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Structural and functional microheterogeneity of rat thyroxine-binding globulin during ontogenesis

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Thyroxine-binding globulin (TBG), the major carrier of thyroid hormones in human and murine sera, is in the rat a developmentally regulated protein, showing a large surge during post-natal growth followed by virtual disappearance in adults. Here we study as a function of age, from the 19-day embryo to 60 days after birth, the structural and binding characteristics of rat TBG microheterogeneity. Serum obtained throughout development, when pre-incubated with ^{125}I-thyroxine (T_4), was shown by isoelectric focusing (IEF; pH range 4–5) to contain six labelled isoforms of TBG, with isoelectric points between 4.25 and 4.55. These isoforms differ in their sialic acid content. The relative labelling densities of the isoforms show age-related changes: in neonates, the bulk of T_4 is bound to the most alkaline (least sialylated) TBG isoforms; then, with advancing age, it shifts to the most acidic isoforms. To understand whether this progressive transfer of ligand reflects developmental changes in the relative abundance of isoforms, we submitted sera from rats of different ages to crossed immunoelectrofocusing analysis. We demonstrate that the relative proportions of the TBG isoforms remain fairly constant, independent of the level of total TBG. The most acidic forms always represented the majority ($\sim$ 50 %), with the most alkaline ones only representing 15 % of total TBG. Experiments based on IEF of charcoal-treated sera, supplemented or not with lipidic serum extracts, further demonstrate that the paradoxical low labelling seen in the neonates for the most abundant highly sialylated isoforms is due to inhibition of their binding abilities by liposoluble components, which are particularly concentrated in the sera at the earlier post-natal ages. These studies represent the first analysis of concentration versus binding functions of rat TBG isoforms in the physiological conditions of normal ontogeny. Our results point to an important influence for the serum environment on the binding properties of TBG isoforms. The physiological significance of such interactions remains to be clarified.

INTRODUCTION

The transport of the thyroid hormones thyroxine (T_4) and tri-iodothyronine (T_3) in human serum relies on two specific carriers, thyroxine-binding globulin (TBG), which is of high affinity and is the major binding protein, and transthyretin (TTR), which contains weaker though saturable binding sites (for a review, see [1]). The rat possesses both TBG and TTR, which are very similar to their human counterparts. However, the rat TBG was recognized only quite recently [2] because, unlike human TBG, it is a dramatically development-regulated protein, highly expressed during post-natal growth but virtually undetectable in normal adults [2–4].

Since its discovery by Gordon *et al.* [5], human TBG has been extensively studied. It is a glycoprotein monomer, its carbohydrate moiety consisting of four oligosaccharide chains terminated by six to ten sialic acid residues [6–8]. It shows a marked microheterogeneity, revealed by isoelectrofocusing (IEF) studies, resulting for the most part from differences in the sialic acid content of isoforms [9,10]. IEF studies in this laboratory using serum from 8-day-old rats showed that rat TBG presents a polymorphism almost identical to that of human TBG, also based, for the most part, on variations in the degree of sialylation of isoforms [11,12].

The present paper is concerned with the study of rat TBG microheterogeneity in the course of ontogenesis. Separation by IEF of TBG isoforms in T_4-prelabelled sera followed by densitometric scanning and immunological quantification of the separated isoforms was used to evaluate the binding properties and concentrations of the isoforms as a function of age. Our evidence indicates that the relative concentrations of the TBG variants are maintained throughout development, with a continued predominance of the most acidic isoforms. However, at the earlier post-natal ages, the sera contain charcoal-adsorbable compound(s) which inhibit strongly the binding of T_4 to these highly sialylated acidic TBG isoforms.

EXPERIMENTAL

Sera, proteins and hormones

Serum was prepared from rats of different ages (Sprague–Dawley, CD strain), supplied by Charles River, St.-Aubin-les-Elbeuf, France. L-[^{125}I]T_4 (specific radioactivity > 1.2 Ci/mg) was from Amersham. Ampholine polyacrylamide gel plates (PAG plates) were obtained from LKB (Bromma, Sweden). Neuraminidase from *Vibrio cholerae* (specific activity > 20 units per mg of protein) was from Calbiochem (San Diego, CA, U.S.A.). Anti-(rat TBG) antiserum was prepared in our laboratory as described [13].

IEF studies

IEF of whole serum was carried out essentially as described [11]. Briefly, freshly thawed serum samples were pre-incubated with tracer amounts of [^{125}I]T_4 (4000 c.p.m./μl) for 1 h at room temperature, then 5 μl of each sample was applied to filter paper at the cathodic site of the PAG plates. Runs were carried out over a pH range of 4–6.5 or 4–5 at 10 °C using an LKB Multiphor chamber, in the following conditions: 2000 V, 25 mA, 25 W for

Abbreviations used: T_4, thyroxine; T_3, tri-iodothyronine; TBG, thyroxine-binding globulin; TTR, transthyretin; IEF, isoelectrofocusing; EID, electroimmunodiffusion.

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2 h 45 min for pH range 4–6.5; 1400 V, 50 mA, 30 W, for 3 h 15 min for pH range 4–5. Immediately after the current was turned off, the gel was transferred to filter paper and dried for 2 h at 60 °C under vacuum on a gel slab dryer (Bio-Rad).

Autoradiography of the focused labelled protein bands was performed by exposure of the dried gels to Kodak X-OMat S film overnight at −80 °C, with X-Omatic rapid screens. Radioactivity of the labelled protein bands was quantified by densitometric scanning, using a dual-wavelength t.l.c. scanner (model CS930, Shamadzu, Kyoto, Japan).

Enzymic desialylation of sera

The immunoreactivity and electrophoretic mobility of a glycoprotein partly depend on its carbohydrate moiety [14]. To validate the accuracy of immunoassay of variously sialylated TBG isoforms, we treated serum for 8-day-old rats (rich in TBG) with different doses of neuraminidase. A 25 μl sample of serum was mixed with 1–25 munits of enzyme, and the volume was made up to 50 μl with acetate buffer (50 mM-sodium acetate, 154 mM-NaCl, 1 mM-$CaCl_2$, pH 5.5). Incubation was at 37 °C for 1 h. The extent of desialylation was assessed by visualization after IEF in the pH 4–6.5 gradient. TBG from untreated and neuraminidase-treated sera was quantified by the electroimmunodiffusion (EID) technique of Laurell [15].

All enzyme doses transformed the TBG isoforms, decreasing their number and causing a shift towards alkaline pI; the most drastic alteration was observed with the highest enzyme dose of 25 munits. EID rocket immunoprecipitates were, however, formed, indicating that TBG immunoreactivity was not affected. The height of the rockets was decreased by ~ 14 % with 25 munits of neuraminidase, but only slightly with the dose of 2.5 munits (~ 4 %). It was inferred that the differences in sialic acid content between isoforms in native sera have only a minor effect, if any, on the precision of their immunoelectrophoretic quantification.

Charcoal treatment (stripping) and replacement studies

Removal of liposoluble compounds from sera was carried out by treatment with charcoal, essentially according to Nanno *et al.* [16]. Native sera were incubated overnight at 4 °C with 80 mg of Norit A charcoal/ml. These conditions were established in preliminary tests as causing maximal changes to the IEF image of TBG, compared with its native image. The TBG concentration was not modified by stripping, as assessed by EID quantification.

Serum organosoluble extracts were prepared as follows: 1 ml of native serum was mixed with 6 ml of solvent (ethyl acetate/cyclohexane, 1:1, v/v), shaken for 30 min at room temperature, and the aqueous phase was removed by freezing (−20 °C). The organic phase was evaporated to dryness and taken up in 1 ml of solvent (benzene/ethanol, 19:1, v/v). The recovery of serum lipids, evaluated by adding radiolabelled heptadecanoic acid to reference serum samples, was 50 ± 10 %.

To reconstitute serum with an approximately native composition, 2 vol. of the liposoluble extract were evaporated and the residue was mixed with 1 vol. of stripped serum.

Crossed immunoelectrofocusing studies

Native serum from rats of different ages was labelled with [^{125}I]T_4 and submitted to IEF in the pH range 4–5. The TBG isoforms were thus separated as labelled bands in the first dimension. Gel strips corresponding to the differential isoelectrofocused sera were cut off and transferred on to the antibody-free section of an agarose gel (1 %) containing 0.5 % anti-(rat TBG) antiserum. They were then submitted to electrophoresis in the second dimensions, overnight at 130 V and 6 °C [17]. The plates were dried and the immunoprecipitated TBG was revealed by autoradiography. The TBG isoforms were quantified by measuring the area of the corresponding immunoprecipitated peaks.

RESULTS

IEF study of TBG microheterogeneity during ontogenesis

Serum from rats of different ages, from 19-day embryos to 60-day post-natal animals, was incubated with radioiodinated T_4 and then submitted to IEF in the pH range 4–5 (Fig. 1). The age-related labelling density pattern of the TBG zone on the autoradiogram was consistent with the developmental trend of the protein, as demonstrated in previous binding, immunological and biosynthetic studies [2,4,13]: absence in the foetus, appearance and increasing concentration in neonates, then decline and finally disappearance in young adults. By contrast, the age-related changes seen in the labelling of TTR were not correlated with its levels, which vary little during the whole period studied [2,18]. The weak labelling of TTR in the growing pups reflects the successful competition of the co-existing TBG for the common T_4 ligand.

The polymorphism of TBG, first observed in serum from 8-day-old rats [11], is maintained throughout development, with respect to both number and isoelectric points (pI) of isoforms. The protein is separated into six bands with pI values between 4.25 and 4.55. The distribution of the T_4 ligand among TBG isoforms is uneven at all ages. Moreover, this pattern of unequal labelling markedly changes during ontogenesis: in the neonates, the greatest labelling is seen with the most alkaline bands, but

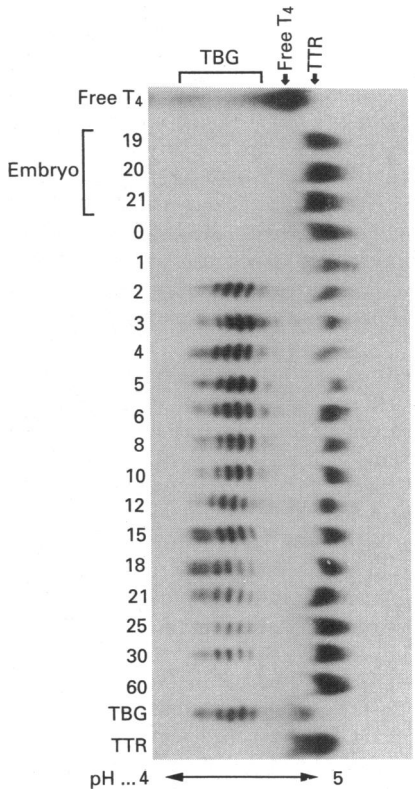

Fig. 1. Autoradiography of [^{125}I]T_4 bound to TBG and TTR in serum from rats of different ages

Ages of embryos or rats (days) are given to the left. IEF was carried out in the pH range 4–5. Free [^{125}I]T_4 and enriched TTR and TBG preparations were used as references.

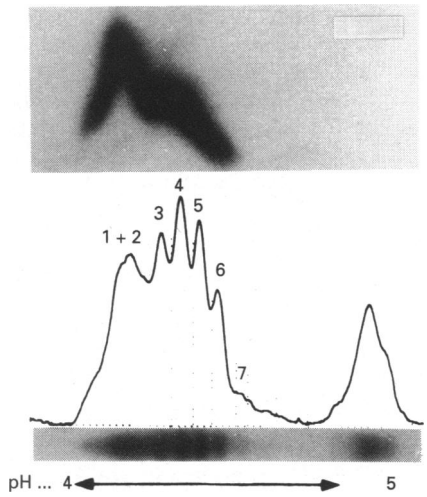

Fig. 2. Crossed immunoelectrofocusing study of TBG isoforms in serum from 8-day-old rats prelabelled with [^{125}I]T_4

Serum was pooled from 12–15 rats. Bottom, autoradiography of the strip cut in the gel after IEF of 8-day serum (10 μl) (pH gradient 4–5); middle, corresponding densitometric scan; top, autoradiography of the immunoprecipitated peaks obtained after immunoelectrophoresis of the labelled bands (from the bottom gel) with 1% anti-(rat TBG). See the text for details.

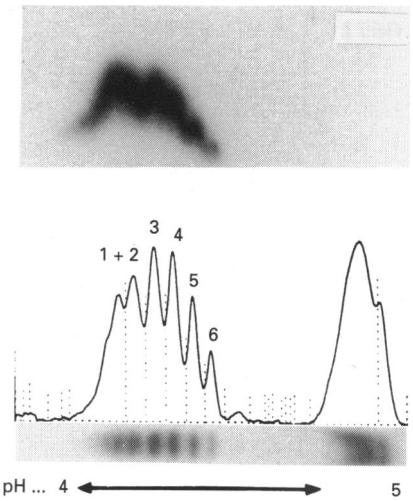

Fig. 3. Crossed immunoelectrofocusing study of TBG isoforms in serum from 30-day-old rats prelabelled with [^{125}I]T_4

Serum was pooled from eight rats. A 15 μl portion of serum was applied to the IEF gel. See Fig. 2 for details.

with increasing age of the animals it shifts gradually to the most acidic bands.

The uneven labelling of TBG variants at a given age and its changes during ontogenesis could result from differences in concentrations of isoforms or/and from differences in their binding properties. The immunological quantification of TBG isoforms in whole serum from rats of different ages was carried out to clarify these points.

Crossed immunoelectrofocusing study of TBG isoforms in whole serum from early and late post-natal rats

TBG isoforms were separated by IEF of T_4-labelled sera in the first dimension, then electrophoresed in the second dimension against anti-(rat TBG) (see the Experimental section). These studies are illustrated in Figs. 2 and 3 for sera from 8- and 30-day-old rats respectively. Each of the Figures shows the autoradiography of the strip cut from the IEF gel, with its six major labelled bands, and the corresponding densitometric scan; in the upper parts of the Figures are presented the immunoprecipitated peaks formed after electrophoresis of these bands against anti-(rat TBG). For each of the variants, the labelling (calculated from the densitometric scan), and the protein concentration (calculated from the immunoprecipitated pattern) are presented as percentages of the total in Table 1. It may be seen that the relative immunoassayed concentrations of the isoforms are fairly well preserved between the 8-day and the 30-day sera. At both ages the most acidic variants (1+2) represent about 50% of total TBG, whereas the most alkaline variants (5+6) represent about 15% of the total. By contrast, the relative labelling of these extreme isoforms is significantly different at the two ages. In the 8-day sera, it is correlated negatively with concentration: indeed the labelling (%)/protein (%) ratios are < 1 for the acidic variants and > 1 for the alkaline variants, suggesting binding hypofunctionality for the former and binding hyperfunctionality for the latter. In the sera from 30-day-old rat sera, labelling of the acidic and basic variants shows a positive correlation with their concentration, with labelling (%)/protein (%) ratios being close to unity.

These results suggested that changes in the binding properties of the isoforms, rather than in their relative concentrations, occur during ontogenesis. A plausible hypothesis is that the serum environment may affect the interaction of T_4 with the TBG isoforms. Indeed, a high sensitivity of TBG binding to inhibition by hydrophobic serum components has been demon-

Table 1. Labelling densities and immunoassayed concentrations of TBG isoforms

Serum was obtained from 8- and 30-day-old rats. Results are calculated as a percentage of the total isoforms and are means ± S.E.M. of values measured with n different serum pools. Where $n = 2$, both values are given in parentheses. Statistical significance of difference from 8-day sera (unpaired Student's t test): $^*P < 0.001$, $^{**}P < 0.01$.

Isoform no.	Labelling (% of total)		Protein (% of total)		Labelling/protein ratio	
	8 days ($n = 7$)	30 days ($n = 3$)	8 days ($n = 5$)	30 days ($n = 2$)	8 days	30 days
1+2†	27.0±3.8	38.0±3.1**	52.6±3.9	48.1 (51.7, 44.5)	0.51	0.79
3	18.5±1.6	22.4±0.6**	17.2±1.0	22.1 (21.5, 22.7)	1.07	1.01
4	22.4±2.7	20.5±1.3	14.3±1.4	14.7 (14.3, 15.0)	1.56	1.39
5	20.3±1.9	13.2±1.4*	9.6±1.4	9.6 (8.6, 10.6)	2.12	1.37
6	11.5±2.1	5.6±0.3**	5.9±0.5	5.5 (3.8, 7.3)	1.93	1.02

† Difficult to evaluate individually.

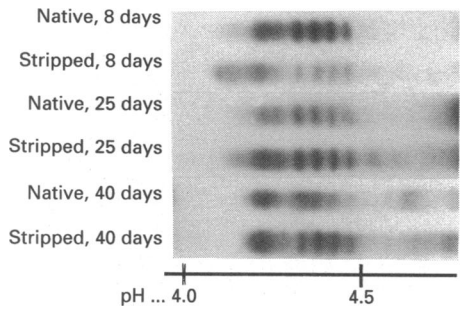

Fig. 4. Autoradiography of [^{125}I]T_4 bound to TBG isoforms

IEF was carried out at pH 4–5. Native and charcoal-treated (stripped) serum was studied from rats of 8, 25 and 40 days of age.

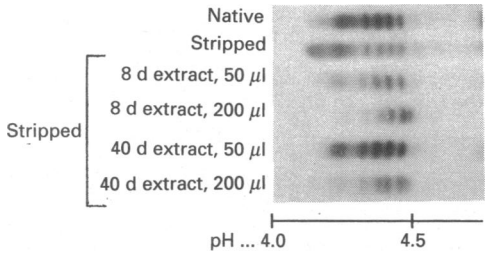

Fig. 5. Replacement experiments

An autoradiogram is shown of [^{125}I]T_4 bound to TBG isoforms, in native, stripped and supplemented serum from 8-day-old rats, following IEF at pH 4–5. 'Supplemented' serum consists of charcoal-treated serum from 8-day-old rats supplemented with 50 μl or 200 μl of an organosoluble extract of serum from 8- or 40-day-old animals.

strated [19,20]. Moreover, significant ontogenic variations in a number of liposoluble components in rat sera have been documented [20–23]. To test this possibility, we compared TBG microheterogeneity in control native sera and in sera stripped of hydrophobic compounds by charcoal treatment.

Age-related effects of charcoal-adsorbable serum components on T_4 binding to TBG isoforms

Sera from rats aged 8, 25 and 40 days were treated with charcoal, incubated with [^{125}I]T_4 and isoelectrofocused in the pH range 4–5 (Fig. 4). At 8 and 25 days of age, the greatest labelling, which in the native sera is to the most alkaline isoforms, is switched over, after stripping with charcoal, to the most acidic ones. Stripping also unmasks very acidic isoform(s), which are hardly visible beforehand. Thus at 8 days the labelling of the acidic isoforms (1+2) in native sera represented ~ 27% of total TBG (cf. table 1). After stripping, the labelling of the acidic isoforms (1+2+unmasked) rose to ~ 55% of total TBG (55.3±7.6% in three different pools). Conversely, the labelling of alkaline isoforms (5+6) fell from ~ 32% in the native serum to ~ 19% (18.7±0.9%) in the stripped serum. By contrast, at 40 days, there were few changes in the pattern of labelling of isoforms after stripping [native, ~ 40%; after stripping ~ 36% for the acidic (1+2) TBG isoforms; native ~ 24%; stripped, ~ 28% for the alkaline (5+6) TBG isoforms]. Crossed immunoelectrofocusing applied to three different pools of stripped serum from 8-day-old rats showed that the negative correlation between labelling and TBG concentration found in native serum (cf. Table 1) becomes a positive correlation after charcoal treatment. The labelling (%)/protein (%) ratio is 1.05 for the (1+2+unmasked) acid TBG zone (compared with 0.51 in the native serum), and 1.14 and 1.3 respectively for alkaline isoforms 5 and 6 (compared with the 2.12 and 1.93 in native serum shown in Table 1).

These results suggested that the serum of the younger rats contains compound(s) which interfere specifically with the most acidic TBG isoforms, and that charcoal treatment restores functional equivalence of isoforms by removing such inhibitors.

The results of the replacement experiments illustrated in Fig. 5 support this hypothesis. When the stripped 8-day sera were supplemented with 50 μl of organosoluble extract from age-matched 8-day sera, maximum labelling of alkaline bands was not only restored but actually exceeded that seen in native sera. In fact, the acidic bands disappeared completely with 200 μl of the 8-day organosoluble extract. Such effects were considerably attenuated when the stripped 8-day serum was supplemented with corresponding amounts of the extract prepared from animals aged 40 days.

Taken together, these results point to the presence in the neonatal serum of hydrophobic charcoal-adsorbable inhibitors of T_4 binding to the most acidic TBG isoforms, and to a decrease in these serum inhibitors with advancing age of the animal.

DISCUSSION

Rat TBG differs from human TBG in that it is a developmentally regulated protein. It appears in the serum about 2 days after birth, peaks at 8–10 days, then declines to virtually disappear beyond 50 days; the ontogenic pattern of serum concentration correlates well with that of hepatic biosynthesis [2,4]. On the other hand, rat and human TBGs have comparable structural and hormone-binding properties. One of the most striking similarities concerns their polymorphism, as revealed by IEF, which is based for the most part in both species on variations in the sialic acid content of isoforms [9–12].

We demonstrated previously the microheterogeneity of rat TBG by IEF studies of serum from 8-day-old rats, which is rich in TBG [11]. We have now further investigated the structural and functional characteristics of rat TBG microheterogeneity during ontogenesis. Our approach combined separation by IEF of TBG isoforms in T_4-labelled serum with subsequent immunoelectrophoretic quantification; labelling densities of isoforms are taken as a measure of their binding function. These methods could be applied to whole serum, thus preserving the native structural and binding characteristics of TBG, which are subject to alteration during the purification procedure [24].

We found that, whatever the level of the circulating TBG, a number of major characteristics of its microheterogeneity are apparent, i.e. the number of isoforms, their isoelectric points and their relative concentrations. With serum from all ages, IEF applied to T_4-labelled sera (pH 4–5) separated TBG into six major variants, with pI values between 4.25 and 4.55. Immunoassay demonstrated the continued preponderance of the most acidic (i.e. most sialylated) over the most alkaline isoforms, the former representing ~ 50% and the latter ~ 15% of total TBG.

However, developmental modifications of TBG microheterogeneity are manifest, and they concern the T_4-binding properties of isoforms. Although the relative concentrations of the isoforms remain unchanged, there occurs with advancing age a marked shift, from preferential T_4 binding on the most alkaline (and less abundant) isoforms, to preferential T_4 binding on the most acidic isoforms. In other words, at the earlier post-natal ages there is a negative correlation between labelling densities

and immunoassayed concentrations of isoforms while at later ages this correlation tends to become positive.

The constancy of the relative concentrations of isoforms throughout ontogenesis indicates that TBG is not affected by the changes of the glycosylation patterns of proteins observed in rat hepatocytes during the perinatal period [25]. It is interesting to note that, in spite of the dramatic developmental regulation of TBG gene expression, no significant age-related modification occurs in the post-translational processing of the protein.

The weak binding to the most sialylated isoforms in the younger rats was surprising, not only because of its inverse relationship to concentration, but also in the light of the existing evidence on the T_4-binding properties of human TBG isoforms. A number of studies indicate preferential T_4 binding to the most acidic variants [26], and the loss of T_4 binding to low-sialylated variants is associated with hepatic disorders or non-thyroidal illnesses [27,28]. Experimental deglycosylation of purified TBG was shown to result in a slight decrease in T_4 affinity constants [29], whereas other authors have found no significant differences in the T_4 affinity constants of the main native TBG isoforms [10]. Altogether, there is no evidence suggesting a decrease of thyroid hormone binding to the human TBG isoforms with highest sialic acid content.

We demonstrate that the paradoxical low labelling of the most acidic TBG isoforms at early post-natal ages is due to inhibitor(s) which interfere preferentially with these acidic variants. Indeed, removal of the hydrophobic serum components by charcoal treatment restored functional equivalence of the isoforms, similar to that observed in older rats even without stripping, i.e. a positive correlation between isoform labelling and concentration. Moreover, supplementation of the depleted serum from younger rats with lipid extracts from age-matched serum restored more readily their native abnormal labelling pattern than did supplementation with corresponding extracts of sera from older animals, pointing to higher levels of inhibitor(s) in the pups.

The nature of the charcoal-adsorbable inhibitor(s) remains to be clarified. The neonatal period involves profound metabolic adaptations, reflected in highly characteristic patterns of a number of liposoluble serum components. Among these, the thyroid hormones and the polyunsaturated non-esterified fatty acids have been described as inhibitors of rat TBG [20]. The fatty acids are plausible candidates as modulators of T_4–TBG interactions in neonates, inasmuch as their serum concentration is high after birth, and then gradually decreases [22]. On the contrary, such a role seems unlikely for the thyroid hormones, which are present at particularly low concentrations in the neonatal period [8,21]. Nutritional aspects of the post-natal period are also worth considering, since milk constitutes a high lipid/low carbohydrate diet [30]; it may thus be envisaged that milk lipids contribute to the characteristic labelling pattern of TBG isoforms in the blood of suckling rats. The identification of the inhibitor would allow us to clarify whether its interference with the acid TBG isoforms results from an inhibitory activity specifically directed against the most sialylated variants, or whether it is due to the IEF behaviour of the inhibitor itself. Overall our results confirm the age-related labelling patterns of TBG isoforms as a meaningful index of normal or deviant serum composition during ontogenesis.

In conclusion, by analysing both concentrations and T_4 labelling patterns of rat TBG isoforms, we have demonstrated that the relative concentrations of isoforms are maintained during ontogenesis. The deviations from functional equivalence of isoforms seen at earlier developmental ages reflect inhibition of the binding of T_4 to the most acidic TBG isoforms by liposoluble serum components; these components decrease markedly in the serum of older rats.

In the human, abnormal labelling patterns of serum TBG have been described in pathophysiological conditions such as obesity, hyperoestrogenic states, non-thyroidal illness and hepatic disease [27,28,31,32]. The nature of these deviations is not altogether understood. Further insight into the mechanisms responsible for aberrant human TBG microheterogeneity might be gained by investigating, along with labelling density patterns, the protein concentrations of isoforms and the possible interference with isoform labelling by components of the serum environment.

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