Some Physical and Chemical Properties of an M-2 Glycoprotein Isolated from Normal Bovine Plasma*

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An acidic glycoprotein has been isolated in an electrophoretically and ultracentrifugally pure form from plasma of normal Holstein cattle. Its molecular weight was determined by the sedimentation-diffusion, the sedimentation-viscosity, and the Archibald methods, which gave virtually identical figures at *p*H 7.0 of 49,000, 48,000, and 48,000 g/mole, respectively. The molecular shape of the glycoprotein, as determined by viscosity and diffusion studies, was very sensitive to changes in *p*H. The glycoprotein formed aggregates when dissolved in aqueous solutions at *p*H 3 or below, or when it was heated at any *p*H value. The aggregation was not reversible. The glycoprotein did not bind thyroxine in native state, but acquired thyroxine-binding properties when heated or subjected to low *p*H. Amino acid and carbohydrate compositions of the glycoprotein were very similar to those of a protein previously isolated.

An acidic glycoprotein has been recently isolated from plasma of "normal" Jersey heifers and was partially characterized (Bezkorovainy and Doherty, 1962a). This protein was in many respects similar to the human M-2 glycoprotein (Mehl *et al.*, 1949; Bürgi and Schmid, 1961), and was termed bovine M-2 glycoprotein. Molecular weight of the bovine M-2 glycoprotein was 39,000 according to the viscosity-sedimentation method, and its thyroxine-binding properties were similar to those of bovine serum albumin.

In an attempt to improve our source of the M-2 glycoprotein, we changed from the young Jersey animals, undergoing slaughter-house procedures, to dairy cattle maintained at nearly optimum conditions at the National Animal Disease Laboratory. It was found that the blood of dairy cattle contained an M-2 glycoprotein which was nearly identical in its physical-chemical properties, except for its thyroxine-binding properties, to the protein previously isolated. The present communication describes additional properties of the M-2 glycoprotein with special emphasis on its physical behavior under the conditions of electrophoresis, sedimentation, diffusion studies, and viscosity studies, and its affinity for thyroxine. It is also shown that the molecular weight of M-2 glycoprotein, previously reported, is in need of revision.

EXPERIMENTAL

Bovine Plasma Source.—Blood donor animals were female Holstein cattle, 7 to 8 years of age, from the National Animal Disease Laboratory's disease-free inbred herd. Blood was collected by venipuncture into glass jars containing 0.1 volume of 5% EDTA[1] in 0.9% NaCl. Plasma was separated from cells and was processed through ammonium sulfate fractionation and chromatography on carboxymethyl cellulose as previously described (Bezkorovainy and Doherty, 1962a). The yield of M-2 glycoprotein was essentially the same as in the previous paper.

Bovine Serum Albumin.—The crystalline bovine serum albumin preparation used in thyroxine-binding experiments was purchased from Pentex Biochemicals, Inc.

Bovine Orosomucoid.—Bovine orosomucoid was prepared by the carboxymethyl cellulose method (Bezkorovainy and Winzler, 1961) either from Cohn's fraction VI (purchased from Mann Laboratories) or from ammonium sulfate fraction P-4 of plasma (Bezkorovainy and Doherty, 1962a).

Thyroxine.—The L-thyroxine used in equilibrium dialysis experiments was purchased from Mann Laboratories.

Ultracentrifugation.—A Spinco Model E ultracentrifuge was operated at its maximum speed of 59,780 rpm at 20° for the determination of sedimentation constants of protein samples. The method of Archibald, as described by Schachman (1957), was used for direct determination of molecular weights of the M-2 glycoprotein under a variety of conditions. All determinations were made at 20°, all synthetic boundary runs were performed at 9945 rpm, and the approach-to-equilibrium runs were made at 3189 or 6569 rpm. Spinco Silicone oil SL722B-12 was used as the synthetic cell bottom. An enlarger was used in processing the ultracentrifugal photographic plates with an enlargement factor of 5–6:1.

Ionic strength of all buffers used to dissolve protein samples prior to ultracentrifugal analysis was 0.1.

Diffusion.—Diffusion constants of proteins in solution were determined in a 2 ml cell with the Spinco Model H Tiselius apparatus. Bath temperature was 1° and diffusion was permitted to take place for at least 36 hours. At least six pictures of the Schlieren boundary were taken during each run. Boundaries were traced from negatives onto graph paper with the aid of an enlarger (enlargement factor 4:1), and areas enclosed by boundaries were computed by the cutting-weighing method.

Viscosity Measurements.—Relative viscosity of protein solutions was measured with an Ostwald-type viscometer at 22.0° and/or 27.0°. Flow time of water at 22° was 310 seconds. Relative viscosities were plotted against per cent of protein in solution, giving a curve whose slope was identical to the intrinsic viscosity of the protein analyzed.

Partial Specific Volume.—Partial specific volume of the M-2 glycoprotein was determined from composition data as previously described (Bezkorovainy and Doherty, 1962a).

Electrophoresis.—Paper electrophoresis was performed in Spinco Model R apparatus with Whatman No.

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[1] The following abbreviations are used: EDTA, sodium ethylene diamine tetraacetate; Tris, tris(hydroxymethyl)-aminomethane.

1 filter paper strips used at 3 volts/cm for 16–18 hours. Dextran samples were included with each run to correct for endosmosis in electrophoretic mobility calculations. Staining with bromphenol blue was performed according to Durrum (1950).

Moving boundary electrophoresis was done in an Antweiler microelectrophoresis apparatus at 65 volts and approximately 10°, and in a Spinco Model H Tiselius apparatus with the 2-ml cell used at 1°.

All buffers used in the paper and Antweiler electrophoresis had ionic strength of 0.05, while those used in the Tiselius apparatus had ionic strength of 0.1.

Spectrophotometric and pH Measurements.—Optical densities were determined in a Beckman DU spectrophotometer, and *p*H was measured with a Photovolt Model 125 *p*H meter.

Composition Analysis.—Amino acids were determined by the method of Moore *et al.* (1958), and the analysis was partially carried out by Oxford Laboratory, Redwood City, Calif. Sialic acid, hexoses, hexosamines, and the *N*-terminal amino acid were estimated as previously described (Bezkorovainy and Doherty, 1962a). Moisture in protein samples was determined by drying the samples at 110° (refluxing toluene) over P_2O_5 at reduced pressure for 4 hours. Ash content of the glycoprotein was determined after incineration in a porcelain crucible in a muffle furnace at 650° for 3 hours. All composition results reported have been corrected for moisture and ash. Tryptophan and tyrosine were determined spectrophotometrically according to Bencze and Schmid (1957). Concentration data used in sedimentation and diffusion constant calculation, and calculations of intrinsic viscosity, were determined by analysis of sialic acid by the direct Ehrlich method (Werner and Oudin, 1952).

Thyroxine-Binding Activity Measurements.—Protein samples were examined for thyroxine-binding activity by the equilibrium dialysis method. One ml of protein solution (1–3 mg/ml) in 0.9% aqueous NaCl at *p*H 9.2 (previously dialyzed against *p*H 9.2, 0.025 M glycine buffer in 0.9% NaCl) was dialyzed (Visking casings, 1 cm i.d.) against 8 ml of $0.5 - 5 \times 10^{-5}$ N L-thyroxine in 0.9% aqueous NaCl buffered at *p*H 9.2 (0.025 M glycine) in the presence of 0.001 M EDTA. Dialysis proceeded for 24 hours at 4° and 27°. Agitation was attained by mounting plastic centrifuge tubes, containing the bag immersed in a solution of thyroxine, on a mechanical stirrer and permitting the latter to rotate at 30–60 rpm, taking care to keep the plastic tubes immersed in a water bath of the appropriate temperature. At the end of a dialysis period 0.7 ml of the contents of each bag was removed and diluted to 3 ml with water. Protein concentration in each diluted sample was determined by sialic acid analysis, and the molar concentration of protein was calculated on the basis of 10% sialic acid content and unit molecular weight of 48,000. Thyroxine was then determined spectrophotometrically at 330 mμ in the diluted protein-bound thyroxine solution, and in the thyroxine solution against which the protein was dialyzed. All diluted protein-bound thyroxine readings were corrected for absorbancy given by an identical concentration of thyroxine-free protein. Association constants and maximum binding sites were computed by plotting data according to Scatchard (1949), and binding energies and entropies were calculated by the free-energy-equilibrium constant equation, the integrated form of the Gibbs-Helmholtz equation, and the $\Delta F - \Delta S$ relationship.

Results

Electrophoresis.—M-2 glycoprotein samples (10–40 mg/ml) were examined by paper electrophoresis at *p*H 2.2 (phosphate), 3.3 (formate), 4.5 (acetate), 6.6 (phosphate), 8.6 (Tris), and 10.5 (glycine). In all cases a single, well-defined zone was observed, indicating homogeneity by this method. When distance migrated by the protein zone was corrected for that of dextran, the calculation of approximate paper electrophoretic mobilities, as described by Block *et al.* (1958), became possible. Figure 1 shows a relationship between *p*H and approximate mobilities of the M-2 glycoprotein and bovine orosomucoid. The isoelectric point of bovine M-2 glycoprotein, according to this method, was at *p*H 3.8, while that of the bovine orosomucoid was at *p*H 2.8. M-2 glycoprotein was also examined by the moving-boundary electrophoresis method at *p*H 2.2, 3.0, 4.5, 6.5, 6.6, and 8.6. All protein concentrations were 10 mg/ml. Figure 2 illustrates some of the patterns obtained in the Spinco and Antweiler machines. It can be seen that an impurity, accounting for some 5% of the total protein present, appears in the Tiselius apparatus at *p*H 3.

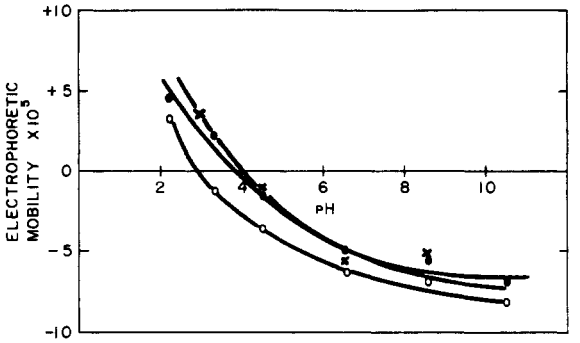

FIG. 1.—Electrophoretic mobility (on paper, corrected for endosmosis) of the bovine M-2 glycoprotein (filled circles), bovine orosomucoid (open circles), and M-2 glycoprotein in the Tiselius apparatus (crosses).

Ultracentrifugal Analysis.—M-2 glycoprotein was analyzed ultracentrifugally at concentrations of 3–11 mg/ml at *p*H 11.3 (0.1 M Na_2CO_3), 7.0 (phosphate), 3.8 (acetate), and 3.0 (formate). Figure 3 shows the ultracentrifugal patterns obtained at the above *p*H values. It can be seen that the preparation was homogeneous at *p*H 11.3, 7.0, and 3.8, but appeared to have at least two components at *p*H 3.0. Figure 4 shows the relationship between sedimentation constants and concentration of M-2 glycoprotein at *p*H 7.0 and 3.8. The curve extrapolated to an $s^\circ_{20,w}$ of 3.8 at *p*H 7.0 and to 3.9 at *p*H 3.8 at infinite dilution. The two peaks, observed when M-2 glycoprotein was examined ultracentrifugally at *p*H 3.0, had sedimentation constants of 3.6 and 8.1 at a total protein concentration of 1%.

Diffusion Studies.—The diffusion constant of M-2 glycoprotein was determined in phosphate buffer at 7.0, ionic strength 0.1, and in acetate buffer at *p*H 3.8, ionic strength 0.1. Observed diffusion constants were corrected for viscosity and temperature and were plotted against concentration. The resulting straight lines (Fig. 5) were extrapolated to infinite dilution giving $D^\circ_{20,w}$ of 6.5×10^{-7} cm²/sec. at *p*H 7.0 and 5.5×10^{-7} cm²/sec. at *p*H 3.8.

Viscosity Studies.—Viscosities of M-2 glycoprotein dissolved in water, in *p*H 7 phosphate buffer ($\mu = 0.1$), in *p*H 3.8 acetate buffer ($\mu = 0.1$), and in *p*H 2.8 phosphate buffer ($\mu = 0.1$) were determined at 22.0° and/or 27.0° (Fig. 6). Intrinsic viscosities of M-2 glycoprotein were 0.0455 at both 22.0° and 27.0°, 0.0440 at 22.0°, and 0.0650 at 22.0° at *p*H values of 7.0, 3.8, and 2.8 respectively. The intrinsic viscosity of M-2 glycoprotein in water at 22.0° was 0.0360.

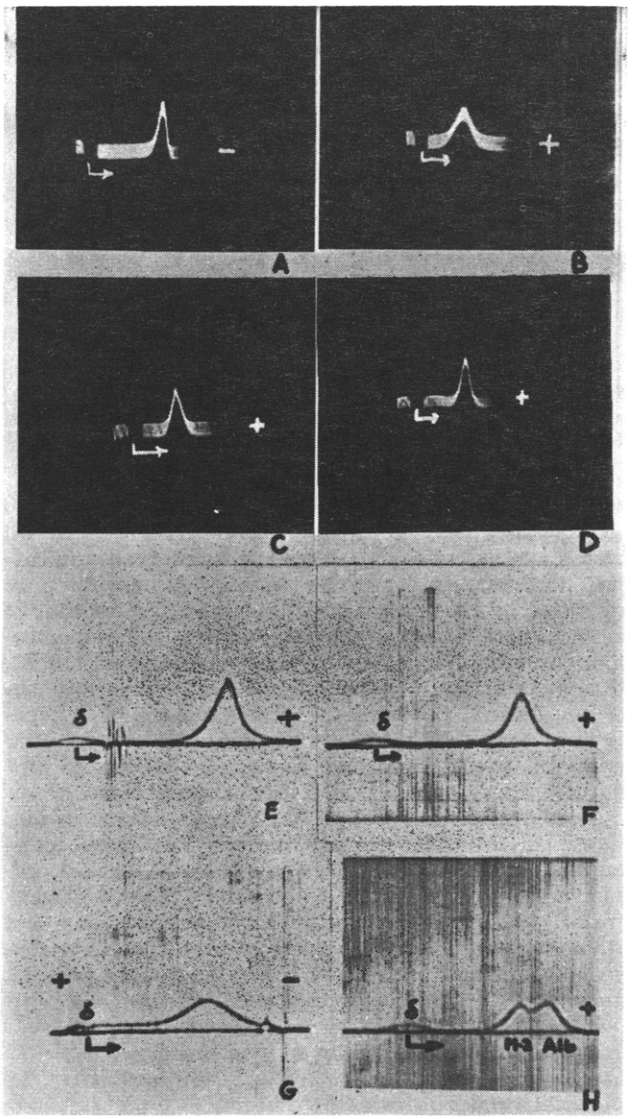

FIG. 2.—Ascending moving boundary (Antweiler and Tiselius) electrophoresis patterns of M-2 glycoprotein. All protein concentrations in the Antweiler experiments were 10 mg/ml, those in Tiselius experiments were 7–8 mg/ml. Antweiler runs were performed at 65 volts, 0.5 mamps, and 10°. *A*, Antweiler run in *p*H 2.2 phosphate buffer, 20-min. picture. *B*, Antweiler run in *p*H 4.5 acetate buffer, 20-min. picture. *C*, Antweiler run in *p*H 6.6 phosphate buffer, 20-min. picture. *D*, Antweiler run in *p*H 8.6 Tris buffer, 10-min. picture. *E*, Tiselius run in *p*H 6.5 phosphate buffer at 2.5 mamps and 165 volts. Concentration 8 mg/ml, 102-min. picture. *F*, Tiselius run in *p*H 8.6 veronal buffer at 4 mamps and 145 volts. Concentration 7 mg/ml, 113-min. picture. *G*, Tiselius run in *p*H 3.0 formate buffer at 13 mamps and 165 volts. Concentration 7 mg/ml, 316-min. picture. *H*, Tiselius run in *p*H 8.6 veronal buffer at 4 mamps and 145 volts of a 1:1 mixture of M-2 glycoprotein and bovine serum albumin at a total concentration of 8 mg/ml, 107-min. picture.

Composition of Bovine M-2 Glycoprotein.—Carbohydrate and amino acid composition of the M-2 glycoprotein is presented in Table I. Moisture content of the protein was 5%, ash content was 3%, and all values in Table I have been corrected for moisture and ash. *N*-terminal amino acid analysis of native M-2 glycoprotein showed the presence of DNP-leucine and, in some preparations, a trace of DNP-aspartic acid in the ether phase.

Partial Specific Volume, Molecular Weight, and Axial Ratio Calculations.—Partial specific volume was calculated from composition data given in Table I and was 0.71 cm^3/g.

Molecular weight of M-2 glycoprotein was calculated from sedimentation-diffusion, sedimentation-viscosity ($\beta = 2.16 \times 10^6$), and Archibald method data (Schachman, 1957). At *p*H 7 (phosphate, $\mu = 0.1$), using intrinsic viscosity of 0.0455, $s^\circ_{20,w}$ of 3.8×10^{-13}, and $\bar{V}$ of 0.71, the molecular weight of M-2 glycoprotein was 48,000. Using $D^\circ_{20,w} = 6.5 \times 10^{-7}$, $S^\circ_{20,w} = 3.8 \times 10^{-13}$, and $\bar{V} = 0.71$, the molecular weight was 49,000. Determination of molecular weight of the M-2 glycoprotein by the Archibald method at *p*H 7.0 in phosphate buffer (6569 rpm and diaphragm angle 80°) gave a value of 48,000 ± 1000 g/mole. In Tris buffer, *p*H 7.4 and $\mu = 0.1$, the molecular weight of the M-2 glycoprotein at the above speed and diaphragm angle was also 48,000 ± 1000. In both Archibald runs the molecular weight was independent of time (up to 2 hours) at both the meniscus and bottom of the cell at 1% protein concentration.

At *p*H 3.8 (acetate, $\mu = 0.1$) the molecular weight of

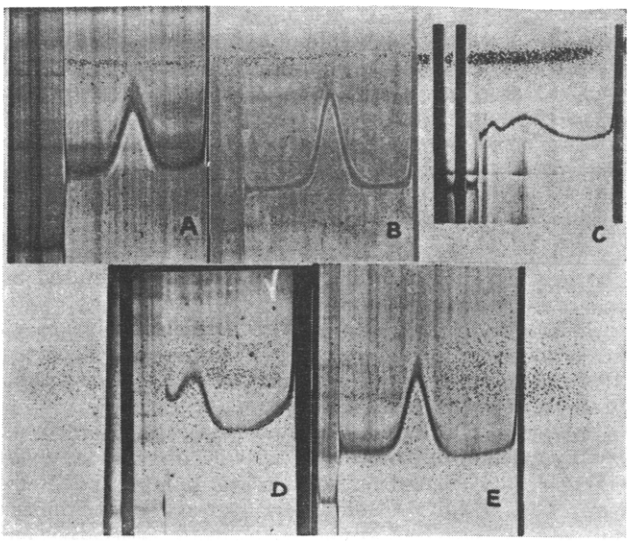

FIG. 3.—Ultracentrifugal analysis of M-2 glycoprotein. All runs proceeded from left to right at 59,780 rpm and 20°. Eight minutes were required to reach speed. *A*, At *p*H 3.8, 8.0 mg/ml, bar angle 45°, 100 min. after commencing run. *B*, At *p*H 7.0, 12.7 mg/ml, bar angle 60°, 129 min. after commencing run. *C*, At *p*H 3.0 (formate buffer), 8 mg/ml, bar angle 50°, 30 min. after commencing run. *D*, In 8 M urea, 11.6 mg/ml, bar angle 50°, 140 min. after commencing run. *E*, At *p*H 11.3, 11 mg/ml, bar angle 60°, 119 min. after commencing run.

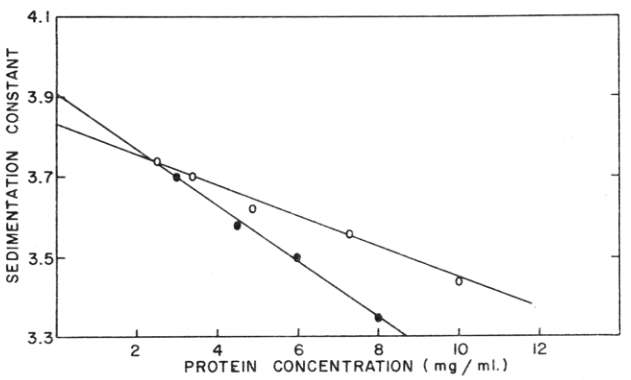

FIG. 4.—Determination of M-2 glycoprotein sedimentation constant at infinite dilution in water at 20° at *p*H 7.0 (open circles) and *p*H 3.8 (darkened circles).

TABLE I
COMPOSITION[a] AND PHYSICAL PROPERTIES OF BOVINE M-2 GLYCOPROTEIN

	22-hr. Hydrolysis (%)	48-hr. Hydrolysis (%)	Probable Value (%)	Residue Weight per 100 g Protein (g)
Composition				
Nitrogen	—	—	13.8	—
Lysine	5.7	7.0	7.0	6.1
Histidine	2.2	2.7	2.7	2.4
Ammonia	1.5	2.2	1.1[b]	1.1
Arginine	4.2	5.2	5.2	4.6
Aspartic acid	8.6	9.8	9.8	8.5
Glutamic acid	12.3	11.8	12.8[c]	11.2
Threonine	4.9	5.2	5.2	4.4
Serine	4.3	3.9	4.7[c]	3.9
Proline	3.4	3.2	3.6[c]	3.0
Glycine	2.1	2.1	2.1	1.6
Alanine	3.8	3.9	3.9	3.1
½ Cystine	0.0	0.0	0.0	—
Valine	4.2	4.5	4.5	3.8
Methionine	1.1	1.7	1.7	1.5
Isoleucine	4.2	4.6	4.6	4.0
Leucine	10.9	10.2	11.6[c]	9.8
Tyrosine[d]	—	2.7	2.7	2.4
Phenylalanine	6.1	6.1	6.1	5.4
Tryptophan[d]	—	—	1.0	0.9
Hexosamine	—	—	6.3	5.7
Hexose	—	—	6.1	5.5
Sialic acid	—	—	10.3	9.7
			113.0	98.6

Physical Properties	
$s^\circ_{20,w}$ (pH 7.0)	3.8
$D^\circ_{20,w}$ (pH 7.0)	6.5×10^{-7} cm²/sec.
Intrinsic viscosity	0.0455
Partial specific volume	0.71
Molecular weight	
$s^\circ_{20,w} - D^\circ_{20,w}$	49,000
$s^\circ_{20,w}$ – intrinsic viscosity	48,000
Archibald method	48,000
Frictional ratio	1.32
Axial ratio	2–4:1
Isoelectric point	3.8–4.0
Extinction coefficient (1% 279 mμ)	5.6

[a] Corrected for 5% moisture and 3% ash. [b] Noncarbohydrate ammonia. [c] Extrapolated to zero time hydrolysis. [d] 2.7% by the spectrophotometric method.

the bovine M-2 glycoprotein was 49,000 according to the sedimentation-viscosity method, but was 60,000 according to the diffusion-sedimentation method, and 58,000 ± 2000 according to the Archibald method (diaphragm angle 80° and 6569 rpm). The molecular weight according to the Archibald method was again independent of time.

Frictional ratio, f/f_0, calculated from the diffusion-average molecular weight data at pH 7.0, was 1.32. Axial ratios of the M-2 glycoprotein were computed by assuming a lower limit of hydration of (0.2 g H_2O)/(g protein) and an upper limit of (0.8 g H_2O)/(g protein). At the lower limit of hydration a/b was 4:1 and at the upper limit of hydration a/b was 2:1. The true value probably lies closer to the 4:1 figure.

Apparent Aggregation of Bovine Serum Acid Glycoproteins in Acid Medium and by Heat.—When bovine M-2 glycoprotein was dissolved in pH 3.0 formate buffer, two peaks were observed when the solution was examined ultracentrifugally (Fig. 3). An identical pattern was observed when a solution of M-2 glycoprotein in 0.9% NaCl was adjusted to pH 3.0 with

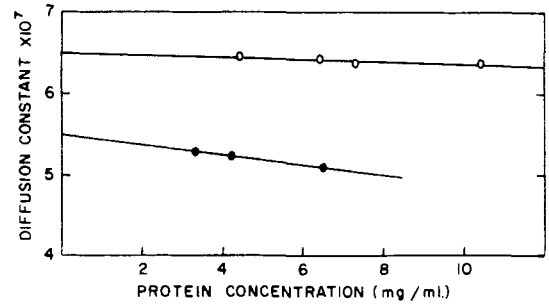

FIG. 5.—Determination of M-2 glycoprotein diffusion constant at infinite dilution in water at 20° at pH 7.0 (open circles) and pH 3.8 (darkened circles).

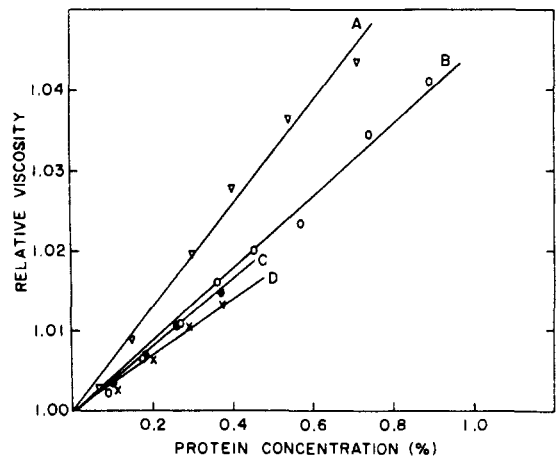

FIG. 6.—Determination of intrinsic viscosity of M-2 glycoprotein at pH 2.8 (curve A), pH 7.0 (curve B), pH 3.8 (curve C), and in distilled water (curve D).

dilute HCl, or when the M-2 glycoprotein was dissolved in pH 2.8 phosphate buffer, $\mu = 0.1$. The ultracentrifugal pattern remained qualitatively the same when the pH of acid solutions of M-2 glycoprotein was brought to pH 7 and to pH 10, or when acidic M-2 glycoprotein solutions were dialyzed, lyophilized, and redissolved in buffers at pH 7.0 or pH 10. Prolonged dialysis of M-2 glycoprotein against pH 9.2 glycine buffer also failed to reverse the effects of low pH. At pH 7 the acid-treated glycoprotein showed two peaks with s_{20} of 3.3 and 9.2 when examined in the ultracentrifuge, while at pH 10 the two peaks had sedimentation constants of 2.9 and 8.5. Attempts to reverse the effects of low pH by dissolving the acid-treated protein in 30% dimethylformamide, dilute HCl at pH 1.4, 2–8 M urea, and salt solutions of ionic strength up to 0.4 were unsuccessful. Presence of glutathione or 0.1 M mercaptoethanol did not prevent the aggregation of M-2 glycoprotein by acidic buffer solutions. After 4 hours of standing at room temperature in pH 3 formate buffer, the weight average molecular weight of the glycoprotein was 615,000 ± 20,000 as determined by the Archibald method at 3189 rpm and a diaphragm angle of 85°. The molecular weight at meniscus decreased and that at the synthetic cell bottom increased with time, and the above molecular weight value was arrived at by extrapolation to zero time.

Ultraviolet absorption curves of native M-2 glycoprotein at pH 7.0 and of acid-treated glycoprotein at pH 3.0 were constructed. Both showed absorption maxima at 279 mμ with identical extinction coefficients of 5.6 at 1% concentration. The acid-treated glycoprotein had an *N*-terminal amino acid picture identical to that of native M-2 glycoprotein.

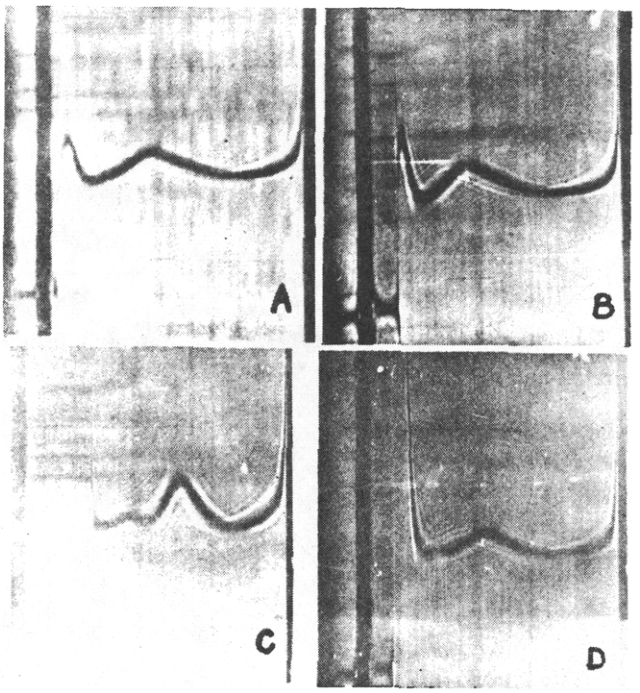

FIG. 7.—Effects of heat treatment on the sedimentation of M-2 glycoprotein and orosomucoid. *A*, M-2 glycoprotein heated for 10 min. at 80° at *p*H 3; 10 mg/ml, bar angle 40°, 17 min. after commencing run. *B*, M-2 glycoprotein heated for 10 min. at 80° at *p*H 7; 10 mg/ml, bar angle 40°, 13 min. after commencing run. *C*, M-2 glycoprotein heated for 10 min. at 80° at *p*H 9.8; 10 mg/ml, bar angle 40°, 47 min. after commencing run. *D*, Orosomucoid heated for 10 min. at 80° at *p*H 3; 10 mg/ml, bar angle 40°, 21 min. after commencing run.

Bovine orosomucoid showed no signs of aggregation when examined ultracentrifugally at *p*H 2.8.

When the M-2 glycoprotein was subjected to heating at 80° for 10 minutes, the presence of larger aggregate particles was observed. Heating the M-2 glycoprotein at *p*H 3 (M-2 glycoprotein in 0.9% NaCl, *p*H adjusted with dilute HCl) resulted in liberation of 14% of its sialic acid; however, no increase in intensity of color given by ninhydrin was observed after the heat treatment. The two ultracentrifugally discernible components observed after heat treatment of the M-2 glycoprotein had sedimentation constants of 3.2 and 28 at a total protein concentration of 1%. A similar pattern in the ultracentrifuge was obtained when the M-2 glycoprotein was analyzed after heating at *p*H 2.8 (phosphate), and at *p*H 7.0 (phosphate buffer) at 80° for 10 minutes. Heating at *p*H 9.8 (glycine, $\mu = 0.1$), however, resulted in formation of aggregates of smaller size; the two components observed in the ultracentrifuge after heating of the protein at *p*H 9.8 had sedimentation constants of 2.8 and 7.1. Changes in the M-2 glycoprotein brought about by heat were not reversible by changing the *p*H, nor were they reversible by urea treatment. Bovine orosomucoid subjected to heat at *p*H 3 and *p*H 2.8 behaved similarly to the M-2 glycoprotein. When examined ultracentrifugally after heating at *p*H 3, orosomucoid showed two components with sedimentation constants of 2.9 and 31 at a total protein concentration of 1%. It was noted, however, that the more rapidly sedimenting material accounted for only a very small portion of the total protein present; the reverse was observed with the M-2 glycoprotein. The presence of aggregates was not observed when bovine orosomucoid solutions were analyzed ultracentrifugally after heating at *p*H 7 and *p*H 9.8.

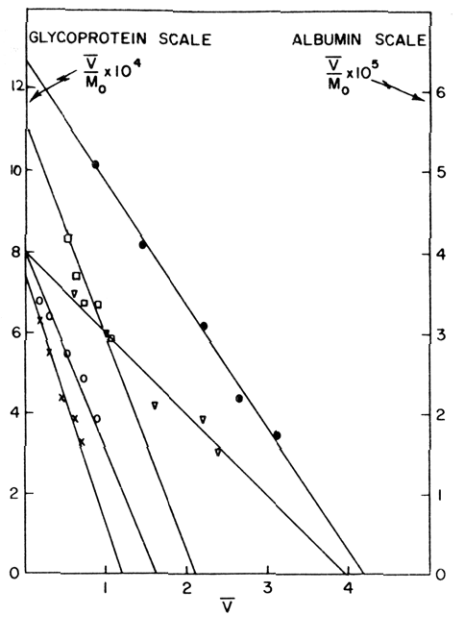

FIG. 8.—Determination of thyroxine-binding constants of bovine serum albumin and heated M-2 glycoprotein by the equilibrium dialysis method. ●, bovine serum albumin at 27°; ▽, bovine serum albumin at 4°; ○, M-2 glycoprotein at 4°; ×, M-2 glycoprotein at 27°; □, M-2 glycoprotein exposed to *p*H 3.0, at 4°. M_0-molarity of thyroxine outside dialysis tubing at equilibrium, $\overline{V}$-average number of thyroxine-binding sites per protein molecule at equilibrium and a certain concentration of free thyroxine (M_0).

Figure 7 illustrates some of the patterns obtained in the ultracentrifuge of bovine glycoprotein solutions treated by heat.

Thyroxine-Binding Properties of Native and Modified M-2 Glycoprotein and of Bovine Serum Albumin.—When native M-2 glycoprotein was examined for thyroxine-binding activity by the equilibrium dialysis method, no binding was observed. No binding of thyroxine by the M-2 glycoprotein was observed in 4 M and 8 M urea, and no binding of thyroxine by the glycoprotein was obtained in the presence of 10^{-4} M Ca^{++}, Mg^{++}, and Zn^{++}. Treatment of the M-2 glycoprotein with viral neuraminidase (loss of 30–50% of its sialic acid) did not alter its inability to bind thyroxine.

However, when M-2 glycoprotein was heated at 80° for 10 minutes at *p*H 3, it acquired thyroxine-binding properties, reacting with 1–2 moles of thyroxine per mole of protein. Acid-treated M-2 glycoprotein (dissolved in *p*H 3 formate buffer, $\mu = 0.1$, left to stand for 4 hours, and dialyzed exhaustively against *p*H 9.2 glycine buffer) showed a thyroxine-binding capacity similar to that of heat-treated M-2 glycoprotein at 4°. Thyroxine-binding properties of M-2 glycoprotein treated with the *p*H 3.0 buffer at room temperature did not differ significantly from those of the glycoprotein exposed to low *p*H at 4°.

Figure 8 shows Scatchard-type plots of data obtained at *p*H 9.2, and Table II summarizes thyroxine-binding data obtained with acid-treated and with heated M-2 glycoprotein, and native bovine serum albumin at thyroxine concentrations of $0.5 - 5 \times 10^{-5}$ M.

Orosomucoid in its native state bound only insignificant amounts of thyroxine, and its binding capacity was not increased by heating it at 80° for 10 minutes at *p*H 3.

DISCUSSION

The major aim of the present work was to extend the physical-chemical characterization of bovine M-2

TABLE II

THERMODYNAMIC CONSTANTS CHARACTERIZING BINDING OF THYROXINE BY BOVINE SERUM ALBUMIN AND M-2 GLYCOPROTEIN TREATED BY HEAT AND BY ACID AT *p*H 9.2

	27°			4°			
Protein	n^a	ΔF (kcal/mole)	ΔS (entropy units)	n	ΔF (kcal/mole)	ΔS (entropy units)	ΔH^b (kcal/mole)
Albumin	4.2	−7.15	33.5	4.0	−6.35	33.4	2.9
Heated M-2 glycoprotein	1.2	−6.6	27	1.6	−6.0	27	1.5
Acid-treated M-2 glycoprotein	—	—	—	2.0	−6.0	—	—

[a] Number of binding sites/mole protein. [b] Independent of temperature.

glycoprotein. However, before such studies could be made, it was deemed necessary to ascertain the degree to which the Holstein cattle and Jersey cattle glycoproteins were similar. Physical-chemical studies carried out previously (Bezkorovainy and Doherty, 1962a) were therefore repeated with the dairy cattle glycoprotein, and the data obtained showed that the two proteins were identical in all respects tested, except for thyroxine-binding properties and molecular weight. These discrepancies are discussed below.

The M-2 glycoprotein preparation described in this communication was reasonably homogeneous by electrophoretic and ultracentrifugal methods; however, end-group analysis and the Tiselius electrophoretic pattern at *p*H 3 suggest that a trace impurity is present. The molecular weight of the M-2 glycoprotein obtained at *p*H 3.8 (isoelectric point) by the viscosity-sedimentation, diffusion-sedimentation, and Archibald methods, was 49,000, 60,000, and 58,000 ± 2000 respectively. This discrepancy leads one to believe that the shape of the M-2 glycoprotein at *p*H 3.8 differs from that in its "native" state and that the sedimentation-viscosity equation is inapplicable in this case. At *p*H 7.0, however, all three methods gave almost identical molecular weights, the average (48,000) of which was used in calculations involving molecular weight.

A molecular weight of 39,000 was reported for the M-2 glycoprotein (Bezkorovainy and Doherty, 1962a). The parameter responsible for this low value is the intrinsic viscosity figure, reported to be 0.033 in distilled water in the previous communication. In view of the data presented above, it appears that the use of this datum was unjustified and hence the molecular weight figure is in need of revision.

Treatment of the M-2 glycoprotein with acid and with heat resulted in the partial formation of aggregates of basically two sizes: those with s_{20} of 7–10 and those with s_{20} of 20–30. The diffuseness of peaks representing these aggregates in the ultracentrifuge (Fig. 7) suggests that these aggregate particles are of many different sizes, and the sedimentation constants herein reported are only average values. Futile attempts to disaggregate these large particles with dimethylformamide, dilute HCl, urea, and high ionic strength solutions demonstrated that the bonds responsible for cohesiveness of these particles are not easily dissociable hydrophobic bonds, sialic acid electrostatic bonds, or hydrogen bonds. Aggregation due to low *p*H may be interpreted as being the result of partial destruction of electrostatic bonds; however, failure to observe aggregation at *p*H 11.3 casts doubt on this possibility. Failure to observe the presence of *N*-terminal amino acid groups in the acid-treated M-2 glycoprotein that are different from those of native M-2 glycoprotein suggests that no fragmentation of the peptide chain occurs at acid *p*H values. The ineffectiveness of reducing agents such as glutathione and mercaptoethanol in preventing or reversing aggregation shows that polymerization due to oxidation probably is not involved. The apparent absence of cysteine from the M-2 glycoprotein makes this possibility even more unlikely. The existence of two peaks, one possessing the sedimentation properties of aggregate particles, the other those of the native protein, in all acid- and/or heat-treated M-2 glycoprotein solutions suggests that an equilibrium between the monomer and polymer exists. Aggregation of protein molecules prior to precipitation during heating is known to occur in serum albumin (Tuchachinskii and Shchagina, 1961).

The thyroxine-binding properties of the dairy cattle glycoprotein and of the Jersey cattle were quite dissimilar. M-2 glycoprotein, previously described, bound thyroxine in its native state with five binding sites and an apparent free energy of binding of −6.2 kcal/mole at 27° (Bezkorovainy and Doherty, 1962b). The protein described above had to be modified before it could acquire thyroxine-binding properties. This "activation" brought about by acid or by heat caused aggregation of the protein molecules present, and exposed two thyroxine-binding sites with a free energy of binding of −6.0 kcal/mole at 4°. Data presented above suggest that this apparent "activation" is physical in nature, and probably does not require the rupture of covalent linkages. The difference in thyroxine-binding activity between the two proteins may be due to factors such as subspecies variation (Holstein cattle *vs.* Jersey cattle), age of animals used (12 years of age *vs.* 2 years of age), and the physical environment of the animals before bleeding.

Thyroxine-binding constants reported for albumin are in good agreement with those reported for the bovine or human proteins by other authors (Lein, 1952; Tritsch *et al.*, 1961; Sterling and Tabachnik, 1961).

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Action of Carboxypeptidase-A on Bovine Insulin: Preparation of Desalanine-Desasparagine-Insulin*

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In order to make use of carboxypeptidase-A as a tool for determining the differences between various insulins (see following paper), it was necessary to develop conditions which would cleave completely the carboxyl-terminal amino acid residues from insulin. With this view in mind, bovine insulin has been subjected to the action of carboxypeptidase-A under a variety of conditions. The rates of release of the carboxyl-terminal amino acids (1) are much greater for zinc-free insulin than for zinc-insulin and (2) decrease with increasing salt concentration at *p*H 7.4. For carboxyl-terminal alanine and asparagine the rate of release increases from *p*H 7.4 to 9.4, while for carboxyl-terminal aspartic acid the rate of release decreases dramatically over this *p*H range. The action of the enzyme on insulin ceases after the removal of one mole of alanine from the carboxyl-terminal position of the B-chain and one mole of asparagine (or aspartic acid) from the carboxyl-terminal position of the A-chain. Conditions were developed for the complete removal of the carboxyl-terminal amino acids to give desalanine-desasparagine-insulin. The latter has been characterized by countercurrent distribution, amino acid composition, and biological activity; it possesses at best less than 5% of the activity of native insulin in the mouse convulsion test.

Lens (1949) was the first to use carboxypeptidase to determine the carboxyl-terminal group of a protein and reported the release of alanine by the action of the enzyme on insulin. Later Harris (1952) reported the release of asparagine as well as alanine and assigned these amino acids to the carboxyl-terminal positions of the A- and B-chains (Sanger, 1949; Ryle *et al.*, 1955), respectively, of insulin. Although an amount of alanine almost equimolar to the insulin was produced by the action of the enzyme, the asparagine was released at a much slower rate and in a total amount equal to only about 20% of the theoretical yield (Harris and Li, 1952). Nicol and Smith (1956) and Nicol (1960) encountered similar difficulties in securing a complete hydrolysis of zinc-insulin but reported nearly complete liberation of alanine and asparagine, as measured by the spot dilution method (Polson *et al.*, 1947), when acetyl-insulin (Fraenkel-Conrat and Fraenkel-Conrat, 1950) was used as a substrate for the carboxypeptidase.

The present studies were initiated in order to find conditions for the complete release of the carboxyl-terminal amino acids from insulin. As it turned out, most samples of crystalline zinc-insulin of bovine origin gave aspartic acid as well as asparagine and alanine upon treatment with carboxypeptidase-A. This fact had been noted previously (Harris, 1952) but had been attributed to partial hydrolysis of the asparagine during the performance of the procedures used to detect the amino acids released by the action of the enzyme. The present investigations were facilitated by the availability of a sample of insulin which, upon digestion with carboxypeptidase, gave about an equal amount of aspartic acid and asparagine. The sum of the amounts of aspartic acid and asparagine was equal to the amount of alanine released, which in turn amounted to one mole per mole of insulin (m.w. 6000). As shown in a subsequent paper, this particular sample of insulin was made up of about equal amounts of insulin-A and desamido-insulin (Harfenist and Craig, 1952; Harfenist, 1953). By the use of this sample, the effect of several variables on the rate of release of each one of the carboxyl-terminal amino acids was followed in the same experiments.

Of particular importance to the performance of the present experiments was an observation first made by Hill and Smith (1957), who had reported that zinc-insulin was particularly resistant to the action of leucine aminopeptidase, while zinc-free insulin was attacked at a moderate rate. We have made a similar observation in our studies on the action of trypsin on insulin (Young and Carpenter, 1961). Preliminary experiments indicated that zinc-free insulin, prepared by precipitation from acid solution as its hydrochloride, was much more susceptible to attack by carboxypeptidase than zinc-insulin. Consequently, insulin hydrochloride rather than zinc-insulin was used as starting material in all of these studies.

Although Harris and Li (1952) had not been able to secure complete cleavage of asparagine from insulin,

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