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PHYLOGENETIC DISTRIBUTION OF THE HEPATIC ENZYME SYSTEM FOR REDUCING NALOXONE TO 6α- AND 6β-NALOXOL IN VERTEBRATES

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Abstract—1. Livers from a variety of vertebrate species were examined for reduction of naloxone to 6α- and 6β-naloxol.

2. Bird and reptile livers possessed relatively high rates of enzyme activity and formed predominantly 6α-naloxol.

3. Livers from fish and amphibia showed generally low rates of naloxone reduction and more equal amounts of 6α- and 6β-naloxol.

4. Naloxone reduction in mammal livers was intermediate in activity and resulted primarily in 6β-naloxol even though guinea pigs were a particularly interesting exception.

5. It was not possible to relate product stereoselectivity to phylogenetic development.

INTRODUCTION

The status of the cytoplasmic aldo-keto reductases as a class of drug metabolizing enzymes was reviewed by Baucher (1976). Among them are enzymes which metabolize warfarin, daunorubicin, cyclohexanone and naloxone. These enzyme systems have been found in liver, kidney, brain, heart, muscle, lung and blood. Because of the similarities among a number of these enzymes he felt that "these enzymes may be related genetically and through evolution". In the present study, the phyletic distribution of the liver cytoplasmic enzymes involved in the reduction of naloxone, a narcotic antagonist, to 6α- and 6β-naloxol is examined on an empirical basis. This reduction reaction for naloxone is of particular interest since product stereospecificity exists for formation of 6α- and 6β-naloxol and seems to depend upon the species of animal used. For example, Fujimoto *et al.* (1975) have shown that rabbit liver cytosol reduced naloxone primarily to 6β-naloxol, while chicken liver cytosol formed exclusively 6α-naloxol as reported by Chatterjie *et al.* (1974). Further studies by Cone (1976) and Roerig *et al.* (1976a) have shown that the rat produced 6β-naloxol while guinea pig formed both 6α-naloxol and 6β-naloxol. A more broad phyletic study therefore serves to give an insight into the distribution of this product stereospecificity. To this end, 52 species, representing the 7 vertebrate classes were examined for reduction of naloxone to 6α- and 6β-naloxol in liver cytosol preparations.

MATERIALS AND METHODS

Materials

Animals used in this study were obtained from a variety of sources. These sources and the locality data for non-domestic forms are available upon request.

Naloxone hydrochloride was provided by Endo Laboratories, Garden City, NY. Standard 6α-naloxol and 6β-naloxol hydrochloride salts were obtained from the National Institute on Drug Abuse (NIDA). Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD) and $NADP^+$ were purchased from Sigma Chemical Company, St. Louis, Missouri. [^{3}H(G)] naloxone (19 Ci/mmol, >95% purity) was purchased from New England Nuclear, Boston, Massachusetts. Regisil II (BSTFA + 1% TMCS) was purchased from Regis Chemical Company, Morton Grove, Illinois. All other chemicals were reagent grade. Thin layer chromatography plates were manufactured by E. M. Merk, Darmstadt, Germany.

Analysis for 6α- and 6β-naloxol formation

Animals were killed and livers removed immediately in most cases and as soon as the circumstances would allow in the few remaining cases. In some situations (e.g. when the animal was killed by a hunter in the field) the livers were put in ice, frozen and processed at a later time. Whenever possible, the livers were immediately processed. The livers were homogenized with 2 ml of 0.05 M potassium phosphate buffer, pH 7.9, per gram wet weight of tissue using a motorized Potter–Elvehjem tissue grinder. Homogenates were centrifuged 10 min at 9000 g and the resulting supernatants were centrifuged 60 min at 100,000 g. The 100,000 g supernatants were assayed for naloxone reducing enzyme activity. Protein content was determined by the method of Lowry *et al.* (1951). Naloxone reducing activity was measured using the analytical procedure published earlier by Roerig *et al.* (1976b). The assay mixture contained about 1 mg protein from the 100,000 g supernatant, 1 mM naloxone (0.1 μCi ^{3}H naloxone), 0.01 M potassium phosphate buffer pH 7.4, and an NADPH generating system consisting of 7.8 mM G6P, 0.125 mM NADP and 2 units G6PD in a final volume of 1 ml. The mixture was incubated generally at 37°C for a predetermined time period in a shaking metabolic incubator. After incubation, tubes were immediately placed in an ice bath and 1 ml of chilled 1 M sodium carbonate buffer pH 10 was added to give a final pH of 8.5–9. The mixture was then extracted with 2 ml ethyl acetate. A 0.4 ml sample of the extract was applied to TLC plates. The plates were developed in

Table 1. Naloxone reducing activity of 100,000 *g* supernatants prepared from livers of selected species of the seven vertebrate classes

Class	Order	Common names	N	Nmol naloxol formed/min/mg protein	α/β	β/α
Agnatha	Petromyzontiformes	Sea lamprey *Petromyzon marinus*	8	0		
Chondrichthyes	Heterodontiformes	Shark *Heterodontus francisci*	1	0.57	1.84	
	Rajiformes	Pacific electric ray *Torpedo californica*	1	0.37	3.81	
Osteichthyes	Amiiformes	Bowfin *Amia calva*	1	0.07		1.72
	Salmoniformes	Rainbow trout *Salmo gardneri*	4	0.01	1.89	
		Northern pike *Esox lucius*	2	0.03	2.38	
	Cypriniformes	Carp *Cyprinus carpio*	2	1.54	all α	
		Goldfish *Carassius auratus*	29	1.89 ± 0.27	all α	
	Perciformes	Largemouth bass *Micropterus salmoides*	1 (2 pooled)	0.04	all α	
		Black crappie *Pomoxis nigromaculatus*	2	0.13	2.19	
	Siluriformes	Yellow bullhead *Ictalurus natalis*	1	0.14	2.13	
Amphibia	Caudata	Hellbender *Cryptobranchus alleganiensis*	2	0.15	2.31	
		Tiger salamander *Ambystoma tigrinum*	2 (pools of 5 each)	0.12		1.66
		Mudpuppy *Necturus maculosus*	1	0.05	7.50	
	Salienta	Bull frog *Rana catesbeiana*	3	0.07 ± 0.04		2.71 ± 0.60
		Southern leopard frog *Rana utricularia*	7	0		
Reptilia	Testudinata	Snapping turtle *Chelydra serpentina*	3	0.24 ± 0.14	2.99 ± 0.78	
		Red-eared turtle *Pseudemys scripta*	2	0.85	all α	
	Crocodilia	Caiman *Caiman crocodilus*	2	0.23	3.19	
	Squamata	Yellow rat snake *Elape obsoleta*	2	3.01	all α	
		Osage copperhead *Agkistrodon contortrix*	1	2.34	all α	
		Iguana *Iguana iguana*	1	0.08	all α	
		Desert iguana *Dipsosaurus dorsalis*	2	0.94	2.33	
		Tokay gecko *Gecko gecko*	2	4.03	all α	
		Chuckwalla *Sauromalus obesus*	1	0.66	all α	
Aves	Anseriformes	Pekin duck *Cairina moschata*	2	2.39	all α	
		Blue wing teal *Anas discors*	4	0.51 ± 0.09	5.20 ± 1.60	
		Wood duck *Aix sponsa*	5	4.05 ± 0.65	all α	
		Mallard *Anas platyrhynchos*	2	6.19	all α	
		Canada goose *Branta canadensis*	3	0.74 ± 0.03	5.96 ± 1.12	
	Galliformes	Chicken *Gallus sp.*	13	6.91 ± 1.90	all α	
		Turkey *Meleagris gallopavo*	1	4.64	all α	
		Bobwhite quail *Colinus virginianus*	4	1.74 ± 0.49	all α	
		Japanese quail *Coturnix japonica*	1 (4 pooled)	0.83	all α	
Aves	Galliformes	Ringneck pheasant *Phasianus colchicus*	1	6.91	all α	
		Spruce grouse *Canachites canadensis*	1	17.49	all α	
	Columbiformes	French pigeon *Columba livia*	4	0.16 ± 0.04	9.26 ± 3.30	
		Common pigeon *Columba livia*	2	0.10	4.13	
Mammalia	Marsupialia	Opossum *Didelphis marsupialis*	1	0.02	2.27	
	Chiroptera	Little brown bat *Myotis lucifugus*	5	0.02	all α	
	Lagomorpha	New Zealand rabbit *Oryctolagus cuniculus*	6	3.34 ± 0.87		8.72 ± 2.98
		Dutch rabbit *Oryctolagus cuniculus*	2	0.96		3.70

Table 1. (*continued*)

Class	Order	Common names	N	Nmol naloxol formed/min/mg protein	α/β	β/α
	Rodentia	Sprague Dawley rat *Rattus norvegicus*	5	0.71 ± 0.17		all β
		Wistar rat *Rattus norvegicus*	4	0.67 ± 0.14		all β
		Swiss Cox mouse *Mus musculus*	11	0.52 ± 0.16		3.03 ± 0.70
		Golden Syrian hamster *Mesocricetus auratus*	1	0.67		1.64
		Hartley guinea pig *Caria porcellus*	7	6.39 ± 1.52	6.34 ± 1.01	
		English smooth hair guinea pig *Caria porcellus*	4	2.64 ± 1.30	1.89 ± 0.53	
	Carnivora	Dog *Canis familiaris*	2	0.04	1.26	
		Cat *Felis catus*	1	0.02	1.58	
	Artiodactyla	Pig *Sus scrofa*	7	1.21 ± 0.41		all β
		Cow *Bos pirmigenius*	1	0.35		all β
		Sheep *Oria ammon*	2	0.77		all β
		Whitetailed deer *Odocoileus virginianus*	2	0.28		2.05
	Perissodactyla	Horse *Equus caballus*	3	0.16 ± 0.02	4.05 ± 1.19	
	Primates	Rhesus monkey *Macaca mulatta*	4	0.21 ± 0.04.		10.25 ± 2.48

chloroform:methanol:ammonia (90:10:4, v/v) and allowed to dry overnight. As the plates dried, yellow spots appeared which corresponded in Rf to standard naloxone (0.70), 6α-naloxol (0.33) and 6β-naloxol (0.27). The separated spots and background areas were scraped into vials containing 10 ml toluene-Triton scintillation solution and the radioactivity was estimated in a Packard Model 2420 scintillation spectrometer. Background counts/min were subtracted from counts/min in the spots. Enzyme activity was estimated by calculating 6α- and 6β-naloxol counts/min as a percent of the total counts/min recovered from the TLC plate. This percent was multiplied by the total amount (nmol) of naloxone added to the assay incubation mixture to obtain the amount (nmol) of product formed. Enzyme activity was expressed as nmol naloxol formed/min/mg protein. Length of the incubation period was determined from a preliminary enzyme assay in which more than 1 mg protein was used and the mixture was incubated for 60 min at 37°C. Time of incubation for the subsequent assay of the same protein mixture (described above) was adjusted to obtain a 5–25% conversion of naloxone to naloxol. These assay mixtures were analyzed by TLC and the results used to calculate the ratio of the amounts of 6α- and 6β-naloxol formed.

The naloxol ratios obtained from TLC results were confirmed by gas chromatographic analysis. Ethyl acetate extracts from assays (in which no ^3H naloxone was added) were evaporated to dryness with nitrogen. 50 microliters Regisil II was added and the mixture was incubated 60 min at 90°C, cooled immediately and evaporated to dryness with nitrogen. The derivatized compounds were dissolved in methylene chloride and the mixture was injected into a Perkin-Elmer Model 900 gas chromatograph equipped with a flame ionization detector. The column was 6′ × 1/4″ glass packed with 3% QF-1 on 80/100 mesh Gas Chrom Q. Temperature was programmed from 200–240°C at 1.5°C/min. Flow rates for nitrogen, hydrogen and air were 55, 45 and 900 ml/min respectively. The retention times were 13.0 min for naloxone, 6.1 min for 6α-naloxol; 6β-naloxol produced two peaks, a small one at 4.8 min and the large one at 7 min. Areas under the peaks were calculated by triangulation and standard curves for naloxone and 6α-naloxol were constructed. Amount of naloxone and reduced metabolites in assay extracts were estimated using the standard curves.

RESULTS

Some of the factors affecting enzyme activity were studied in various ways. Not all the *in vitro* incubations in the present study were performed at 37°C; liver cytosols from goldfish, carp, bass, ray, shark, bullhead, pike, crappie, trout, turtle, iguana, caiman, frog, tiger salamander, copperhead and Tokay gecko were incubated at 26°C as well as 37°C. In these cases, the rate of naloxone reduction at 26°C was less than or the same as that at 37°C. Because the enzyme activity did not deteriorate at 37°C, this temperature was used for incubation of the preparations from these species even though Dewaide (1971) reported that another enzyme system, the hepatic microsomal mixed function oxidase system of fish showed higher activity at 26°C. Liver cytosols from pheasant, chicken, grouse, wood duck, turkey, Bobwhite quail, Canada goose and mallard were incubated at 42°C and compared to identical assays performed at 37°C. In all these species, incubation at 42°C produced a higher rate of naloxone reduction than incubation at 37°C. Since these birds all showed relatively high naloxone reducing activity at 37°C, further comparisons between species were made at the standard 37°C incubation temperature.

Most assay mixtures were incubated for 30 min to obtain enzyme activity values. However, if the preliminary assay (incubated for 60 min) indicated the protein mixture was very active, time of incubation was decreased to 15 min in the subsequent assay. This short incubation time was used only in a few bird and reptile species, which formed only 6α-naloxol. If in preparations from other species little naloxone reducing activity was found after 60 min of incubation, the protein mixture was subsequently incubated for as

long as 6 hr (in trout) to detect the small amount of naloxone reduction.

Assay mixtures using 1 mg chicken, goldfish, rabbit and guinea pig proteins and incubating for 5, 10, 20, 30, 60 and 120 min showed linearity of naloxone reduction with time between 10 min to 120 min. Experiments on the effect of protein concentration of 0.2–2.6 mg for the chicken, rabbit, guinea pig, goldfish and Tokay gecko indicated that the rate of naloxone reduction was linear between approximately 0.5 and 1.5 mg protein in these 5 species. Therefore, in all species, protein amounts of around 1 mg were used in assays for determining enzyme velocity.

Since all livers could not be processed immediately after removal from the animal, studies were carried out to assess enzyme activity of fresh and frozen livers in a few selected species. Livers from chicken, rabbit, guinea pig, cow and pig were removed and divided into two parts. One part was processed immediately and the remainder was frozen, then several days later thawed and processed. There was no appreciable change in naloxone reducing activity using fresh or frozen livers from rabbit, guinea pig, or pig. Chicken liver lost approximately 30% of its naloxone reducing activity after freezing and thawing. Therefore, fresh chicken livers were used for subsequent comparisons. The turkey, quail, pigeon and Pekin duck livers were fresh. Naloxone reducing activity from cow liver was greater in the frozen liver for both 6α and 6β formation but this difference was not investigated further. Livers from most mammals were obtained fresh, except for monkey, deer and opossum.

To check the stability of the 100,000 *g* supernatant stored at $-20°C$, chicken and rabbit liver supernatants were used as representative samples. These 100,000 *g* supernatants were divided into 1 ml aliquots and stored at $-20°C$. After 1, 2, 7, 10, 20, 30, 40, 60 and 90 days the samples were thawed and assayed for naloxone reducing activity. Supernatants from livers of both species failed to lose activity even after 90 days at $-20°C$. The enzyme velocity was 2.77 ± 0.08 nmol/min/mg for the rabbit preparation over the 90 day period and 7.28 ± 0.67 for the chicken preparation.

Table 1 shows the rate of naloxone reduction and the ratio of $6\alpha/6\beta$- or $6\beta/6\alpha$-naloxol. The values are means with $\pm$SE in some cases. For the ratio, the predominant product is always the numerator and the product formed in smaller quantity the denominator. This manner of expression is used so that the ratio is

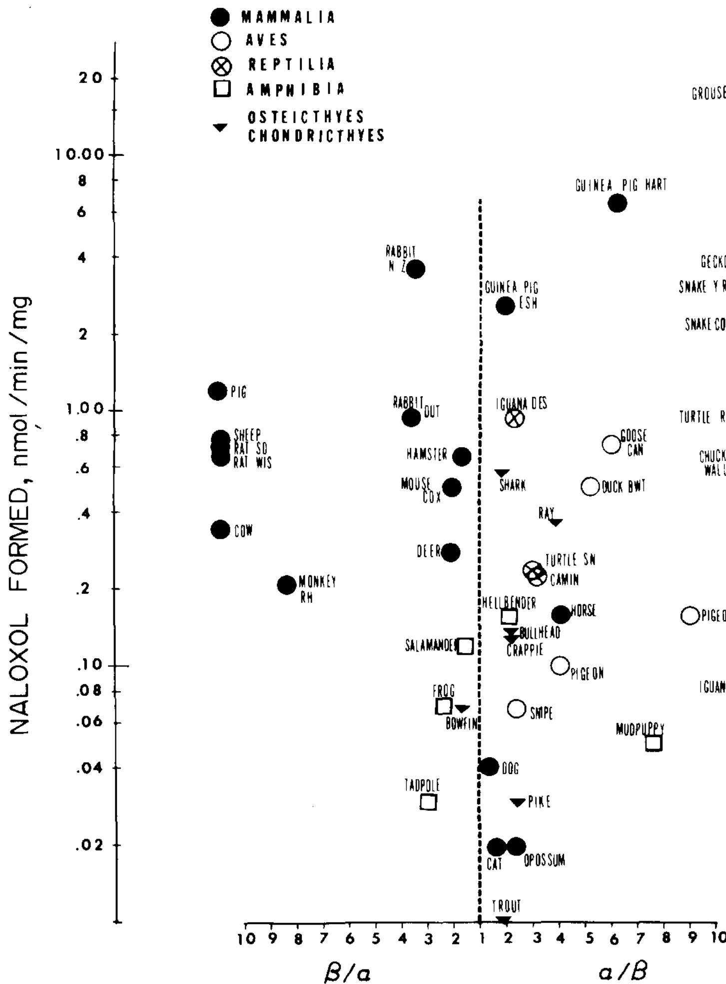

Fig. 1. Naloxone reducing activity of 100,000 *g* supernatants prepared from livers of various animals plotted as the total rate naloxone reduction (ordinate) versus the ratio of the α to β or β to α naloxol (abscissa).

greater than one and avoids values less than one. It avoids the obvious difficulty of comparing a ratio of, for example 2 vs 1/2. That is, an α/β-ratio of 2 is equal in magnitude to a β/α-ratio for 2. The data in Table 1 are shown in illustration form in Fig. 1 to provide a simpler format in which to compare the species. Note in this figure the overall rate is plotted on a logarithmic scale which tends to squeeze the higher values together. Also, we empirically chose a value for the ratio of 10 as the cut off where the product was predominantly one or the other product.

In terms of overall rate, aves and then reptilia were notable for their generally high rates. Mammals were intermediate and amphibia and fishes had low rates. Since there were sufficient exceptions to these general statements, no obvious relationship could be perceived.

Inclusion of the ratio, β/α- or α/β-, as an added parameter, tended to further differentiate between species. Mammals which possessed intermediate naloxone reducing activity were characterized by the predominance of species, which produced more 6β- than 6α-naloxol. The horse and guinea pigs were interesting in that they reduced naloxone rapidly but they produced more α- than β-naloxol. The Hartley guinea pig had the highest enzymatic activity among the mammals. Note that the other mammals that produced more α- than β-naloxol, the dog, cat, opossum and bat, all had very slow rates.

The grouse, chicken, pheasant, Mallard duck, turkey, Wood duck, Pekin duck, and Bobwhite and Japanese quails were notable for forming solely 6α-naloxol at high rates. However there were other birds that formed both 6α- and 6β-naloxol; these had much slower rates.

The amphibia formed both isomers, but their overall rate was slow and their values seemed to segregate in an area different from most other species. The distribution of data points for reptilia were similar to the aves. The goldfish and carp both from the order cypriniformes were predominant 6α-naloxol formers at high rates but other fish had much slower rates.

DISCUSSION

It is obvious from the results that it was not possible to relate the product stereospecificity in formation of 6α- and 6β-naloxol to phylogenetic development. At best, enzyme activity in mammalian species segregate toward predominance in formation of 6β-naloxol while fish, reptilia and birds have predominantly 6α-naloxol forming activities.

This situation was not improved by considering such factors as habitat, food source, body size, general metabolism, etc. Thus, it was not clear why mammals in general should be different from the other animals.

Some specific points are of interest. The Hartley guinea pig was predominantly a 6α-naloxol former and its total enzyme activity was high. Because of these factors, a separate study was done earlier by Roerig *et al.* (1977) to characterize its enzymes further. The guinea pig showed other species differences. A previous study by Fujimoto *et al.* (1975) in the rabbit showed that the conversion of naloxone to 6β-naloxol was inhibited completely by 10^{-3} M morphine added to the incubation mixture; the formation of 6α-naloxol was not affected. The formation of 6α-naloxol in the chicken, an animal which produces no 6β-naloxol, was also not affected by morphine as shown by Roerig *et al.* (1976b). Thus, it appeared that the ability of morphine to differentially inhibit 6β-naloxol formation could be used to compare and characterize enzyme systems. Since the guinea pig produced both 6α- and 6β-isomers, we expected morphine to inhibit 6β-naloxol formation. However, Roerig *et al.* (1977) showed that addition of morphine *in vitro* to guinea pig liver preparations stimulated the formation of 6α-naloxol. In the present study, the horse like the guinea pig was a mammal which produces predominantly 6α-naloxol. Therefore, morphine was added *in vitro* to horse liver preparations but the data are not given because the magnitude of the stimulatory effect observed was questionable due to the low control values.

In conclusion, the wide range of enzyme activities for forming 6α- and 6β-naloxol were insensitive as phyletic indicators.

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