BINDING OF CORTICOSTEROIDS BY PLASMA PROTEINS. IV. THE ELECTROPHORETIC DEMONSTRATION OF CORTICOSTEROID BINDING GLOBULIN [1, 2]

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The usefulness of equilibrium paper electrophoresis to detect the binding of corticosteroids by plasma proteins was described in an earlier paper of this series (2). Plasma was dialyzed initially against the electrophoretic buffer to which carbon labeled corticosteroids had been added. Later this buffer was used to saturate the electrophoretic paper. Thus, the concentration relationship between the buffer steroid and the protein bound steroid was maintained throughout the development of the electrophoresis. Consequently, there was little tendency for the bound steroid to trail the advancing protein components as occurs with customary electrophoresis of steroids (3). In the experiments carried out with this method using barbital buffer, pH 8.8, both corticosterone-4-C^{14} and cortisol-4-C^{14} appeared to migrate with the albumin peak of plasma.

Subsequent experiments with carbon labeled steroids using dialysis equilibrium suggested the possibility that there were two important corticosteroid binding proteins of human plasma (4). The first of these binding systems probably accounts for nearly all the binding of cortisol at normal physiologic concentrations. Under such circumstances about 99 per cent of the cortisol in plasma is protein-bound. As the concentration of hormone in the plasma rises, the degree of binding falls rapidly at first and then very much more slowly. Like plasma, plasma Fraction IV-4 has also been demonstrated to have a high affinity for corticosterone and cortisol. The affinity of this fraction for progesterone, testosterone, estrone, and estradiol is much less than for cortisol and corticosterone when tested under conditions of low hormone concentration.

The second corticosteroid binding system is operative at high plasma concentrations of cortisol. Most of this binding can be attributed to albumin whose binding characteristics have been adequately described (5–7). Cortisol and corticosterone are bound much less firmly by albumin than the other steroid hormones mentioned above.

The failure to observe two corticosteroid binding zones in our equilibrium paper electrophoresis experiments can be attributed to the fact that these experiments were carried out with amounts of corticosterone and cortisol far in excess of those needed to saturate the corticosteroid binding globulin. Binding, predominantly by albumin, could be anticipated under these conditions. It was not possible to carry out equilibrium paper electrophoresis at physiologic cortisol concentrations because the small amount of plasma which can be applied to the electrophoretic paper limits the amount of radioactive steroid available for counting. In the present paper, this difficulty was circumvented by using continuous flow paper electrophoresis of equilibrated plasma, which has permitted the demonstration of a corticosteroid binding globulin that is distinct from human albumin.

METHODS

Serum was collected from laboratory workers and medical students, two to three hours after breakfast. Before subjecting this serum to continuous flow electrophoresis, it was dialyzed extensively against the electrophoretic buffer in the presence of the radioactive steroid under study. Ten ml. of serum in a Visking cellophane bag was dialyzed against 1,500 ml. of buffer. The amount of steroid added to the serum and buffer was estimated to provide about 0.5 μg. of steroid bound to the serum and an equilibrium concentration in the buffer. To speed the attainment of equilibrium, 0.5 μg. of the labeled steroid was added to the plasma directly. To the buffer was added 2.3 μg. of cortisol-4-C^{14} or corticosterone-4-C^{14}.

[1] A preliminary report of these findings has been published (1).

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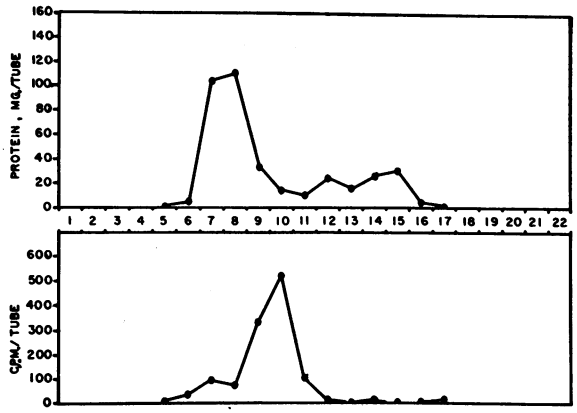

FIG. 1. CONTINUOUS FLOW ELECTROPHORESIS OF HUMAN SERUM CARRIED OUT WITH BARBITAL BUFFER, pH 8.8, IONIC STRENGTH 0.045 IN THE PRESENCE OF CORTICOSTERONE-4-C^{14}

Serum was applied to curtain above tube 14. The elution tubes are indicated by the Nos. 1 through 22 between the two curves.

In the experiments with progesterone-4-C^{14}, 1.87 μg. of this steroid was added to the buffer. Nonradioactive cortisol, 350 μg., was added in certain experiments with progesterone-4-C^{14}.

Continuous flow electrophoresis was carried out using the Karler-Misco apparatus at 4° C. Two electrophoretic buffers were used—barbituric acid-sodium barbiturate, pH 8.8, ionic strength 0.045; acetic acid-sodium acetate, pH 5.2, 0.02 M. A fractionation plate, similar to that described by Shetlar, Cahill, Stidworthy, and Shetlar (8), gave improved separation of the protein fraction. The curtains were made from Schleicher and Schuell paper No. 588. The upper reservoirs of the apparatus, which provide the flow of buffer for the curtain, were filled with the buffer which had been dialyzed against the serum in the presence of the radioactive steroid. The electrode vessels were filled with stock buffer. As soon as the curtain was wet with buffer, the current was turned on for three to five hours to create a steady state. In the barbital buffer the current flow was 13 milliamperes with 700 volts and 4 to 6 milliamperes with 800 to 900 volts with the acetate buffer.

Serum was applied to the paper through a 2 mm. wick cut from Schleicher and Schuell paper No. 470A. In a period of from 36 to 40 hours, 5 to 10 ml. of serum were fractionated. The eluent was collected in 22 small test tubes. Each tube contained between 3 and 4 ml. of eluent at the end of a run. The curtain was removed and dried rapidly with a hair drier. Drying was completed and the proteins fixed to the paper by heating to 100° C. for 30 minutes in an oven. The positions of the protein bands were identified by staining with bromphenol blue.

The eluent tubes were made up to a total volume of 5 ml. each. Aliquots were analyzed for protein using the method of Lowry, Rosebrough, Farr, and Randall (9). The protein bound hexose was determined in several experiments by the method of Winzler (10). Radioactivity was determined in each tube by extracting 3 ml. of eluent with 25 ml. of chloroform in a large stoppered tube. The extracts were washed with 2.5 ml. of 0.1 N sodium hydroxide and 2.5 ml. of water. Twenty ml. of the final chloroform extracts was evaporated in 40 ml. centrifuge tubes. The residues, of negligible mass, were transferred to small brass cups in alcohol. Radioactivity was determined in a gas flow counter operating in the proportional range.

The cortisol-4-C^{14} and the corticosterone-4-C^{14} used in these experiments were obtained from the Endocrinology Study Section of the National Institutes of Health. Both compounds had a specific activity of 1.467 millicuries per millimole. The progesterone-4-C^{14} was obtained from Tracer Lab, Inc., and had a specific activity of 1.63 millicuries per millimole.

The general reproducibility of the electrophoretic experiments was excellent, although slight changes in the rate of migration and the exactness of definition occurred. The accuracy of counting was better than ± 10 per cent.

RESULTS

The binding of cortisol-4-C^{14} was first examined electrophoretically in barbital buffer. When the electrophoresis was performed in a buffer with an ionic strength of 0.045, the peak of radioactivity of the bound steroid followed the main albumin peak (Figure 1). Only a slight asymmetry with a shoulder of increased radioactivity in the albumin containing tubes was apparent. The position

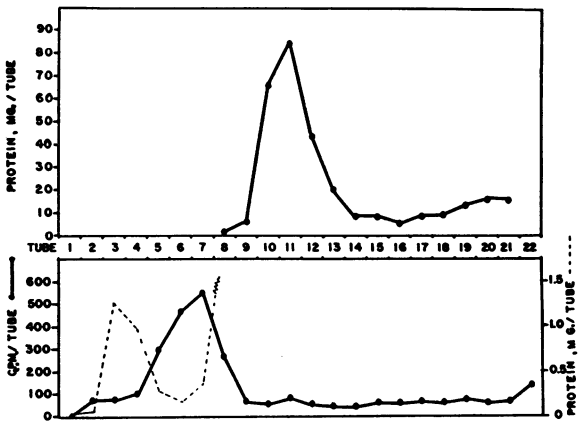

FIG. 2. CONTINUOUS FLOW ELECTROPHORESIS OF HUMAN SERUM IN THE PRESENCE OF CORTICOSTERONE-4-C^{14}

Acetate buffer, pH 5.2, 0.02 M was used. Serum was applied to curtain above tube 12. The dotted line on the lower graph indicates protein concentration measured by the Lowry, Rosebrough, Farr, and Randall procedure (9).

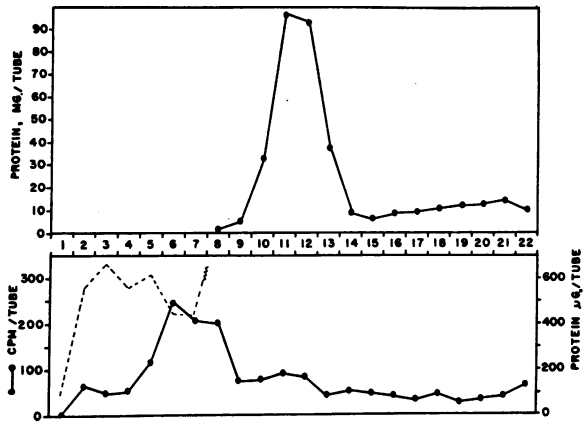

FIG. 3. CONTINUOUS FLOW ELECTROPHORESIS OF HUMAN SERUM IN THE PRESENCE OF CORTISOL-4-C^{14}

Acetate buffer, pH 5.2, 0.02 M was used. Serum was applied to curtain above tube 12. The dotted line on the lower graph indicates the protein concentration measured by the Lowry, Rosebrough, Farr, and Randall procedure (9).

of the radioactivity in respect to the albumin peak was influenced by minor experimental variations. Lowering the ionic strength of the barbital buffer increased the mobility of the bound radioactive steroid. With a buffer of ionic strength of 0.02, the bound steroid slightly preceded the albumin peak.

To gain further information concerning the binding of corticosterone, experiments have been conducted using 0.02 M acetate buffer, pH 5.2. Albumin migrates little at this pH and descends directly from the point of application above tube 12. In control experiments in which cortisol-4-C^{14} was added directly to the curtain in the absence of serum the steroid descended somewhat cathodally from the point of application with peak concentrations in tubes 14 and 15. The displacement of the noncharged steroid can be attributed to electroendosmotic movement of the buffer. When the experiment was carried out with serum the distribution of radioactivity was very different from the control experiment (Figure 2). The greatest concentrations of the labeled cortisol were located in tubes 5, 6, and 7. These tubes are on the anodal side of the albumin which was concentrated in tubes 10 and 11. The tubes containing the greatest amounts of the labeled steroid were found to contain only small quantities of protein.

When the electrophoresis of normal serum was carried out with cortisol-4-C^{14}, a similar distribution of radioactivity was observed (Figure 3). In this particular experiment there was a greater concentration of the anodally migrating protein components in the region of the cortisol binding than observed in the experiment with corticosterone.

The binding pattern with progesterone-4-C^{14} is of considerable interest (Figure 4) in that there is a clearly defined double peak of binding of radioactivity. One peak is associated with the anodally migrating component which binds corticosteroids, but in addition there is significant binding in the region of the albumin peak. This finding is consistent with the interpretation of the binding of progesterone-4-C^{14} in dialysis equilibrium experiments with different quantities of carrier nonradioactive progesterone (4). It was suggested that the binding of progesterone was relatively less for corticosteroid binding globulin and relatively greater for albumin as compared to cortisol. To support this view, the ability of cortisol to displace progesterone-4-C^{14} from the corticosteroid binding globulin has been studied. In the electrophoretic experiments, depicted in Figure 5, 350 μg. of nonradioactive cortisol was added to the preliminary dialysis carried out with 10 ml. of serum, 1,500 ml. of acetate buffer and

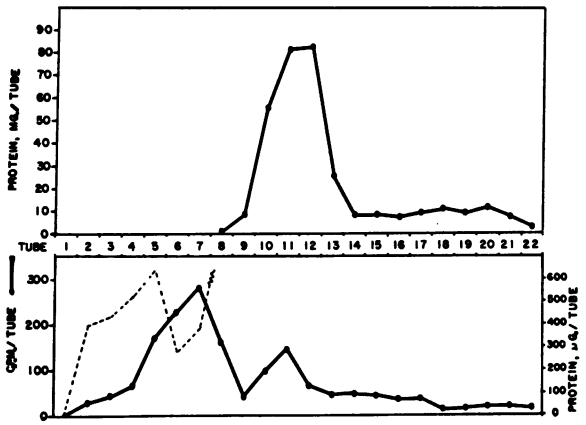

FIG. 4. CONTINUOUS FLOW ELECTROPHORESIS OF HUMAN SERUM IN THE PRESENCE OF PROGESTERONE-4-C^{14}

Acetate buffer, pH 5.2, 0.02 M was used. Serum was applied to curtain above tube 12. The dotted line on the lower graph indicates protein concentration measured by the Lowry, Rosebrough, Farr, and Randall procedure (9).

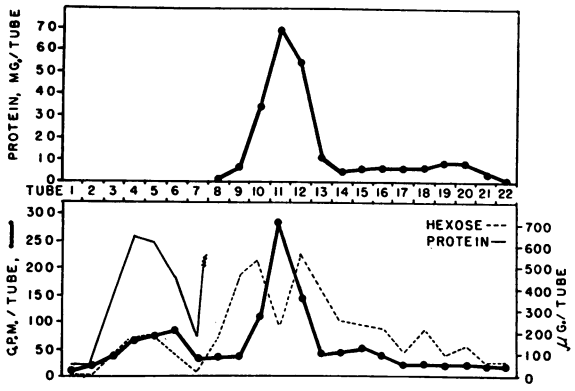

FIG. 5. CONTINUOUS FLOW ELECTROPHORESIS OF HUMAN SERUM IN THE PRESENCE OF PROGESTERONE-4-C^{14} AND NONRADIOACTIVE CORTISOL.

Acetate buffer, pH 5.2, 0.02 M. was used. Serum applied above tube 12.

progesterone-4-C^{14}. Under these conditions, the binding of the progesterone-4-C^{14} by the anodally moving protein was virtually eliminated and the binding by the albumin peak greatly accentuated. Analyses of the bound hexose distribution are also included in this graph.

DISCUSSION

The application of the principle of equilibrium electrophoresis to continuous flow electrophoresis has presented no particular problems. The apparatus selected for use has been favorable for such studies because of the small capacity of the reservoirs feeding the buffer to the curtain. Other designs of this type of instrument have larger buffer reservoirs which make the initial dialysis of buffer with serum more troublesome.

The existence of corticosteroid binding globulin with mobility characteristics distinct from albumin seems evident from the experimental results. In the customary barbital buffer system this protein has the mobility of an alpha globulin. Anodal mobility of the corticosteroid binding globulin in acetate buffer, pH 5.2, is indicative of a low isoelectric point. These features suggest an acidic glycoprotein. At this pH, two anodally migrating glycoproteins have been recognized. The fastest component has been described by Mehl, Golden, and Winzler (11) as M-1 and the second component with a mobility only slightly faster than albumin has been called M-2. The thyroxine binding globulin is believed to be associated with M-2. The fastest minor protein peak in our experiments probably corresponds to M-1, and M-2 was not clearly separated from albumin. The presence of bound hexose and hexosamine in these fractions has been confirmed by analysis. The anodal migration of corticosteroid binding globulin occupies a position intermediate between that reported for M-1 and M-2, and in certain experiments, such as in Figure 1, these tubes contain only minute amounts of protein. This observation cannot be construed as evidence that the corticosteroid binding material is other than protein. The ease with which corticosteroid binding globulin of plasma is saturated with small additions of corticosteroids indicates that only minute amounts of the protein are normally present. The preliminary binding experiments which have been done with plasma protein fractions indicate that corticosteroid binding globulin may be contained in Fraction IV-4, a fraction known to be rich in glycoproteins (2).

The relatively low affinity of progesterone for the corticosteroid binding globulin has been experimentally useful in permitting the demonstration of binding of this protein and albumin. The change in distribution of progesterone with the addition of cortisol is explained by the higher affinity of the corticosteroid binding globulin for cortisol.

SUMMARY

1. The binding of cortisol-4-C^{14}, corticosterone-4-C^{14}, and progesterone-4-C^{14} by human serum proteins has been studied by continuous flow electrophoresis. Careful predialysis of the serum, the steroid, and the electrophoretic buffer has prevented elution artifacts.

2. With barbital buffer, pH 8.8, the peak of corticosterone binding migrates more slowly than albumin and is present in the alpha globulin region. In acetate buffer, pH 5.2, the corticosteroid binding globulin moves anodally away from the albumin component.

3. Binding studies conducted with progesterone-4-C^{14} have permitted the demonstration of binding both by the anodally moving binding protein and by albumin. The bound progesterone could be displaced off the anodally moving component onto the albumin peak by adding cortisol to the system.

4. These observations provide direct evidence of the existence of a corticosteroid binding globulin which is separate from albumin and suggest its glycoprotein nature.

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