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DISTRIBUTION AND PERCENTAGES OF NON-PROTEIN BOUND CONTRACEPTIVE STEROIDS IN HUMAN SERUM

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SUMMARY

The percentages of non-protein bound ethinyl-estradiol (EE_2)†, norethisterone (NET)†, D-norgestrel† and cyproterone-acetate (CA)† have been measured by centrifugal ultrafiltration-dialysis in undiluted serum at 37°C. The respective percentages (mean ± SD) of these steroids which are non-protein bound in serum from normal men ($n = 5$) and women ($n = 10$) are as follows: EE_2, 1.44 ± 0.15, and 1.54 ± 0.16; NET, 4.71 ± 0.55 and 3.69 ± 0.90; D-norgestrel, 3.05 ± 0.34 and 2.50 ± 0.68; CA, 6.62 ± 0.43 and 7.22 ± 0.96. Similar values were found for samples taken during follicular and luteal phases of the menstrual cycle. Percentages of non-protein bound serum EE_2 and CA were similar in both sexes, while those for NET ($P < 0.05$) and D-norgestrel ($P < 0.2$) appear to be higher in serum from men than women. Furthermore, the percentages of non-protein bound EE_2 (1.58 ± 0.14, $n = 4$) and CA (6.33 ± 1.01, $n = 5$) in third trimester pregnancy sera were similar to those in serum samples from men and non-pregnant women, while those of NET (1.06 ± 0.21, $n = 5$) and D-norgestrel (1.19 ± 0.29, $n = 4$) were much lower ($P < 0.001$) in pregnancy serum than in any of the other samples. These differences are probably a consequence of NET and D-norgestrel binding to sex-hormone binding globulin (SHBG), since correlations were observed between serum SHBG binding capacities and percentages of non-protein bound NET and D-norgestrel, and because heat denaturation of SHBG binding capacity by >90% almost completely abolished these differences. By comparing the non-protein bound percentages of steroids in native and heat treated (60° for 1 h) serum, it was calculated that in native serum both EE_2 and CA are almost exclusively bound by the heat stable component (predominantly albumin), which binds >98.5% of EE_2 and >93.0% of CA in both men and women. In contrast, the respective percentages of NET and D-norgestrel bound to SHBG are 20.57 ± 11.38 and 37.73 ± 7.66 in men, and 35.49 ± 13.64 and 47.54 ± 11.69 in women, while the percentages of these steroids bound to albumin are 74.72 ± 10.94 and 59.21 ± 7.33 in men, and 60.82 ± 12.94 and 49.96 ± 11.05 in women, respectively. These data indicate that the distributions of NET and D-norgestrel between various serum binding proteins are determined largely by SHBG binding capacity, and that knowledge of the latter may be useful in assessments of the lowest effective dose of these two progestins in different individuals.

INTRODUCTION

Synthetic steroids, like natural steroids, interact with serum binding proteins which determine the fraction of non-protein bound steroid that circulates in blood. Although it is generally assumed that only non-protein bound steroids in blood enter target cells, it has been shown that albumin bound steroids are also taken up by the rat brain [1]. In addition, it has been suggested that cortisol binding globulin (CBG) may facilitate progesterone uptake by the rat uterus but not the brain [2], and recently serum sex-hormone binding globulin (SHBG) has been identifed in the cytoplasm of sex steroid target cells [3]. Thus, the distribution of synthetic steroids between various protein bound and non-protein bound components in serum may influence their bio-availability at different target tissues.

The binding of several contraceptive steroids to SHBG has recently been studied in detail [4–7], and there is some information about the relative binding of ethinyl-estradiol (EE_2), norethisterone (NET), and D-norgestrel to serum albumin [6, 7], but it is not known what effects these interactions may have on their serum distribution under physiological conditions. Recently, estimates have been reported of the percentages of EE_2 [7], D-norgestrel [6], and NET [6, 8] that are non-protein bound in plasma. However, these measurements have relied on the use

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† *Trivial and systematic steroid nomenclature*: Ethinyl-estradiol (EE_2), 17-ethinyl-1,3,5-estratriene-3, 17β-diol; norethisterone (NET), 17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one; D-norgestrel, 13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one; cyproterone acetate (CA), 17α-acetoxy-6-chloro-1α,2α-methylene-4,6-pregnadiene-3,20-dione; 5α-dihydrotestosterone (DHT), 17β-hydroxy-5α-androstan-3-one.

of equilibrium dialysis which often yields an overestimation of values, because of predilutions of samples, dilutions which occur during dialysis due to fluid shifts, or degradation of the labeled steroid during prolonged incubations [9, 10]. Furthermore, incubations not conducted at physiological temperature result in unphysiological changes in steroid distribution between different binding proteins and the non-protein bound fraction [10].

We have therefore employed a newly developed technique, centrifugal ultrafiltration-dialysis [10], which not only permits determinations of the percentages of steroids that are non-protein bound in undiluted serum at 37°C, but can also be used to calculate the distribution of steroids between various protein species, under conditions which mimic the physiological situation [11].

EXPERIMENTAL

Radiolabeled compounds

[6,7-^{3}H]-Ethinyl-estradiol (SA 59 Ci/mmol), [15,16-^{3}H]-norethisterone (SA 13 Ci/mmol), [15,16-^{3}H]-D-norgestrel (SA 39 Ci/mmol) and [^{3}H]-cyproterone acetate (SA 20 Ci/mmol) were kindly supplied by Schering AG (Berlin, W. Germany). [1,2-^{3}H]-5α-Dihydrotestosterone (51 Ci/mmol) and [1,2-^{3}H]-cortisol (53 Ci/mmol) were purchased from NEN Chemicals GmbH (Dreieich, W. Germany). Radiolabeled steroids (100 pmol) were purified immediately prior to use by chromatography on Lipidex-5000 (Packard-Becker, B.V., The Netherlands) microcolumns, using appropriate mixtures of light petroleum ether (b.p. 66–69°C) and chloroform as the elution solvents. The purity of the labeled steroids was ascertained to be >99% by repeated chromatography on Lipidex-5000. [^{14}C]-Glucose (13.9 mCi/mmol) was obtained from NEN Chemicals GmbH, and was stored and used as supplied.

Serum SHBG and CBG binding capacities

The methods used to determine serum SHBG and CBG binding capacities rely on the use of dextran coated charcoal (DCC) for the separation of protein bound from non-bound radiolabeled steroid, and details of the assays are to be published elsewhere [12]. In brief, 50 μl of serum was incubated (30 min at room temperature with intermittent mixing) with 5 ml DCC solution (1.25 g Norit A + 0.125 g Dextran T-70 in 500 ml 0.1 M phosphate buffered saline containing 0.1% gelatin (PBS), pH 7.4). The mixture was then centrifuged (2,000 *g*, 10 min) to sediment the DCC. The resulting supernatant (1:100 dilution) was used as such to measure SHBG binding capacity in serum from men and non-pregnant women, but was further diluted (1:2) in PBS to measure SHBG binding capacity in pregnancy serum, and also diluted (1:5) in PBS to measure CBG binding capacity in all samples. Duplicated aliquots (100 μl) of the respective serum dilutions were then added to dried tubes containing ^{3}H labeled steroids (1 pmol [^{3}H]-cortisol for the CBG assay and 1 pmol [^{3}H]-5α-dihydrotestosterone + 200 pmol cortisol for SHBG assay), and also to tubes containing a 200-fold excess of the respective non-radiolabeled steroid in addition to the above. After vortex mixing, the mixtures were incubated at room temperature for 1 h, and then at 0° for 15 min, before the addition of 900 μl of DCC solution at 0° with a nine-channel pipette (Labsystems OY, Helsinki, Finland). The tubes were further incubated at 0° for 10 min, and then centrifuged at 3,000 *g* for 3 min. Aliquots (750 μl) of the supernatant were then counted in a liquid scintillation spectrometer in vials containing 5 ml RiaLuma (LKB/Wallac, Turku, Finland). The concentration of specifically bound ^{3}H labeled steroid was calculated and extrapolated to zero serum dilution to give the binding capacity in pmol/ml. All samples were measured in the same assays for SHBG and CBG, and the precision (CV%) of the measurements were <10%.

Determination of percentage non-protein bound steroid.

Details of the equipment required for the centrifugal ultrafiltration-dialysis method have been published [10]. Serum samples (450 μl) were incubated (1 h at 37°) with 1.5–2 pmol of the purified ^{3}H labeled steroid under investigation, together with approximately 10,000 d.p.m. [^{14}C]-glucose (the exact concentrations of radiolabeled compounds are not important, since only isotope ratios are used). Duplicate aliquots (200 μl) of these incubations were then placed in centrifugal ultrafiltration vials and centrifuged at 3,000 *g* for 1 h at 37°C. The percentage of non-protein bound steroid was determined by the ratio of ^{3}H labeled steroid: [^{14}C]-glucose in the ultrafiltrate divided by the corresponding ratio in the serum retained by the dialysis membrane.

Serum distribution of steroids

Heat treatment of serum samples (700 μl) at 60° for 1 h results in greater than 90% losses in the binding capacities of SHBG and CBG, while the binding of steroids to pure human serum albumin solutions remains unchanged [11, 13]. Thus by measuring the percentage of non-protein bound steroid in heat treated serum samples, it is possible to determine the relative amount (%) of steroid that interacts with heat stable serum binding proteins, in the absence of high affinity binding by SHBG and/or CBG. Since all four synthetic steroids studied interact very poorly with CBG [14], the heat labile binding component can be almost entirely attributed to SHBG. For many 3-oxo-4-ene steroids the heat stable component may include binding to α_1-acid glycoprotein as well as to albumin, but because its concentration and steroid binding capacity are small relative to those of albumin [13] its contribution is probably insignificant, and the heat stable binding can therefore be attributed to albumin. Thus, in heat treated serum, we can estimate that: percentage of steroid bound to

albumin ($\alpha\%$) = 100%—non-protein bound steroid %. Furthermore, since the dissociation constants (K_D) of the binding of steroids which interact significantly with SHBG and CBG are 3–4 orders of magnitude lower than for albumin, the SHBG and/or CBG bound fraction can be considered to be unavailable for interaction with albumin at equilibrium in native serum. Moreover, because of the low affinity and extremely high capacity nature of the albumin-steroid interaction, the percentage of available steroid (i.e. not bound to high affinity sites) that albumin is capable of binding is probably the same in native and heat treated serum (i.e. $\alpha\%$). We can therefore use the following equations to calculate the distribution of steroid between SHBG and/or CBG (heat labile component: B), albumin (heat stable component: A) and non-protein bound (F) components in native serum:

$$100\% = B\% + A\% + F\% \quad \text{(i)}$$

or, since $A\% = (100\% - B\%)(\alpha\%)$ in the presence of B:

$$100\% = B\% + (100\% - B\%)(\alpha\%) + F\% \quad \text{(ii)}$$

Knowledge of α and F obtained by measurements of the percentages of non-protein bound steroid in heat treated and native serum, respectively, can thus be used to solve for B% using the abstraction of Eqn (ii):

$$B\% = 100\% - \frac{F\%}{1 - \alpha\%} \quad \text{(iii)}$$

B% can then be used to calculate the amount of steroid bound to albumin (A%) in native serum from Eqn (i).

RESULTS

Percentages of non-protein bound steroids and serum SHBG and CBG binding capacities

A summary of the mean ± SD percentages of non-protein bound EE_2, NET, D-norgestrel and cyproterone acetate (CA) in serum from men, women during follicular and luteal phases of the menstrual cycle, and from women during late pregnancy is presented in Table 1, together with the respective mean ± SD serum SHBG and CBG binding capacities. It is clear from these data that there are no differences in percentages of non-protein bound EE_2 and CA with respect to sex or serum SHBG and CBG binding capacities. However, serum percentages of non-protein bound NET ($P < 0.05$) are significantly lower in women than in men. This is probably also the case with D-norgestrel, but the numbers of subjects studied may be insufficient to observe a significant sex difference ($P < 0.2$). However, the percentages of non-protein bound NET and D-norgestrel are both very much lower ($P < 0.001$) in serum from pregnant women when compared to non-pregnant women.

These differences appear to be inversely related to serum SHBG binding capacity (Table 1), and this is clearly demonstrated in Fig. 1, in which inverse correlations are observed between SHBG binding capacity and the percentages of non-protein bound NET ($r = -0.94$, Fig. 1, a) and D-norgestrel ($r = -0.85$, Fig. 1, b). Although serum CBG binding capacity is also increased in late pregnancy serum, it did not correlate significantly with the percentages of non-protein bound NET or D-norgestrel in pregnancy serum samples or any of the other samples investigated. Further evidence that SHBG is responsible for the differences in percentages of non-protein bound NET and D-norgestrel observed in different samples can also be inferred from the observation that heat denaturation of SHBG by >90% (data not shown) almost abolishes these differences (Table 1). Thus the percentages of non-protein bound NET and d-norgestrel in heat treated serum from men and non-pregnant women are virtually identical, and largely represent the contribution of albumin binding alone. Although

Table 1. Percentage of non-protein bound ethinyl-estradiol (EE_2), norethisterone (NET), D-norgestrel and cyproterone acetate (CA) in native (N) and heat treated (H) serum samples, and their relationship to serum SHBG and CBG binding capacities

Subjects	*n*	Mean ± SD binding capacity (pmol/ml)		Mean ± SD % non-protein bound steroid							
				EE_2		NET		D-Norgestrel		CA	
		SHBG	CBG	N	H	N	H	N	H	N	H
Men	5	49	458	1.44	1.44	4.71	5.96	3.05	4.91	6.62	6.41
		±	±	±	±	±	±	±	±	±	±
		15	27	0.15	0.08	0.55	0.55	0.34	0.15	0.43	1.02
Women	5	67	432	1.50	1.49	3.91	5.70	2.42	4.62	6.86	6.96
(Follicular)		±	±	±	±	±	±	±	±	±	±
		30	84	0.21	0.06	0.89	0.67	0.69	0.37	1.16	1.71
Women	5	85	439	1.52	1.45	3.48	5.82	2.59	4.87	7.57	6.78
(luteal)		±	±	±	±	±	±	±	±	±	±
		32	132	0.09	0.12	0.95	1.00	0.75	0.54	0.65	1.13
Women	5	390	1046	1.58*	1.51	1.60	4.63	1.19*	2.48	6.33	6.27
3rd trimester		±	±	±	±	±	±	±	±	±	±
		140	236	0.14	0.22	0.21	0.92	0.29	0.56	1.01	0.86

* $n = 4$; N = native serum; H = heat treated serum (60° for 1 h).

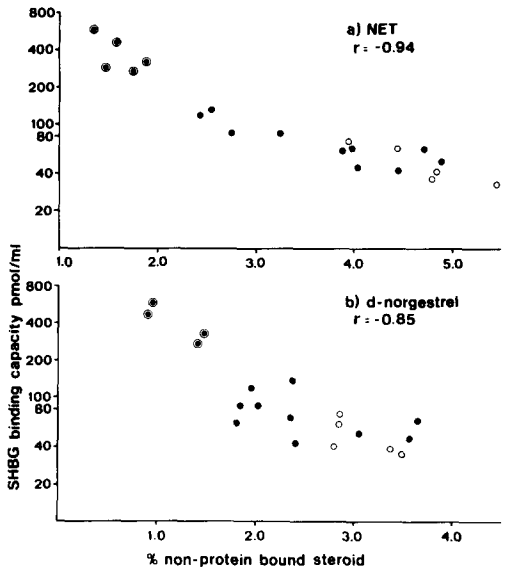

Fig. 1. Relationship between serum SHBG binding capacity (pmol/ml, note log scale) and percentage non-protein bound NET (a) and D-norgestrel (b). Symbols represent serum samples from: (○) men; (●) women and (⊙) women during 3rd trimester of pregnancy. r = correlation coefficient.

the percentages of non-protein bound NET and D-norgestrel in heat treated pregnancy serum are lower than in heat treated serum from men and non-pregnant women, they are much higher than the respective values in untreated pregnancy serum.

Relative binding of steroids to albumin

As mentioned above, the heat stable binding component in serum can be attributed to albumin, and from Table 1 it can be deduced that the relative affinities of the steroids investigated for albumin are $EE_2 \gg$ D-norgestrel > NET > CA.

Distribution of serum steroids

The data in Table 1 indicate that heat labile binding proteins do not appear to influence the percentages of non-protein bound EE_2 and CA, because no differences in these parameters are observed between native and heat treated serum samples. It can therefore be inferred that EE_2 and CA are almost exclusively bound by albumin in native serum, and from the percentages of non-protein bound EE_2 and CA in native serum it can be calculated that >98.5% of EE_2 and > 93.0% of CA are bound to albumin (Table 2). In contrast, the percentages of non-protein bound NET and D-norgestrel in native serum are inversely related to SHBG binding capacity (Fig. 1), and the respective mean percentages of these steroids bound by this component under physiological conditions were calculated to be 20.57% and 37.73% in men, and 35.49% and 47.54% in non-pregnant women (Table 2). As a consequence of this, the proportions of NET and D-norgestrel bound to albumin in native serum are much lower ($P < 0.001$) than the albumin bound proportions of EE_2 and CA (Table 2). Although the small number of male samples studied undoubtedly limits the statistical evaluation of our observations, the proportions of NET and D-norgestrel bound to SHBG appear to be higher ($P < 0.1$ and $P < 0.2$, respectively) in serum from women than from men, while the proportions of these steroids bound to albumin are correspondingly lower ($P < 0.1$ and $P < 0.2$, respectively) in female serum as compared to male serum. This may in part be explained by the slightly higher ($P < 0.2$) serum SHBG capacity in women than in men.

Table 2. The distribution of ethinyl-estradiol (EE_2), norethisterone (NET), D-norgestrel and cyproterone-acetate (CA) between different serum steroid binding protein and non-protein bound components of serum from normal men and women

Subjects	Serum component	Mean ± SD % distribution of steroids			
		EE_2	NET	D-Norgestrel	CA
Mean	SHBG + CBG bound	NM	20.57	37.73	NM
$n = 5$	(heat labile)		±	±	
			11.38	7.66	
	Albumin bound	98.56	74.72	59.21	93.59
	(heat stable)	±	±	±	±
		0.07	10.94	7.33	1.01
	Non-protein bound	1.44	4.71	3.05	6.62
		±	±	±	±
		0.15	0.55	0.34	0.43
Women	SHBG + CBG bound	NM	35.49	47.54	NM
$n = 10$	(heat labile)		±	±	
			13.64	11.69	
	Albumin bound	98.52	60.82	49.96	93.13
	(heat stable)	±	±	±	±
		0.07	12.94	11.05	1.37
	Non-protein bound	1.54	3.69	2.50	7.22
		±	±	±	±
		0.16	0.90	0.68	0.96

NM = not measurable.

DISCUSSION

The technique we have used to estimate the percentages of non-protein bound steroids in serum has several advantages over other published methods. The most important being that serum samples are not diluted either prior to, or during measurements, and that labeled steroids are incubated with serum samples for relatively short periods at 37°C. These features of the present method probably contribute to the lower percentages of non-protein bound EE_2, D-norgestrel and NET reported here, as compared to previous estimates [6, 7, 8]. Another factor that could explain these differences is related to the purity of the ^3H labeled steroids used, particularly as previous reports have either not remarked upon the purity of tracers [7, 8], or only estimated them to be more than 95% pure [6]. Since impurities are generally non-steroidal in nature, and are probably not bound by serum proteins, even small percentages of impurities (1–2%) will represent a considerable source of error. In the present study, the labeled steroids were therefore purified in small batches immediately prior to use in order to ensure a greater than 99% purity.

Although the present measurements were made under conditions which simulate a physiological environment, and every effort was made to minimize errors due to tracer impurities, the accuracy of our data can not be assessed against any other method that is not subject to the same limitations we have strived to overcome. Indeed, to date, no direct method for the estimation of non-protein bound steroids in serum has been reported. However, interest has recently focused on the possibility of comparing the concentrations of steroids in saliva and serum samples taken concomitantly, in order to obtain a direct measurement of non-protein bound steroids in the blood circulation [15]. Recently, saliva and serum concentrations of NET have been measured in women after ingestion of a contraceptive preparation containing this steroid [8, 16]. In both studies, the percentage of non-protein bound NET was calculated in this way to be 3–5%, and a comparison between saliva NET concentrations and non-protein bound NET concentrations in serum (i.e. total serum concentrations multiplied by the non-protein bound percentage estimated by equilibrium dialysis) indicated a close association between these two parameters [8]. The present values for the percentages of non-protein bound NET in serum are very similar to those calculated by comparing saliva:serum concentrations measured by radioimmunoassays, but slightly lower than those obtained by equilibrium dialysis [8]. This may be a reflection of small dilutions of serum samples that occur during equilibrium dialysis, and which lead to overestimations in the percentages of non-protein bound steroids [9].

Our data support binding studies which have shown that D-norgestrel and NET bind with relatively high affinity to SHBG [4, 6, 14], while EE_2 and CA bind to this protein with much lower affinity [5, 7, 14]. A 17β-hydroxyl-group is known to be important for steroid binding to SHBG [17], and the introduction of an ethinyl-substituent at the 17α-position obviously reduces the affinity of SHBG for steroids [14]. In the case of ethinyl-estradiol, however, it appears that the presence of the ethinyl-group has a more marked effect in this respect, as compared to NET and D-norgestrel. The reason for this is unknown, but it may be a consequence of the orientation of the ethinyl-substituents relative to the A ring of the steroid molecules in question. The present data also indicate that the proportions of non-protein bound D-norgestrel and NET in serum are inversely related to SHBG binding capacity. This relationship seems to be more pronounced in the case of NET than D-norgestrel, which was unexpected considering the greater affinity of SHBG for D-norgestrel than NET [4], but probably reflects the fact that NET binds less well to serum albumin than does D-norgestrel [6, and present data].

The degree of destruction of SHBG and CBG binding in serum from men and non-pregnant women by heat treatment is difficult to accurately determine, because of limitations in the sensitivity of the competitive steroid binding assays. However, in pregnancy sera it was found that >90% of the binding capacity of these serum steroid binding proteins was destroyed. This is probably sufficient to eliminate any effects of SHBG in normal male and female serum, but because of the higher levels of SHBG in pregnancy serum, a small amount remains after heat treatment.

In serum from normal men and women, it is likely that the heat stable steroid binding component almost exclusively reflects the contribution of serum albumin, which appears to bind EE_2 much more effectively than D-norgestrel, NET and especially CA. Previous data on the interaction between albumin and these steroids are limited, but our observations agree well with Scatchard analyses of the relative binding constants obtained for EE_2 [7], D-norgestrel and NET [6], with albumin at 4°C.

The percentages of non-protein bound EE_2 and CA were found to be very similar in both native and heat treated serum samples from men and women, which indicates that albumin is the predominant, if not only, protein responsible for the binding of EE_2 and CA in serum. In contrast, the data indicate that the percentages of NET and D-norgestrel bound to SHBG are lower in men than in women, and consequently the proportions of these steroids bound to albumin are correspondingly higher in men. Although not statistically significant, these apparent differences may be explained by the fact that the concentration of available SHBG binding sites is much lower in male serum than in female serum, due to the presence of higher concentrations of testosterone in male serum, and because serum SHBG binding capacity is generally lower in men than in women [17].

The exact physiological implications of our observations cannot be deduced from the present data, but they indicate that the non-protein bound and albumin bound fractions of NET and D-norgestrel may vary by as much as 2–3-fold between women who are known to have subnormal or supranormal levels of serum SHBG binding capacity, for example as a result of obesity [18] or hyperthyroidism [17], respectively. Therefore, it is suggested that measurements of serum SHBG binding capacity may provide a method of assessing the lowest effective dose of these two progestins in individual subjects, and that this may consequently help to reduce side-effects occasionally associated with their use. It must be remembered, however, that the actual amounts of non-protein bound steroids are also determined by total serum steroid concentrations. In this respect, it is pertinent to note that changes in serum SHBG capacity, induced by treatment with contraceptive preparations, may influence the serum concentrations of both NET [8] and D-norgestrel [4]. It should also be mentioned that in this study constant amounts of labeled steroids were added to normal serum samples. Although this provides an indication of the serum distribution of these steroids under the experimental conditions used, it must be remembered that serum concentrations of synthetic steroids may vary considerably between different treatment regimens, and methods of administration. Therefore, the effect of differences in serum SHBG binding capacity, and serum steroid concentrations, on the actual non-protein bound serum concentrations and distribution of these progestins is an important question to be addressed in future studies.

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