Starch-Gel Electrophoresis—Application to the Classification of Pituitary Proteins and Polypeptides

By K. A. Ferguson

HISTORICALLY, the isolation of pituitary hormones has been preceded by the identification of the physiologic processes regulated by these hormones. Thus the classification of pituitary hormones is based essentially on their biological activities. It is now well established that the substances possessing particular hormonal activities are in most cases different for each animal species; furthermore, multiple forms with the same activity have been found within species.[1,2] The classification based on biological responses does not distinguish among species, nor does it distinguish the circulating hormone from possible active precursors or degradation products. Consequently, in no case can the identity of the circulating substance or true hormone be said to have been established with certainty.

Zone electrophoresis in starch gel has played a major part in demonstrating the lack of specificity of the biological responses and provides a classification of pituitary hormones based on substances rather than activities. Until the circulating forms of the pituitary hormones have been identified, the classification remains one of pituitary proteins and peptides rather than of hormones; since the physiologic functions of the hundreds of proteins and peptides extractable from pituitary tissue are for the most part unknown. It may be expected that more specific hormonal responses will be progressively discovered but unless a prior classification of possible hormones based on substances is available, such increase in biological specificity will be more difficult to detect.

The mobilities of different proteins in starch gel electrophoresis are dependent on a number of factors which have not always been controlled and specified. As a result it is sometimes impossible to identify the corresponding components in analyses of the same protein mixture carried out in different laboratories.

In the present investigation the effects of two of these factors on mobility have been measured and an attempt made to derive a procedure for a classification based on three parameters which largely determine differences among proteins in their migration through starch gel.

Methods

Starch Gel Electrophoresis

Electrophoresis was carried out essentially as described by Ferguson and Wallace 1963.[2] However the following modifications were introduced:

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1. The cooling system was made an integral part of the mould on which the starch gel was cast in order to provide more even cooling across the gel. Two toughened glass sheets, 65 cm. x 42 cm. x 0.32 cm., were separated by a strip of foam rubber (0.8 cm. x 0.5 cm. in section) glued around the edges to form a chamber enclosing a Perspex sheet 64 cm. x 41 cm. x 0.32 cm. On this sheet, baffles projected to channel the flow of cooling fluid back and forth across the undersurface of the glass plate on which the gel was cast.

2. The power supply was modified to deliver approximately constant power so that as the resistance of the gel increased during electrophoresis, producing a fall in current, there was an increase in voltage gradient.

3. The heating time and temperature of the starch solution were more rigorously standardized during preparation of the gel. The starch buffer suspension was put into a boiling water bath and stirred rapidly for 15 minutes. Stirring speed was reduced for the next 15 minutes at the end of which the temperature of the solution was 88 $\pm$ 2 C. After degassing by water pump, the temperature was allowed to drop to 80 C. when the gel was poured.

4. The Perspex sample slot-former used previously was replaced by a more durable one of nickel-plated brass.

For the experiments reported in this paper, except where otherwise noted, the buffer in which the gel was prepared contained 10 per cent 0.02 M lithium hydroxide, 0.076 M boric acid and 90 per cent 0.0033 M citric acid, 0.025 M trishydroxymethylaminomethane (Tris). Most analyses were carried out with the temperature of the cooling fluid maintained at 30 C. The basic components of sheep and ox growth hormone resolve better at this temperature but for most proteins, lower temperatures give equally good results.

Gel Filtration

Gel filtration of pituitary extracts was carried out in Perspex colums packed with the cross-linked dextran Sephadex (Pharmacia, Uppsala) in 0.15 M ammonium bicarbonate. This volatile salt was chosen to avoid the necessity for dialysis prior to freeze drying.

Large bed volumes of Sephadex provided sufficient material for subsequent physiologic investigation and further fractionation. In the calculation of distribution coefficients (Kd), wet density of the gel was derived from a dry density value of 1.645 for all grades of Sephadex and the water regain of the batch of Sephadex used. The amount of dry gel in a column was then derived from measured bed volume, void volume, water regain, wet density.[3]

The pituitary extracts fractionated by gel filtration were obtained as follows: Frozen sheep pituitaries were ground with solid carbon dioxide to a coarse powder and extracted with 1 per cent sodium chloride. The residue after centrifugation was extracted with 0.025 M sodium hydroxide, 0.095 M boric acid in 1 per cent sodium chloride. The extracts were dialyzed and freeze-dried. They are subsequently referred to as the sodium chloride and sodium borate extracts respectively.

Results

The Relation of Mobility to Starch Concentration

In the size range of protein molecules and in the range of ionic strengths of the buffers generally employed, Henry's equation includes a term allowing for differences in mobility for molecules of different size. The effect of viscosity in this equation, however, is independent of molecular size and the differential retarding effect of starch gel according to size[4] suggests either that the gel provides a different kind of impediment or Henry's equation does not accurately describe the mobility of different proteins at different viscosities.

Smithies[4] found that the migration of proteins was linearly related to the reciprocal of starch concentration over a range from 11.4 to 15.6 Gm./100 ml. buffer. Migration also depends on charge and the effect of starch concentra-

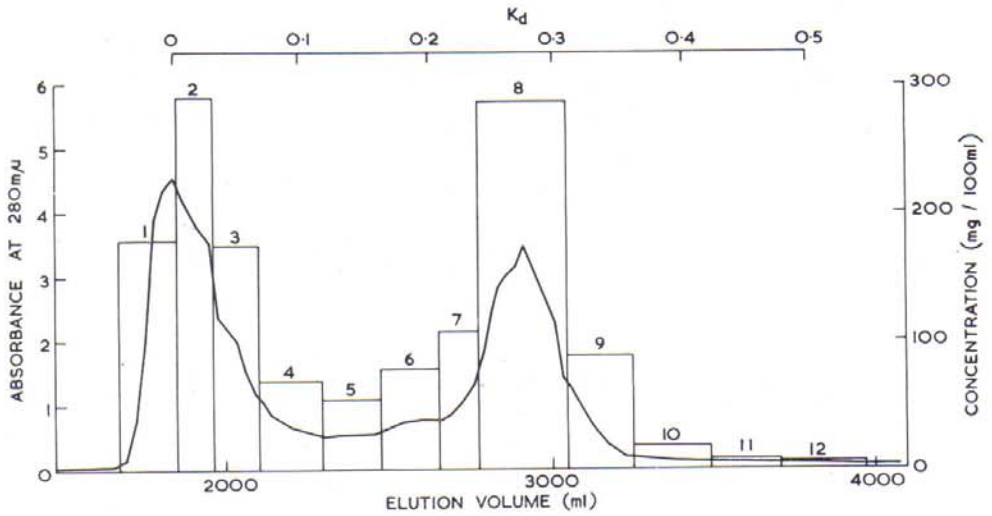

Fig. 1.—Gel filtration on Sephadex G75 of 5 Gm. of an 0.025M NaOH, 0.095M H_3BO_3 in 1% NaCl extract of sheep pituitary glands previously extracted with 1% NaCl. Bed volume 6150 ml., height 86.4 cm. Elution with 0.15M NH_4HCO_3.

tion was isolated by expressing the change in migration with starch concentration as a fraction of the migration at a reference starch concentration.

Smithies found it difficult to make starch gels with Tris and borate buffers that were reproducible over a wide enough starch concentration and resorted to an acid gel system containing 8 M urea and mercaptoethanol. Such conditions cause the unfolding of many proteins which take up a random coil configuration. This may be taken as an advantage in eliminating aggregation and asymmetry and providing a better relation between the retardation coefficient and molecular size. However, for purposes of characterization, the retardation coefficients of the native proteins are just as valid and the contribution of aggregation and asymmetry may actually increase the discriminating power of the measurement.

For uncertain reasons the difficulty found by Smithies in preparing gels over a sufficient range of starch concentration with alkaline buffers has not been experienced. Gels ranging in starch concentration from 10 to 22 per cent have been made without varying the procedure.

The relation of the mobility of proteins of different size to starch concentration was examined by subjecting a series of sheep pituitary fractions to electrophoresis at five different starch concentrations. The fractions were produced by gel filtration of the sodium borate extract of sheep pituitaries. The absorbance of the eluate from Sephadex G75 is shown in figure 1; the eluate was combined into fractions as indicated. The electrophoretic distribution of components in these fractions at one starch concentration is given in figure 2.

The main component reaching a peak concentration in fractions 8 and 9 is the principal component of prolactin. The second most intense component in these fractions is an active conversion form of prolactin, labeled 112 in the paper by Ferguson and Wallace.[2] Albumin appears in low concentration in fraction 3. The most intense component migrating faster than prolactin in fraction 10 is a peptide which appears in high concentration in sheep posterior

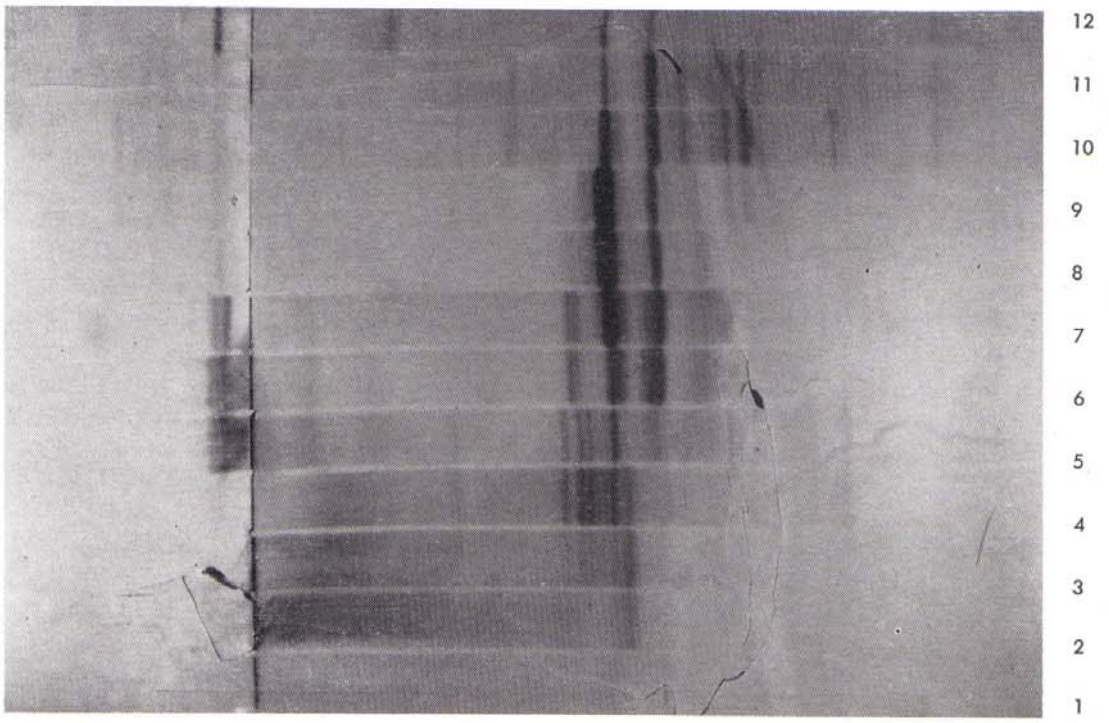

Fig. 2.—Electrophoresis of fractions obtained from gel filtration of an Na_3BO_3 extract of sheep pituitaries (fig. 1). Mean voltage gradient 21 volts per cm. 250 minutes. Starch concentration 14 Gm. per 100 ml. Cooling temperature 30 C.

lobe extracts. The mobility of these four components is related to starch concentration in figure 3 employing only a single determination at each starch concentration.

Several mathematical functions of both mobility and starch concentration may be used to rectify the relationship. However, the relation of log mobility to starch concentration accords more closely with the basic equations relating free solution mobility to the zeta potential and viscosity More importantly, the log function provides that the slope of the relation is independent of the actual mobility.

Extrapolation to zero starch concentration gives an estimate of free solution mobility which is 18.4 (cm. x 10^{-5}/sec.)/(volt/cm.) for prolactin and 26.3 (cm. x 10^{-5}/sec.)/(volt/cm.) for albumin. No published measurement of free solution mobility are available for the buffer composition, ionic strength and temperature (30 C.) used. However, adjusting from 0.02 to 0.1 ionic strength by the ratio of the square roots of these ionic strengths and to a temperature of 4 C. by the ratio of viscosities of water at the two temperatures, values of mobility of 4.2 and 6.0 respectively are obtained for the two proteins. The value of 6.0 for albumin is within the range of published values of free solution mobility using sodium diethylbarbiturate buffer at pH 8.6. In extrapolating to zero starch concentration, the effects of electro-osmosis, adsorption and pH change due to the starch should disappear, avoiding the need for any correction for these factors. However, a discrepancy between the extrapolated values and true free solution mobility may arise from the use of the mean of the initial and final voltage gradient in the calculation of mobility,

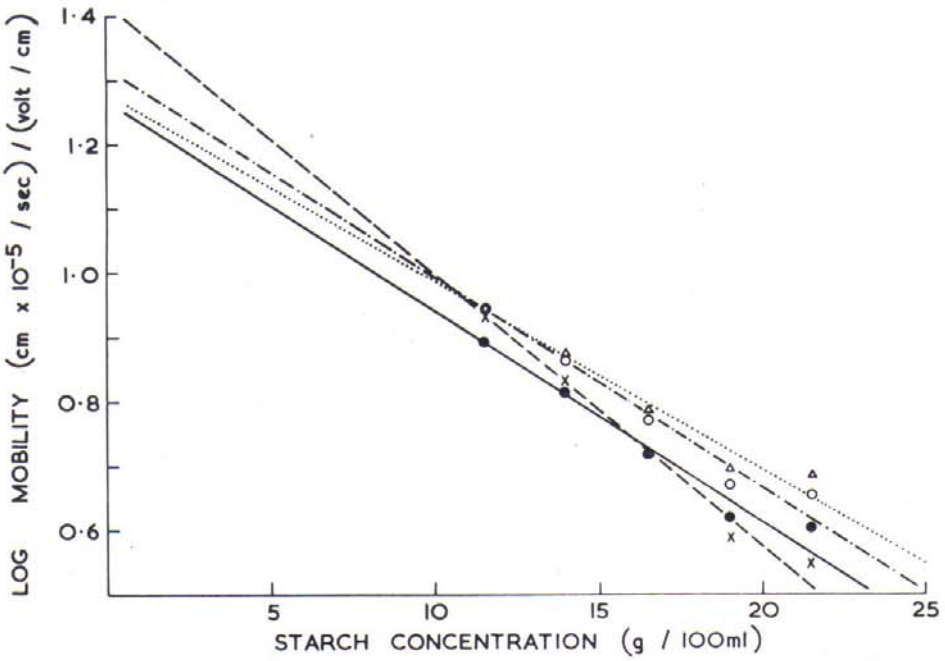

Fig. 3.—Relation of electrophoretic mobility to starch concentration. ●——● prolactin; ×——× albumin; ○—.—○ prolactin second component; △....△ main component fraction 11 in figure 2.

when using the partially discontinuous buffer system. Further research is required on the measurement of the true mean voltage with moving non-linear voltage gradients. The expression of mobility relative to that of a reference protein avoids the problem of exact voltage control and measurement although the effects of possible differences between components in the voltage gradient and temperature to which they have been exposed are not removed.

The use of a reference protein also avoids some of the errors introduced by uncontrolled variations in technic by providing an internal standard. Using the main component of sheep prolactin as a reference, the relation of the logarithm of the ratio of mobilities to starch concentration is shown in figure 4 for the same four proteins.

The data are well fitted by the linear relation

$$\log \frac{m_x}{m_p} = a' + b'S \qquad (1)$$

where S = the starch concentration,

m_x = the mobility of any protein,

m_p = the mobility of the reference protein prolactin;

a' = a constant characteristic for each protein representing the logarithm of the ratio of its mobility to that of prolactin at zero starch concentration,

and b' = a constant characteristic for each protein representing the retardation of mobility imposed by increasing starch concentration.

The value of a' for any protein is subject to the errors involved in extrapolation. Its antilogarithm as a measure of mobility at zero starch concentration

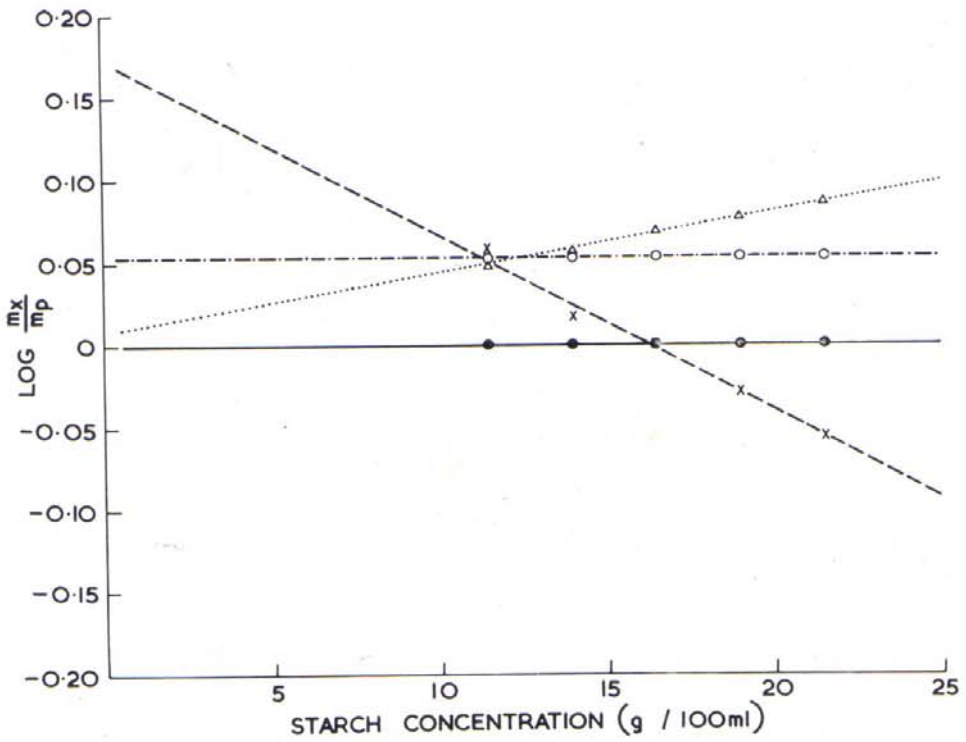

Fig. 4.—Relation of log $\frac{m_x}{m_p}$ to starch concentration ●——● m_x = prolactin; ×——× m_x = albumin; ○—.○ m_x = prolactin second component; △....△ m_x = main component fraction 11 in figure 2.

(as a fraction of the mobility of prolactin) is subject to the validity of equation (1) outside the range of experimental data.

It may be seen that the use of relative mobility has increased the accuracy of distinction between the constants for different proteins compared with the constants describing the relations shown in figure 3.

The value of b′ may be expected to depend on the gelling properties of the particular batch of starch employed and this was confirmed experimentally. However, as may also be expected, the value of a′ was found to be independent of the batch of starch. This dependence of b′ was found to be removed if the relative mobility of two reference proteins was used as an index of the starch concentration or gel porosity. Necessarily the two reference proteins had to show different degrees of retardation due to increasing starch concentration.

Taking prolactin and albumin as reference proteins, the relationship assumes the form shown in figure 5.

The relation is expressed by the equation

$$\log \frac{m_x}{m_p} = a + b \log \frac{m_a}{m_p} \qquad (2)$$

where m_a = the mobility of albumin and

a = a constant characteristic for each protein representing the loga-

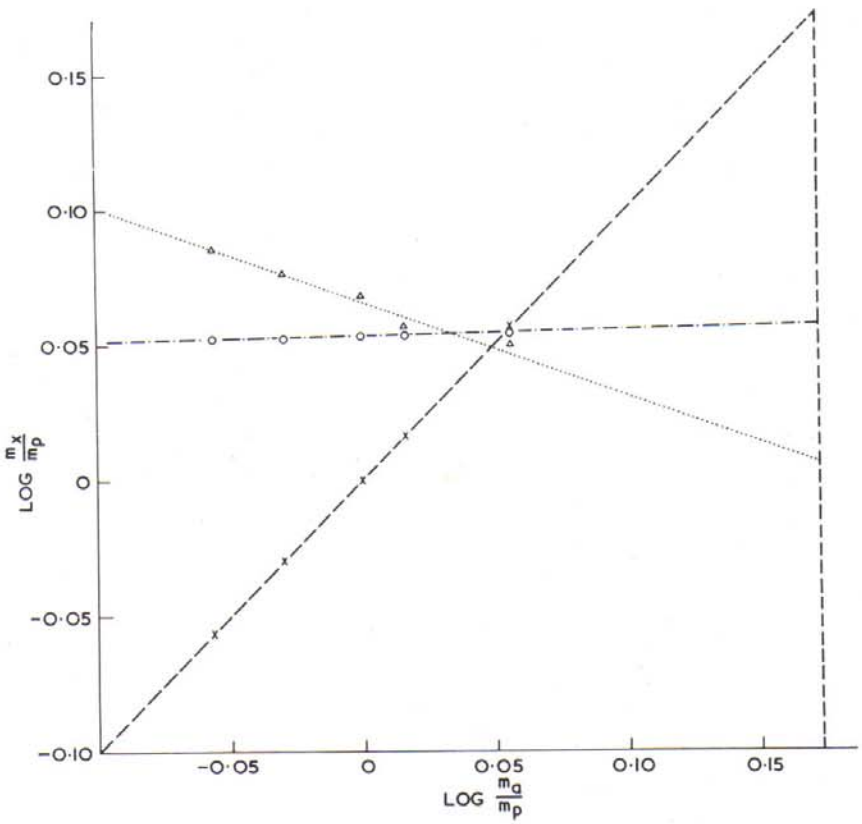

Fig. 5.—Relation of log $\frac{m_x}{m_p}$ to log $\frac{m_a}{m_p}$ ×——× m_x = albumen; ○–·–·–○ m_x = prolactin second component; △. . . .△ m_x = main component fraction 11 in figure 2.

rithm of the ratio of its mobility to that of prolactin at a starch concentration producing equal mobilities of the two reference proteins.

b = a constant characteristic for each protein representing the change in the logarithm of the relative mobility per unit change in the logarithm of the relative mobility of the two reference proteins where this has been changed by altering the starch concentration. b will have positive values for proteins more retarded than prolactin by increasing starch concentration and negative values for proteins less retarded than prolactin. The value of b presumably largely reflects molecular size, but molecular shape and adsorption might also be concerned. It is of interest to note that the conversion form of prolactin has a value of b close to zero indicating a similar molecular weight to the main component of prolactin.

Equation (2) does not specify any value of the relative mobility of the two reference proteins corresponding to zero starch concentrations. However, from equation (1), a′ for albumen was 0.174: hence the antilogarithm of (a + .174b) represents the mobility of proteins relative to prolactin at zero starch concentration. More accurate estimates of the retarding power of the starch gel are given by the relative mobility of two reference proteins that are more affected by starch concentration than albumen and prolactin. However, the

Table 1.—*Distribution Coefficients (Kd) of Proteins in Different Grades of Sephadex*

Protein	G25	G50	G75	G100	G200
Albumin	0	0.00	0.05	0.17	0.44
Growth hormone (basic components)	0	0.07	0.17	0.34	0.59
Prolactin	0	0.14	0.28	0.51	0.67

0.15 M NH_4HCO_3 used as eluant except in case of G100 where 0.15 M NaCl used. Ox pituitary extract instead of sheep used for experiment on G50.

common occurrence of these two proteins in pituitary extracts makes them convenient markers.

For proteins moving to the cathode, basic reference proteins should be selected because variations in gel pH will have reverse effects on the mobility of basic and acidic proteins. However, the procedure for the calculation of the constants is similar. The classification of basic proteins and peptides is not considered in detail in this paper.

The Relation of the Starch-Gel Retardation Coefficient to the Distribution Coefficient on Sephadex G75

Under suitable conditions, proteins and peptides are eluted from columns of cross-linked dextran largely in the reverse order of their molecular weights although adsorption of aromatic and basic substances or exclusion of acidic substances play a part.[5]

Gel filtration is primarily a preparative procedure and the absorbance distribution in the eluate does not distinguish large numbers of proteins or peptides with similar distribution coefficients. However, when starch gel electrophoresis is carried out on successive fractions, distribution coefficients can be calculated for particular components characterized by electrophoresis.

Experiments with commercially available grades of Sephadex G25, G50, G75, G100 and G200 were carried out to find the grade giving optimum separation of the majority of proteins and peptides in pituitary extracts. The molecular weight limits for complete exclusion of polysaccharides[3] appear to be too low for proteins. Thus albumin (mol. wt. 65,000) is slightly retarded in G75 with Kd = 0.05. Dextrans with molecular weights above 40,000 were found by Flodin[3] to be unretarded in G75.

The distribution coefficients of sheep albumin, growth hormone (basic components) and prolactin on different grades of Sephadex are given in table 1. The differences in distribution cofficients do not reveal the best grade for separating these three proteins apparently due to increasing EHTP (equivalent height of a theoretical plate) with increasing retardation. Thus best separation was obtained with G75 despite greater differences in Kd for G100. All known pituitary hormones appear to have greater Kd values than albumin so that the use of G100 or G200 to expand the upper molecular weight range is not required for the fractionation of known pituitary hormones. Figure 6 shows the absorbance distribution of the eluate after gel filtration of the sodium chloride extract of sheep pituitaries on G75. The distribution of electro-

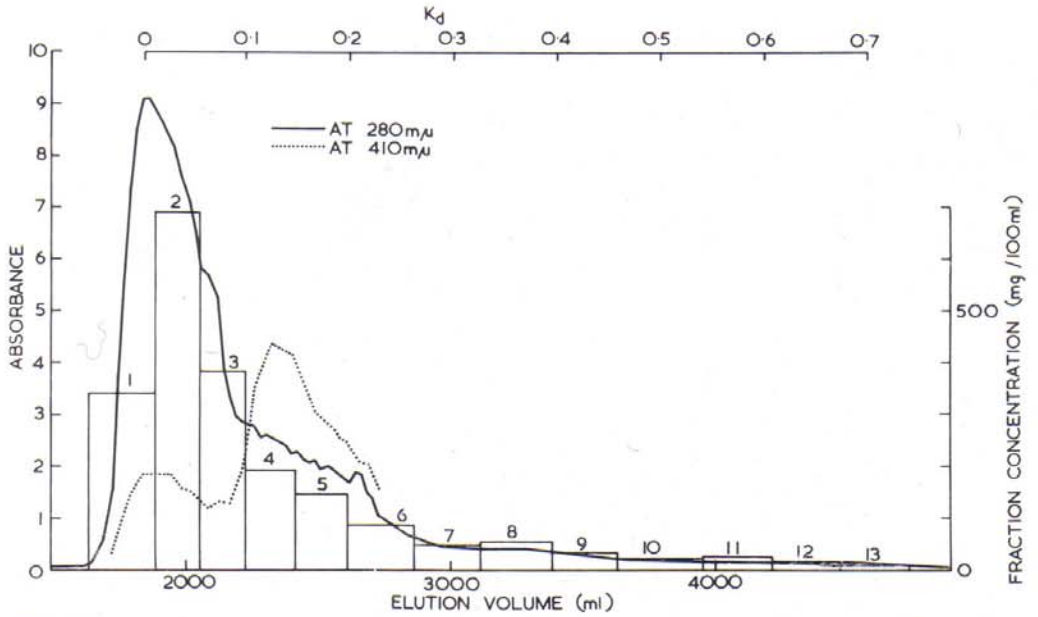

Fig. 6.—Absorbance distribution after gel filtration on Sephadex G75 of 5 Gm. of a 1 per cent NaCl extract of sheep pituitaries. Bed volume 6150 ml., height 86.4 cm. Elution with 0.15 M NH_4HCO_3.

phoretic components in successive fractions in a starch gel containing 14 Gm. starch per 100 ml. is shown in figure 7. Electrophoresis in a gel containing 19 Gm. starch per 100 ml. allowed calculation of relative retardation coefficients (b) and extrapolated relative mobilities at zero starch concentration (antilog a + 0.174 b) as defined earlier. The values were calculated only for the main components which could be definitely identified at both starch concentrations.

The relation of the relative retardation coefficients of the components to the distribution coefficients on G75 is shown in figure 8. The correlation coefficient is −0.59 (95 per cent fiducial limits −0.76 to −0.35) which indicates that both parameters are largely dependent on the same property of the molecules, presumably molecular size. However, the correlation is not as high as one would expect if only errors of measurement of both variables were present. Factors other than molecular size thus probably contribute differentially to the values obtained by the two procedures. However, this increases the discriminating power of a two-way classification by both criteria.

The correlation of the relative retardation coefficient (b) and relative mobility at zero starch concentration (antilog a + 0.174b) was found to be small ($r = 0.34$) and statistically not significant for the same components. This result suggests that net charge does not substantially influence the value of b and that the two parameters are essentially independent.

The Relation of Mobility to Temperature

On theoretical grounds the effect of temperature in increasing electrophoretic mobility may be expected to be mediated by the effect of viscosity, charge density being assumed to remain constant.[6] As with viscosity, Henry's equation makes no allowance for differential effects of temperature on the mobility of different proteins.

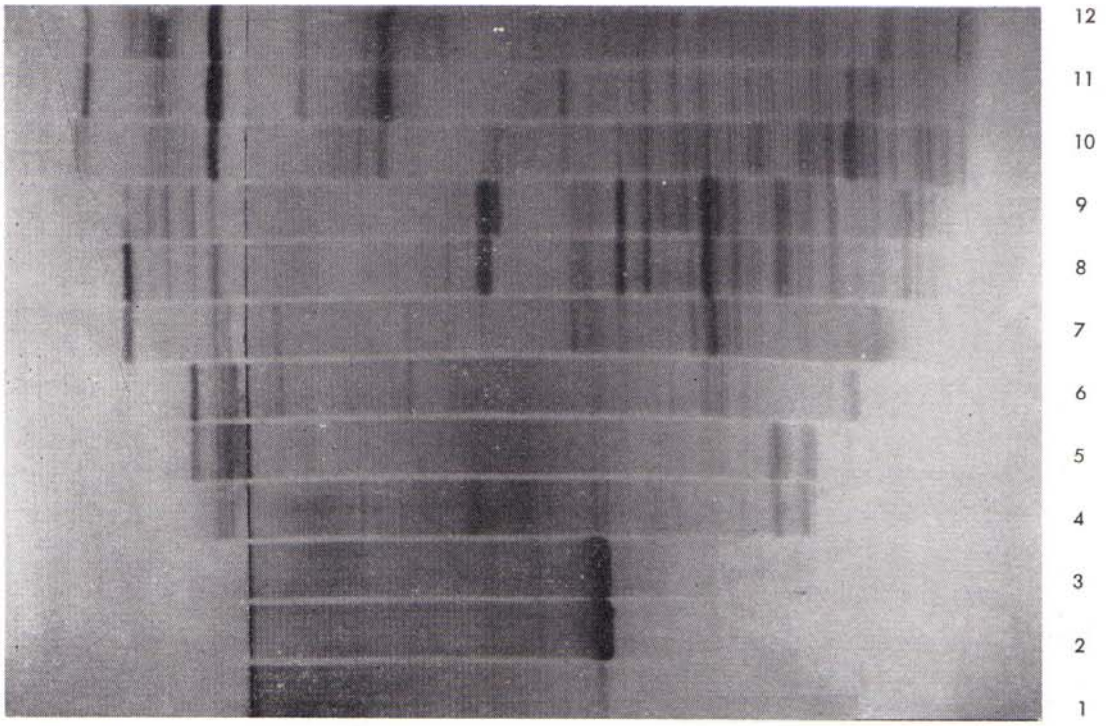

Fig. 7.—Electrophoresis of fractions obtained from gel filtration of a 1 per cent NaCl extract of sheep pituitaries (fig. 6.). Mean voltage gradient 20.3 volts per cm., 267 minutes. Starch concentration 14 Gm. per 100 ml. Cooling temperature 30 C.

The Tiselius apparatus is generally operated to give a temperature of 4 C. to minimize convection and few studies have been reported on differential effects of temperature. Using zone electrophoresis on paper, McDonald et al.[7] did not find appreciable differences between the temperature coefficients of substances with different molecular weights but did not study a range of different proteins.

Ferguson and Wallace[2] reported differential effects of temperature on the relative mobility of the active components of sheep prolactin and human growth hormone. Although it was suggested that the temperature coefficient might be related to molecular size, the log function of relative mobility was not used and no correction was made for the effect of the mean mobility on the coefficient.

The relation of mobility to the cooling temperature for the four proteins examined earlier is shown in figure 9. The data were derived from the experiments of Ferguson and Wallace[2] and the electrophoretic conditions were slightly different to those of the other experiments described in this paper. Based on single determinations the data are not sufficiently accurate to distinguish between several alternative mathematical forms for the relationship, and the simplest function of temperature has been used.

By comparison of figures 3 and 9, it may be seen that a change in temperature from 0 to 30 C. produces about the same effect on mobility as a change in starch concentration from 11.5 to 21.5 Gm. per 100 ml. However, effects on relative mobility are less marked for temperature. This is made more ap-

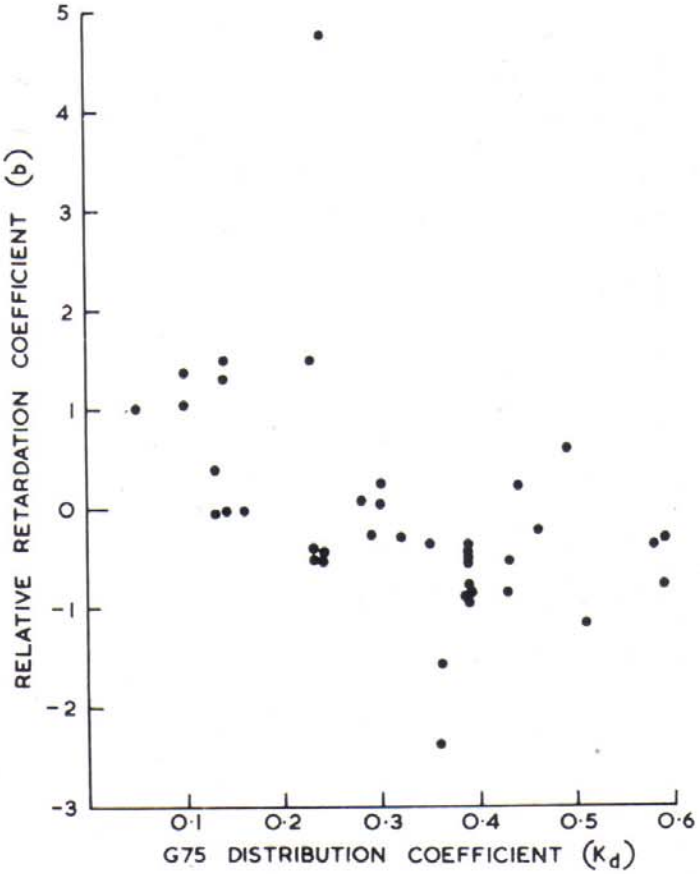

Fig. 8.—Relation of relative retardation coefficient (b) to distribution coefficient on G75 (Kd) for some of components shown in figure 7.

parent in fig. 10 where the mobility of the proteins relative to that of prolactin is related to temperature. Comparison with figures 4 and 5 shows that not only is the effect of temperature on relative mobility less marked, but also the different proteins are not affected in the same way by changes in temperature and starch concentration.

The temperature coefficients, i.e., the change in log relative mobility per C. (b_t), were calculated for the same proteins shown in figure 7 for which the relative retardation coefficients and relative mobilities at zero starch concentration had been calculated.

The data for the three parameters together with the corresponding G75 distribution coefficients are listed in table 2 in order of increasing relative mobility. The absence of any marked correlation between the three electrophoretic characteristics is illustrated in figure 11. High temperature coefficients occur more in the proteins with low relative mobilities and high retardation coefficients. However, the correlation between temperature and retardation coefficients is low ($r = 0.28$) and not statistically significant, confirming that starch concentration and temperature do not affect relative mobility in the same manner.

Albumin has the lowest temperature coefficient of those measured. The essential independence of retardation coefficient and relative mobility may be seen as noted earlier.

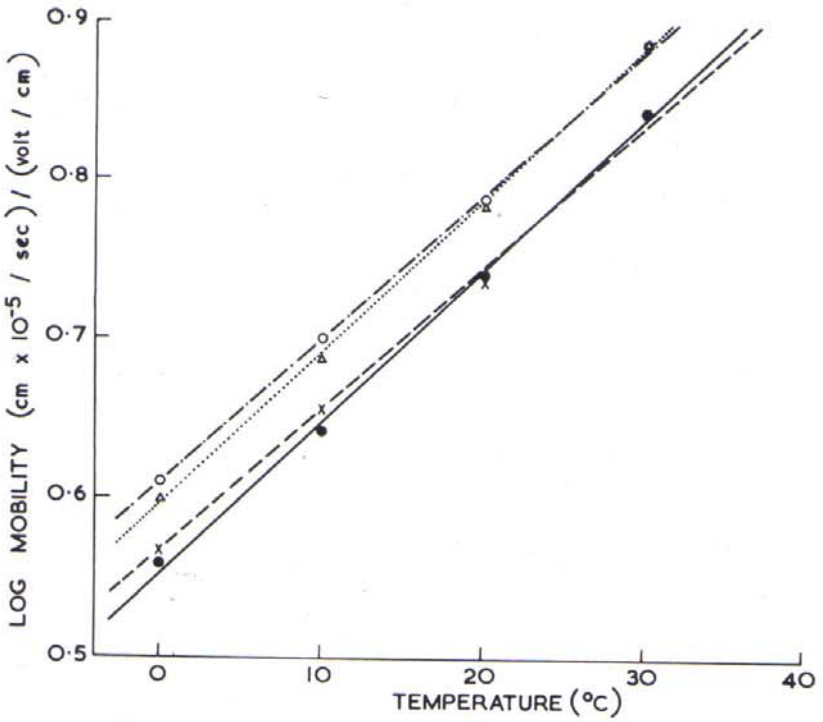

Fig. 9.—Relation of log mobility to cooling temperature. ●——● prolactin; ×– – –× albumin; ○–.–.–○ prolactin second component; △. . .△ main component fraction 11 in figure 2. Starch concentration 13 Gm. per 100 ml. 0.16 M Tris buffer system. Constant voltage gradient 23.3 volts/cm.

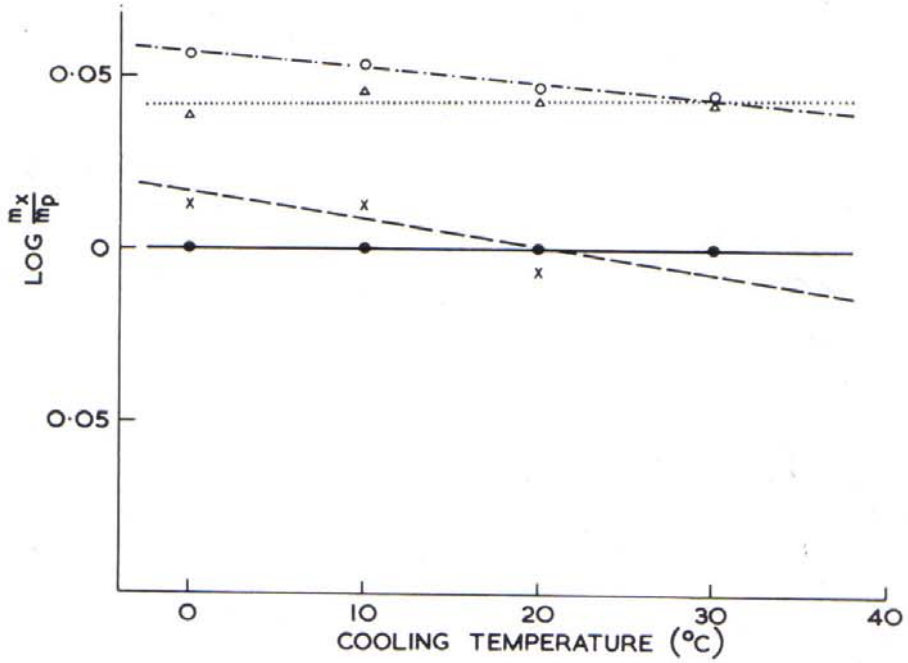

Fig. 10.—Relation of log $\frac{m_x}{m_p}$ to cooling temperature. ○——○ m_x = prolactin; ×– – –× m_x = albumin; ○–.–.–.○ m_x = prolactin, second component; △. . .△ m_x = main component fraction II in figure 2.

Table 2.—*Electrophoretic and Gel Filtration Parameters for Some Acidic Proteins and Peptides in Sheep Pituitary Extracts*

Relative Mobility Antilog a + 0.174b	Relative Retardation Coefficient b	Temperature Coefficient $b_t \times 10^3$	Distribution Coefficient Kd(G75)	Relative Mobility Antilog a + 0.174b	Relative Retardation Coefficient b	Temperature Coefficient $b_t \times 10^3$	Distribution Coefficient Kd(G75)
0.16	−2.38	—	0.36	1.08	−0.53	0.52	0.23
0.27	−1.56	—	0.36	1.11	0.09	—	0.28
0.32	0.24	5.51	0.30	1.17	−0.54	0.56	0.39
0.38	−0.30	6.72	0.59	1.17	1.49	0.33	0.14
0.42	0.57	0.88	0.49	1.18	−0.47	0.54	0.39
0.51	−0.95	1.28	0.39	1.19	−0.87	1.07	0.39
0.52	0.15	1.48	0.24	1.19	−0.45	0.81	0.24
0.53	0.39	4.37	0.13	1.20	−0.54	−0.32	0.43
0.54	−0.89	1.53	0.39	1.23	−1.16	1.20	0.51
0.55	1.35	5.48	0.10	1.23	−0.79	0.79	0.39
0.55	−0.36	0.37	0.35	1.23	−0.30	0.18	0.32
0.56	1.02	3.81	0.10	1.25	−0.20	0.29	0.46
0.69	−0.95	1.23	0.58	1.25	−0.42	1.08	0.23
0.75	1.48	3.19	0.23	1.31	−0.25	0.54	0.29
0.83	0.22	2.88	0.44	1.32	1.28	0.41	0.14
1.00	−0.77	0.48	0.59	1.33	−0.86	1.04	0.43
1.00*	0.00	0.00	0.28	1.49†	1.00	−1.34	0.05
1.01	−0.55	−0.17	0.39	1.53	−0.54	1.86	0.24
1.01	−0.36	0.14	0.39	1.59	−0.04	0.99	0.14
1.06	0.06	—	0.30	1.69	−0.03	1.72	0.16
1.07	−0.04	0.83	0.13				

*Prolactin.
†Albumin.

Electrophoretic Parameters of Some Pituitary Proteins and Peptides with Hormonal Activity

The three parameters antilog (a + 0.174b), b and b_t were calculated for some proteins and peptides which possess hormonal activity. In no case, however, is it certain that they are the circulating hormones.

The electrophoretic patterns of the preparations used are shown in figure 12. Preparations of sheep and ox growth hormone are included for comparison although the parameters of the components of these preparations have not been calculated. The three characteristics for the components marked in figure 12 are listed in table 3. Where the parameters are given for more than one component in a preparation the components are listed in order of increasing migration in figure 12.

The active components of the pig and whale growth hormone preparations have not been positively identified but the components marked are those which have been found to be present in all preparations of these hormones examined. Likewise the activities of the components believed to be pig ACTH A2 and βMSH have not been assayed biologically. The activity of the components in the preparations of pig peptide I and human FSH have been reported.[8,9] However, in the latter case the correspondence of the components

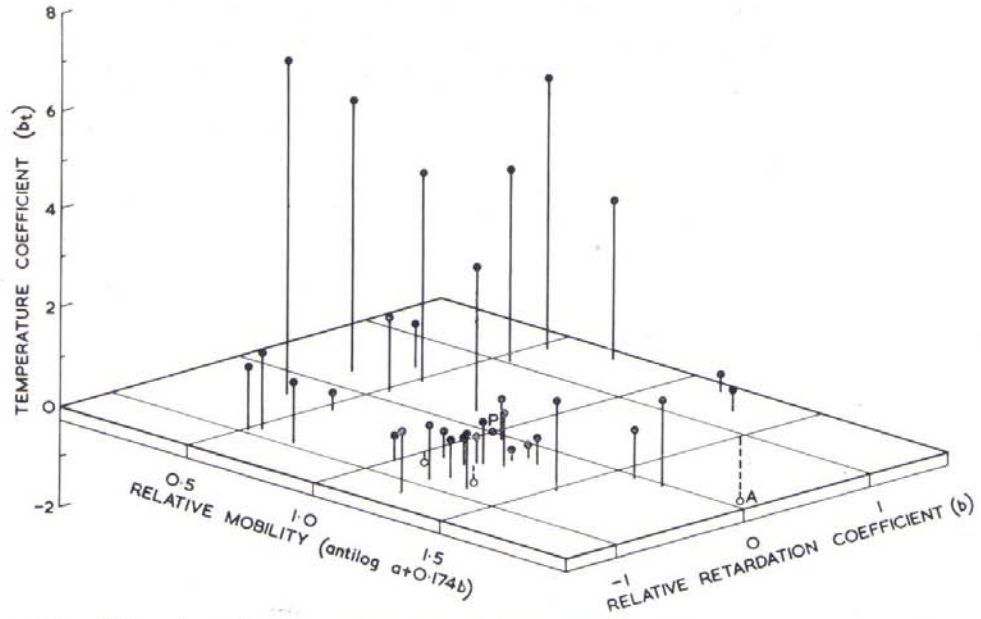

Fig. 11.—The distribution of values of relative mobility at zero starch concentration antilog (a + 0.174b), relative retardation coefficient (b) and temperature coefficient (b_t) in some acidic proteins extracted from sheep pituitaries.

assayed by Butt et al.[9] and the one marked for the FSH preparation in figure 12 is uncertain. Butt obtained a greater number of components in this very potent FSH preparation than shown in figure 12. This is probably due to the use of a greater quantity of sample (5 mg.) and a shorter migration than used in the present experiment. Active components of human growth hormone and of ox and sheep prolactin have been identified.[2]

Also shown in table 3 are published values for the molecular weights of some hormone preparations. The molecular weight determinations were probably made on preparations as heterogeneous as those illustrated in figure 12 and do not necessarily represent the weights of the components for which the parameters have been calculated.

The relation of the relative retardation coefficient (b) to molecular weight is shown in figure 13 and suggests that b is a linear function of molecular weight. The two points which do not fit the relationship are those of pig and whale growth hormone. The retardation coefficients for these proteins indicate higher molecular weights than those published. However, the relationship requires examination for a greater number of proteins than shown in figure 13. The molecular weight for sheep albumin has been taken as the same as that for human albumin since the albumins of these two species have similar retardation coefficients. Following the discussion of Hughes,[10] the value of 65,000 has been taken rather than the higher values that have been reported for albumin. As Phelps and Putman[11] point out, the wide range in values found for albumin is a serious reflection on the present status of methods for the determination of molecular weight of proteins.

Referring again to table 3 and figure 12, it may be seen that the two most prominent components of sheep and ox prolactin have similar values of b and

Table 3.—*Electrophoretic Parameters for Some Proteins and Peptides with Hormonal Activity*

Species	Component	Relative Mobility Antilog $a + 0.174b$	Relative Retardation Coefficient b	Temperature Coefficient $b_t \times 10^3$	Molecular Weight
Pig	peptide I	1.32	−0.51	−0.29	5,000[8]
Pig	βMSH	0.95	−0.70	0.31	2,177[13]
Pig	ACTHA2	0.30	−0.52	−0.25	4,551[14]
Sheep	Albumin	1.49	1.00	−1.22	65,000[10]
Human	FSH	1.63	0.84	−0.84	—
Ox	prolactin	1.19	0.49	−0.33	—
	prolactin	1.12	0.02	0.14	26,000[15]
	prolactin	1.23	−0.02	−0.26	—
Sheep	prolactin	1.36	0.51	−0.32	—
	prolactin	1.00	0.00	0.00	23,300[12]
	prolactin	1.16	0.03	−0.28	—
Human	GH	0.94	−0.01	−0.41	—
	GH	0.99	−0.01	−0.18	27,100[15]
	GH	1.15	0.04	−0.21	—
Whale	GH	0.24	1.04	7.92	39,900[15]
Pig	GH	0.29	1.32	8.89	41,600[15]

are presumably the same in molecular size whereas the minor marked components of both preparations have values of b corresponding to molecular weights double that of the major components. This suggests that the minor components may be dimers although the prolactin activity of these components remains to be fully established. Unpublished experiments on the electrophoresis of fractions of sheep serum obtained by gel filtration showed that the albumin dimer is substantially if not completely split into the monomer by the electrophoretic conditions employed. There is evidence in figure 2 of the formation of sheep prolactin dimer which is also split on electrophoresis. Thus there may be both reversible and irreversible forms of the prolactin dimer. However, the degree of polymerization of sheep prolactin after freeze drying found by Squire et al.[12] was not seen in these experiments.

The three active components of human growth hormone have similar retardation coefficients to those of the major components of sheep and ox prolactin and are presumably similar in molecular size. There is no evidence of dimer formation.

The main components of human growth hormone and sheep prolactin are similar in relative mobility and retardation coefficient but they are distinguished by different temperature coefficients. The phylogenetic relations of human growth hormone and sheep prolactin are still uncertain. The components of sheep prolactin possess only slight growth activity in the hypophysectomized rat (Wallace and Ferguson, unpublished) but sheep prolactin produces metabolic responses in humans similar to those caused by human growth hormone.[16,17] The growth activity of sheep prolactin in the sheep has not been reported.

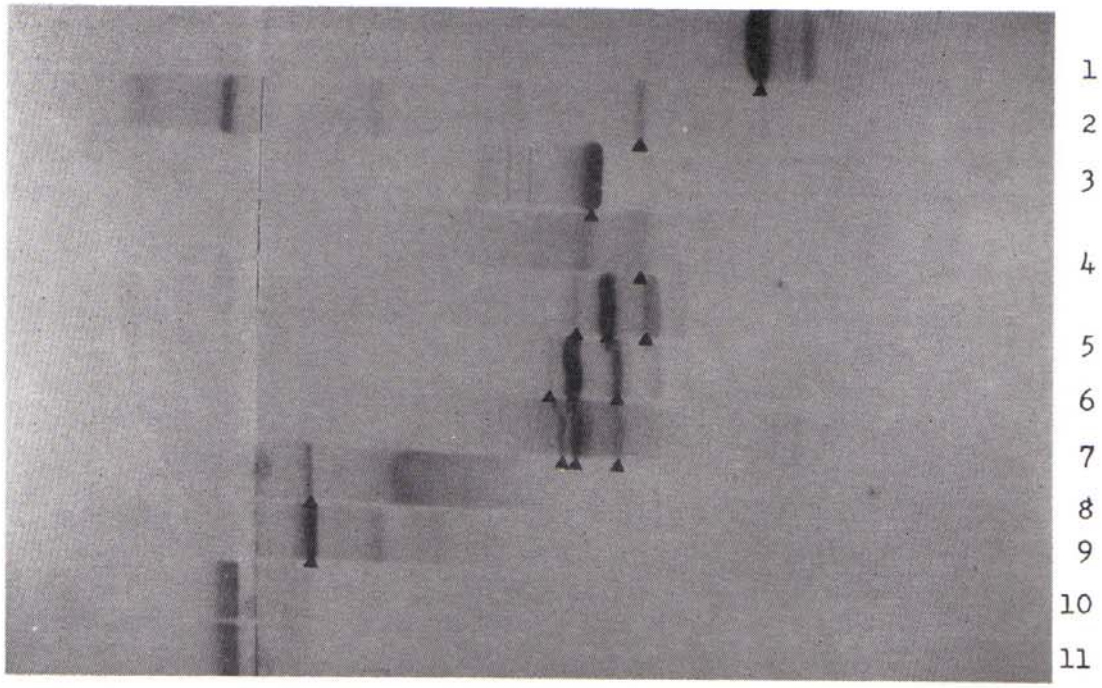

Fig. 12.—Electrophoresis of preparations possessing hormonal activities. (1) 0.5 mg. pig peptide I Astwood; (2) 1 mg. pig ACTH A 5602 Ferring; (3) 0.5 mg. sheep albumin 20 II; (4) 1 mg. human FSH CPI Butt; (5) 1 mg. ox prolactin 11 II; (6) 1 mg. sheep prolactin 14 IV; (7) 1 mg. human GH $42F_4$; (8) 1 mg. whale GH 51C; (9) 1 mg. pig GH 50C; (10) 0.5 mg. ox GH 49B; (11) 0.5 mg. sheep GH 71S:1. Starch concentration 14 Gm. per 100 ml. Mean voltage gradient 19.2 volts per cm. for 240 minutes.

Discussion

Three parameters for the characterization and classification of pituitary proteins and peptides based on starch gel electrophoresis have been proposed. The relative mobility at zero starch concentration and relative retardation coefficient are clearly functions of net charge and molecular size respectively but the temperature coefficient is more empirical and to this extent less informative.

The simple mathematical relations concerned enable the calculation of the constants from three electrophoretic experiments for a very large number of proteins and peptides. The present paper describes the derivation of the constants and the calculation of their approximate values for a number of proteins and peptides. Calculation of precise values and their fiducial limits has not been attempted. However, preliminary evidence suggests that the values of antilog $(a + 0.174b)$ and b are estimated more accurately from single determinations than corresponding estimates of free solution mobility in the boundary apparatus and molecular weight from sedimentation and diffusion data.

Although the starch gel electrophoretic parameters apply only to specified experimental conditions, this is also true of the classical criteria. The use of basic units in terms of reference proteins to characterize mobility and mo-

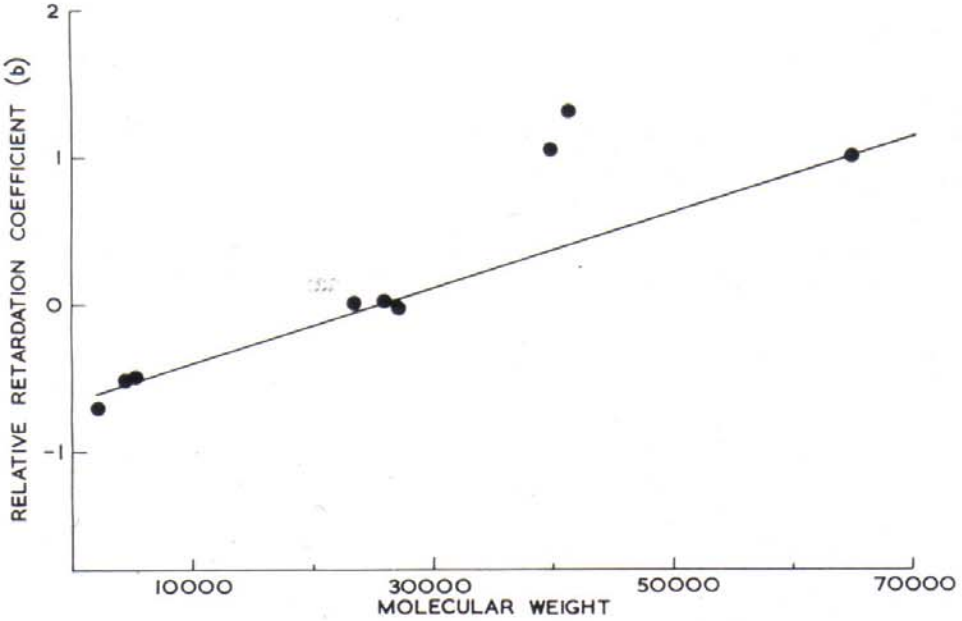

Fig. 13.—The relation of relative retardation coefficient (b) to published values of molecular weight for some proteins and peptides with hormonal activity.

lecular size eliminates some sources of experimental error. Apart from misidentification and remote possibilities of genetic polymorphism, the reference proteins must remain perfectly constant in behavior whereas attempts to express mobility and size in absolute terms have to contend with problems of calibration and instrumental error.

Although identical values for the three characteristics do not prove identity of primary, secondary and tertiary structure, additional specificity is provided by the species of pituitary and the extraction and fractionation procedure carried out prior to electrophoresis. Gel filtration has been found to be particularly useful in providing such preparative characterization.

Starch gel electrophoresis shows a greater capacity to split aggregates than gel filtration and the combination of both procedures is useful in detecting polymerization and the binding of peptides to proteins.

The electrophoretic parameters proposed can be as readily derived for the soluble proteins of tissues other than the pituitary but in such cases it may be convenient to select reference proteins from the tissue concerned.

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