation of the same molecule.

Rabbits immunized with serum proteins from other rabbits produce isoprecipitins which react with the sera of some but not all normal rabbit sera. These genetic variants of serum proteins identified by their antigenic properties with isoantibodies are designated as allotypes. The allotypic rabbit proteins thus far identified and characterized are the γG-, γA-, and γM-immunoglobulins[1] (Oudin, 1960; Dray *et al.*, 1963a; Hamers *et al.*, 1966; Dubiski and Muller, 1967; Kelus and Gell, 1967; Conway *et al.*, 1968), low-density lipoproteins (Albers and Dray, 1968), and α_2-macroglobulin (Knight and Dray, 1968). The most extensive studies have been done with the γG-immunoglobulin allotypes which are controlled by two series of allelic genes at two unlinked genetic loci (Dray *et al.*, 1963a). The allotypic specificities for the immunoglobulins serve as a genetic marker for the protein and reflect differences in the amino acid sequence (Small *et al.*, 1965, 1966; Reisfeld *et al.*, 1965). The antiallotype antisera have been used to study the structure and biosynthesis of γG-immunoglobulin molecules (Dray and Nisonoff, 1963; Lark *et al.*, 1965; Harris *et al.*, 1963; Adler *et al.*, 1966; Pernis *et al.*, 1965; Cebra *et al.*, 1966). Precipitation of radioiodinated γG allotypes has shown that only 70–90% of the γG molecules carry the allotypic specificities of the heavy- and light-chain loci,[2] even in animals heterozygous at these loci (Dray and Nisonoff, 1963; Dray *et al.*, 1963b). In a b^4b^5 heterozygous rabbit, only one allotypic specificity is present in each γG molecule. That is, the allelic genes b^4 and b^5 do not contribute polypeptide chains to the formation of the same molecule. Fluorescent antibody techniques have revealed that in a b^4b^5 heterozygote, and also in an a^1a^2 heterozygote, only one allele appears to be operating in the synthesis of γ-globulin within a single cell (Pernis *et al.*, 1965; Cebra *et al.*, 1966), a situation which has been termed "allelic exclusion." A somewhat different situation occurs with the hemoglobin molecule. Allelic exclusion does not occur since both alleles are expressed in the same cell. Nevertheless, in the heterozygous sickle cell trait, both β-polypeptide chains in an individual molecule are either β^A or β^S (Baglioni and Colombo, 1964).

The experiments reported here were designed to determine if two alleles can contribute to the synthesis of a single α_2-macroglobulin molecule. Two allotypic specificities for the α_2-macroglobulin have been identified and have been shown to be controlled by allelic genes at an autosomal locus designated *Mtz* (Knight and Dray, 1968). Little is known about the structure, function, or synthesis of α_2M. The distribution of the two allotypic specificities in molecules of α_2M from a heterozygous animal was determined by the method described previously (Dray and Nisonoff, 1963) for successive precipitation of specific allotypes of ^{131}I-labeled γG-immunoglobulin with antiallotype antisera. Preparations of ^{125}I-labeled α_2M from homozygous z1,[3] homozygous z2, and heterozygous z1z2 rabbits were precipitated with the antiallotype antisera, anti-Mtz-1 and anti-Mtz-2. This paper presents evidence that the products of the two allelic genes for α_2M can occur in the same α_2M molecule. Also, it appears that all α_2M molecules have the allotypic specificity of the *Mtz* locus.

Methods

Isolation of α_2M. The α_2M was isolated from the sera of individual rabbits of the following genotypes: z^1z^1,

* From the Department of Microbiology, University of Illinois at the Medical Center, Chicago, Illinois 60612. *Received June 3, 1968.*

[1] The nomenclature for the immunoglobulins is that suggested in *Bull. World Health Org. 30*, 447 (1964).

[2] Notation for γG-immunoglobulin allotypes is as described by Dray *et al.* (1962). The light-chain alleles are abbreviated to b^4, b^5, and b^6 and the heavy-chain alleles are abbreviated to a^1, a^2, and a^3.

[3] The *Mtz* locus for α_2-macroglobulin (α_2M) is abbreviated to *z*.

z^2z^2, and z^1z^2. The first step was to remove the low-density lipoproteins by ultracentrifugation as described previously (Knight and Dray, 1968). After centrifugation in the 65K rotor for 20 hr at 40,000 rpm, the upper 1–2 ml containing the low-density lipoprotein was discarded. The remaining 10–11 ml in each tube was combined and dialyzed overnight against 0.02 M phosphate buffer (pH 6.0). Approximately 125 ml of the serum, free of low-density lipoprotein, was then placed on a DEAE-cellulose (Whatman DE23) column (2.5 × 30 cm) in 0.02 M phosphate buffer (pH 6.0). The α_2M was eluted from the column by the use of a salt gradient prepared in a varigrad (Buchler Co.). The gradient was prepared in nine chambers of the varigrad starting with 120 ml of 0.02 M phosphate buffer (pH 5.0) and ending with 0.1 M phosphate–0.15 M NaCl (pH 5.0). The eluate from the column was monitored at 280 mμ. Samples collected from the column were concentrated by ultrafiltration and examined by immunoelectrophoresis for the presence of α_1M and α_2M with a goat antiserum to rabbit α-macroglobulin. Samples containing α_2M and no α_1M were passed through a column of Sephadex G-200 in borate–saline buffer (pH 8.0). Purified α_2M was obtained in the first protein fraction from these Sephadex columns.

Iodination of α_2M. To minimize loss of antigenic activity due to aging, the preparations of α_2M were iodinated immediately following the purification steps. The purified α_2M was iodinated with ^{125}I by the method of McFarlane (1958). Assuming 900,000 as the molecular weight of α_2M (Schonenberger *et al.*, 1958), 40 moles of iodine/mole of α_2M was used and the coupling efficiency was approximately 20–40%. The use of less than 20 moles of iodine/mole α_2M resulted in a coupling efficiency of less than 1%. Following iodination, the α_2M was dialyzed overnight against borate saline buffer (0.14 M NaCl–0.23 M borate) (pH 8.0). The samples were then placed on a Dowex 1-X2 column in 0.1 M sodium acetate buffer (pH 4.8) to remove the unbound radioactivity. The eluate was dialyzed overnight against the borate–saline buffer (pH 8.0). Following precipitation in 5% trichloroacetic acid, more than 98.5% of the radioactivity was found in the precipitate.

Antisera. The two rabbit antiallotype antisera, anti-Mtz-1 and anti-Mtz-2, were those described previously (Knight and Dray, 1968). Each antiserum resulted in a single precipitin arc on immunoelectrophoresis and on double-diffusion plates. The anti-α-macroglobulin antiserum had been prepared by injecting a goat with macroglobulins isolated from disc electrophoresis (Knight and Dray, 1968). The goat antiserum revealed two precipitin arcs on immunoelectrophoresis, one due to antibody to α_2M and the other due to antibody to α_1M. The antiserum was absorbed with purified α_1M to obtain an antiserum which gave only the α_2M precipitin arc on immunoelectrophoresis with whole serum. This absorbed antiserum revealed only one band by Ouchterlony analysis.

Radioprecipitation of the Allotypes. To minimize the possible loss of antigenic activity due to radiation damage, the radioprecipitations were begun within 1 day after the iodination procedure was completed. The iodinated α_2M samples were precipitated with the goat anti-α_2M and with each antiallotype antiserum, anti-z1 and anti-z2, in a manner similar to that described for the immunoglobulins (Dray and Nisonoff, 1963). After the first precipitation, additional antiserum and also a known amount of unlabeled carrier α_2M of the appropriate allotype were added to the supernatant of the previous precipitation. Each sample was precipitated in this manner at least two or three times to ensure that all the precipitable allotype was precipitated. When less than 4% of the original total label was precipitated, the analysis was considered complete.

Approximately 50 μg of α_2M, assuming the extinction coefficient $E^{1\%}_{1\,cm}$ 9 (Dunn and Spiro, 1967), was mixed with the appropriate antiserum, incubated at 37° for 1 hr, and finally kept at 4° overnight. The amount of antibody used was in excess of the equivalence as determined by preliminary experiments. The reactions were done in the borate–saline buffer (pH 8.0) in a volume of 0.2–0.5 ml.

Following the precipitation, the tubes were centrifuged and a portion of the supernatant fluid was removed and carefully weighed. The precipitate was washed three times with cold borate–saline buffer (pH 8.0) and twice with cold 0.85% NaCl. The precipitate was then dissolved in approximately 0.05 M NaOH. The radioactivity of the dissolved precipitate and the supernatant fluid was measured in a well-type γ scintillation counter. A minimum of 20,000 counts was recorded for each sample. The radioactivity of the total supernatant fluid was calculated from the radioactivity of the weighed portion of supernatant fluid using specific gravity = 1.02 and assuming the contribution of the precipitate to the volume was negligible. Successive precipitations were performed on the portion of the supernatant fluid which was removed and weighed from the previous precipitation. A known amount of unlabeled α_2M and an appropriate amount of additional antisera (excess of equivalence) were added for each successive precipitation. The total volume and the amount of antigen and antibody present were kept approximately the same for each successive precipitation.

The per cent of total radioactivity precipitated in each precipitation was determined. For example, if 90% of the radioactivity was found in the precipitate of the first reaction, then 10% was in the total supernatant fluid. If the second precipitation revealed 60% of the radioactivity in the precipitate and 40% in the supernatant fluid, then the total amount of radioactivity precipitated was $90\% + (60 \times 10\%) = 96\%$.

Following complete precipitation of α_2M from a heterozygous animal with one antiallotype antisera, the other antiallotype antisera could be added to the supernatant fluid of the previous precipitation to precipitate any remaining α_2M which did not have the allotypic specificity that the first antibody was directed against. Duplicate samples of all reactions were done simultaneously.

Several control experiments for the radioprecipitations were carried out as follows. Labeled α_2M-z1 was precipitated by anti-z2 and labeled α_2M-z2 was pre-

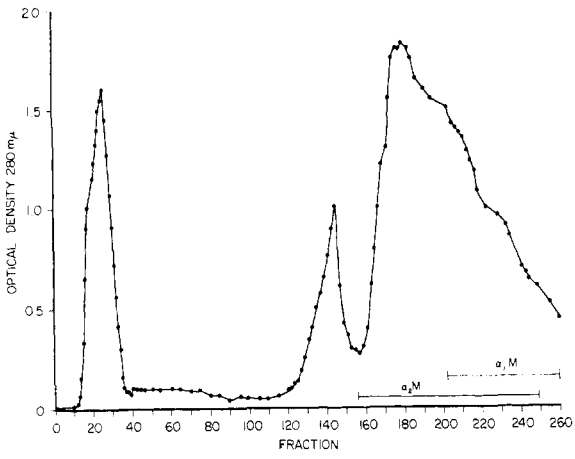

FIGURE 1: Elution of serum, free of lipoprotein, on DEAE-cellulose uing a salt gradient from 0.02 M phosphate buffer (pH 5.0) to 0.1 M phosphate–0.15 M NaCl (pH 5.0).

cipitated by anti-z1 to show that cross-reactivity was negligible. The amount of antigen and antibody used in the experiments was similar to that used for the precipitation of α_2M-z1 with anti-z1 or α_2M-z2 with anti-z2. To determine the amount of nonspecific coprecipitation of the α_2M, unlabeled α_2M-z1 was precipitated with an excess of anti-z1 in the presence of labeled α_2M-z2; also, unlabeled α_2M-z2 was precipitated with an excess of anti-z2 in the presence of labeled α_2M-z1. Approximately 20–40 μg of both antigens was used and the total volume was 0.3–0.6 ml. Finally, an artificial mixture of α_2M-z1 and α_2M-z2 was labeled and separate portions were precipitated with anti-z1 and anti-z2 to determine if the iodination procedure might cause aggregation of z1 and z2 molecules. The α_2M-z1, isolated from rabbit D26-3 and α_2M-z2, isolated from rabbit D119-5, were mixed in a ratio of *ca.* 2:3 and iodinated with ^{125}I. The radioprecipitations with anti-zl, anti-z2, and goat anti-α_2M were carried out as described above.

Analytical Methods. Immunoelectrophoresis was performed in 1% Agarose in 0.05 M sodium barbital buffer (pH 8.6) (Grabar and Williams, 1955). Sedimentation velocity was determined in the Spinco Model E ultracentrifuge using the An-D rotor with 12-mm double-

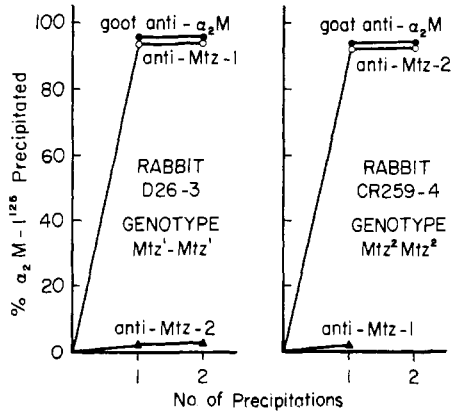

FIGURE 2: Precipitation of [^{125}I]α_2M from homozygous z^1z^1 and homozygous z^2z^2 rabbits with goat anti-α_2M and with the antiallotype antisera, anti-Mtz-1 and anti-Mtz-2.

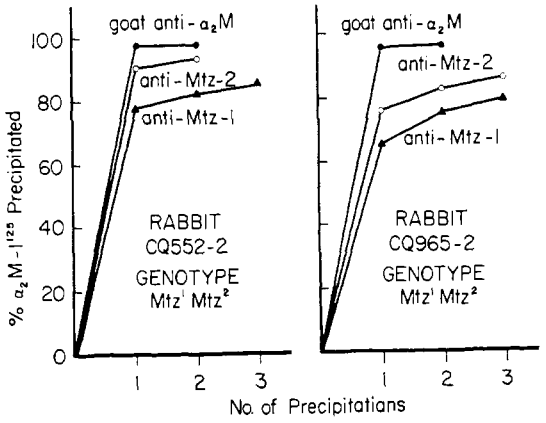

FIGURE 3: Precipitation of [^{125}I]α_2M from two heterozygous z^1z^2 rabbits with goat anti-α_2M and with the antiallotype antisera, anti-Mtz-1 and anti-Mtz-2.

sector cells. Centrifugation was performed at 4° in borate–saline buffer (0.14 M NaCl–0.23 M borate) (pH 8.0) at 52,000 rpm.

Results

Isolation of Macroglobulins. A representative elution pattern of protein from a DEAE-cellulose column is shown in Figure 1. By immunoelectrophoresis, fractions 156–192 were found to contain many serum proteins, including α_2M; however, no α_1M was detected. For further purification of α_2M, fractions 156–192 were pooled and placed on Sephadex G-200 columns for gel filtration. Separation into three protein fractions was obtained and the first of these fractions eluted from the Sephadex contained α_2M. Immunoelectrophoresis of this first fraction revealed only a single precipitin arc when tested against a potent sheep antiserum to whole rabbit serum. Ultracentrifugation revealed a single symmetrical peak with a sedimentation coefficient of 19 S, similar to the preparations described previously (Knight and Dray, 1968). This purified α_2M was used for the iodination experiments.

Allotype Precipitations from Homozygous Rabbits. The precipitation of [^{125}I]α_2M prepared from rabbits homozygous for each allotype z^1z^1 and z^2z^2 is graphically shown in Figure 2. The anti-z1 precipitated 95% of the [^{125}I]α_2M from rabbit D26-3, genotype z^1z^1; the anti-z2 precipitated 93% of the [^{125}I]α_2M from rabbit CR259-4, genotype z^2z^2. The goat anti-α_2M precipitated 95% and 93% of the radioactivity, respectively. Similar results were obtained with α_2M from two other homozygous rabbits, D22-3, z^1z^1, and D119-5, z^2z^2. Nearly all of the precipitable α_2M was removed from the reaction mixture with the first precipitation. The second precipitation removed less than 4% of the radioactivity, thus eliminating the need for more than two precipitations. The anti-z1 precipitated less than 3% of the [^{125}I]α_2M having only the z2 specificity, and likewise anti-z2 precipitated less than 3% of the [^{125}I]α_2M having only the z1 specificity. These results show that highly purified preparations of α_2M have been obtained and that cross-reactivity is negligible. Also, apparently all the α_2M

TABLE I: Precipitation of [^{125}I]α_2M from a z^1z^2 Rabbit (CQ965-2) with Goat Anti-α_2M and Anti-z1 Followed by Anti-z2

Reaction Mixture	Total Vol	cpm of Total Supernatant Cor for Vol Loss	cpm of Ppt	% Radioactivity Pptd	Cumulative % Radioactivity Pptd
Antiallotype					
(1) 25 μg of [^{125}I]α_2M + 0.1 ml of anti-zl (D129-2)	0.20	808,400	1,428,571	63.9 65.5[a]	64 66[a]
(2) 0.11 ml of supernatant of 1 + 25 μg of α_2M (unlabeled) + 0.1 ml of anti-z1	0.23	287,485	138,699	11.7 10.8[a]	76 76[a]
(3) 0.13 ml of supernatant of 2 + 50 μg of α_2M (unlabeled) + 0.1 ml of anti-z1	0.28	141,068	28,077	4.0 4.1[a]	80 80[a]
(4) 0.16 ml of supernatant of 3 + 25 μg of α_2M (unlabeled) + 0.1 ml of anti-z2 (E292-4)	0.28	10,479	59,303	17.3 14.3[a]	97 95[a]
Goat anti-α_2M					
(a) 25 μg of [^{125}I]α_2M + 0.1 ml of goat anti-α_2M	0.20	78,411	2,045,454	96.3 96.2	93 93[a]
(b) 0.14 ml of supernatant of a + 25 μg of α_2M (unlabeled) + 0.1 ml of goat anti-α_2M	0.25	42,788	10,504	0.7[a] 0.8[a]	97 97[a]

[a] Duplicate values obtained in separate experiments.

molecules in these preparations have the allotypic specificity of the *Mtz* locus, z1 and z2, depending on the genotype of the animal.

Allotype Precipitations from Heterozygous Rabbits. The precipitation of [^{125}I]α_2M from two heterozygous animals is graphically shown in Figure 3. The method for precipitation of the α_2M obtained from a heterozygous animal is similar to that used for α_2M from a homozygous animal, except that the supernatant fluid following exhaustive precipitation with one antiserum may then be precipitated with the other antiserum. Table I illustrates the method used for precipitation of the allotypes, using as a typical experiment the precipitation of [^{125}I]α_2M from rabbit CQ965-2 with goat anti-α_2M and with antiallotype antisera. The first precipitation with anti-z1 resulted in 63.9% of the total radioactivity precipitated. The second precipitation, using unlabeled α_2M from rabbit CQ965-2 with a portion of the supernatant fluid from the first precipitation and additional anti-z1, resulted in an additional 11.7% total radioactivity precipitated. A third precipitation with anti-z1 precipitated 4% of the radioactivity. Following this third precipitation, the addition of anti-z2 to the supernatant fluid of the third precipitation resulted in the remaining 17% of the radioactivity precipitated. Duplicate samples were run on all precipitations and the values for these determinations are indicated in Table I. The close agreement of the duplicate values can be noted. Precipitation with goat anti-α_2M resulted in 96.3% of the total radioactivity precipitated. A second precipitation, using labeled α_2M from rabbit CQ965-2 with a portion of the supernatant fluid from the first precipitation and additional goat anti-α_2M, resulted in 0.7% of the total radioactivity precipitated.

Figure 3 reveals that neither one of the antiallotype antisera precipitates all of the α_2M from a heterozygous animal. However, each antiserum precipitates at least 80% of the radioactivity. This indicates that many of the α_2M molecules contain both allotypic specificities. For example, in the experiment shown in Table I, three precipitations with anti-z1 precipitated 80% of the radioactivity; subsequently, anti-z2 precipitated the remaining radioactivity, 14–17%. The reverse experiment, in which the α_2M was precipitated first with anti-z2, revealed that 87% of the radioactivity was removed by anti-z2. An additional 4% was precipitated by anti-z1. These results suggest that approximately 70% of the α_2M molecules have both allotypic specificities, that 15–20% of the molecules have only z2, and that 0–10% have only z1. The experiments with α_2M obtained from homozygous animals revealed that anti-z2 did not precipitate α_2M-z1, and that anti-z1 did not precipitate α_2M-z2 (Figure 2). Also, in other experiments, less than 4% of radioactive α_2M-z2 coprecipitated with the precipitation of unlabeled α_2M-z1 by anti-z1. However, in two experiments, anti-z2 did coprecipitate 7.0 and 9.5% of the labeled α_2M-z1.

The possibility that both allotypic specificities might occur in the same molecule of the heterozygote as a result of aggregation during isolation or iodination of molecules carrying only z1 with those carrying only z2 was considered. Ultracentrifugation of the iodinated

preparation of α_2M from rabbit CQ965-2, genotype z^1z^2, revealed only a single peak. The sedimentation coefficient of this peak was approximately 19 S, identical with that for unlabeled α_2M (Knight and Dray, 1968). No faster sedimenting peak was observed. Also, an artificial mixture of α_2M-z1 and α_2M-z2 was prepared, and iodinated and individual portions subsequently were precipitated by anti-z1 and anti-z2; the sum of the radioactivity precipitated by anti-z1 plus anti-z2 was 103%. Goat anti-α_2M precipitated 95% of the total radioactivity. The 8% excess of radioactivity precipitated by the two antiallotype antisera can be attributed to the observation that anti-z2 precipitates 7–10% of α_2M-z1 molecules in the presence of α_2M-z2 molecules. Therefore, the number of α_2M-z1 plus α_2M-z2 molecules is essentially equal to the total α_2M molecules.

Following exhaustive precipitation of the z^1z^2 heterozygote's α_2M with either anti-z1 or anti-z2, the supernatant fluid was examined for the presence of soluble complexes containing [^{125}I]α_2M by precipitation with γ-globulin antiallotype antisera. The anti-z1 and anti-z2 were prepared in rabbits with γ-globulin genotype b^4b^4, and the reagent used to precipitate any soluble complexes was anti-b4. Less than 1% of the radioactivity was precipitated by anti-b4 following exhaustive precipitation with either anti-z1 or anti-z2, showing that there were no soluble complexes containing [^{125}I]α_2M present in the supernatant fluid.

Discussion

Antiallotype antisera have been used to precipitate labeled α_2M isolated from rabbits of known genotypes to determine the contribution of allelic genes in the formation of α_2M molecules. More than 93% of the [^{125}I]α_2M isolated from animals homozygous for z1 and z2 was precipitated by the corresponding antiallotype antiserum. Assuming that the amount of radioactivity precipitated by goat anti-α_2M (93–95%) indicates the total amount of α_2M present, the antiallotype antisera precipitated 98–99% of the [^{125}I]α_2M, revealing that essentially all α_2M molecules have the allotypic specificity of the *Mtz* locus. This is unlike the immunoglobulin molecules in which only 70–90% of the molecules carry the allotypic specificity of the light-chain *b* locus.

In z^1z^2 heterozygotes, approximately 60–80% of the molecules have both z1 and z2 since the sum of the radioactivity precipitated from separate portions by anti-*z1* and anti-*z2* equals approximately 160–190%. To ensure that the remaining 20–40% of the molecules were not *z1–z2* hybrids remaining in the supernatant fluid as soluble [^{125}I]α_2M–anti-α_2M complexes following exhaustive precipitation with anti-z1 or anti-z2, the antiallotype antibody, anti-z1 or anti-z2 (each possessing the b4 allotypic specificity), was precipitated with an anti-γG allotype antibody, anti-b4. The failure of this anti-γG antibody to precipitate any additional radioactivity indicates that these 20–40% of the α_2M molecules were not *z1–z2* hybrids. That these 20–40% of α_2M molecules all have either the z1 or z2 allotypic specificity was indicated by experiments which showed that following exhaustive precipitation with anti-z1 or anti-z2, subsequent precipitation with anti-z2 or anti-z1 precipitated the remainder of the radioactivity.

The possibility that the z1 and z2 specificities might occur in the same molecule, following iodination, as a result of aggregation of molecules having only one specificity with molecules having the other specificity was examined. Ultracentrifugation of an iodinated α_2M-z1z2 revealed only a 19S peak, characteristic of α_2M. If aggregation had occurred, a faster sedimenting peak should have been observed. Also, a mixture of α_2M-z1 and α_2M-z2 was iodinated and separate portions were precipitated with anti-z1, anti-z2, and goat anti-α_2M. The total radioactivity precipitated by anti-z1 plus that precipitated by anti-z2 was essentially equal to that precipitated by goat anti-α_2M. If significant aggregation of the α_2M-z1 and α_2M-z2 molecules had occurred during iodination, the total radioactivity precipitated by the two antiallotype antisera would have been much greater than 100%. Therefore, the results showing that 60–80% of the α_2M molecules from a heterozygous rabbit have both allotypic specificities cannot be due to aggregation during the iodination procedure. However, we cannot exclude the unlikely possibility that during the isolation procedure, molecules carrying only one specificity might dissociate and the subunits reassociate with subunits of the other specificity.

The anti-z1 precipitated only α_2M-z1 molecules; α_2M-z2 was not precipitated either in the absence or presence of carrier α_2M-z1. On the other hand, although the anti-z2 did not precipitate α_2M-z1 in the absence of carrier α_2M-z2 molecules, 7–10% labeled α_2M-z1 was coprecipitated in the presence of unlabeled α_2M-z2. The explanation for the coprecipitation of 7–10% is not known, but one possibility is that the anti-z2 antiserum contained an antibody to an unknown allotypic specificity associated with α_2M molecules. The 7–10% coprecipitation is consistent with the observation that in a z^1z^2 heterozygote, the anti-z2 precipitates 7–10% more radioactivity than anti-z1. It seems reasonable to correct the observed data by subtracting 7–10% from the total radioactivity precipitated by anti-z2 with α_2M-z1z2. Then, the anti-z1 and anti-z2 would each be considered to precipitate 80–90% of the total radioactivity showing that 60–80% of the molecules carry both specificities and that 10–20% carry only the z1 specificity and the remaining 10–20% carry only the z2 specificity.

The isolation of α_2M resulted in highly purified preparations as indicated by precipitation of approximately 95% of the radioactivity of [^{125}I]α_2M by unispecific goat anti-α_2M. Difficulty in obtaining α_2M uncontaminated by α_1M resulted in large losses (approximately 50%) of α_2M during the isolation procedure. Thus, the distribution of allotypes observed among our isolated α_2M molecules may not necessarily reflect the distribution in the entire population of α_2M molecules.

The presence of both allotypic specificities, z1 and z2, in the same α_2M molecules reveals that allelic genes are contributing to the formation of the same molecule. This is in contrast to the immunoglobulin system in which the allelic allotypic specficities of the light chain, b4 and b5 (Dray and Nisonoff, 1963), do not occur in the same molecule. On the other hand, the allelic *A* and *B* genes of

the *ABO* system do contribute to the formation of a single complex mucopolysaccharide heteropolymer molecule. These genes are presumably controlling the synthesis of specific glycosyl transferase enzymes which add sugar units to a preformed glycoprotein molecule (Watkins, 1966). The role of the *Mtz* locus in the biosynthesis of α_2M is not yet known. The antigenic differences between z1 and z2 could result from differences in amino acid sequence in analogy to the immunoglobulins or to differences in prosthetic groups such as the carbohydrate moiety in analogy to the blood group substances. Should it turn out that the *Mtz* locus directly controls the synthesis of a polypeptide chain, the mechanism of hybrid formation could represent the association of monomer units each having only the z1 or z2 specificity. The chemical basis, *i.e.*, amino acid or carbohydrate, for the differences between the z1 and z2 α_2M molecules is under investigation and should provide the information necessary to relate genetic control to biosynthesis of α_2M.

The site of α_2M synthesis is not yet well identified, and it would be of interest to determine if both Mtz^1 and Mtz^2 are being expressed in the same or different cells. The expression of only Mtz^1 or Mtz^2 in a cell would suggest some extracellular mechanism involved in the synthesis of α_2M. This problem is presently being investigated by fluorescent antibody methods.

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