Density Gradient Centrifugation of Bovine Thyroid-Stimulating Hormone

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The sedimentation rate of bovine thyroid-stimulating activity has been compared in sucrose gradients with those of lysozyme and pepsinogen. Examination of several preparations of the hormone ranging in potency from 0.2 to 40 IU/mg has shown that in the neutral *p*H range the sedimentation rate of the activity is independent of the degree of purity. The method of sucrose gradient centrifugation employed was found to be a sensitive technique for assessing the heterogeneity of the various preparations that were studied. On the assumption that the partial specific volume of the hormone is close to that of lysozyme and pepsinogen, its sedimentation coefficient was estimated to be 2.82 *S*. Its molecular weight most probably lies within the range of 26,000 to 31,000.

Bovine thyroid-stimulating hormone (TSH) was originally reported to have a sedimentation coefficient of 1.0 *S* (White, 1944). On the basis of this figure it was suggested that the molecular weight of the hormone was approximately 10,000. Later studies with pituitary fractions purified by chromatography on the weak cation exchanger IRC-50 suggested that the sedimentation coefficient was 3.0 *S* (Pierce and Nyc, 1956). After chromatography on CM-cellulose and DEAE-cellulose the sedimentation coefficient of preparations with potencies of 30 to 40 IU/mg was found to be 2.6 *S* (Bates and Condliffe, 1960). All of these findings were obtained by following the sedimentation of the protein in the analytical ultracentrifuge by optical methods. While preparations of TSH with potencies of 30 to 40 IU/mg display a single peak in the ultracentrifuge, gel filtration of the hormone on Sephadex G-100 revealed that these potent fractions can be further fractionated and that the activity is associated with only a small portion of the protein (Condliffe and Porath, 1962). We therefore sought to ascertain the sedimentation coefficient and the molecular weight of the hormone by examining the behavior of the biological activity during centrifugation in sucrose density gradients by the method of Martin and Ames (1961). By their technique it is possible to determine the distribution of hormonal activity in the gradient so that its sedimentation rate rather than that of the contaminating protein can be estimated.

Experimental Methods and Materials

1. Biological Assays.—Thyroid - stimulating activity was assayed by the iodine-depletion method of Bates and Cornfield (1957), using baby chicks treated with propylthiouracil to block recirculation of iodine discharged from the thyroid, and with thyroxine to prevent discharge of endogenous TSH from the chick's own pituitary gland. Only chicks with I^{131} uptakes of over 15% of the administered dose were employed for the assay. Dilution of samples for injection was done with a solution of 0.1% bovine serum albumin to which chloretone was added as a preservative. The USP reference TSH was used as a standard in all assays. Since the USP unit is equipotent with the international unit (IU) of TSH activity, all results have been expressed in international units.

2. Protein Determinations.—The protein concentration was measured in 50-μl aliquots of each fraction by the modified Folin method described by Lowry *et al.* (1951). In the case of tubes containing TSH, an absorbancy of 0.250 above the background level was found to correspond to about 0.5 mg/ml.

3. Preparation of TSH.—TSH preparations of several degrees of purity were studied. Two crude preparations, having potencies of less than 1 IU/mg, were obtained by a modification of the acetone fractionation procedure described by Ciereszko (1945) and by percolation as described by Bates *et al.* (1959). The specific activities of these two preparations were 0.6 IU/mg and 0.15 IU/mg, respectively.

A more purified fraction was prepared from crude TSH by chromatography on CM-cellulose as described by Condliffe and Bates (1956). The original method was modified by the use of gel filtration on Sephadex G-50 instead of dialysis for the equilibration of samples before chromatography. Recovery of TSH was also done on G-50 in a volatile buffer system (0.1 M NH_4HCO_3). The unretarded peak, which contained the hormonal activity, was lyophilized. The lyophilized powder had a potency of 5 IU/mg.[1]

Further purification was obtained by chromatography on DEAE-cellulose as described by Condliffe *et al.* (1959). The change of buffers between CM-cellulose and DEAE-cellulose and recovery of the purified TSH were again carried out by means of gel filtration on Sephadex G-50. The TSH prepared this way had a potency of 35 IU/mg. When examined by starch gel electrophoresis the preparation displayed several bands. No bands associated with luteinizing hormone were found in the 35 IU/mg preparation used in this study. TSH prepared this way is usually free of significant contamination by luteinizing hormone (Condliffe *et al.*, 1959).

4. Density Gradient Centrifugation.—The sucrose gradient method of Martin and Ames (1961) was employed. The gradients were linear from 20% sucrose at the bottom of the lusteroid centrifuge tube to 5% sucrose at the top. The volume in each tube was 4.5 ml; the buffer employed for most experiments was Tris-HCl (*p*H 7.5, 0.05 M Tris). Between 1 and 2 mg of protein in 0.1 ml of buffer was layered on top of the gradient, making a total volume of 4.6 ml. Centrifugation was carried out using the swinging bucket head (SW-39 rotor) in a Spinco Model L centrifuge. In a typical run, three gradients were centrifuged simultaneously, one containing the TSH preparation, the other two proteins of known molecular weight. After centrifugation at 38,000 rpm and 5°, the gradients were fractionated as described by Martin and Ames

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[1] Further experiments with gel filtration of TSH on Sephadex will be discussed in another publication.

(1961). The 4.6 ml of solution gave on the average 34.6 fractions of 10 drops each.

5. *Reference Proteins.*—Pepsinogen (Worthington Biochemical Co., recrystallized) and lysozyme (Nutritional Biochemicals Company) were chosen as reference proteins since their respective molecular weights of 41,000[2] and 14,400 (Sophianopoulos *et al.*, 1962) bracket the probable value of 25,000 to 30,000 that has been suggested for TSH (Pierce and Carsten, 1958). The sedimentation coefficients were taken as 3.4 for pepsinogen[2] and 1.91 for lysozyme (Sophianopoulos *et al.*, 1962).

6. *Calculations.*—The sedimentation coefficient was calculated from the distance traveled by the TSH peak relative to the distance traveled by the reference proteins pepsinogen and lysozyme. These distances were readily estimated on the basis that 1 ml of gradient volume corresponded to 0.79 cm. Each 10-drop fraction (0.133 ml) thus corresponded to 0.105 cm.

In order to estimate the sedimentation coefficient of TSH the ratio R was determined by the following relationship:

$$R = \frac{\text{distance traveled from top of gradient by TSH}}{\text{distance traveled from top of gradient by standard}}$$

For proteins of the same partial specific volume:

$$R = \frac{S_{20,w} \text{ of unknown (TSH)}}{S_{20,w} \text{ of standard}} \qquad (1)$$

Thus the sedimentation coefficient of TSH was estimated relative to pepsinogen and lysozyme.

On the assumption that the TSH and standards are not too different in shape, a crude estimate of the molecular weight (mw) was obtained by formula (2) (Schachman, 1959):

$$\frac{mw_2}{mw_1} = \left(\frac{S_2}{S_1}\right)^{3/2} \text{ where } S_2/S_1 = R \qquad (2)$$

This formula is only an approximation; for instance, in the case of the standards:

$$\frac{\text{mw pepsinogen}}{\text{mw lysozyme}} = 2.84 \text{ and } \left(\frac{S_p}{S_l}\right)^{3/2} = 2.37$$

where S_p and S_l are the sedimentation coefficients of pepsinogen and lysozyme respectively.

Results

1. Patterns Obtained with Different Preparations of TSH

(*a*) *Crude TSH (< 1 IU/mg).*—With the Ciereszko TSH, the bulk of the protein sedimented relatively slowly, while the hormonal activity moved faster. For example, after centrifugation for 17.85 hours, the pattern shown in Figure 1 was obtained in which the TSH peak was at 1.35 cm, while the slow-moving protein was 0.66 cm from the meniscus. The specific biological activity in the peak fraction at 1.35 cm was 2 IU/mg of protein. Thus, a 3-fold purification was obtained. In the case of the percolate a similar pattern was obtained, but the purification was 8-fold since the starting material had a potency of 0.16 IU mg and in the peak fraction the potency was 1.3 IU mg.

(*b*) *Partly Purified TSH (5 IU/mg).*—Most of the slow-sedimenting impurities were eliminated by gel filtration on Sephadex G-50 and chromatography on CM-cellulose, but the TSH and protein peaks still occupied different positions, as shown in Figure 2.

[2] Personal communication from Dr. Gertrude Perlmann, Rockefeller Institute.

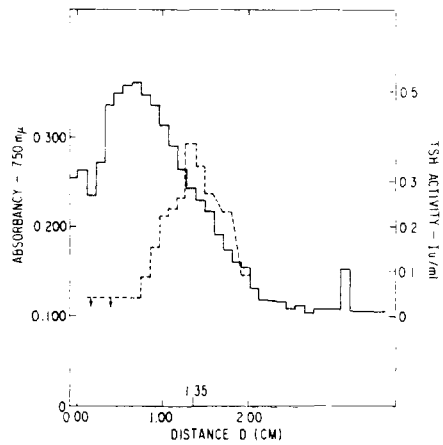

FIG. 1.—Sucrose gradient centrifugation of crude bovine TSH (0.6 IU/mg). Centrifuged at 38,000 rpm, 5°, for 17.85 hours. Experimental details given in text. Solid line denotes absorbancy at 750 mμ developed in 50-μl aliquots by use of the modified Folin reaction of Lowry *et al.* (1951). TSH concentration is represented by dashed line.

Some further purification was realized in this experiment, too, the specific activity at the TSH peak being 10 IU/mg, compared with 5 IU/mg for the starting material. The position of the TSH activity fell between those of lysozyme and pepsinogen.

(*c*) *Highly Purified TSH (35 IU/mg).*—Even in the case of highly purified TSH, obtained by sequential chromatography on CM-cellulose and DEAE-cellulose, the experiment depicted in Figure 3 showed that the distribution of hormonal activity did not correspond with that of the protein. However, the contaminating protein that was still present sedimented slightly faster than the TSH.

The specific activity at the TSH peak was about 41 IU/mg, which is only slightly higher than that of the starting material, but the lack of fit between the curves of protein and TSH concentration showed that the preparation was heterogeneous.

A similar pattern was obtained when this preparation was centrifuged at *p*H 4.5. In this experiment the sucrose gradient was prepared with a sodium acetate buffer containing 0.044 M Na^+.

2. Sedimentation Rate of TSH

As previously mentioned, the distance traveled by TSH relative to the distance traveled by lysozyme or pepsinogen (R) was determined for each individual experiment in order to minimize the influence of variations in experimental conditions between centrifuge runs. However, it is interesting that the reproducibility from run to run is quite good, as shown in Figure 4, where the absolute distance traveled by the standard proteins is shown as a function of the time of centrifugation. In accord with theory the points for each of the standard proteins fall on a straight line through the origin. It is therefore possible to calculate mean rates of sedimentation of 0.053 ± 0.0005 cm/hour (mean ± standard error of the mean) for lysozyme and 0.091 ± 0.001 cm/hour for pepsinogen at 38,000 rpm. The ratio of these figures (1.71) is very close to the ratio of the actual sedimentation coefficients (1.78).

The individual values of R for TSH are tabulated in Table I, together with the calculated values for $S_{20,w}$ in each case. The mean values for $S_{20,w}$ at *p*H 7.5 are 2.78 ± 0.05 (mean ± standard error of the mean) relative to lysozyme and 2.85 ± 0.08 relative to pepsino-

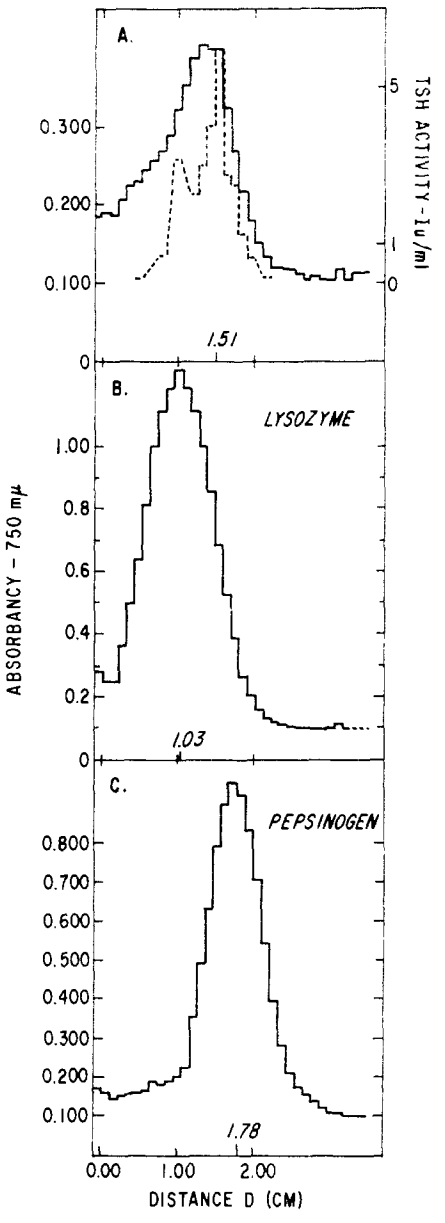

FIG. 2.—Sucrose gradient centrifugation of partly purified TSH (5 IU/mg). Three gradients centrifuged at the same time: (*A*) TSH, (*B*) lysozyme, (*C*) pepsinogen. Centrifuged at 38,000 rpm, 5°, for 20 hours. Experimental details given in text. Solid line denotes absorbancy at 750 mμ developed in 50-μl aliquots by use of the modified Folin reaction of Lowry *et al.* (1951). TSH concentration is represented by the dashed line.

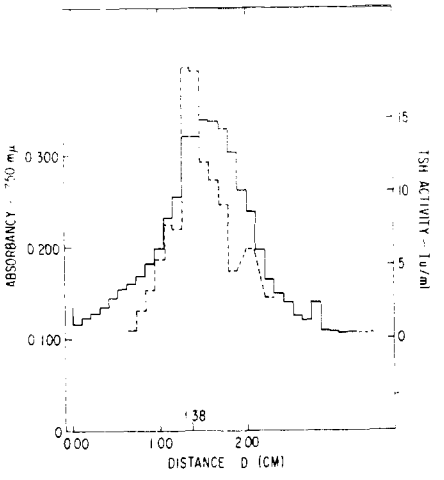

FIG. 3.—Sucrose gradient centrifugation of highly purified bovine TSH (35 IU/mg). Centrifuged at 38,000 rpm, 5°, for 20.22 hours. Experimental details given in text. Solid line denotes absorbancy at 750 mμ developed in 50-μl aliquots by use of the modified Folin reaction of Lowry *et al.* (1951). TSH concentration is represented by dashed line.

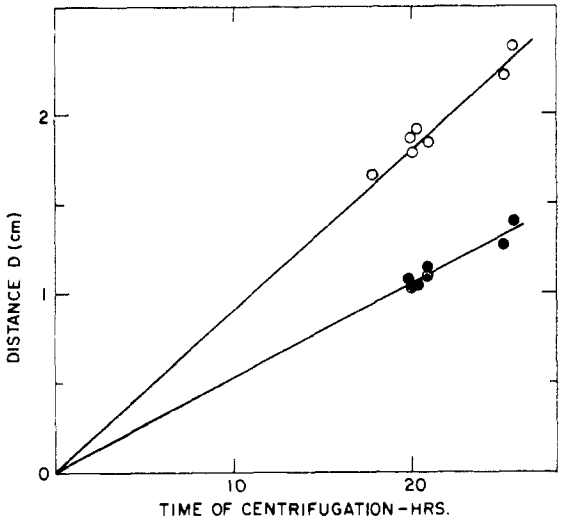

FIG. 4.—Distance traveled by lysozyme and pepsinogen, as a function of the time of centrifugation at 38,000 rpm. Each point represents the result of an experiment similar to that in Fig. 2*A* or 2*B*. O, Pepsinogen; ●, lysozyme.

gen. They obviously are not different, and the general mean is 2.82 ± 0.04. The approximate molecular weight was estimated from equation (2) as 25,300 relative to lysozyme and 31,400 relative to pepsinogen.

DISCUSSION

The principal advantage of using the density gradient method of centrifugation is that it is possible to recover fractions for both biological and chemical assay. Thus, given a sufficiently sensitive bioassay procedure such as the one employed here for TSH, one can examine the distribution both of hormonal activity and of protein in the gradient. By superimposing the concentration curves that are obtained, one can readily assess the heterogeneity of a given preparation in a single experiment. By conventional methods of ultracentrifugation, using optical techniques to observe either a sedimenting boundary as in sedimentation velocity or the distribution of solute as in sedimentation equilibrium, it is not possible to tell whether the biologically active material sediments with the principal protein component that is present. Thus, a partially purified hormone preparation may exhibit a single peak in sedimentation velocity experiments but in reality the biologically active substance may comprise only a fraction of the total protein that is present. In the case of TSH, preparations having potencies of 30 to 40 IU/mg have been shown to have but one visible component in the ultracentrifuge but to be heterogeneous in starch gel electrophoresis. Despite the apparent homogeneity in conventional ultracentrifugation, it is clear from the patterns obtained here, as exemplified in Figure 3, that preparations of this level of potency are also heterogeneous with respect to the sedimentation rates of the biological activity and the proteins present. In fact, one of the uses to which density gradient centrifugation can be put is to test the homogeneity of

TABLE I
SEDIMENTATION COEFFICIENT OF TSH

TSH Preparation	Standard Lysozyme R	Standard Lysozyme $S_{20,w}$	Standard Pepsinogen R	Standard Pepsinogen $S_{20,w}$
Percolate 0.2 IU/mg	1.52	2.90	0.91	3.09
Ciereszko 0.6 IU/mg	1.40	2.67	0.83 0.87	2.82 2.96
CM-cellulose 5 IU/mg	1.46	2.79	0.85	2.89
CM-cellulose DEAE-cellulose 35 IU/mg	1.45	2.77	0.73 0.84	2.48 2.85
Mean ± S.E.	—	2.78 ± 0.05	—	2.85 ± 0.08

protein hormones, or other biologically active proteins, for which suitable bioassay methods exist.

Direct assay of material can also be carried out in a separation cell of the type described by Yphantis and Waugh (1956). However, the density-gradient technique enables one to obtain a complete distribution pattern in a single run, whereas with the separation cell several runs must be carried out in order to accumulate the necessary data for the calculation of sedimentation rates.

The findings presented here fit in well with those obtained in gel filtration of TSH on Sephadex G-100 and G-200, where the biological activity present in preparations having potencies of 30 to 40 IU/mg is retarded more than the major part of the protein (Condliffe and Porath, 1962). There can be no doubt that pure TSH must have a considerably higher potency than has been obtained so far by fractionation with CM-cellulose and DEAE-cellulose.

The values, calculated by the use of equation (2), of 25,300 and 31,400 for the molecular weight of TSH against lysozyme and pepsinogen, respectively, are in good agreement with the range of 26,000 to 30,000 estimated by Pierce and Carsten (1958). They used an entirely different approach in which they studied the electrodialysis of the hormone through membranes of different porosities which had been calibrated with proteins of known molecular weight. The agreement between the two methods suggests that, at least in the case of TSH, there is no significant error in the estimate of molecular weight as a result of membrane potential effects in the electrodialysis technique that Pierce and Carsten (1958) devised.

It should be pointed out that, in the present work, while the estimates of sedimentation coefficients are reasonably reliable the calculation of molecular weight by equation (2) can give only an approximate estimate, since the effect of shape factors is not taken into account. The limitations of this method of density-gradient centrifugation have been discussed at length elsewhere (Martin and Ames, 1961). It should be emphasized that the estimate of the sedimentation coefficient of bovine TSH rests upon the assumption that its partial specific volume is close to those of pepsinogen and lysozyme. Provided that the values lie within the range 0.725 ± 0.025 cm³/g, the error in the estimate of $S_{20,w}$ will be less than 10%, since the change of 0.025 cm³/g in $\bar{V}$ will lead to a change in the factor $(1-\bar{V}\rho)$ of from 0.275 to 0.25. In fact the imprecision of the method itself, as it has been applied here, is of this order of magnitude. Apart from the error due to this basic assumption, error can arise from the difficulty of locating the precise fraction in which the peak is centered. Another source of error lies in the estimate of the distance traveled by the peak.[3]

By taking these different sources of error into account, the variability to be expected between different determinations of the sedimentation rate of TSH relative to a standard protein can be estimated. The standard deviation to be expected in such a group of experiments was reckoned to be about 6% of the mean value. As can be seen from the individual values of $S_{20,w}$ presented in Table I, the actual variation between the experimental results is of this order of magnitude.

Finally, it is of interest that the values obtained here for the sedimentation coefficient and molecular weight of bovine TSH are very close to those that have been found for the ovine and human luteinizing hormones (Ward *et al.*, 1959; Squire *et al.*, 1962). TSH and LH of bovine origin behave similarly in salt fractionation and other procedures, including gel filtration on Sephadex G-100.[4] Indeed, the only practicable procedure that has been found for the separation and recovery of both hormones at once is by means of chromatography on DEAE-cellulose (Condliffe *et al.*, 1959).

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[3] The error in this latter case was evaluated from the variation in the number of drops obtained from many gradients of the same total volume.

[4] Condliffe and Porath, unpublished observations.