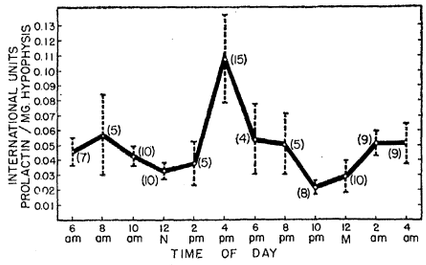

Fig. 1. Circadian variation in the concentration of prolactin in the hypophysis. The figures in parentheses represent the number of rats used at each point. The vertical bars represent standard errors of the mean.

The product of the mean response of the three pigeons multiplied by 3 was considered equivalent to the total activity of prolactin in the gland. In most instances, the pituitary homogenates were injected on one side of the crop sac and various concentrations of a prolactin preparation of known activity (*4*) on the opposite side. From the observations made with the prolactin preparation, a regression equation was obtained by the method least squares. Calculations of unknown concentrations were made from the equation

$$\log Y = -1.724 + 0.608X$$

where Y is the log of the dose, and X is the pigeon response.

The highest concentration of prolactin in the hypophysis occurred at 4 P.M. (Fig. 1). The concentration had decreased significantly by 10 P.M. ($p < .02$) and by midnight ($p < .05$). Also, the concentration at 4 P.M. was considerably higher than that at noon ($p < .05$). Except for the peak at 4 P.M., significant changes in the concentration of prolactin were not detected at other times of day. When data from rats killed at various times of day were grouped, the concentration at 4 P.M. was higher than the mean concentration for all rats taken from 6 A.M. to 2 P.M. ($p < .05$) and for those from 6 P.M. to 4 A.M. ($p < .05$). The concentration of prolactin in individual rats bore no consistent relationship to the period of estrus as determined from microscopic sections of the vaginal wall, rats in most stages of the cycle being represented in each group.

Thus, a circadian periodicity in the concentration of prolactin in the hypophysis was demonstrated, with a 325-percent increase occurring between 12 noon and 4 P.M. and the concentration reaching its lowest point at 10 P.M.

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Hybridization of Half Molecules of Rabbit Gamma Globulin

Abstract. *Specifically purified rabbit antiovalbumin and normal γ-globulin labeled with I-131 were dissociated into half molecules by reduction and acidification. When a mixture of the two preparations was neutralized, a large proportion of mixed molecules having active combining sites and the same sedimentation coefficient as the original γ-globulins was formed. Since the sulfhydryl groups were inactivated after reduction, the recombined subunits appear to be linked by noncovalent bonds.*

It was recently reported (*1*) that molecules of 6.2*S* rabbit γ-globulin are split into two subunits of molecular weight 75,000 to 80,000 by reduction with 0.1*M* mercaptoethylamine followed by acidification to *p*H 2.5 in 0.1*M* sodium chloride. Supporting evidence included the agreement between the weight and *Z*-average molecular weights; the fact that the subunits migrate almost entirely as a symmetrical peak in the ultracentrifuge; and that the splitting can be accomplished without proportionate release of free "B chains" (*2–5*). The results indicated that each subunit contains an "A chain" and a "B chain." The molecular weights of the chains are 55,000 and 20,000 to 25,000, respectively (*6, 7*). After neutralization, most of the combining sites of an antihapten antibody were shown by equilibrium dialysis to be intact. No appreciable formation of insoluble material was associated with these procedures.

After neutralization of the acidified protein, the major component has a sedimentation coefficient of 6*S*. Smaller amounts of a 4*S* component and some faster-moving material are ordinarily present. The $s_{20,w}$ value of the major component has been found in numerous experiments to agree within 3 percent with that of the untreated γ-globulin, which strongly suggests that most of the half molecules recombine in pairs at neutral *p*H. Since the −SH groups are inactivated with *p*-chloromercuribenzoate (CMB) after reduction, the recombined molecules are evidently held together by noncovalent bonds, as is the case prior to acidification. More than 90 percent of the protein can be redissociated, to give a product having a sedimentation coefficient characteristic of half molecules, by dissolving a recombined preparation in 0.025*M* NaCl, *p*H 2.4 (*8*).

Since each of the subunits obtained by reduction and acidification appears to contain an "A chain" and a "B chain," the possibility was suggested that they are univalent (*1*). The evidence reported here provides support for this hypothesis. The data also indicate that the subunits of different γ-globulin molecules associate to form 6*S* molecules after neutralization of a mixture of two reduced, acidified preparations.

A pool of antiserum obtained from several rabbits hyperimmunized with ovalbumin was specifically purified by partial dissociation of a specific precipitate at *p*H 2.5 and fractionation at this *p*H with sodium sulfate (*9*). The product had a sedimentation coefficient, $s_{20,w}$, of 6.1*S*; approximately 8 percent of faster moving material (about 10*S*) was also present (*10*). An optimal concentration of ovalbumin precipitated 89 percent of the protein. The method used for estimating amounts of antigen in precipitates near equivalence has been described (*11*). In view of the slight solubility of antigen in the presence of excess rabbit antibody, contamination of the purified antibody by antigen was probably very small.

A γ-globulin fraction was prepared from the serum of a nonimmunized rabbit by three precipitations with sodium sulfate (*12*), followed by passage through DEAE-cellulose (diethylamino-

cthyl) (*13*) in 0.0175*M* phosphate buffer, *p*H 6.9. The product ($s_{20,w}$ = 6.2*S*) consisted entirely of γ-globulin by the criterion of immunoelectrophoresis. A portion was labeled with I^{131} (1.2 atoms of iodine per molecule) by the method of McFarlane (*14*). After repeated dialysis more than 99 percent of the radioactive iodine was precipitable by trichloroacetic acid, added to a final concentration of 5 percent. The specific activity at the start of the experiments reported in Table 1 was approximately 2×10^5 count/min per milligram of protein. Portions of each preparation (about 70 mg) were reduced with 0.1*M* mercaptoethylamine hydrochloride for 75 minutes at 37°C, in 0.1*M* acetate buffer, *p*H 5.0. The reducing agent was removed on a ion-exchange column, excess CMB was added, and portions were back-titrated with recrystallized L-cysteine, in the presence of 0.5 percent sodium lauryl sulfate, to determine the number of −SH groups liberated. These methods have been described in detail (*15*). After correcting for the small −SH content of the unreduced proteins (0.6 and 1.0 −SH groups per molecule), the values for −SH groups liberated by the reduction were 8.4 and 9.2 groups per molecule, for the specifically purified antiovalbumin and labeled normal γ-globulin, respectively. Each preparation was dialyzed for 2 days against two 4-liter portions of cold 0.025*M* sodium chloride. Less than 3 percent of the protein was lost as insoluble material during dialysis. The *p*H was then lowered to 2.4. In each case there was essentially complete breakdown to components with $s_{20,w}$ = 3.1*S*, which corresponds to the *S* value of half molecules at this *p*H and salt concentration (*1*, *16*). The use of 0.025*M*, as compared with 0.1*M* sodium chloride, has been found to result in more nearly complete cleavage into half molecules.

During ultracentrifugation which was carried out at 10.0°C, the remainder of each sample was stored in a constant-temperature bath at the same temperature. This was done to insure that conditions in the ultracentrifuge also applied to the rest of the protein. Immediately after the run, mixtures were prepared of the reduced antiovalbumin and I^{131}-γ-globulin preparations with proportions as indicated in Table 1. A total of 4 mg of protein was present in each mixture and the final volume was 0.7 ml. Four-milligram samples of each protein preparation were also

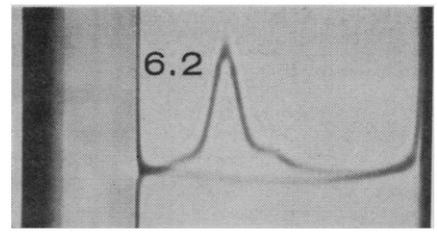

Fig. 1. A schlieren pattern obtained with a Spinco Model E ultracentrifuge after 32 minutes at 59,780 rev/min (20°C). Sedimentation is from left to right. Reduced, specifically purified antiovalbumin and reduced labeled normal γ-globulin were acidified separately in 0.025*M* NaCl, then mixed in a 3:1 weight-ratio (antibody: labeled globulin) and neutralized. The solvent is saline-borate buffer, ionic strength 0.16 *p*H 8.0. The numeral is the $s_{20,w}$ value of the major peak.

treated separately (without mixing). Sodium chloride was then added to each solution to give a final concentration of 0.5*M*; this was done to reduce internal electrostatic repulsions associated with low ionic strength. Mixtures were then dialyzed for 2 days against two 4-liter portions of cold saline-borate buffer, ionic strength 0.16, *p*H 8.0. A representative schlieren pattern of a dialyzed sample is shown in Fig. 1. The major component, comprising 70 percent of the area in the schlieren pattern in Fig. 1, had a sedimentation coefficient, $s_{20,w}$, of 6.2*S*. Approximately 7 percent of slower-moving material and 23 percent of faster components were present. In the other mixtures the $s_{20,w}$ values of the major component were 6.1 to 6.3*S*. This component comprised 64 to 81 percent of the total areas. The amounts of faster-moving components varied from 19 to 23 percent of the total areas. Slower-moving components made up 0 to 14 percent of the areas.

To test for hybridization of subunits, adsorptions were carried out with an ovalbumin-antiovalbumin precipitate made in antigen excess. It was expected that hybridization would be reflected by removal of radioactivity, associated with the normal γ-globulin, by the adsorbent. Three-milligram quantities of precipitate were suspended in saline-borate buffer, ionic strength 0.16, *p*H 8.0, and washed repeatedly. Each was then shaken gently, in a test tube (10 × 75 mm) containing two glass beads, with 0.5 ml portions of buffer. Shaking was done with a Burrell shaker at room temperature, and was repeated until the amount of protein liberated in a 30-minute shaking period was less than 10 μg, as shown by the optical density of the supernatant fluid at 280 mμ (extinction coefficient, 1.5 optical density units per milligram per milliliter). Portions of the dialyzed mixtures of antiovalbumin and labeled γ-globulin described above were then added to 3 mg quantities of washed precipitate, volumes were adjusted to 0.5 ml with buffer, and the mixtures were shaken for 30 minutes. The amount of protein solution used for the adsorption was sufficient to contain 300 μg of antiovalbumin. Control adsorptions were carried out with the labeled, reduced normal γ-globulin, acidified and neutralized in the absence of antiovalbumin, and also with the recombined antiovalbumin. After shaking, the test tube was centrifuged, and the supernatant solution transferred to another tube and weighed. The amount of liquid remaining with the precipitate was similarly determined by weighing and subtracting the weight of the precipitate (3 mg). The percentage of radioactivity adsorbed was determined by counting the supernatant and the wet precipitate. The value for the latter was corrected for radioactivity due to the residual supernatant fluid. Results of the experiments were as follows.

Adsorption of a 300-μg portion of the acidified, neutralized antiovalbumin resulted in a decrease in the total amount of protein in the supernatant to 39 μg, as indicated by the optical density at 280 mμ. A second adsorption removed less than 5 μg of protein.

The results of adsorptions of varying amounts of reduced, labeled normal γ-globulin, after acidification and neutralization (controls), are shown in the first four rows of Table 1. The nonspecific adsorption indicated in the table (5 to 11 percent of the radioactivity) was confirmed by measurements of optical density of the supernatants at 280 mμ. The results agreed closely with those of the radioactivity measurements. The latter were used for correcting data obtained with mixtures (experiments 2, 3, 4, and 5, Table 1). Experiment 5 represents another control experiment, in which antiovalbumin and labeled normal γ-globulin were recombined separately and then mixed. It is evident that the percentage of radioactivity adsorbed from this mixture did not significantly exceed that expected for the labeled normal γ-globulin alone.

Adsorption of mixtures of the two reduced proteins, prepared at low *p*H

Table 1. Adsorption of mixtures of reduced antiovalbumin and I^{131}-γ-globulin, prepared at low *p*H and neutralized. *Unless specified otherwise, the reduced, CMB-treated proteins were mixed at *p*H 2.4 in 0.025*M* NaCl and brought to neutrality by dialysis. In experiment 5 the proteins were mixed after neutralization. Adsorptions of the mixed proteins were carried out with 3 mg of washed ovalbumin-antiovalbumin precipitate (made in antigen excess), and an amount of protein containing 300 μg of the antiovalbumin. The data in the first four rows (experiment 1) were obtained with reduced I^{131}-γ-globulin alone (acidified and neutralized), and were used to correct for nonspecific adsorption (next to last column).

Expt. No.	Composition of mixture		Adsorption		Radioactivity adsorbed* (% total)	Radioactivity adsorbed corrected† (% total)	Cumulative (%)
	I^{131}-γ-globulin (mg)	Antiovalbumin	Wt. used (μg)	No.			
			Mixed before neutralization				
1	4	0	40	1st	10.8		
			80	1st	9.9		
			300	1st	6.0		
			900	1st	4.8		
2	3	1	1200	1st	22.8	18	
				2nd‡	4.1	0	18
3	2	2	600	1st	38.6	34	
				2nd‡	8.3	3	36§
4	1	3	400	1st	56.0	46	
				2nd‡	17.4	8	50‖
				3rd‡	5.5	0	50
			Mixed after neutralization				
5	2	2	600	1st	6.8	1	

* A minimum of 6000 counts was recorded for each sample. † Corrected by subtracting values for nonspecific adsorption for an amount of reconstituted I^{131}-γ-globulin equivalent to that in the mixture. ‡ Carried out with the supernatant of the previous adsorption and 3 mg of adsorbent. § 34 percent + 3 percent (1.00–0.34). ‖ 46 percent + 8 percent (1.00–0.46).

and neutralized, resulted in removal of significantly greater amounts of radioactivity (experiments 2, 3, and 4, Table 1). The supernatant of each adsorption was readsorbed with 3 mg of adsorbent and the percentage of radioactivity removed was again determined. In experiments 2 and 3, significant quantities of radioactivity were not removed by the second adsorption (Table 1). In experiment 4, a third adsorption was required.

The results indicate that the products contained significant proportions of molecules in which subunits derived from both proteins were present. The fraction of the total radioactivity adsorbed increased with increasing ratio of antiovalbumin to labeled γ-globulin in the mixtures, as would be predicted on the basis of probability. Since about 70 percent of the molecules in each mixture consisted of 6.1 to 6.3*S* protein, this component must have included a large proportion of the hybridized molecules. The possibility that the 6*S* molecules consisted preferentially of antiovalbumin seems improbable in view of the fact that the antiovalbumin and labeled γ-globulin had very similar sedimentation patterns when recombined separately.

If the antiovalbumin were pure, and if completely random recombination to form whole molecules proceeded to 100 percent of completion, the percentages of radioactivity adsorbed in experiments 2, 3, and 4 of Table 1 should have been 25, 50, and 75 percent, respectively. The impurity of the antibody and the fact that the slower (about 4*S*) component probably represents unrecombined subunits are factors which would tend to give low results. On the other hand, the presence of protein with a higher sedimentation rate may increase the observed values, if it represents molecules containing active antiovalbumin sites and labeled γ-globulin. The average sedimentation coefficient of the faster material is about 10*S*. It is significant that the actual values for percentage adsorbed are in direct proportion to the "idealized" values discussed above. The ratios in experiments 2, 3, and 4 are 0.72 (18/25), 0.72, and 0.67, respectively.

In another experiment, a mixture of the two proteins was recombined and adsorbed at *p*H 6, rather than *p*H 8. A saline-phosphate buffer, ionic strength 0.2, was used. At *p*H 6 smaller amounts of the fastest-moving component were formed, but more of the slowest-moving (4*S*) component remained. A second preparation of labeled normal γ-globulin, containing 1.1 atoms of iodine per molecule, was used. The nonspecific adsorption of reconstituted labeled normal γ-globulin was found to be somewhat lower than in the experiments described above. The weight-ratio of specifically purified antiovalbumin to labeled normal γ-globulin in the hybridization was 3 to 1. After recombination, a 6.0*S* component accounted for 55 percent of the total area; 34 percent of a slower-moving component and 11 percent of faster-moving material were present. The amount of radioactivity adsorbable at *p*H 6, corrected for nonspecific adsorption, was 46 percent of the total present. Three adsorptions were necessary.

To determine whether any exchange of iodine occurs between side chains of labeled and unlabeled protein under the conditions of these experiments, equal quantities of labeled normal γ-globulin and unlabeled ovalbumin were mixed in 0.025*M* NaCl, *p*H 2.4, and allowed to stand overnight at 10°C. The proteins were then separated on a column of Sephadex G-200. The ovalbumin, which is eluted from the column last, was found to be free of label. (An experiment testing exchange between two 6.5*S* γ-globulins was not practical because of the formation of some protein of higher sedimentation rate upon neutralization.)

The results indicate that 6*S* hybrid molecules are formed and suggest that recombination may be random. A more rigorous quantitative interpretation will require the isolation of the 6*S* protein. Attempts to do this by fractionation on Sephadex G-200 have so far been unsuccessful.

Interpretation of the results of precipitin reactions of recombined preparations are complicated by the presence of components of higher molecular weight. However, preliminary experiments indicate that the recombination of purified antiovalbumin alone results in a product which is 70 to 80 percent precipitable, whereas recombination in the presence of a large excess of reduced normal γ-globulin yields antibody with specific blocking activity.

The hybridization of half molecules contrasts with that of univalent fragments (*17*) in that noncovalent bonds appear to link the half molecules, whereas the fragments separate after reduction of a single disulfide bond. The noncovalent bonds joining the half molecules may also account, at least in part, for the spontaneous aggregation of free "A chains" at neutral *p*H (*6*).

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Iodine-131 Thyroid Dose from Milk in Italy during the Period September 1962 to February 1963

Abstract. *During the period September 1962 to February 1963, iodine-131 was measured in milk sampled in 14 Italian cities. At the end of the period the average intake was 9100 pc, corresponding to a thyroid dose of 0.31 rad; the maximum intake (at Bari) was 16,300 pc, corresponding to a thyroid dose of 0.56 rad.*

After the renewal of the nuclear tests in 1961 the Laboratory for Environmental Radioactivity carried on a program of monitoring the concentration of iodine-131 in milk. First results, referring to the period October to December 1961, have already been published (see *1*). In the following months no iodine-131 activity could be detected in milk up to the end of August 1962.

This report deals with the values measured from the end of August 1962 till the end of February 1963, when the concentration of iodine-131 in milk dropped again below the minimum detectable value (15 pc/lit.).

Cow's milk distributed to the population was sampled daily in Milan, Rome, Bari, and Catania, and weekly in Turin, Alessandria, Varese, Verona, Venice, Genoa, Florence, Ancona, Naples, and Palermo.

The samples were collected in the milk distribution centers of each city and are representative of the whole production of these centers. Each sample was measured in our laboratory. Such whole production has been called "measured" amount of milk. For each milkshed a surrounding area has been defined, taking into account orographical, meteorological, and agricultural features, so that the milk sample could be considered to be representative for the defined area. The production of milk in such an area has been called "surveyed" amount.

With reference to the Italian production of milk for direct consumption as liquid milk, the "measured" amount was nearly 15 percent and the "surveyed" amount ranged between 65 to 70 percent (*2*).

Measurements were carried on with gamma-ray spectrometers (*1*). In order to calculate the average intake of iodine-131 in Italy, we obtained an average of iodine-131 concentration in milk, weighted for the local consumption (Fig. 1).

In Fig. 1 the results for Milan and Bari are also recorded. The difference between the two patterns (representative of the situations in northern and southern Italy, respectively) is due both to the difference in cow's diet and meteorological conditions.

The integral intakes were calculated under the assumption of an ingestion of 1 liter/day (this figure is taken as an upper limit to the possible ingestion). In Fig. 2 the integral intakes for the weighted Italian average and Bari (where highest values were reached) are recorded.

Table 1. Iodine-131 intake from milk and delivered dose to the thyroid, in Italian cities.

City	Total intake (pc)	Thyroid dose (mrad)
Milan	6,800	230
Venice	9,500	320
Genoa	8,800	300
Florence	7,600	260
Ancona	8,700	300
Rome	9,900	340
Naples	11,300	390
Bari	16,300	560
Catania	7,900	270

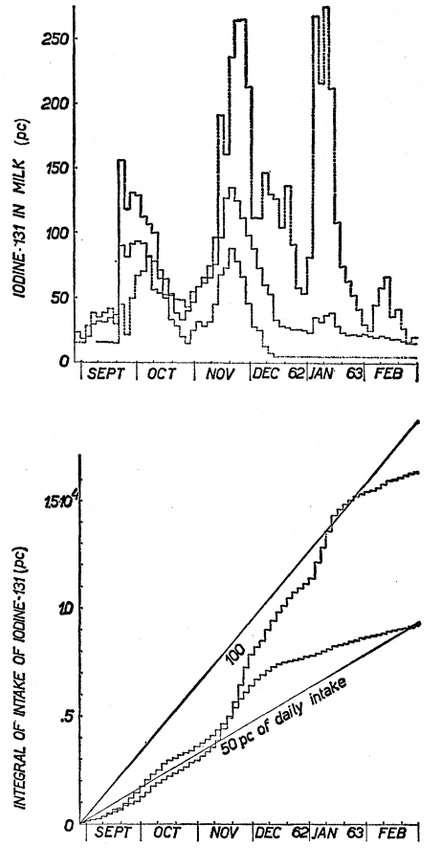

Fig. 1 (top). Concentration of iodine-131 in milk distributed by the milk distribution center of Milan (dotted line) and Bari (dashed line). The Italian weighted-average concentration is recorded by a solid line. The histograms concerning the period after 23 December for Milan and the Italian average must be regarded as representing an upper limit to the possible concentration. Fig. 2 (bottom). Graph of the integral of the intake of iodine-131 (by persons fed 1 liter of milk a day). Italian weighted average, solid line; Bari, dotted line.

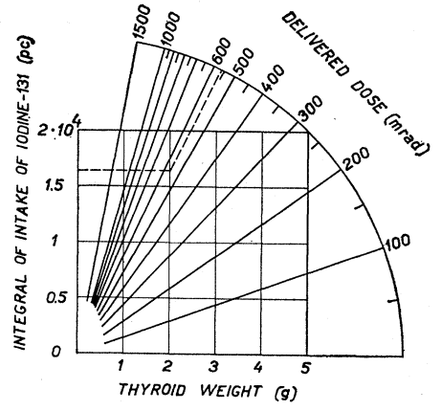

Fig. 3. Graph for calculating the dose delivered to the thyroid. The dashed line refers to the dose delivered to the babies of Bari, according to the assumptions previously reported.