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Enhancement of Somatomedin Titers of Normal and Hypopituitary Sera by Addition of L-Triiodothyronine *In Vitro* at Physiological Concentrations

(somatomedin potency of serum/sulfation of mucopolysaccharides/chick embryo sternum)

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ABSTRACT **Somatomedin potencies of sera were assayed by following sulfation of mucopolysaccharides in chick embryo sterna *in vitro*. Apparent potencies of sera from hypophysectomized rats, maintained on a low-iodine diet, were restored to levels above normal by addition to the incubation medium of L-triiodothyronine at 10^{-7} mol/liter of serum. The potencies of normal rat, human, and fetal calf sera were raised 1.3- to 3-fold by addition of triiodothyronine at 10^{-9}–10^{-7} mol/liter of serum. L-Thyroxine was about 10 times less effective than triiodothyronine. Triiodothyronine alone did not stimulate sulfation to nearly the same extent as triiodothyronine plus serum, even at higher concentrations. Serum could not be substituted in this system by any of six purified hormones, nor by trace metals or amino acids not included in the incubation mixture. It is concluded that triiodothyronine, in combination with a factor in serum, causes a rapid stimulation of sulfation in chick embryo cartilage, and that thyroid hormones may be involved in the action of normal serum on this tissue.**

Incorporation of sulfate into mucopolysaccharides of cartilage is stimulated by one or more factors in serum (1). These factors, which have been named somatomedins (2), are thought to be produced peripherally in response to growth hormone (1, 3). Part of the evidence for the involvement of growth hormone is the markedly lower stimulation of sulfation by serum from hypophysectomized rats (1, 3, 4) or hypopituitary humans (5–9) as compared to serum from normal individuals. Administration of purified growth hormone increased the titers of somatomedin in serum from hypopituitary rats (1, 3) or humans (6, 7, 9).

We report here that in one system that has been used for assaying somatomedin, namely, cartilage from 12-day chick embryos (8, 10), somatomedin titers of serum from hypophysectomized rats were restored to values within or exceeding the normal range by addition *in vitro* of L-3,5,3′-triiodothyronine (T_3) at physiological concentrations. Addition of T_3 to normal serum also increases its apparent somatomedin content.

MATERIALS AND METHODS

T_3 and L-thyroxine (T_4) (Calbiochem) were dissolved in alkali and diluted rapidly in incubation medium before use. Bovine growth hormone was prepared according to Ellis (11) and purified by isoelectric focussing in a pH 7–9 gradient (12). Acid-soluble nonsuppressible insulin-like activity (NSILA), purified approximately 2000-fold from serum by acid ethanol extraction and chromatography on Sephadex G-75 (13), was a gift of Dr. E. R. Froesch. Purified relaxin (14) was provided by Dr. C. Schwabe.

Hypophysectomized rats from which serum was obtained were operated on as weanlings and maintained for 6–8 weeks on a low-iodine diet, with or without daily maintenance doses of thyroid hormones, according to the protocol described by Denckla (15). In some groups of animals, cortisol was removed from the drinking water one week before blood was collected. Judged by the criterion of minimal oxygen consumption, all hypophysectomized rats were hypothyroid unless maintained on thyroid hormones (15). T_4 determinations by binding assay, using a commercially available kit ($StaT_4$; Oxford Laboratories), showed that serum from these rats contained less than 1.0 μg of T_4 per 100 ml if no thyroid hormones were administered. The same assay, when applied to the normal rat, human, and fetal calf sera used in this work, gave values between 4.5 and 5.5 μg of T_4 per 100 ml.

Somatomedin was assayed by incubating sterna from 12-day chick embryos for 5 or 6 hr in a synthetic medium (8) containing bovine serum albumin (Sigma; 2× crystallized) and $Na_2{}^{35}SO_4$, with or without addition of serum, and measuring incorporation into mucopolysaccharide after digestion with papain and precipitation with alcian blue. Results were expressed as pmol of SO_4^{2-} per μg of chondroitin sulfate, based on the calculated specific radioactivity of the precursor inorganic sulfate. Full details of the experimental technique and justification for the method of calculating the incorporation are given elsewhere (10). Apparent potencies of sera were estimated by dilution assay (16), and expressed relative to an unsupplemented normal serum of the appropriate species, whose potency was set equal to 1.0. Unless otherwise noted, at least two concentrations of each serum were used in each assay; the concentrations were chosen to fall on the linear portion of the log-dose response curve [between 3 and 20% (v/v) for normal human serum, between 3 and 40% for normal rat serum, and between 5 and 70% for fetal calf serum]. An assay was regarded as valid if the linear portions of the log-dose response curves of all preparations being tested satisfied statistical criteria for parallelism (16), with $P > 0.05$. The relative potency of each preparation was estimated in the customary manner (16), by taking the antilogarithm of the horizontal distance between the linear portions of the log-dose response curves of the standard serum and the prepara-

Abbreviations: T_3, L-3,5,3′-triiodothyronine; T_4, L-thyroxine; NSILA, acid-soluble nonsuppressible insulin-like activity from serum.

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TABLE 1. *Effect of freezing on somatomedin potency of hypothyroid serum*

Serum	Relative potency
Normal	1.00
Hypothyroid (stored at 4°)	0.39 (0.23–0.59)
Hypothyroid (stored at −20°)	0.16 (0.08–0.27)

Two concentrations (10 and 40%, v/v) of each serum were used in a 5-hr incubation. Log-dose response curves satisfied criteria for parallelism ($P > 0.5$); the index of precision, λ, of the assay was 0.19. Normal rat serum was from 4-week-old females. Serum from hypothyroid hypophysectomized rats was stored overnight either at 4° or at −20° before assay. Ninety-five percent fiducial limits for potency estimates are in parentheses in column 2.

tion in question. Ninety-five percent fiducial limits for potency estimates, and other statistical information, were calculated by standard methods, as described elsewhere (10, 16).

RESULTS

When assayed immediately after preparation, pooled sera from hypophysectomized rats that had received no thyroid hormone had potencies in the range 0.35–0.60 relative to normal rat serum, which is the same as the relative potency of serum from rats given daily maintenance doses of T_3 or T_4 (10). However, after storage overnight at −20°, the potencies of the hypothyroid sera fell by 50% or more (Table 1); this is in marked contrast to serum from normal rats, or from hypophysectomized rats maintained on thyroid hormones, which is stable to freezing over long periods. Addition of T_3, at a concentration of 10^{-7} mol/liter of serum, to incubation mixtures containing either of two hypothyroid sera that had been stored at −20° resulted in a dramatic enhancement of the stimulation of sulfation (Fig. 1). The apparent somatomedin

TABLE 2. *Enhancement of somatomedin potencies of hypothyroid and normal rat sera by addition of T_3**

Serum	Addition	Relative potency
Normal	—	1.00
	T_3	2.31 (1.94–2.78)
Hypothyroid†	—	0.32 (0.26–0.40)§
	T_3	1.79 (1.51–2.14)
Hypothyroid‡	—	0.19 (0.15–0.24)§
	T_3	1.25 (1.06–1.48)

* Relative potencies were calculated from the data in Fig. 1. All points in Fig. 1 were used except the lowest concentration of each hypothyroid serum without T_3. The four two-point assays satisfied criteria for parallelism ($P > 0.5$). The index of precision, λ, for the assay was 0.08. Ninety-five percent fiducial limits for potencies are in parentheses in column 3. T_3 was added at 10^{-7} mol/liter of serum where indicated.

† Cortisol was removed from drinking water 1 week before collection of blood.

‡ Cortisol was not removed from drinking water.

§ Independent assays established that the linear portions of the log-dose response curves of the two hypothyroid sera extended to concentrations below 20% (v/v) and were parallel to the log-dose response curve for normal serum; hence, the incorporation at this concentration could be used to provide approximate potency estimates for these sera, which agreed with the potency estimates obtained independently.

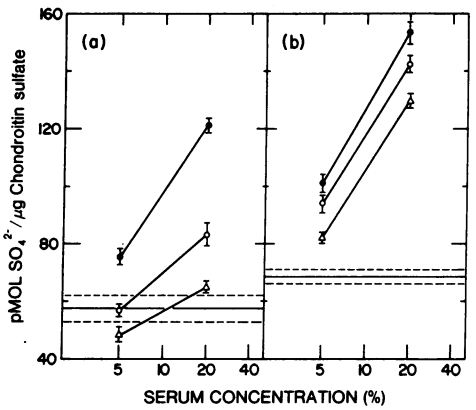

FIG. 1. Log-dose response curves of normal rat serum and serum from hypothyroid hypophysectomized rats. Incubations ($n = 5$) were for 5 hr. Serum from hypophysectomized rats was stored at −20° for 3–5 weeks before use. ●, serum from normal 4-week-old female rats; △, serum from hypophysectomized female rats maintained on a low-iodine diet; ○, serum from hypophysectomized rats maintained on a low-iodine diet, with no cortisol in the drinking water for 1 week before removal of blood. (a) No addition of thyroid hormone; (b) T_3 added to incubation medium at 10^{-7} mol/liter of serum. Bars represent SEM. The horizontal lines in panel (a) show mean ± SEM incorporation in control incubations lacking T_3 or serum; the lines in panel (b) show mean ± SEM incorporation in the presence of 10^{-9} M T_3 without serum.

contents of the two sera were increased by a factor of six, to values greater than that of the standard normal rat serum (Table 2). The titer of the normal serum was also increased by a factor of two. Addition of T_4 to hypothyroid serum also raised the apparent somatomedin titer; however, on a molar basis, T_4 was at least 30 times less effective than T_3 (data not shown).

Addition of T_3 to a sample of human serum produced a significant increase in apparent somatomedin potency at a concentration of 10^{-9} mol/liter of serum (Table 3). This is within the range of T_3 concentrations in euthyroid sera (17).

TABLE 3. *Enhancement of somatomedin potencies of human serum by addition of T_3 or T_4*

Assay no.	λ	Addition	Concentration (mol/liter of serum)	Relative potency
1	0.11	—	—	1.00
		T_3	10^{-9}	1.55 (1.24–1.99)
		T_3	10^{-8}	2.27 (1.79–2.95)
		T_3	10^{-7}	3.05 (2.38–4.05)
2	0.21	—	—	1.00
		T_3	5×10^{-9}	1.40 (0.91–2.21)
		T_3	5×10^{-8}	4.87 (1.33–8.96)
		T_4	5×10^{-8}	2.05 (1.33–3.34)
		T_4	5×10^{-7}	3.63 (2.26–6.52)

Two concentrations of serum (5 and 20%, v/v) were used for each estimate of potency. Parallelism was moderately satisfactory, with $P > 0.05$ for the first assay and $P > 0.25$ for the second. The sera used in the two assays were both reconstituted from lyophilized pooled human serum (Miles Laboratories). Ninety-five percent fiducial limits for potencies are in parentheses in column 5.

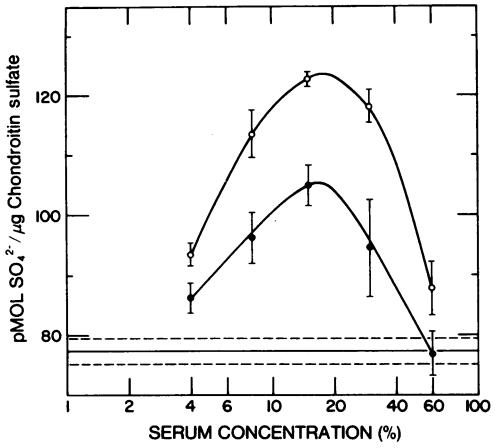

FIG. 2. Log-dose response curves of human serum. Incubations (n = 5) were for 6 hr. ●, unsupplemented serum (Miles Laboratories); ○, serum plus T_3 (5×10^{-8} mol/liter of serum). Bars represent SEM. Horizontal lines show mean ± SEM incorporation in the presence of 3×10^{-8} M T_3 without serum.

Increasing the amount of added T_3 to 10^{-8} or 10^{-7} mol/liter of serum produced a corresponding rise in the somatomedin titer, up to about 3-fold at the highest concentration of T_3 tested. In another assay of human serum, T_4 was about 10 times less effective than T_3 in raising the somatomedin titer (Table 3). The relative potency of a sample of fetal calf serum (Grand Island Biological) was also raised from 1.00 to 1.30 (95% fiducial limits: 1.10–1.58) by addition of 10^{-8} mol of T_3/liter of serum (data not shown).

The log-dose response curve for normal rat serum is linear over the range 3–40% (v/v), while with normal human serum, the range of linearity extends only up to about 20%, and stimulation declines above this level (10). Addition of T_3 did not alter the shape of either log-dose response curve, but merely raised the incorporation at all serum levels. This is shown in Fig. 2 for human serum. Both in the presence and absence of added T_3 (5×10^{-8} mol/liter of serum), sulfation was maximally stimulated by 20% serum, and declined markedly above 50% serum. With a lower concentration of added T_3 (5×10^{-9} mol/liter of serum), the incorporation of sulfate lay between the values in Fig. 2 at all serum levels. In a similar experiment in which normal rat serum was assayed at concentrations of 5, 10, 20, and 40% (v/v), with or without addition of T_3 (5×10^{-8} mol/liter of serum), both log-dose response curves satisfied statistical criteria for linearity ($P > 0.9$) and parallelism ($P > 0.1$), and the addition of T_3 enhanced the apparent somatomedin potency of the serum by a factor of 2.93 (95% fiducial limits: 2.29–3.88).

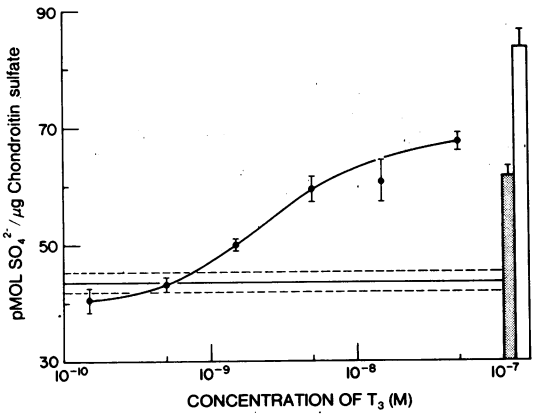

FIG. 3. Log-dose response curve to T_3 in the absence of serum. Incubations (n = 5) were for 5 hr; bars represent SEM. The histogram at the right shows the incorporation in the presence of 5% (v/v) human serum (Miles Laboratories) alone (stippled bar), or of 5% serum and 5×10^{-10} M T_3 (open bar). Horizontal lines show mean ± SEM incorporation in control incubations (no addition).

In the absence of serum, there was a slight rise in sulfation, up to a value of about 60% of the control at 5×10^{-8} M T_3 (Fig. 3). This degree of stimulation was about equal to that caused by 5% (v/v) human serum. Addition of 5×10^{-10} M T_3 and 5% serum together gave about twice as much stimulation as did 5% serum alone; however, T_3 at this concentration, in the absence of serum, did not stimulate sulfation significantly (Fig. 3).

With experimental error, no lag period could be detected before the stimulation of sulfation by T_3 became evident, and rates of incorporation were nearly constant for 6 hr (Fig. 4). This was true both in the presence and absence of serum. The rate of sulfation in the presence of unsupplemented serum is also constant over this time period (8, 10).

Inorganic sulfate was isolated from the incubation media in some experiments, including the one described in Fig. 2, and its specific radioactivity was determined (10). The specific activities of the inorganic sulfate pools were not significantly altered from their values at the beginning of the incubation period. This shows that addition of T_3 caused no detectable breakdown or turnover of mucopolysaccharides, and the observed incorporation of sulfate into mucopolysaccharides very probably represents synthesis of new sulfate ester groups.

Serum could not be replaced by Ca^{2+}, Fe^{3+}, Zn^{2+}, and Mn^{2+} at appropriate concentrations, nor by a mixture of nonessential amino acids not normally included in the incubation medium. The following purified hormones at the indicated concentrations, in the presence of 10^{-9} M T_3, caused no further stimulation of sulfation: bovine growth hormone (10 ng/ml), glucagon (Sigma; 20 ng/ml), relaxin (10 ng/ml), norepinephrine bitartrate (Winthrop Laboratories; 10 ng/ml), insulin (Sigma; 10 μU/ml), or NSILA (10–25 μg/ml).† Some activity was present in human Cohn fractions III (Schwarz/Mann) and IV (Miles Laboratories), but neither fraction completely reproduced the stimulation observed with serum. The activity of human serum or Cohn fraction IV was stable to heating at 90° for 10 min, but was partially destroyed on longer heating; it was also lost slowly during dialysis.

DISCUSSION

Our results suggest that T_3 by itself has little effect on chick embryo cartilage, but that T_3 acts synergistically with some factor(s) in serum to stimulate the incorporation of sulfate into mucopolysaccharides. The concentrations of T_3 used in this work were mostly in the range characteristic of thyrotoxicosis or slightly higher (17); however, in several experiments significant effects were seen at euthyroid concentrations (Table 3). In undiluted serum, more than 99.5% of the total T_3 is bound to albumin or thyroxine-binding globulin (18), and

† This preparation of NSILA stimulated sulfation 40–50% above control values at concentrations between 5 and 100 μg/ml, but did not stimulate further in the presence of T_3.

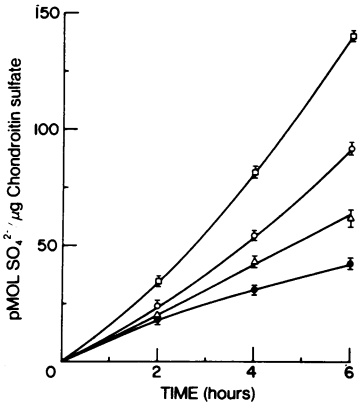

FIG. 4. Time course of incorporation into mucopolysaccharide. $Na_2{}^{35}SO_4$ was added after a 30-min preincubation period in which all other components were present. Bars represent SEM (n = 5). ●, control (no addition); △, 10^{-8} M T_3; ○, 5% human serum (Miles Laboratories) and 2.5×10^{-9} M T_3; □, 20% human serum and 10^{-8} M T_3.

the molar concentrations of free thyroid hormones remain approximately constant as the serum is diluted up to 150-fold (19). From these considerations, and from equilibrium dialysis experiments not reported here, we conclude that the concentration of free T_3 did not exceed 5×10^{-10} M in any experiment in which T_3 and serum were present together. In the work reported here, the medium contained bovine serum albumin at a concentration of 0.5 mg/ml. In the absence of serum, about 75% of any T_3 added to the medium would be bound to albumin under our experimental conditions, the remainder being in the free form (ref. 18; and our unpublished experiments). Thus, in the experiment shown in Fig. 3, in which T_3 was added at increasing concentrations with no serum present, the free T_3 concentration would have reached 5×10^{-10} M when the total concentration was 2×10^{-9} M. However, the stimulation of sulfation at this concentration of T_3 was marginal in the absence of serum; hence, the enhancement of sulfation is probably not a direct action of free T_3 alone, even though this is generally thought to be the physiologically important form of the hormone (20). The similarity of the dose–response curves of normal sera in the presence and absence of added T_3 suggests that the stimulation of sulfation by unsupplemented sera may also involve thyroid hormones.

Attempts to identify the factor in serum that acts synergistically with T_3 have been unsuccessful so far. Neither the trace metals required for chondrogenesis (21) nor the nonessential amino acids not included in the incubation medium seem to be involved directly. The factor does not appear to be replaceable by growth hormone, insulin, glucagon, or NSILA. Our results to data are compatible with an effector of relatively low molecular weight, which is loosely bound to a high-molecular-weight compound and becomes inactive when so bound. One possibility is that the effector is one of the minor T_4-binding proteins in serum discovered recently by Hoch and Lewallen, which might play some part in promoting the physiological action of thyroid hormones (22). Another possible role of serum in this system may be to prevent degradation of T_3 by maintaining a low concentration of free T_3 in equilibrium with bound T_3 throughout the incubation period.

Our results do not necessarily contradict the somatomedin hypothesis (2). Perhaps sulfation can be stimulated in chondrocytes by several apparently unrelated hormones or combinations of hormones, in the same way that lipolysis is stimulated in adipocytes by many unrelated hormones (23). We anticipate that there will be much variation in response to thyroid hormones by cartilage from animals of different ages, and even by different cartilages from the same animal, depending on the stage of chondrogenesis or osteogenesis, since the effect of thyroid hormones on cartilage is to promote maturation rather than growth (24). In this connection, preliminary experiments indicate that adult rat costal cartilage may not respond to T_3, while pelvic fragments from 12-day chick embryos respond in a manner similar to that observed with sterna from the same embryos. However, there is a distinct possibility that in some of the cases reported in the literature, part of the action of purified growth hormone in raising serum somatomedin titers of hypopituitary individuals was due to contamination with thyrotropin (25).

Reports of rapid *in vitro* effects of thyroid hormones abound in the literature, but the concentrations of T_3 or T_4 that produce the effect exceed the known concentrations of circulating free hormones by a factor of 10^2–10^6. Recently, Samuels, Tsai, and Cintron showed that growth and glucose metabolism of cultured GH_1 cells, in medium containing hypothyroid fetal calf serum, could be stimulated up to 3-fold by addition of T_3 or T_4 at physiological concentrations (26). However, these parameters were monitored only at intervals of 12 hr or longer, and it is not clear how long a period elapsed before the action of T_3 was expressed. By contrast, the effect we describe here is produced by physiological concentrations of T_3 or T_4 within an hour at most. The rapidity of the response suggests that stimulation of sulfation is not mediated solely by the high-affinity nuclear binding mechanism for T_3 (27, 28); and the low concentrations of hormones involved make it likely that none of the known *in vitro* actions of T_3 or T_4 on cytoplasmic components (29–32) or permeability and uptake mechanisms (33, 34) is at work, either.

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