The Transport of 25-Hydroxycholecalciferol in a New World Monkey

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(Received 9 July 1975)

Albumin is responsible for the transport of 25-hydroxycholecalciferol in the Capuchin monkey. This was confirmed by gel filtration, analytical polyacrylamide-disc-gel electrophoresis, polyacrylamide-gel isoelectric focusing and a competitive protein-binding assay. Association constants of the serum transport proteins of a New and an Old World monkey towards 25-hydroxycholecalciferol were calculated; the transport protein in the New World monkey has a lower affinity for the vitamin D metabolite than the transport protein in the Old World primate.

The antirachitic activity of plasma is almost entirely due to cholecalciferol and 25(OH)D₃* (Lawson *et al.*, 1969; Mawer *et al.*, 1971), and it has been established that both steroids are bound to plasma proteins (Chalk & Kodicek, 1961; Rijkers & DeLuca, 1967; Edelstein *et al.*, 1972, 1973; Hay & Watson, 1975*a,b*).

Protein binding increases the solubility of steroids; however, the hormones testosterone and oestradiol are water soluble at their physiological concentration (Baulieu *et al.*, 1970). It has been suggested that the plasma proteins act as a reservoir of bound steroid (Baird *et al.*, 1969) and that the biological activity of oestradiol and testosterone is exerted by the unbound fraction in plasma (Burke & Anderson, 1972).

The steroid-binding globulins may, however, possess a more active role, for instance in the transport of hormones into their respective target organs or in the passage of secreted steroid from endocrine cells into the blood (Baulieu *et al.*, 1970). In chick serum there are two β -globulin cholecalciferol-binding proteins, one of which binds mainly cholecalciferol and the other binds 25(OH)D₃ (Edelstein *et al.*, 1972). This observation in the chick may suggest an active role for the steroid-binding globulins, namely transport of cholecalciferol to the liver for metabolism to 25(OH)D₃ and subsequent transport of this steroid to kidney for further metabolism to 1,25(OH)₂D₃.

A survey of cholecalciferol-binding proteins in vertebrates indicated considerable variation in the type of protein used, which included α -globulins, β -globulins and lipoproteins (Edelstein *et al.*, 1973; Hay & Watson, 1975*a,b*). Most mammals use a single α -globulin for the transport of cholecalciferol and 25(OH)D₃; however, some species, including the

New World Capuchin monkeys (*Cebus*), transport these two steroids on a protein which exhibits mobility similar to that of albumin on polyacrylamide-disc-gel electrophoresis (Edelstein *et al.*, 1973; Hay & Watson, 1975*a*).

It remained to be shown whether albumin itself is the carrier protein for cholecalciferol in these monkeys or whether the binding protein merely exhibited the same mobility as that of albumin on gel electrophoresis. In view of the reverse correlation between the metabolic clearance rate of androgens in the human and their affinity to testosterone-oestradiol-binding globulin (Baird *et al.*, 1969) and the fact that albumin has a low affinity for testosterone and oestradiol, in contrast with the high affinity of the testosterone-oestradiol-binding globulin (Burke & Anderson, 1972), further resolution of the transport protein in the *Cebus* monkeys was required to confirm its identity and to measure its affinity towards 25(OH)D₃.

The present paper reports the results of tests which confirm that in one species of *Cebus* monkey, *C. albifrons*, albumin is indeed the 25(OH)D₃ transport protein in blood.

Experimental

Animals. Capuchin monkeys (*Cebus albifrons*) were obtained from Tarpon Zoo (Tarpon Springs, Fla., U.S.A.). They were fed on a diet (Herbert, 1971) deficient only in vitamin D. Patas monkeys (*Erythrocebus patas*) were purchased from Shamrock Farms, Small Dole, Henfield, Sussex, U.K., and were fed on a vitamin D-replete diet (Herbert, 1971).

Radiochemicals. 25-Hydroxy[26,27-³H]cholecalciferol (sp. radioactivity 11 Ci/mmol) was obtained from Dr. D. E. M. Lawson, Dunn Nutrition Laboratory, Cambridge.

Sample preparations. The Capuchin monkeys

* Abbreviations: 25(OH)D₃, 25-hydroxycholecalciferol; 1,25(OH)₂D₃, 1,25-dihydroxycholecalciferol.

were bled by venepuncture at intervals over a period of 2 months; the serum was separated and the samples were pooled. Serum (10.0 ml) was incubated *in vitro* with $1 \mu\text{Ci}$ of $[26,27\text{-}^3\text{H}]25(\text{OH})\text{D}_3$ at 4°C for 16 h to ensure complete uptake of the steroid. Blood was obtained from the Patas monkey by venepuncture and the serum separated.

Electrophoresis. Analytical polyacrylamide-disc-gel electrophoresis with a continuous buffer system (Hjerten *et al.*, 1965) was performed on two samples of serum by using 7 mm (internal diam.) tubes. One gel was stained for protein with Naphthalene Black; the other was frozen with solid CO_2 , 1 mm segments were cut with a macrotome (Hay & Ray, 1975), and the radioactivity was extracted from the slices with NCS tissue solubilizer (Amersham/Searle, High Wycombe, Bucks., U.K.) as described by Hellung-Larsen (1971). The reference marker was reconstituted human serum (Versatol; William Warner and Co. Ltd., Eastleigh, Hants., U.K.).

Gel gradient. A discontinuous 5–20% polyacrylamide gel gradient was prepared by the method of Neuhoﬀ (1970). Bovine serum albumin was used as a reference marker.

Serum fractionation. The albumin fraction of the serum incubated with $[26,27\text{-}^3\text{H}]25(\text{OH})\text{D}_3$ was separated by zinc and barium precipitation by using Method 10 of the Cohn fractionation procedure (Cohn *et al.*, 1950). The fraction was dialysed against 0.02 M-sodium phosphate buffer (pH 7.6) for 72 h with frequent changes of the buffer, freeze-dried and redissolved in 7.0 ml of 0.05 M-sodium phosphate buffer (pH 7.6) containing 0.2 M-NaCl, 1 mM-mercaptoethanol and 1.5 mM-EDTA.

Column chromatography. Gel filtration was carried out on a column (1.54 cm $\times$ 79.00 cm; bed volume 147 ml) packed with Sephadex G-100 (Pharmacia Fine Chemicals Ltd., London W.5, U.K.) equilibrated with 0.05 M-sodium phosphate buffer (pH 7.6) containing 0.2 M-NaCl, 1 mM-mercaptoethanol and 1.5 mM-EDTA. A portion (2.0 ml) of the dialysed albumin fraction was applied to the column and elution was carried out at 4°C with the same buffer and with a constant flow rate of 4 ml/h maintained by an LKB peristaltic pump. Fractions (2.5 ml) were collected, and effluent was continuously monitored in an LKB Uvicord absorptiometer at 280 nm to measure protein content. All the fractions containing radioactivity were combined, freeze-dried and redissolved in 1.0 ml of water.

Isoelectric focusing. Polyacrylamide disc gels were conditioned with 0.3 ml of 10% (w/v) sucrose containing 2.5% ampholyte (pH 3–10; LKB, Croydon, Surrey, U.K.) for 45 min. The Sephadex G-100 protein eluate and reference bovine serum albumin in 10% sucrose solutions, after application on the gels, were over-layered with 0.1 ml of 5% sucrose containing 2.5% ampholyte. The initial current of 2.0 mA per

tube, raised to 3.3 mA after 15 min, had fallen to 0.65 mA after the 3 h run. Radioactivity and protein were located as for electrophoresis. Each run included two tubes with ampholyte only. These were sectioned as 5 mm segments, the ampholyte was extracted with 1.0 ml of water and the pH measured.

25-Hydroxycholecalciferol-protein binding affinity
A competitive protein-binding assay for $25(\text{OH})\text{D}_3$ (Edelstein *et al.*, 1974) was used to compare the transport proteins of *C. albifrons* and *Erythrocebus patas* (an Old World monkey which uses an α -globulin fraction for vitamin D transport) (Edelstein *et al.*, 1973; Hay & Watson, 1975a). Bovine serum albumin was used as a reference protein.

Measurement of radioactivity. Radioactivity was measured by using a Packard Tri-Carb automatic liquid-scintillation spectrometer no. 3003. The extracts of gel slices were counted for radioactivity in a toluene-dioxane-ethanol solution (Hellung-Larsen, 1971). For aqueous samples, 0.5 ml was removed from each 2.5 ml gel-filtration fraction, and the radioactivity counted in a 1:2 (v/v) Triton X-100-toluene [0.5% 2,5-diphenyloxazole, 0.03% 1,4-bis-(5-phenyloxazol-2-yl)benzene scintillation liquid].

Results and discussion

Polyacrylamide-gel electrophoresis was used to test for binding of $[26,27\text{-}^3\text{H}]25(\text{OH})\text{D}_3$ to Capuchin serum plasma proteins after incubation of serum and steroid at 4°C for 16 h. The radioactivity was found with a protein fraction exhibiting similar mobility to *C. albifrons* and human serum albumins.

This albumin fraction of the Capuchin serum incubated with the radioactive $25(\text{OH})\text{D}_3$ was separated by Cohn fractionation and applied to a Sephadex G-100 column. The elution profiles of protein and radioactivity are shown in Fig. 1. Clearly the radioactivity is migrating with a single protein fraction. This fraction was collected, freeze-dried and redissolved in water.

The reconstituted column eluate was further characterized by using gel electrophoresis with a discontinuous gradient and isoelectric focusing in polyacrylamide gels (Wrigley, 1968). A single peak of radioactivity was observed in both tests. In the gel electrophoresis the radioactivity migrated with a protein fraction which exhibited similar mobility to bovine serum albumin, and in the isoelectric focusing with a protein fraction with pI 4.6–4.7. Bovine serum albumin exhibited pI 4.8–4.9, as reported previously (Gainer, 1973). The pI of human serum albumin is 4.7 (White *et al.*, 1964).

Most mammals have α -globulin transport proteins for 25-hydroxycholecalciferol (Hay & Watson, 1975a). If albumin is used to transport this steroid in Capuchin monkeys it should have a different affinity for $25(\text{OH})\text{D}_3$ compared with α -globulin

Fig. 1. Gel filtration on Sephadex G-100 of serum albumin fraction from Capuchin-monkey serum obtained by Cohn fractionation

For details see the text. $\bullet$, ^3H radioactivity; $\circ$, E_{280} .

Fig. 2. Competitive displacement of 25-hydroxy[26,27- ^3H]-cholecalciferol from vitamin-D deficient *C. albifrons* serum and from *E. patas* serum by increasing amounts of non-labelled 25-hydroxycholecalciferol

For details see the text. Each point is the mean of two determinations. $\circ$, *C. albifrons*; $\bullet$, *E. patas*.

transport proteins in other species. The binding affinities of *Cebus albifrons* and *Erythrocebus patas* serum proteins for $25(\text{OH})\text{D}_3$ were tested by using a competitive protein-binding assay (Edelstein *et al.*, 1974) (Fig. 2). There was no binding of radioactive $25(\text{OH})\text{D}_3$ to bovine serum albumin.

Association constants for the binding proteins towards $25(\text{OH})\text{D}_3$ were calculated from Scatchard

(1949) plots of these displacement curves, and the values obtained were: *E. patas*, K_A $4.0 \times 10^9 \text{ M}^{-1}$; *C. albifrons*, K_A $1.6 \times 10^8 \text{ M}^{-1}$. From these values it is apparent that the binding protein in *E. patas* serum has a considerably higher affinity for $25(\text{OH})\text{D}_3$ than the protein in *C. albifrons* serum. The association constants are consistent with the use of an α -globulin protein for $25(\text{OH})\text{D}_3$ transport in the Patas monkey (Edelstein *et al.*, 1973; Hay & Watson, 1975a) and albumin as the transport protein in the Capuchin monkey.

These results confirm that in the New World Capuchin monkey (*Cebus albifrons*), albumin is the serum protein fraction responsible for $25(\text{OH})\text{D}_3$ transport. As the kidney must remove $25(\text{OH})\text{D}_3$ from the circulation and convert it into $1,25(\text{OH})_2\text{D}_3$ there must be some mechanism for the transfer of $25(\text{OH})\text{D}_3$ from the circulating protein to the kidney. For oestrogens it has been suggested that binding to membrane precedes the transfer of the steroid from plasma protein to cytoplasm. Because of the magnitude of binding of oestrogens to plasma membrane, the membrane must be considered a separate compartment as a carrier for oestrogens, distinct from the extracellular fluid and cytoplasm (Jacobsen *et al.*, 1975). If the cholecalciferol carrier proteins play an active role in vitamin D metabolism then it would seem reasonable to assume that the binding affinity of the proteins for cholecalciferol and its metabolites might be important in the regulation of the transfer of steroid to membrane.

The metabolic clearance rate of steroids is in part dependent on their binding to specific plasma proteins (Baird *et al.*, 1969); testosterone, which is

strongly bound to testosterone-oestradiol-binding globulin, is cleared from plasma at a higher rate than 5α -dihydrotestosterone, which is more strongly bound (Mahoudeau *et al.*, 1971). If the metabolism of vitamin D is similarly dependent in part on the binding affinities of the plasma transport proteins, then the differences in the association constants for the transport proteins towards $25(\text{OH})\text{D}_3$ in *E. patas* and *C. albifrons* sera respectively may influence their rate of cholecalciferol metabolism.

Other animals known to have transport proteins which exhibit mobility similar to that of albumin on gel electrophoresis include two other species of Capuchin monkey (*Cebus capucinus* and *Cebus apella*), the African elephant (*Loxodonta africana*) and Indian elephant (*Elephas maximus*), the Pacific dolphin (*Delphinus bairdi*) and killer whale (*Orcinus orca*) (Hay & Watson, 1975a). In *C. capucinus* and *C. apella* the binding affinity of the serum transport proteins for $25(\text{OH})\text{D}_3$ is similar to that of the albumin transport protein described in this paper (A. W. M. Hay, unpublished work).

This work was supported by a grant from the Wellcome Trust. I am grateful to Dr. D. E. M. Lawson for supplying the labelled $25(\text{OH})\text{D}_3$, to Dr. S. Edelstein for assistance with the gel filtration, to Miss M. Charman for assistance with the competitive protein-binding assay and to Mr. D. G. Taylor for the photograph.

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