Purification and Characterization of Human Pituitary Interstitial Cell-Stimulating Hormone*

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The procedure used in this laboratory for the purification of human pituitary growth hormone provides a side-fraction with a high interstitial cell-stimulating hormone (ICSH) activity. This fraction is further purified by chromatography on carboxymethyl-cellulose followed by column zone electrophoresis. The final product migrates as a single peak in boundary electrophoresis and possesses an isoelectric point at *p*H 5.4. In sedimentation velocity studies, a single symmetrical boundary was observed with a sedimentation coefficient of $S_{20,w} = 2.71$ *S*. The weight average molecular weight as determined by sedimentation equilibrium experiments is 26,000.

In a recent publication (Li *et al.*, 1960) from this laboratory, we reported the results of our earlier efforts toward the purification of the interstitial cell-stimulating hormone (ICSH) and the follicle-stimulating hormone (FSH) from human pituitary glands, with certain side-fractions obtained during the purification of human growth hormone used as starting materials (Li and Papkoff, 1956; Li, 1960). We have since conducted further studies in an attempt to purify ICSH by chromatography on carboxymethyl-cellulose (CM-cellulose), on diethylaminoethyl-cellulose (DEAE-cellulose), and by zone electrophoresis on starch troughs and by column electrophoresis. As a result of these studies, we have found that a two-step process—specifically, CM-cellulose chromatography, and column zone electrophoresis in a formate buffer of *p*H 3.6—yields a product with a higher biological activity than has been reported previously and a high degree of physicochemical and biological homogeneity. Although the most exacting criteria of homogeneity are not fulfilled, much has been learned of the properties of the hormone which have a bearing on its purification, and the state of purity of the final product is sufficient to permit valid conclusions to be drawn concerning the properties of the purified hormone.

EXPERIMENTAL

Materials and Methods.—The human ICSH concentrate used as starting material in these studies was the material, designated fraction HA, which had been described previously (Li *et al.*, 1960; Li, 1960). It is obtained by extraction of lyophilized human pituitaries with a calcium oxide solution followed by ammonium sulfate fractionation and chromatography on IRC-50 resin. This fraction has a biological activity of 51 ± 41 units/mg (*vide infra*). The wide variation indicated by the standard deviation is a result of considerable variation in the condition of the glands as they are received from the various sources of supply.

The carboxymethyl-cellulose was prepared by the method described by Ellis and Simpson (1956). The content of acidic groups was 0.56 meq per g of cellulose.[1] The chromatographic buffers are prepared by dilution of a 1 M solution of ammonium acetate to which enough acetic acid is added to reduce the *p*H to 6.1.

The methods used in this laboratory for starch trough electrophoresis have been detailed elsewhere (Fønss-Bech and Li, 1954). The equipment and operational procedures used in column zone electrophoretic experiments were essentially as described previously (Jutisz and Squire, 1958; Jutisz and Squire, 1961), except that we have turned to the use of Pevikon[2] (Glomset and Porath, 1960) instead of ethanolyzed cellulose as a stabilizing medium. The Pevikon, a polyvinylchloride–polyvinyl acetate copolymer obtained commercially, was further processed as recommended by Glomset and Porath (1960), and the columns were packed under a hydrostatic head of 10 ft. After being packed, the column was equilibrated with formate buffer of *p*H 3.6 and ionic strength 0.05, and the flow properties of the column were tested with ε-dinitrophenylated lysine (ε-DNP-lysine). In these tests, a 1-ml

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[1] In other experiments in this series of studies, in which Selectacel Standard CM-cellulose (lot no. 1160, 0.7 meq/g, obtained from Schleicher and Schuell Company, Keene, N. H.) was used, there was almost complete loss of biological activity. Presumably this was due to some contaminant in this CM-cellulose preparation which might be removed by proper washing, but we have not investigated this question further.

[2] Pevikon C-870, obtained from Fosfatbolaget, Stockholm 5, Sweden.

sample of the ε-DNP-lysine solution was allowed to pass halfway through the column and the flow was then stopped overnight. Failure of the yellow band to move relative to solvent provided evidence of flow-stabilization. Flow was then resumed until the yellow band had passed through the column and into tubes in a fraction collector. As the band passed through the column, its shape was carefully noted in order to detect possible imperfections in packing. The volume passing through the column between application of the sample and the appearance of the optical density maximum was taken as the retention volume. In calculating the "origin" of electrophoretic migration in the zone electrophoretic experiments, the volume used to wash the sample onto the column was simply subtracted from the retention volume, *i.e.*, no corrections were made for electro-osmotic flow. All zone electrophoretic experiments were done at 4° in the cold room. The most useful buffer for the analytical and preparative zone electrophoretic experiments reported here was a formate buffer of *p*H 3.6 and ionic strength 0.05, containing 0.05 mole of formic acid and 0.05 mole of sodium formate per liter.

The boundary electrophoretic experiments were conducted in the Spinco Model H electrophoresis-diffusion apparatus. Electrophoresis and conductivity measurements were made at 1°; hence, the calculated mobilities correspond to that temperature. Because of the scarcity of material, the protein concentrations were quite low; consequently, the Rayleigh fringe diagrams were used to determine boundary shape and position. The buffers were of 0.05 ionic strength, and consisted entirely of the buffer acid (or base) and its conjugate salt. A rather low current density, 6 ma., was used in order to avoid convective disturbances which might otherwise arise at this low protein concentration. Measurements of the photographed Rayleigh fringes were made by means of the Gaertner two-dimensional microcomparator. Alignment of plates and the method of recording fringe positions were essentially as described by Longsworth (1951). In order to represent the boundary shape in terms of the refractive index gradient, the reciprocals of the Rayleigh fringe separations were plotted as a function of the mean fringe position. It has been shown experimentally (Longsworth, 1951) that this method of calculating the refractive index gradient from Rayleigh fringes gives results which are comparable to the photographed Schlieren diagram, yet which are more accurate, especially when the protein concentration is very low.

The sedimentation studies were carried out in the Spinco Model E ultracentrifuge. Schlieren optics, with a phaseplate as the Schlieren diaphragm, were used in the sedimentation velocity experiments, and the Rayleigh interference optical system was used in the sedimentation equilibrium experiments. The details of operational procedure and calibration of the instrument are essentially as described previously (Squire and Li, 1961).

The bioassay method used routinely to determine the ICSH activity of the various fractions was the ventral prostate test, which was conducted essentially as described by Greep *et al.* (1941), except that the strain of rats used was Long-Evans (Lostroh *et al.*, 1958). The unit which is used to express activity is defined as the amount of ICSH which will result in a 100% increase in ventral prostate weight above the terminal controls. Since we have found no significant seasonal variation in control ventral prostate weights over a period of several years, we have taken the mean value, 7.7 mg, as a constant of the bioassay; thus, the amount of ICSH resulting in a mean ventral prostate weight of 15.4 mg is taken as one unit.

Results

Chromatography on Carboxymethyl-Cellulose.—The use of CM-cellulose chromatography for the purification of human ICSH has been reported by Steelman *et al.* (1959). Exploratory experiments were conducted with fraction H-C prepared by chromatography on IRC-50 resin (Li *et al.*, 1960) used as starting material. It was found that about 30% of this fraction passed through the CM-cellulose column unabsorbed from the 0.01 M ammonium acetate buffer of *p*H 6.1, whereas the active fractions, eluted with higher concentrations of buffer, had no higher activity than fractions subsequently obtained by CM-cellulose chromatography of the crude ICSH fraction H-A (Li *et al.*, 1960). Since no clear advantage from the use of IRC-50 resin preceding the CM-cellulose chromatography was apparent, this step has been eliminated, thus minimizing the amount of handling of the hormone required during its purification.

In further exploratory studies, elution of proteins absorbed from 0.01 M ammonium acetate buffer was carried out by means of a continuous gradient as well as by stepwise elution. In the experiments using the continuous gradient the ionic strength of the eluate was measured by conductivity. From these experiments, it was evident that fractions with the highest specific activity were eluted when the buffer concentration became approximately 0.08 M, although considerable spreading of the ICSH activity was observed. As a result of these studies, a procedure involving stepwise elution by *p*H 6.1 ammonium acetate buffer at concentrations of 0.04 M, 0.08 M, 0.20 M, and 1.0 M was chosen for our studies, and the results of experiments carried out in this manner will be discussed in more detail.

Analytical chromatography experiments were carried out on a 10-ml column (0.9 × 18 cm), and preparative experiments, on a 117-ml column (1.6 × 63 cm). The recovery and specific activity data obtained from the two types of experiments were indistinguishable, and no decrease in resolution was observed when the amount of starting

material was as high as 10 mg per ml of column volume. A typical elution diagram[3] is presented in Figure 1, and the yield and bioassay data obtained from seven of the experiments are recorded in Table I. In all experiments the ICSH activity of the 0.08 M fraction was the highest of all the fractions. The activity of the 0.04 and 0.20 M fractions varied considerably from experiment to experiment, and we have not been able to determine what the variable might be which is responsible for this; however, the results of zone electrophoretic experiments with the three active fractions (*vide infra*) suggest that the ICSH activity may be associated with an inactive protein or proteins, and the variability in the chromatographic behavior might result from variations in the association behavior of the different preparations studied.

All the fractions were run again under similar conditions of chromatography, but on an analytical scale. The unabsorbed 0.01 M fraction was also unabsorbed on the second run, with the exception of a small 0.04 M peak amounting to less than 1% of the protein; this demonstrates that the preparative column is not overloaded under the conditions reported here. The elution diagrams obtained from the second run of the 0.04 M, 0.08 M, and 0.20 M fractions are presented in Figures 1B, 1C, and 1D, respectively. In each instance it may be seen that the chromatographic integrity of each of the fractions is retained.

Zone Electrophoresis.—We have previously reported (Li *et al.*, 1960) preparative zone electrophoretic experiments with crude ICSH fraction H-A in a carbonate-bicarbonate buffer of *p*H 8.6

[3] Fluctuations of *p*H occur when buffers of increasing ionic strength pass through the column. The *p*H has been observed to decrease 0.3 *p*H units at the breakthrough point of the 0.08 M buffer, rising again to *p*H 6.1 after the passage of about one-half of the retention volume of the column. Sometimes this has resulted in bimodal peaks, the first maximum occurring with the increase in ionic strength and the second with the transient rise of *p*H. These phenomena are considered artifacts of chromatography, and the fractions are pooled according to the ionic strength of the buffer and are designated as the 0.01 M, 0.04 M, 0.08 M, 0.20 M, and 1 M fractions, respectively.

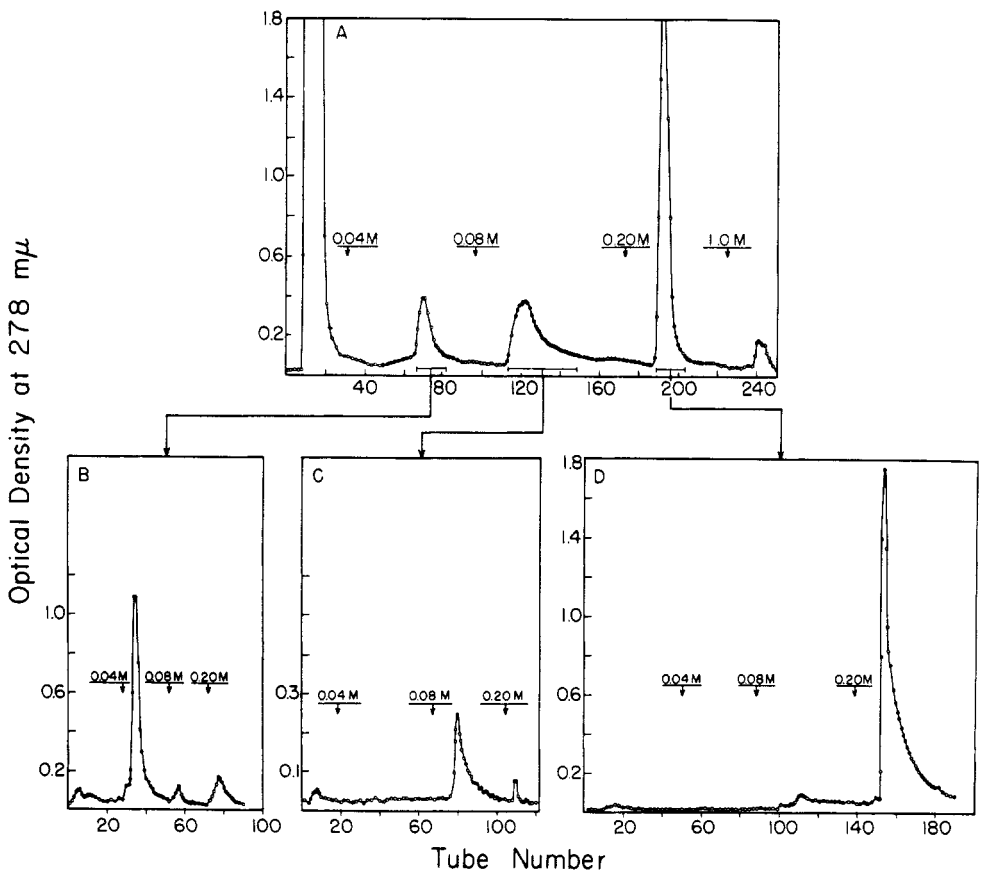

FIG. 1.—*A*, preparative chromatography of 1.0 g human ICSH fraction A on a CM-cellulose column measuring 1.3 × 63 cm. The various fractions were eluted by means of stepwise increases in the ionic strength of *p*H 6.0 ammonium acetate buffer; the fractions are designated by the ionic strength of the eluting buffer. Bioassay data are recorded in Table I. *B*, re-run of the 0.04 M fraction. *C*, re-run of the 0.08 M fraction. *D*, re-run of the 0.20 M fraction.

TABLE I

BIOASSAY AND RECOVERY DATA FROM PREPARATIVE CM-CELLULOSE CHROMATOGRAPHY OF HUMAN ICSH CONCENTRATES[a]

Expt. No.	0.01 M		0.04 M		0.08 M		0.20 M	
	%	units/mg	%	units/mg	%	units/mg	%	units/mg
4				340		620		332
5	33	10	5	10	5	530	10	180
6	(40)	38	10	125	7	418	11	445
8	49		4	320	9	270	12	
17		51		44		417		282
18	26	10	18	166	11	450	15	442
22	44	34	4	186	8	478	15	392

[a] It was found that approximately 30% of these concentrates were not soluble in 0.01 M buffer and that the insoluble fractions had practically no ICSH activity. It was further noted that not more than 3% of the absorbed protein was eluted with 1.00 M buffer, and this fraction was also without ICSH activity.

and 0.1 ionic strength, on cellulose columns. In these experiments, the sample was partially resolved into two major peaks, one of relatively high mobility which was inactive, followed by a slower-moving peak which contained most of the biological activity. Further study of the zone electrophoresis of ICSH fractions has demonstrated that a much better separation of ICSH from the inactive contaminants may be obtained by the use of acidic buffers. When an acetate buffer of *p*H 4.2 and ionic strength 0.05 was used, a rather broad complex peak was observed. The entire peak displayed ICSH activity, but the specific activity decreased as the distance from the origin increased. Analysis in a *p*H 3.6 formate buffer of ionic strength 0.05 resulted in the resolution of an inert peak of high mobility from a zone of greatly enhanced specific activity. This experiment was repeated on a preparative scale, and the protein was recovered from the ICSH-active zone. When this fraction was re-run by zone electrophoresis in the same buffer, a single peak with the same mobility and the same specific activity as the isolated active zone was observed. There was no evidence of any inactive material of higher mobility; hence, we were able to rule out the possibility that the active ICSH was being converted into an inert protein with a higher mobility. Also, it should be added that the ICSH fractions studied do not lose specific activity in this buffer on standing at 4° for 3 days. Consequently, we have chosen the *p*H 3.6 formate buffer for the further purification of the ICSH fractions prepared by CM-cellulose chromatography.

The three fractions obtained by CM-cellulose chromatography have all been submitted to preparative column zone electrophoresis in formate buffer, *p*H 3.6 and 0.05 ionic strength. The results obtained with the 0.04 and the 0.08 M fractions are virtually indistinguishable, and a typical elution diagram is presented in Figure 2. Here it may be seen that, in agreement with the starch zone electrophoresis experiments, these fractions are resolved into a major peak with essentially no activity, followed by a small peak of greatly enhanced activity. As indicated, the fractions were pooled to form an inactive fraction, comprising tubes 1-24, a fraction of intermediate activity, tubes 25–35, and a fraction of high activity, tubes 36–62. The fraction of intermediate activity, pooled from several experiments, has been re-run under the same conditions, with results very similar to those observed here, indicating that this fraction is simply a mixture of the proteins which constitute the active and inactive peaks. The rather broad distribution of biological activity displayed in Figure 2 suggests that relatively mild interactions between ICSH and the inactive protein of high mobility occur even in the formate

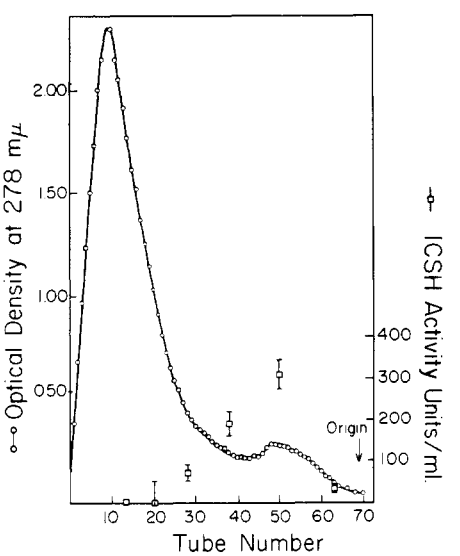

FIG. 2.—Column zone electrophoresis of the chromatographic fraction eluted by 0.08 M ammonium acetate buffer. The distribution of protein is given by the solid line, the distribution of ICSH activity is indicated by the squares, with the vertical bars indicating the standard error of the bioassay, and the origin of electrophoretic migration is indicated by the arrow. Electrophoresis was conducted for 19 hours at a field strength of about 5 v/cm in a column 54 cm long and 2.0 cm in diameter. The buffer was formic acid–formate of *p*H 3.6 and ionic strength 0.05.

buffer, and the components in the intermediate active zone (tubes 25–35) are in a state of reversible association equilibrium.

Zone electrophoresis of the 0.2 M CM-cellulose fraction results in a somewhat different elution diagram. Once again there is a partial separation of an inactive fraction of higher mobility, but the slower, active zone contains approximately half of the total protein and there is little increase in specific activity. This suggests that the interactions between the ICSH and inactive proteins in this fraction are much stronger, and some way must be found to dissociate the components in order to obtain highly purified ICSH from this fraction.

Recovery of protein from the column zone electrophoretic experiments is better than 90%, but the recovery of activity is more difficult to assess owing to inaccuracies in the bioassay. The results of the experiment reported here are, however, typical: the total recovery of activity amounts to 78%.

Analysis by Boundary Electrophoresis.—The highly purified ICSH fraction obtained by column zone electrophoresis of the 0.08 M CM-cellulose fraction was submitted to analysis by boundary electrophoresis in order to assess the electrophoretic homogeneity of the fraction and to determine the isoelectric point. The refractive index gradients, calculated from the Rayleigh fringe positions after $3^1/_2$ hours of electrophoresis in *p*H 3.6 formate buffer, are plotted in Figure 3. A high degree of electrophoretic homogeneity is indicated by the appearance of a single, almost gaussian peak. Mobilities, calculated from the rate of movement of the central Rayleigh fringe in three experiments, are plotted against *p*H in Figure 4. The isoelectric point, estimated from Figure 4, is located at *p*H 5.4 in acetate buffer of 0.05 ionic strength.

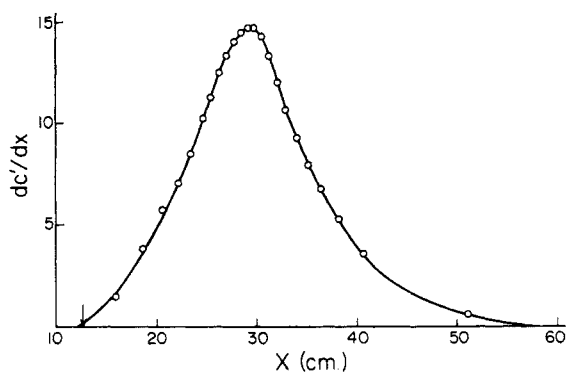

FIG. 3.—Boundary electrophoresis of ICSH obtained by column zone electrophoresis of the fraction eluted from the CM-cellulose column by 0.08 M buffer. In this figure, taken after 196 minutes of electrophoresis at a field strength of 3.20 v/cm, the refractive index gradient, calculated from the Rayleigh fringes, is plotted as a function of cell position. The origin of electrophoretic migration is indicated by the arrow. The buffer was formic acid–formate of *p*H 3.6 and ionic strength 0.05.

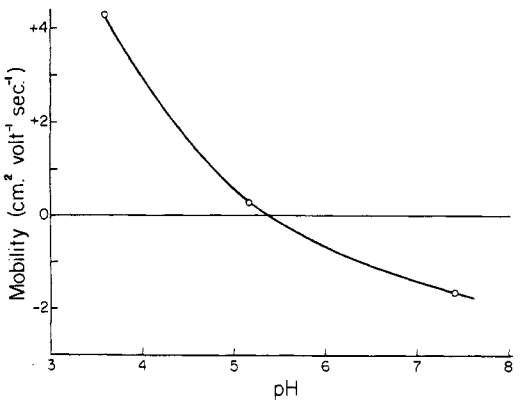

FIG. 4.—Mobility of highly purified human ICSH as a function of *p*H.

Sedimentation Analysis.—Purified human ICSH obtained by column zone electrophoresis of the 0.08 M chromatographic fraction was submitted to sedimentation velocity and sedimentation equilibrium analyses in *p*H 3.6 formate buffer of 0.100 ionic strength. Sedimentation velocity at 59,780 rpm revealed a slightly skewed single peak with a somewhat steeper leading edge; the asymmetry was detectable only by plotting the microcomparator data (Fig. 5). The sedimentation coefficient at a protein concentration of 0.005 g/ml, corrected to the usual standard conditions, is $s_{20,w} = 2.71$ *S*.

The molecular weight of the human hormone, and further information on the homogeneity of the preparation, was obtained from sedimentation equilibrium experiments. The formate buffer of *p*H 3.6 and ionic strength 0.100 was also used in these experiments. The results of three experiments performed on two samples are recorded in Table II, and a plot of the logarithm of the protein concentration as a function of X^2 is recorded in Figure 6. The upward curvature of the points in

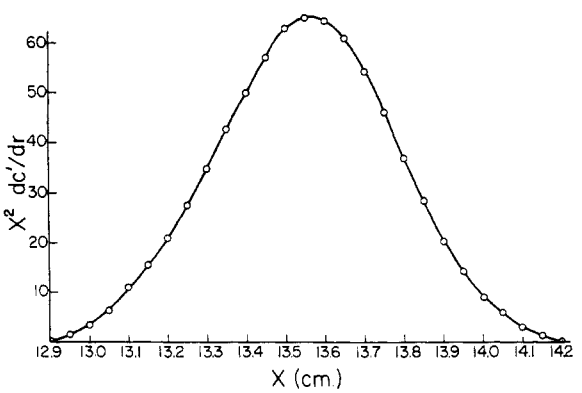

FIG. 5.—Sedimentation velocity of highly purified human ICSH in formate buffer of *p*H 3.6 and 0.1 ionic strength. The refractive index gradient has been multiplied by the square of the radial distance in order to correct for radial dilution and the effect of increasing field. This picture was taken after 64 minutes of sedimentation at 59,780 rpm.

TABLE II

MOLECULAR WEIGHT[a] OF HIGHLY PURIFIED HUMAN ICSH AS DETERMINED BY SEDIMENTATION EQUILIBRIUM

Expt.	Protein Conc. (g/100 ml)	$\overline{M}_w$	$\overline{M}_z$
S-42 A	0.66	27,100	64,000
S-42 B	0.32	25,700	53,000
S-45	0.38	26,400	43,700

[a] The partial specific volume was assumed to be v = 0.730.

Figure 6 could result from either concentration dependence or irreversible association of the protein, or from heterogeneity. A concentration dependence of $\overline{M}_w$ or $\overline{M}_z$, as demonstrated by the data from experiments S 42 A and B (Table II), would explain the upward curvature, but is insufficient to account for the entire effect[4]; consequently, one must recognize the presence in the sample of some higher-molecular-weight material, either irreversible association products or simply a contaminant. At any rate, our best estimate of the molecular weight of the human hormone is 26,000.

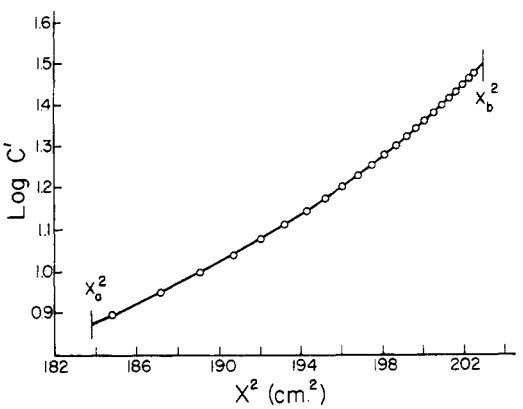

FIG. 6.—Sedimentation equilibrium of highly purified human ICSH in formate buffer of *p*H 3.6 and 0.1 ionic strength. The logarithm of concentration is plotted as a function of the square of radial distance multiplied by the square of the radial magnification factor.

Biological Studies.—It may be seen in Table I that the 0.08 M chromatographic fraction had an average potency of approximately 450 units per mg. When a total dose of 1.0 μg of the final product obtained by zone electrophoresis on the column was injected into seven hypophysectomized male rats and assayed by the ventral prostate test (Greep *et al.*, 1941; Lostroh *et al.*, 1958), an increment of the ventral-prostate weight from 7.7 mg to 18.0 mg was obtained. It was estimated that the purified human ICSH had an ICSH potency of approximately 1500 units per mg.

Preliminary estimates of the extent of contamination of the purified human ICSH by other known pituitary gonadotropic hormones have been made by standard bioassay methods. No lactogenic hormone activity was detected by the local crop sac test (Lyons, 1937) at a dosage of 100 μg. Since 0.5 μg of ovine lactogenic hormone may be detected by this method, the results indicate that the extent of contamination is less than 0.5%.

The same fraction was also assayed for FSH contamination in female rats according to the procedure of Simpson *et al.* (1956). In this experiment two female rats were given a total dosage of 83 μg of human ICSH and the ovaries were weighed and examined histologically for features which might be ascribable to FSH activity. Slight follicular stimulation was observed, but in no case had this stimulation proceeded to the point of antrum formation. Since preparations of FSH currently prepared in this laboratory have a minimal effective dose, as defined by Woods and Simpson (1956), of 1 μg, we may conclude that the level of FSH, either in the form of contamination or as intrinsic activity, is a few tenths of a percent or less.

DISCUSSION

In our studies directed toward the purification of sheep pituitary ICSH (Squire and Li, 1959; Jutisz and Squire, 1958; Jutisz and Squire, 1961) we observed several biologically active components which differ from each other in their chromatographic and electrophoretic behavior. This multiplicity is at least partially attributable to protein-protein interactions. These observations have been confirmed and extended by Ward *et al.* (1959, 1961). Similar observations were made in the present study of human ICSH with respect to three chromatographically distinct biologically active fractions which could be obtained by chromatography on CM-cellulose at *p*H 6.1; however, subsequent zone electrophoresis of two of these fractions, those eluted by 0.04 and 0.08 M buffers, respectively, results in the separation of the ICSH activity from a large amount of inactive protein. On the other hand, zone electrophoresis of the fraction eluted by the 0.20 M buffer does not given a clear-cut fractionation. We have inferred from these results that CM-cellulose chromatography resolves three chromatographically distinct interaction products of ICSH. In two of these fractions, those eluted with 0.04 M and 0.08 M buffer, respectively, the complexes are dissociated at *p*H 3.6, and the ICSH is separated from inactive protein, owing to their different mobilities. The results of column zone electrophoresis of the fraction eluted with 0.20 M buffer indicate, however, that the interaction products in this fraction are

[4] Detailed arguments for the interpretation of sedimentation equilibrium data in terms of concentration dependence on the one hand, and irreversible association or heterogeneity on the other, have been presented in a previous paper (Squire and Li, 1961).

insufficiently dissociated at *p*H 3.6 to permit good resolution and that some other method must be found to separate highly purified ICSH from the inactive proteins in this fraction.

If it can be demonstrated that the highly purified ICSH preparations obtained by column zone electrophoresis of the 0.04 M and 0.08 M fractions are similar, then the purification procedure can be simplified by omitting the 0.04 M elution, thus combining the 0.04 and 0.08 M fractions.

The heterogeneity in molecular weight demonstrated by the sedimentation equilibrium experiments remains a vexing problem. Further work will be required, as material becomes available, to determine whether this is due to the presence of irreversibly associated ICSH aggregates or whether inactive contaminants may yet be detected in the preparations obtained from column zone electrophoresis.

While this manuscript was in preparation, Butt *et al.* (1961) have reported the results of their studies in the purification of human urinary and pituitary gonadotropins. They found that chromatography on carboxymethyl-cellulose as described by Steelman *et al.* (1959) yields a product which upon analysis by sedimentation velocity at *p*H 8.6 displays a major component (95%) with $s_{20,w} = 4.17\,S$. In view of our results on materials obtained from zone electrophoresis at *p*H 3.6 of a similar product, we suggest that the major component observed by Butt *et al.* (1961) may be a product of interaction between ICSH and inert protein.

TABLE III
COMPARISON OF SOME PROPERTIES OF OVINE AND HUMAN ICSH

Properties	Ovine	Human
$s_{20,w}$	2.70 *S*	2.71 *S*
Molecular weight	30,000	26,000
Isoelectric point	7.3	5.4
"Leak point" IRC-50	6.2	5.6
Specific activity (units/mg)	300	1,500

Some of the properties of human and ovine ICSH are summarized in Table III. Owing to the fact that neither hormone has been prepared in a state of complete mass homogeneity, both values for the molecular weight are subject to at least 5% error; therefore, no significance can be attached to the observed difference in molecular weight. The sedimentation coefficients are virtually identical. The isoelectric points, on the other hand, differ markedly, with that of the human hormone located at *p*H 5.4, as compared with 7.3 for the ovine. This difference in isoelectric point was predicted by the chromatographic behavior of the two hormones on IRC-50 resin, when it was observed that the "leak point," *i.e.*, the critical *p*H above which the biological activity is no longer absorbed by IRC-50 resin from a 0.2 M phosphate buffer, was 5.6 for human ICSH and 6.2 for the ovine hormone. The specific activity of human ICSH is about five times as high as that of the sheep ICSH on the basis of results of the ventral prostate test.

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