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β-PHOCACHOLIC ACID IN BILE; BIOCHEMICAL EVIDENCE THAT THE FLAMINGO IS RELATED TO AN ANCIENT GOOSE[1]

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Abstract. The biliary bile acid composition of 10 anseriform and three flamingo species was determined using a variety of chromatographic techniques. Some bile samples contained an unusual 23-hydroxylated derivative of chenodeoxycholic acid, (23R)-3α,7α,23-trihydroxy-5β-cholan-24-oic acid (β-phocacholic acid). The proportion of this unusual bile acid correlated highly with the order of evolutionary appearance. β-Phocacholic acid was found to constitute a major proportion of biliary bile acids in three flamingo species. The bile acid spectra present in these flamingos were similar to those of several ducks from the subfamily Anatinae; those of the Greater Flamingo (*Phoenicopterus ruber*) were virtually identical to those of the Nene or Hawaiian Goose (*Branta sandvicensis*). It is suggested that the common presence of a new biochemical character, β-phocacholic acid, in both ducks (Anatinae) and flamingos (Phoenicopteridae) provides evidence for a close evolutionary link between these bird families.

Key words: *Bile acids; β-phocacholic; flamingo; phylogeny.*

INTRODUCTION

Traditional morphological considerations (Wetmore 1960, Cracraft 1981) and a recent DNA hybridization study (Sibley et al. 1988) have been used as evidence for assigning flamingos together with storks, herons, and ibises to the order Ciconiiformes. Nonetheless, others have proposed that flamingos are more closely related to the Charadriiformes (Olson and Feduccia 1980) or Anseriformes (Delacour 1961). Although it is hazardous to use a single biochemical character as a criterion for evolutionary relationships, the available bile acid analyses of vertebrate species have been shown to agree well with assignments based on morphology and palaeontology (Haslewood 1978a). We reasoned that bile acid structure could provide useful information for defining the phylogenetic position of the flamingo. To test this hypothesis, conventional and spectroscopic techniques developed in the process of identifying the unusual bile alcohols of the manatee (Kuroki et al. 1988) were used to analyze the bile salts of a variety of birds and flamingos.

MATERIALS AND METHODS

Anseriform and flamingo gall bladder bile samples obtained from dead animals were provided by the pathology laboratories of the San Diego Zoo and by Sea World Florida. Samples were placed in several volumes of isopropanol immediately after collection to prevent bacterial degradation.

Thin layer chromatography (TLC) of the whole bile was performed on silica gel G (E. Merck, Darmstadt, Federal Republic of Germany) using two solvent systems: (1) isoamylacetate: propionic acid : 1-propanol : water, 4:3:2:1, v/v (Hofmann 1962), for free and conjugated bile acids; (2) a double development system for the taurine conjugates, in which plates were developed first in chloroform : acetone : methanol : propionic acid : water, 10:4:2:2:1, v/v and after drying overnight, in 1-butanol: propionic acid : water 10:1:1 v/v (Elferink et al. 1989). (The latter system separates glucuronide conjugates from taurine conjugates.) Bile acids were isolated by scraping selected bands off a TLC plate and eluting with $CHCl_3$: MeOH, 2:1, v/v or collecting selected peaks in the high pressure liquid chromatography (HPLC) eluent. Bile acids were visualized by spray reagents for hydroxyl groups (phosphomolybdic acid), oxo groups (2,4 dinitrophenyl-hydrazine), glucuronides (naphthore-

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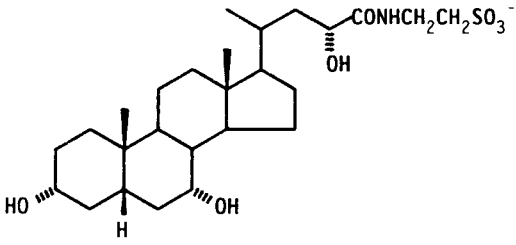

FIGURE 1. Structural formula of β-phocacholic acid present in flamingo bile as its taurine (N-acyl) conjugate.

sorcinol), or vicinal hydroxyl groups (lead tetraacetate) (Dawson et al. 1969). Conjugated bile acids were analyzed by HPLC of whole bile using a modification of the technique of Rossi et al. (1987) which uses a reversed phase octadecylsilane column (RP C-18) and elution at 0.75 ml/min with an isocratic buffer, pH 5.4, composed of a mixture of methanol (67.4%) and 0.01 M KH_2PO_4; the effluent was monitored at 205 nm (amide bond). Bile acids were deconjugated chemically (1.0 NaOH, 130°C, 4 hr), and the resulting unconjugated bile acids were analyzed by gas liquid chromatography (GLC) as their methyl ester acetate derivatives using a Hewlett-Packard 5840A instrument fitted with a 0.63 cm × 2 m SP-2250 (methyl : phenyl) column packed on Supelcoport 100–120 at 270°C (isothermal). ^{1}H-NMR was recorded in Cl_3CD on a 360 MHz instrument equipped with a modified Varian MR-220 console, Oxford magnet and Nicolet 1180-E computer system. Chemical shifts are in ppm relative to tetramethylsilane.

RESULTS AND DISCUSSION

The chemical identity of β-phocacholyltaurine (Fig. 1) was determined from the material obtained by collecting the appropriate peak from an HPLC chromatogram of flamingo bile. By low resolution secondary ion mass spectroscopy (SIMS) the compound's quasimolecular ion at M/2 514 indicated that it was the taurine conjugate of a trihydroxy C_{24} bile acid (Whitney and Burlingame 1985). It had a TLC (Rf 0.12 System 1, Rf 0.37 System 2) and HPLC (RRT 0.34 compared to chenodeoxycholyl-glycine) identical to that of an authentic sample of β-phocacholyltaurine. Following deconjugation and formation of the methyl ester acetate derivative, material from peak (A) (Fig. 2) had a GLC RRT identical to that of the triacetyl derivative of the methyl ester of 23R-phocacholic acid (RRT 1.45 relative to cholic methyl ester acetate). This derivative was found by ^{1}H-NMR to be methyl (23R) 3α,7α,23-triacetyloxy-5β-cholan-24-oate: δ 0.663 (s, 3H, Me-18), 0.933 (s, 3H, Me-19), 0.967 (d, 3H, J = 6.1 Hz, Me-21), 2.030 (s, 3H, CH_3COO-), 2.051 (s, 3H, CH_3COO-), 2.145 (s, 3H, 23-CH_3COO-), 3.732 (s, 3H, -$COOCH_3$), 4.587 (m, 1H, H-3), 4.880 (m, 1H, H-7), 5.05 (dd, 1H, J = 11.0 and 1.6 Hz, H-23). This spectrum was identical to that reported by Klinot et al. (1986). Especially diagnostic is the signal of H-23 which appeared as a double doublet (J = 11.0 and 1.6 Hz) at 5.05 ppm, indicating a configuration of 23R for the acetate and the original hydroxyl group. In contrast, in 23S derivatives H-23 appears as a triplet with J = 11 Hz.

Analyses of the (biliary) bile acids from the Greater Flamingo (*Phoenicopterus ruber*), Lesser Flamingo (*Phoeniconaias minor*), and Chilean Flamingo (*Phoenicopterus chilensis*) indicated the presence of a substantial proportion of β-phocacholic acid (Fig. 1) in each of the three species. This compound was present, as were the other identified bile acids, in the form of its N-acyl taurine conjugate.

Unlike the great majority of vertebrate species in which dihydroxy bile acids are further hydroxylated at position 12 (on the steroid nucleus) to form cholic acid, β-phocacholic acid is hydroxylated in position 23 (on the side chain), a trait shared in common with the Anatinae subfamily (ducks) of Anseriformes (Klinot et al. 1986).

Table 1 lists the biliary bile acid composition of these three flamingo species, as well as other selected wading birds with which flamingos have been traditionally associated. From this table, it is quite clear that the presence of β-phocacholic acid, as well as the low proportion or absence of cholic acid, groups the flamingos with the anseriforms and differentiates them from other ciconiiforms and the charadriforms. Additional support in favor of this grouping is the sharing of common anatomical and physiological features between ducks and flamingos, including lamellate bill, feather structure, voice patterns, a large cecum, preen wax composition, and mallophagan parasites (Sibley et al. 1969).

In Table 2, 10 anseriforms are listed in order of evolutionary appearance, as assembled by Livezey (1986), based on his phylogenetic analysis using morphological characters. A high cor-

TABLE 1. Biliary bile acid composition[a,b] of flamingos and other selected wading birds by HPLC.

Order	Species			Biliary bile acid composition, %			
	Common name	Latin name	*n*	3α7α[c]	3α7α12α[c]	3α7α23R[c]	Compound E[d]
	Chilean Flamingo	*Pheonicopterus chilensis*	6	25.2	7.1	62.3	0
	Lesser Flamingo	*Phoeniconaias minor*	2	60.8	7.3	29.5	2.4
	Greater Flamingo	*Phoenicopterus ruber*	7	82.8	1.3	13.6	2.3
Anseriformes	Nene	*Branta sandvicensis*	2	84.7	0	10.5	4.8
Anseriformes	Ringed Teal	*Callonetta leucophrys*	1	64.3	2.9	30.1	2.7
Charadriformes	American Avocet	*Recurvirostra americana*	2	30.8	69.2	0	0
Ciconiiformes	Marabou Stork	*Leptoptilos crumeniferus*	1	35.3	64.7	0	0
Ciconiiformes	Scarlet Ibis	*Eudocimus ruber*	1	21.5	78.5	0	0

[a] Bile acids were present in bile in all birds as the taurine (N-acyl) conjugates.
[b] Biliary bile acid composition has been normalized to 100%.
[c] The position of the hydroxyl substituents is indicated. 3α7α, chenodeoxycholic acid; 3α7α12α, cholic acid; 3α7α23R, β-phocacholic acid.
[d] A preliminary determination of the structure of this compound indicates that it is allo-chenodeoxycholic acid (3α,7α-dihydroxy-5α-cholan-24-oic acid).

relation between the relative percentage of β-phocacholic acid and the ranking of the birds is readily apparent (Spearman's rank correlation test, $P < 0.001$) (Brown and Hollander 1977). Including the flamingo in this statistical test (rank value of 0 assigned) resulted in a P-value < 0.015, still statistically significant. In this correlation, the relative amount of β-phocacholic acid increases in bile over time in a manner which is independent of diet and morphology; evidently the genes encoding enzymes responsible for 23-hydroxylation have been evolutionarily successful.

If it is true that the flamingos and anseriforms are directly related (as evidenced by the common presence of a bile acid 23-hydroxylase), the relatively low level of β-phocacholic acid expression in the Greater Flamingo suggests that among the flamingos it is closest to the branch point of the flamingo-duck families. This is true even if one assumes that the genetic course of 23-hydroxylase development proceeded differently in flamingos and geese once these two families diverged.

Using rank correlation based on the percentage of phocacholic acid, it is possible to assign the origin of the Phoenicopteridae within the phylogeny of extant anseriforms. Figure 2 contains an abbreviated phylogenetic tree of anseriform family names assembled by Livezey (1986) into which the Phoenicopteridae has been inserted next to the *Branta*. In addition to the presence of β-phocacholic acid, a high degree of concordance is also present in the overall bile acid profiles of the Greater Flamingo and the Nene, two birds with low but proportionately equal amounts of β-phocacholic acid. As shown by Figure 3, this similarity included not only the common pres-

TABLE 2. Biliary bile acid composition[a,b] of selected anseriforms[c] and the Greater Flamingo by HPLC.

Species			Biliary bile acid composition, %[d]		
Common name	Rank	Latin name	3α7α	3α7α23R	Compound E
Southern Screamer	1	*Chauna torquata*	27	0	63
Pied Goose	2	*Anseranus semipalmata*	93	0	7
Java Tree Duck	3	*Dendrocygna javanica*	98	0	2
Black-Neck Swan	5	*Cygnus melanocoryphus*	96	0	4
Bar-Head Goose	5	*Anser indicus*	87	9	4
Red-Breasted Goose	5	*Branta ruficollis*	90	5	5
Nene	5	*Branta sandvicensis*	86	10	4
Greater Flamingo		*Phoenicopterus ruber*	84	13	3
Mandarin Duck	9	*Aix galericulata*	68	27	5
Crested Duck	12	*Anas specularidides*	62	34	4
Tufted Duck	13	*Aythya fuligula*	27	69	4

[a] Bile acids were present in bile in all birds as the taurine (N-acyl) conjugates.
[b] Biliary bile acid composition has been normalized to 100%.
[c] Anseriforms are listed in order of evolutionary appearance.
[d] For abbreviations, see Table 1.

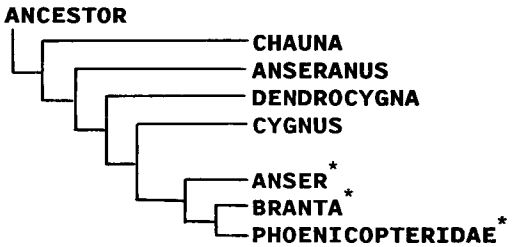

FIGURE 2. Addition of the flamingo family Phoenicopteridae into the phylogenetic tree of recent anseriform genera adopted from Livezey (1986). The presence of β-phocacholic acid in bile is indicated by an asterisk.

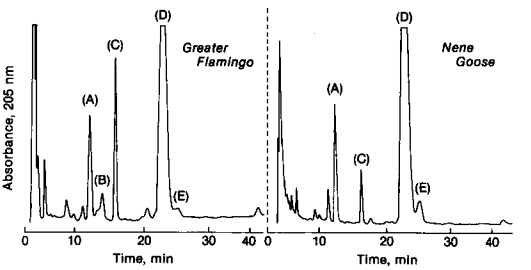

FIGURE 3. Similar high pressure liquid chromatography (HPLC) bile acid profiles of the Greater Flamingo and Nene. Peak identification: (A) phocacholyl-taurine, (B) phocadeoxycholyl-taurine, (C) unknown, (D) chenodeoxycholyl-taurine (chemical structure is given in Table 1), and (E) allo-chenodeoxycholyl-taurine.

ence of a dominant amount of chenodeoxycholic acid (Peak D) and the unusual bile acid β-phocacholic acid (Peak A), but in addition included most of the unidentified peaks seen in the HPLC chromatograms. The unexpected resemblance between these two biliary profiles offers further support for both a close relationship and a similar time of divergence from a common ancestor.

The occurrence of a similar bile acid spectrum (presumably inherited from a common ancestor) in two birds which exhibit dissimilar morphology and diets implies that morphological and dietary changes are evolving at a faster rate than are changes in bile acids. This stability in the bile acid profile is present even though steady state biliary bile acid composition is the result of not only hepatic biosynthesis rate, but also canalicular secretion rate, as well as intestinal conservation and subsequent bacterial and hepatic biotransformation pathways (Hofmann 1989). In vitro, microorganisms can be shown to hydroxylate bile acids at a variety of positions on the steroid ring (Hayakawa 1982), but such compounds are never observed in significant proportions in bile. Side-chain hydroxylation of bile acids by microorganisms has not been observed (MacDonald et al. 1983).

The presence of 23-hydroxylated bile acids in only a few genera that belong to unrelated groups (ducks, pinnipeds, snakes) is further evidence that phocacholic acid is derived, rather than primitive. Although it is possible that β-phocacholic acid could have arisen independently in flamingos and anseriforms, the rarity of 23-hydroxylation in vertebrate bile would appear to justify our proposal of the existence of one common ancestor that experienced the event de novo.

It is also of interest to note that the presence of compound E (tentatively identified to be 3α,7α-dihydroxy-5α-cholan-24-oic acid), so prominent in the relatively "ancient" screamer, and shared by all the anseriforms (including the Greater and Lesser flamingos), is also observed in certain "older" members of the Galliformes.

The hepatic biosynthesis of bile acids from cholesterol and the formation of urea from ammonia (Mommsen and Walsh 1989) are examples of stable end products of metabolism which appear useful to deduce taxonomic relationships. Bile acids are the metabolic end products of cholesterol found in all vertebrates, and their formation is essential to life (Haslewood 1978b). Moreover, there are a variety of vertebrate bile acids, and all of these function adequately as digestive surfactants (Hofmann and Mysels 1988). The biliary bile acid pattern observed in each species is determined predominantly by hepatic synthesis, in which specific hepatic hydroxylases operate on a finite number of sites on the steroid molecule. The relationship between biosynthetic intermediates and functioning bile acids is biochemically very close and appears to conform to a biochemical analogy of Haeckel's law in which the bile acids present in adults of older vertebrates have become intermediates in the synthesis of the more complex bile acids of more recently evolved vertebrates. Whether the precise molecular differences seen in the stable bile acid spectrum can ultimately be directly related to the genes of each species can only be decided by further investigation.

While evolutionary biologists have long distrusted conclusions based on a single character, the bile acid profiles reported here are objective

and quantifiable data that link the origins of the flamingo to the Anseriformes, albeit a currently unfashionable view.

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