Studies on the Structure of 27S Thyroid Iodoprotein*

G. Vecchio,† H. Edelhoch, J. Robbins, and B. Weathers

ABSTRACT: The 27S iodoprotein, isolated from bovine and rat thyroid glands, was dissociated into slower sedimenting components, *i.e.*, 19S, 12S, and 6S units, by exposure to low ionic strength, alkaline pH, or heat. Under mild conditions, the predominant species was 19 S. The further dissociation of this derived 19S iodoprotein was different from that of thyroglobulin, since 6S units are formed from the latter only by reduction of disulfide bonds. The data are compatible with two possible structures for the 27S iodoprotein: (1) it is composed of one 19S molecule plus smaller subunits which after liberation recombine to form 19S protein, or more likely (2) it is a dimer of 19S molecules. Studies with doubly labeled rat thyroids revealed differences in the dissociation of equilibrium- and pulse-labeled 27S iodoprotein.

Recently a soluble thyroid component, the 27S iodoprotein, which sediments in the ultracentrifuge faster than the main thyroid protein, thyroglobulin,[1] has been obtained in a homogeneous form, and some of its properties have been described (Salvatore *et al.*, 1965). The similarity in amino acid composition and immunochemical properties of thyroglobulin and the 27S iodoprotein suggests that the two macromolecules are made up of the same basic units. Since their molecular weights differ by a factor of about 2 (665,000 and 1,220,000) and they have similar frictional ratios it appears possible that the 27S iodoprotein is a polymer of thyroglobulin or of its subunits.

The aim of the present experiments was to clarify the structure of the 27S iodoprotein and its relationship to thyroglobulin. The structure of thyroglobulin itself is still uncertain, but from early studies (Edelhoch, 1960; Edelhoch and Lippoldt, 1960; Metzger and Edelhoch, 1961; Edelhoch and Metzger, 1961) it is known to contain readily dissociable subunits of approximately one-half the molecular weight of the parent compound. In more recent studies (Edelhoch, 1965; Pierce *et al.*, 1965; deCrombrugghe *et al.*, 1966) molecular species of even smaller size (about one-quarter the molecular weight) were obtained by reduction of disulfide bonds.

In the study of the structure of 27S iodoprotein, dissociation was produced by exposure to one or more of the following conditions: low ionic strength, slightly alkaline pH, and mild heating. In one group of experiments, the dissociation of highly purified bovine 27S iodoprotein was examined in the analytical ultracentrifuge. In a second group, preparations of rat 27S iodoprotein, which had been "equilibrium" and "pulse" labeled *in vivo* with ^{125}I and ^{131}I, respectively, were studied by sucrose gradient centrifugation. The latter technique also offered the possibility of comparing the behavior of the newly formed iodoprotein with that stored in the thyroid gland.

Methods

Preparation of Iodoproteins

Bovine Iodoproteins. Preparations of bovine thyroglobulin and 27S iodoprotein were obtained either by agar gel filtration or by a combination of this technique and sucrose density-gradient centrifugation. Details concerning the two procedures have been described elsewhere (Salvatore *et al.*, 1964, 1965).

Rat Iodoproteins. Groups of Sprague-Dawley rats weighing from 100 to 150 g were kept for 3 weeks on a low iodine diet (Remington, 0.1 μg of iodine/g) to which NaI was added to give a final concentration of approximately 0.6 μg of iodine/g of diet. After this period the rats were fed a diet having the same concentration of iodine labeled with ^{125}I (0.55 μc/g of diet at the start). Both the unlabeled and labeled diet were mixed according to the method of Schneider (1964) and were fed *ad lib.* In a few instances the rats were put directly on the radioactive diet. The animals were used after periods varying from 4 to 9 weeks after starting the radioactive feeding. Prior to killing (30 or 90 min), a single injection of 10–40 μc of carrier-free ^{131}I was administered intraperitoneally to each rat in a volume of 1.0 ml of 0.9% NaCl. For the preparation of 27S iodoprotein, the thyroid glands of up to

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[1] Thyroglobulin is the classical, iodinated protein of the thyroid gland, which comprises about two-thirds of the extractable protein and has a sedimentation constant of 19 S. Other thyroidal components and thyroglobulin subunits are referred to by their sedimentation constants ($s^0_{20,w}$). The subunits derived from 27S iodoprotein are referred to as 19, 17, 12, and 6 S. The three former species have been well characterized by analytical ultracentrifugation since they agree with the properties of thyroglobulin or species derived from it. A species similar to the 6 S is observed only after limited reduction of the disulfide bonds of thyroglobulin.

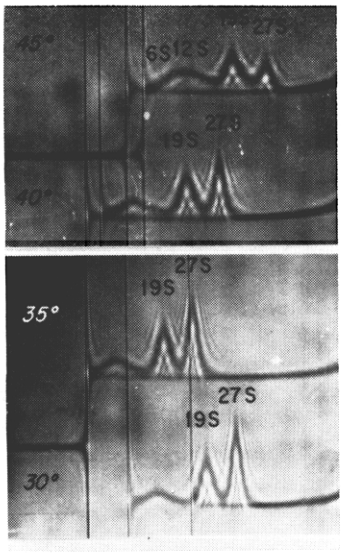

FIGURE 1: The effect of heat on bovine 27S iodoprotein. Solutions of 27S iodoprotein (0.5%) in 0.01 M KNO_3, 0.02 M glycine, pH 9.6, were heated for 10 min at the indicated temperatures, cooled, and centrifuged at room temperature. Pictures taken 8 min after reaching speed of 56,100 rpm.

20 rats were pooled. When only thyroglobulin was desired, one or two rats were used.

Isolation of iodoproteins by sucrose density-gradient centrifugation was done by previously described techniques (Salvatore *et al.*, 1964, 1965). Iodoprotein distribution was detected by counting each tube in a well-type γ-scintillation counter provided with an automatic sample changer and a double-channel pulse-height analyzer. Both isotopes were counted simultaneously and the interference of ^{131}I in the ^{125}I channel was corrected with the aid of a standard containing ^{131}I as the only isotope. Heterogeneity of labeling in thyroglobulin was evidenced by a shift in the ^{125}I 19S peak, relative to the ^{131}I peak, toward the bottom of the tube. The thyroglobulin isolated for these experiments was obtained from the region of the top of the peak, and included each isotope in roughly the same proportion to the total amount of that isotope in thyroglobulin. The significance of the heterogeneous labeling of thyroglobulin is reported in detail elsewhere (Robbins *et al.*, 1966). The isolated 27S protein was obtained from the entire 27S peak and was carried through one additional centrifugation. The 19S and 27S protein preparations were shown to be essentially monodisperse by density-gradient ultracentrifugation.

Unlabeled rat thyroglobulin was prepared in a similar way from rats fed a regular diet of Purina Lab Chow and the protein distribution detected by absorbance at 280 or 210 mμ, as previously described (Salvatore *et al.*, 1964).

Dissociation Experiments

The starting solutions of iodoprotein were in 0.1 M KCl and 0.02 M sodium phosphate buffer, pH 7.4.

Bovine Iodoproteins. Dissociation by alkaline pH at 0.1 M salt was carried out by adjusting the pH of small samples of protein (0.5 ml of a 0.5% solution containing a suitable buffer) with small amounts of base with continuous magnetic stirring at room temperature. In the case of experiments at low ionic strength, the protein samples were first dialyzed extensively at 4° against the desired salt solutions, which contained a buffer for the pH range to be studied. Then the protein samples were brought to the desired pH as described above. In the experiments employing dissociation by heat, the protein solutions were heated at the desired temperatures in a water bath and centrifuged immediately thereafter. The dissociation was analyzed by velocity sedimentation in the Spinco Model E ultracentrifuge. All the runs were done between 20 and 25°.

Rat Iodoproteins. The protein samples, containing small concentrations of labeled iodoprotein, were dialyzed extensively at 4° against a buffer at the desired salt concentration and pH. Heating, when required, was performed in a water bath. The dissociation was analyzed by sucrose density-gradient centrifugation in the SW25 or SW39 rotors of the Spinco Model L ultracentrifuge with the chamber temperature at −5° according to methods previously described (Salvatore *et al.*, 1964), except that the sucrose was dissolved in the same buffer solution used to produce dissociation. The protein distribution was detected by radioactivity measurement as described above (see preparation of rat iodoproteins). In some experiments, the concentration of iodoprotein was sufficient to permit its measurement either by absorbance at 380 or 210 mμ or by the Lowry procedure (Lowry *et al.*, 1951). The distribution of protein and radioactivity was found to be in agreement. In the sucrose gradient analyses, the 19S and 27S species are designated by their sedimentation constants, whereas the slower species are designated by the values calculated from the sucrose gradient runs (Salvatore *et al.*, 1964).

Results

Studies with Bovine 27S Iodoprotein

1. Thermal Effects. The effect of temperature on 27S iodoprotein in 0.01 M KNO_3 and 0.02 M glycine, pH 9.6, appears in Figure 1. The 27S component constituted about 95% of the protein in 0.1 M KCl at pH 7.4. After heating at 30° for 10 min in 0.01 M KNO_3, pH 9.6, two new boundaries appeared, one with a sedimentation coefficient of about 19 S and a slower boundary with a broad peak. The relative areas of the new components increased with increasing temperature. At 45°, the slower peak resolved into two peaks with sedimentation coefficients of about 6 and 12 S. The area of 6 S + 12 S increased more between 40 and 45° than that of 19 S.

In order to produce significant dissociation or alteration of native thyroglobulin at pH 9.6 (0.01 M KNO_3–0.02 M glycine) (Edelhoch and Metzger, 1961), it is

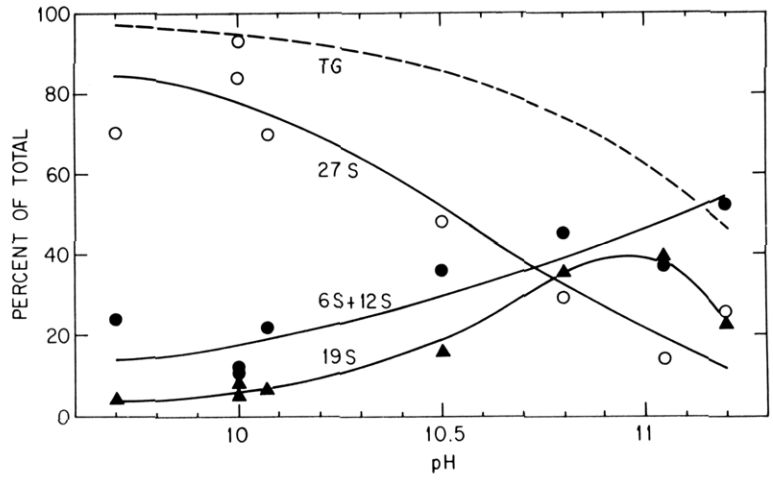

FIGURE 2: The effect of pH on bovine 27S iodoprotein. The protein solution (0.4%) in 0.1 M KNO_3–0.1 M lysine was brought to the desired pH at 20° and centrifuged at room temperature. The relative areas under the respective schlieren boundaries, uncorrected for Johnston–Ogston or radial dilution effects, are plotted. The dotted line (TG) represents a similar experiment done with bovine thyroglobulin as reported by Edelhoch and Lippoldt (1962) and depicts the disappearance of the 19S component.

necessary to heat solutions either for longer periods than 10 min or at temperatures above 45°. There was no clear evidence in the dissociation of 27S protein for the formation of a 17S component. This component, believed to be an unfolded form of thyroglobulin, is produced during heating of native 19S solutions.

2. Alkaline pH. The effect of alkali on 27S iodoprotein in 0.1 M KNO_3 is shown in Figure 2. The 27S protein fell to 10–20% of the starting material by pH 11, and was accompanied by increasing amounts of 19S and 6S + 12S components. The latter exceeded the 19S component at all pH levels tested, and the relative amount of 19S protein was maximum at about pH 11. In comparison with bovine thyroglobulin the 27S protein was less stable at alkaline pH.

3. Low Ionic Strength. In order to determine whether the slow-sedimenting species, *i.e.*, 6 S + 12 S, were present as discrete units in the 27S molecule or were formed from dissociation of 19S units, milder conditions for the dissociation reaction were investigated. Results of experiments at pH 7.6 and 8.3 in 0.01 M KNO_3 are presented in Figure 3. Under only one of the four conditions illustrated, at pH 7.6 and 20°, the 19S component appeared to be the sole product of the dissociation of 27S iodoprotein. At lower ionic strength (0.0025 M KNO_3–0.005 M histidine, pH 7.5) there was further dissociation of 27S protein to slower sedimenting components. At a still lower pH, pH 6.7 in 0.01 M salt, 19 S appeared to be the sole product of dissociation at both 20 and 45°. Since only from 10 to 30% of the 27S protein dissociated under these conditions, the possibility was considered that small amounts of slower components were present but unresolved. This appears to be unlikely in the experiment shown in Figure 3.

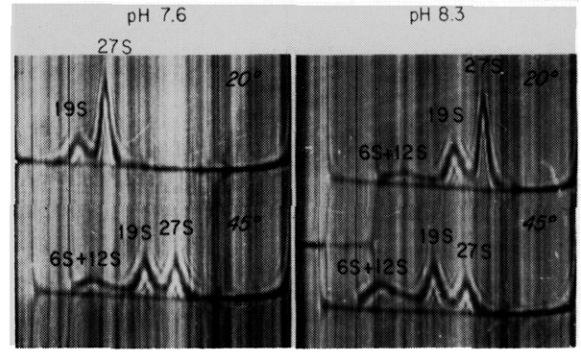

FIGURE 3: The effect of low ionic strength on bovine 27S iodoprotein. The protein solution (0.5%) was brought to 0.01 M KNO_3–0.02 M glycylglycine at the indicated pH values and then heated for 10 min at the temperatures shown, cooled to 20°, and centrifuged. Pictures were taken after centrifugation for 12 min at 52,640 rpm.

Studies with Rat 27S Iodoprotein

In the experiments with rat 27S iodoprotein, sucrose density-gradient centrifugation was used to analyze the dissociation mixtures and also to isolate the products.

1. Dissociation. The dissociation of 27S iodoprotein in 0.01 M KNO_3 and 0.005 M histidine at pH 7.8 is shown in Figure 4. The entire procedure was carried out between 0 and 4°. The extent of dissociation of different preparations of 27S protein was variable. In some experiments dialysis against 0.005 M KNO_3, pH 7.8, for 3–7 days was required for extensive dissocia-

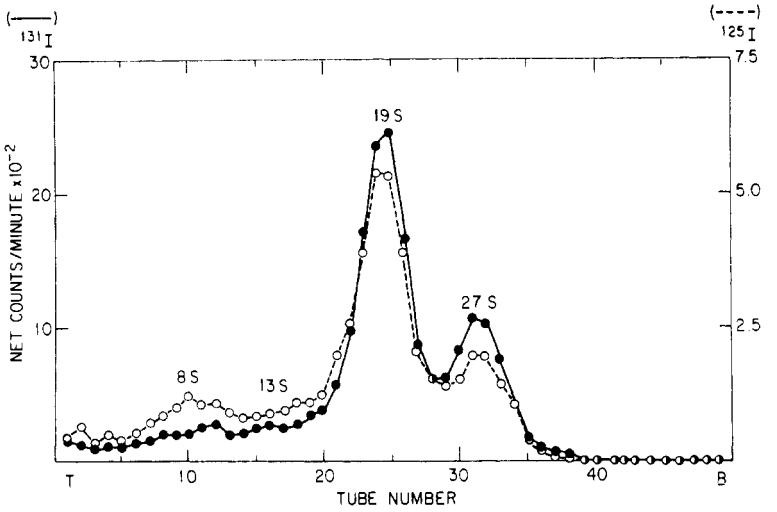

FIGURE 4: The effect of low ionic strength on rat 27S iodoprotein. Sucrose density-gradient centrifugation of purified rat 27S iodoprotein in 0.01 M KNO_3–0.005 M histidine, pH 7.8; SW25 rotor; equivalent time of centrifugation at 23,000 rpm is 22 hr. T and B indicate top and bottom, respectively.

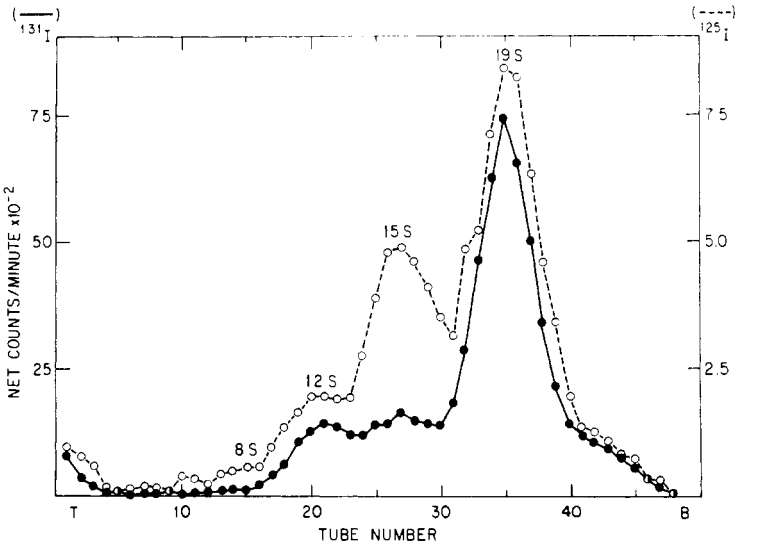

FIGURE 5: The effect of heat on rat thyroglobulin. Sucrose density-gradient centrifugation of a purified preparation of rat thyroglobulin (19 S) which had been heated at 45° for 10 min in 0.01 M KNO_3–0.02 M glycine, pH 9.6. SW39 rotor; equivalent time of centrifugation at 38,000 rpm is 6 hr. T and B as in Figure 4.

tion. The 27S protein was converted mainly to 19S molecules. A relatively small amount of lighter species was also formed, and these had a higher $^{125}I/^{131}I$ ratio than the 27S protein from which they were derived. The ratio of the two isotopes was approximately the same in the derived 19S and in the 27S peaks, whereas in the original thyroid extract thyroglobulin had a much lower $^{125}I/^{131}I$ ratio than did the 27S protein (Robbins *et al.*, 1966).

Thyroglobulin was stable under the conditions described in Figure 4, but dissociated when heated at alkaline pH. The dissociation of doubly labeled rat thyroglobulin in 0.01 M KNO_3 and 0.02 M glycine, pH 9.6, is shown in Figure 5. The dissociation was similar to that obtained with bovine thyroglobulin under similar conditions, the 15S peak probably being analogous to the 17S peak in the latter (Edelhoch and Metzger, 1961). There was, however, a striking difference between the amount of the two labels present in the 15S peak, the ^{125}I yielding a much larger amount of this species than the ^{131}I label. This was a consistent finding in a number of experiments. A less striking difference in the behavior of the two labels was present in the zones corresponding to 12S and 8S species.

2. Stability of Derived 19 S. From a mixture such as the one presented in Figure 4, it is possible to isolate

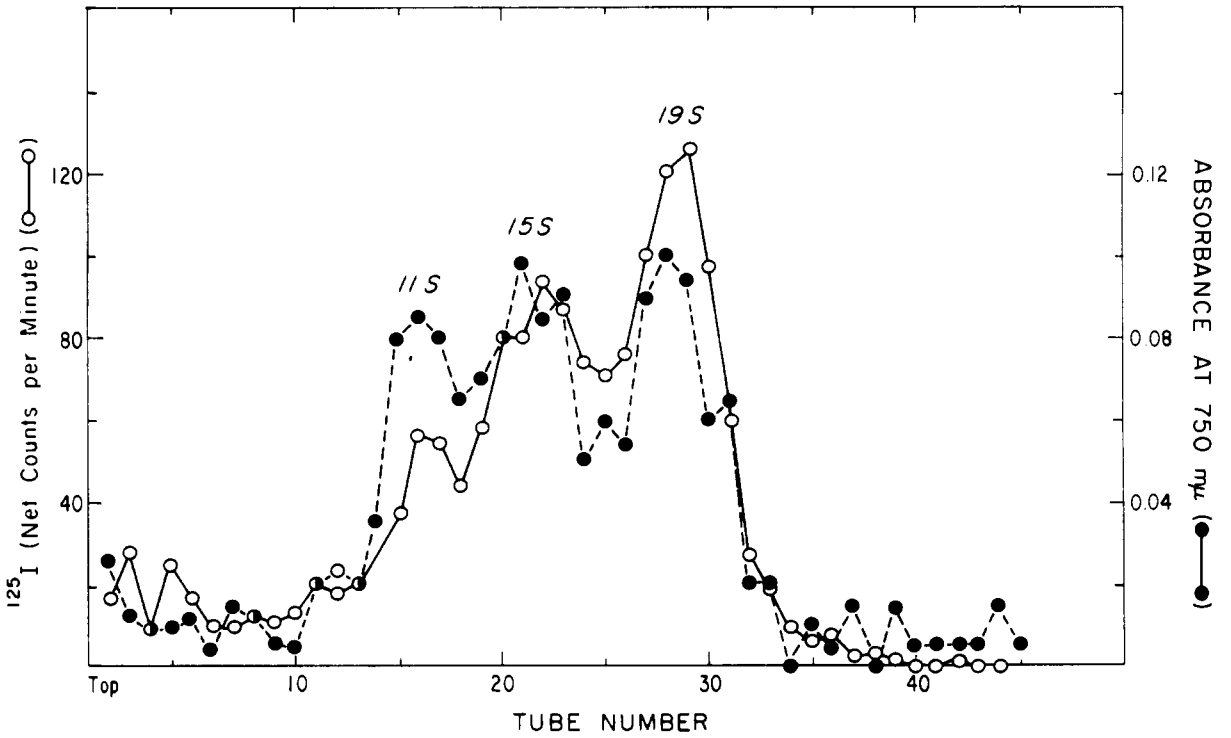

FIGURE 6: The effect of heat on rat thyroglobulin and on the 19S unit derived from rat 27S iodoprotein. Sucrose density-gradient centrifugation of a mixture of the native and derived 19S proteins which had been heated at 45° for 100 min in 0.01 M KCl–0.02 M glycine, pH 9.6. Conditions of centrifugation as in Figure 4.

a small amount of 19S protein derived from the dissociation of 27 S molecules. The stability of this 19S protein was compared with that of native thyroglobulin as reported in Figure 6. Unlabeled native rat thyroglobulin was added to radioactive[2] derived 19S protein during the concentration of the latter in order to minimize losses, as well as to serve as an internal control. Prior to the addition, the thyroglobulin was exposed to the same conditions as was the derived 19S protein. The mixture was then dialyzed against 0.01 M KCl–0.02 M glycine, pH 9.6, and heated at 45° for 10, 100, or 300 min. The derived 19S protein was found to be at least as stable as the native thyroglobulin under these conditions. The centrifugal analysis of the mixture heated for 100 min is presented in Figure 6.

3. Reversibility. In earlier studies on bovine or porcine thyroglobulin it had been shown that dissociation induced by low ionic strength (Lundgren and Williams, 1939; O'Donnell *et al.*, 1958), urea (Edelhoch and Lippoldt, 1964), or organic reagents (Edelhoch and Lippoldt, 1960; O'Donnell *et al.*, 1958) could be reversed by increasing the salt concentration or by removing the dissociating agent. One method of determining the subunit structure of 27 S is to see if reversal of the dissociation could be accomplished with only 19S molecules or whether 6S and 12S units are necessary. Consequently 19S protein was isolated from a mixture similar to that shown in Figure 4. In a second set of experiments 27 S was removed from the dissociation mixture leaving 6, 12, and 19 S. The third test was with the complete mixture of components. The ionic strength was then increased to 0.1 M or 0.2 M KCl. In repeated attempts with all three systems, no significant amounts of 27S protein were formed.

Discussion

Similarity in amino acid composition and immunochemical properties (Salvatore *et al.*, 1965) strongly suggested that the 27S iodoprotein was a polymer of thyroglobulin or of its subunits. A 12S molecule (mol wt ~330,000) is certainly one of the subunits of thyroglobulin (Edelhoch, 1960) and probably represents its immediate precursor during thyroglobulin biosynthesis (Seed and Goldberg, 1963, 1965). The molecular weight of the 27S protein (mol wt ~1,220,000), however, is in better accord with the hypothesis that it is made up of two 19S molecules (mol wt ~665,000) than three 12S units. The results obtained here with bovine 27S iodoprotein, while confirming the hypothesis that 19 S units constitute part of the molecule, also provided evidence for other constituents. The 27S iodoprotein not only yielded the 12S component, which could be accounted for as a product of the further dissociation of the 19S molecule, but also

[2] In this experiment, labeled iodoprotein was prepared from thyroids of rats given a single injection of ^{131}I 48 hr before killing.

another component with a sedimentation coefficient of about 6 S. It is of interest that proteins which sediment between 3 and 8 S in a sucrose gradient have been identified as possible intermediates in thyroglobulin biosynthesis (Seed and Goldberg, 1963, 1965; Sellin and Goldberg, 1965; Lissitzky *et al.*, 1964). The presence of this latter component, which has not been detected after dissociation of thyroglobulin (Edelhoch and Lippoldt, 1960; Edelhoch and Metzger, 1961), introduces another possibility for the composition of the 27S iodoprotein. If the reasonable assumption is made that the 6S species is not an unfolded form of 12 S and, therefore, has a molecular size smaller than 12 S, one can assign to it a molecular weight of about one-quarter (mol wt ~165,000) of thyroglobulin. One may then postulate that the 27S protein is formed from equimolar amounts of 19, 12, and 6 S (mol wt ~1,160,000). This approximates the molecular weight found for the 27S protein as closely as does the hypothesis that it is composed of two 19S molecules (mol wt ~1,330,000). In this case the weight ratio, after dissociation, of 6 S + 12 S/19 S should be 0.75, or larger if 19 S dissociates into 6S and 12S components. If the weight ratio is less than 0.75 one would have to conclude that 27 S is formed from two 19S molecules, provided that the small units do not reassociate to form 19 S once they are released from 27 S.

Experimental conditions have been found for bovine 27S iodoprotein in which very little if any 6 or 12 S was observed (see Figure 3). Since 19 S represented about 20% of the total protein composition it seems unlikely that 10% of 12 S and 5% of 6 S would not have been detected. With rat 27S iodoprotein, conditions have been found in which the ratio 6 S + 12 S/19 S was significantly less than 0.75 (see Figure 4). In this case most of 27 S was dissociated to 19 S and only very small amounts of slower sedimenting material were observed by the sucrose gradient method. Since the rat experiments were based on radioactivity measurements, it is possible that unequal labeling of the subunits might have given misleading results. This was, however, ruled out in several instances in which protein measurements were done simultaneously. These results tend to favor the point of view that the 27S iodoprotein is a dimer of 19S units, but they are not conclusive since the conditions for the dissociation of 27S protein into 19S units are those in which the subunits of 19S would spontaneously recombine. The possibility that 12S or smaller units released from 27 S might have reassociated to form 19S molecules has not been excluded. Attempts to reform 27S protein by reversing the conditions of dissociation were uninformative, since no 27S protein could be formed either from the isolated 19S molecules or from the entire dissociation mixture.

The 27S iodoprotein is considerably less stable than thyroglobulin, although the 19S protein derived from the dissociation of rat 27 S has a stability comparable to that of native rat thyroglobulin. The 6S units are released by dissociation of 27S protein, but apparently not from thyroglobulin except after reduction of disulfide bonds. In the reduction of thyroglobulin (Edelhoch, 1965; Pierce *et al.*, 1965; deCrombrugghe *et al.*, 1966), the 6S molecules that are produced show a very strong tendency to associate, presumably by noncovalent bonding. A speculative proposal for the synthesis of 27S iodoprotein would, therefore, be that the 6S units do not have free sulfhydryl groups needed for the formation of the disulfide-linked 12S molecule, but are associated by noncovalent interactions. If this is so, the 27S protein and thyroglobulin would be composed of the same basic units, but have different interchain linkages. Indeed, the difference in structure between noncovalent and disulfide-linked 6S units might result in the different degrees of polymerization observed in the native 19S and 27S molecular forms.

The studies on the rat gave additional information about the properties of the 27S protein since it was labeled with two iodine isotopes administered for different times. The ^{125}I-labeled 27S iodoprotein more closely approximated the total 27S protein contained in the gland, whereas that labeled with ^{131}I represented the newly formed iodoprotein. Although the number and types of components formed from the dissociation of each were the same, they differed in amount; the "old" protein was less stable and gave rise to a somewhat larger amount of small units. This indicates a degree of heterogeneity among the molecules which comprise the group called 27S iodoprotein.

An analogous heterogeneity has been found in the case of thyroglobulin. Exposure of doubly labeled thyroglobulin to low ionic strength and heat at pH 9.6 resulted in the formation of a larger amount of the 15S component in the case of the equilibrium-labeled material, and a slight increase in the dissociation of thyroglobulin. Lissitzky *et al.*, (1964), on the other hand, found that newly labeled thyroglobulin was more readily dissociated to 12S units by the action of 0.01 M NH_4OH, followed by centrifugal analysis in "Tris" buffer at pH 8.6. Presumably these differences are related to matters of technique.

The 19S unit which was obtained from the dissociation of rat 27S iodoprotein had the same $^{125}I/^{131}I$ ratio as the 27S protein, and differed from that of the native thyroglobulin in the same glands. This provides additional evidence for the individuality of the 27S iodoprotein.

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Specific Anion Binding to Fructose Diphosphate Aldolase from Rabbit Muscle*

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ABSTRACT: The binding of sulfate, phosphate, and hexitol diphosphate (mixture of D-mannitol and D-sorbitol 1,6-diphosphates) to native fructose 1,6-diphosphate (FDP) aldolase each was measured by equilibrium dialysis against the radioactive anion at pH 7.8 in 0.02 M Tris–0.017 M chloride. In addition, phosphate and hexitol diphosphate ion binding to the inactive β-glycerophosphate aldolase derivative of Horecker and co-workers [Horecker, B. L., Rowley, P. T., Grazi, E., Cheng, T., and Tchola, O. (1963), *Biochem. Z. 338*, 36] was examined under the same conditions. The results indicate that the native protein at this pH has approximately 2.6 highly specific binding sites for phosphate (k_A' = 28,000, where k_A' is the apparent association constant) and about 2.9 additional sites with a lower affinity for phosphate (k_A' = 1100). The enzyme binds sulfate under these conditions to an apparent extent of 5.5 ions/molecule with k_A' = 2100 for all sites.

About 2.7 hexitol diphosphate molecules/molecule of native aldolase are bound to apparently equivalent sites with k_A' = 830,000. (The binding of fructose 1,6-diphosphate at 10^{-4}–10^{-3} M concentrations duplicates that of the hexitol diphosphate analogs.) The inactive derivative, formed here with ~2.6 equiv of dihydroxyacetone phosphate covalently attached (presumably to the active sites of the aldolase molecule), does not exhibit the very specific binding of either phosphate or hexitol diphosphate. The magnitude of the affinity constants measured for the anion binding to aldolase implicates cooperating positive charges clustered at each site as proposed by Velick, Saroff, and Loeb and Saroff [Velick, S. F. (1949), *J. Phys. Colloid Chem. 53*, 135; Saroff, H. A. (1957), *J. Phys. Chem. 61*, 1364; Loeb, G. I., and Saroff, H. A. (1964), *Biochemistry 3*, 1819]. Thus, it appears that native FDP aldolase has *three* highly organized active sites, each of which contains two phosphate binding sites.

Rabbit muscle aldolase is a globular protein with a molecular weight of 142,000 and a hydrodynamic frictional ratio of ~1.26 (Stellwagen and Schachman, 1962). It is composed of three polypeptide chains of about equal size, one of which appears to differ in its carboxyl-terminal sequence (Kowalsky and Boyer, 1960; Stellwagen and Schachman, 1962; Deal *et al.* 1963; Winstead and Wold, 1964). Aldolase is inactive when *reversibly* dissociated into its constituent polypeptide chains under fairly mild conditions (Swenson and Boyer, 1957; Schachman, 1960; Stellwagen and Schachman, 1962; Deal *et al.*, 1963). Thus, the secondary and tertiary structure of this protein, while governed by the primary structure, is an integral part of the configuration of the active site(s).

The question arises as to the actual number of active

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