Proc. Natl. Acad. Sci. USA
Vol. 73, No. 8, pp. 2904–2908, August 1976
Medical Sciences

Nonsuppressible insulin-like activity and thyroid hormones: Major pituitary-dependent sulfation factors for chick embryo cartilage*

(somatomedins/growth hormone)

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Communicated by Sidney Udenfriend, May 27, 1976

ABSTRACT **Serum from hypothyroid hypophysectomized rats did not stimulate sulfation or incorporation of amino acids into chick embryo sterna. When such rats were treated for a short time with growth hormone (somatotropin), their serum stimulated incorporation both of sulfate and of amino acids. The different actions of the two types of sera were not due to changes in thyroid state. The results support the existence in serum of a sulfation factor for chick embryo cartilage that is dependent upon growth hormone.**

Highly purified preparations of nonsuppressible insulin-like activity from human serum stimulated incorporation of amino acids, and of uridine into RNA, in chick embryo sterna *in vitro*; chondrocytes prepared from this tissue had specific high-affinity binding sites for this insulin-like activity. However, sulfate incorporation was stimulated very little, unless serum from hypothyroid hypophysectomized rats was also present. When L-3,5,3′-triiodothyronine was added as well, the stimulation was enhanced further. From these and other experiments, we conclude that (*i*) nonsuppressible insulin-like activity or a closely related peptide is the growth-hormone-dependent growth and sulfation factor for chick embryo cartilage; (*ii*) a second, unidentified factor must be present for the insulin-like activity to stimulate sulfation; and (*iii*) stimulation of sulfation by thyroid hormones *in vitro* is additive to that of nonsuppressible insulin-like activity.

Nonsuppressible insulin-like activity (NSILA) of serum is attributable to two peptides, NSILA-I and NSILA-II, which have a molecular weight of 5800 and are presently being characterized in detail (ref. 2; R. Rinderknecht, J. Zapf, and R. E. Humbel, in preparation). The insulin-like properties of NSILA have been reviewed recently (3, 4). Growth-promoting effects of NSILA have been demonstrated in chick embryo and human fibroblast cultures (5, 6); these appear to be mediated by a highly specific membrane receptor for NSILA (7). NSILA is also a potent sulfation factor in rat costal cartilage (8).

Thyroid hormones appear to be responsible in part for the stimulation by normal serum of sulfation in chick embryo cartilage (9). Stimulation of sulfation by L-3,5,3′-triiodothyronine (T_3) and serum reflects increased synthesis of at least the major proteoglycan in the tissue (10). The effect of serum on sulfation in this tissue has also been attributed to two small peptides, which have been named somatomedins A_1 and A_2 (11). These two peptides are clearly distinct from NSILA since they have a different amino acid composition and apparently do not contain disulfide bridges, which are essential for the activity of NSILA. Published reports of the biological activities of these peptides (12, 13) are insufficient to allow assessment of their physiological significance as growth-promoting or sulfation factors.

In this report, we present evidence that NSILA is a growth and sulfation factor for chick embryo sternum. Our results suggest that the stimulation of sulfation by normal serum can be explained in terms of its content of NSILA and thyroid hormones and of an unidentified factor that is not under pituitary control. Implications for the somatomedin hypothesis are discussed.

MATERIALS AND METHODS

Partially purified NSILA from human serum [specific activity, 3.8–17 milliunits (mU)/mg; ref. 2] and homogeneous NSILA-I and NSILA-II (specific activity, 400 mU/mg), were generous gifts from Dr. R. E. Humbel. ^{125}I-Labeled NSILA was prepared as described (7). Crystalline insulin was from Sigma; sources of other materials are listed elsewhere (9, 10).

Procedures for hypophysectomy of weanling rats, with formaldehyde cauterization of the *sella turcica*, and for their subsequent care and maintenance, have been described by Denckla (14). Hypophysectomized rats were kept on a low-iodine diet; under these conditions, the animals remain hypothyroid for several weeks, as judged by measurements of minimal and maximal oxygen consumption (15). Bovine growth hormone (somatotropin; GH) was administered to such rats, 8–10 weeks after hypophysectomy, by subcutaneous injection as a suspension in 0.05 ml of peanut oil, at a dose of 0.5 mg/kg of body weight per day for 5 days. Blood was collected on the sixth day.

Insulin-like activity of NSILA preparations was estimated with the rat fat-pad assay, using porcine insulin as standard (16). Concentrations of NSILA are expressed as microunits (μU)/ml of insulin-like activity, based on the results of this assay. NSILA in serum was determined by submitting the serum to chromatography on Sephadex G-50 with 1 M acetic acid as eluant (17); the fractions eluting between 0.6 and 0.85 bed volume were assayed for NSILA with a protein binding assay (18). Thyroid hormones in serum were determined by radioimmunoassay, using commercially available kits (RIAMAT, Mallinckrodt, for T_3; Roche T_4 RIA, Hoffmann-La Roche, for thyroxine). Comparative estimates of the thyroid hormone binding capacity of sera are based on results of StaT3 assays (Oxford Laboratories).

Labeled precursors were incorporated into sterna isolated from 12-day chick embryos, which were incubated individually in 1 ml of synthetic medium, as described (19). The composition of the medium, which contained a full complement of amino acids, is given in ref. 10. In all experiments, incubations were continued for 6 hr after addition of label. For assays of sulfation, incorporation of $^{35}SO_4^{2-}$ (0.5–2 μCi/μmol) into glycosamino-

Abbreviations: NSILA, acid-soluble insulin-like activity from human serum not suppressible by antibody against insulin; T_3, L-3,5,3′-triiodothyronine; GH, growth hormone (somatotropin); U, unit of insulin-like activity relative to porcine insulin.

* Part of this work has been presented in abstract form (ref. 1).

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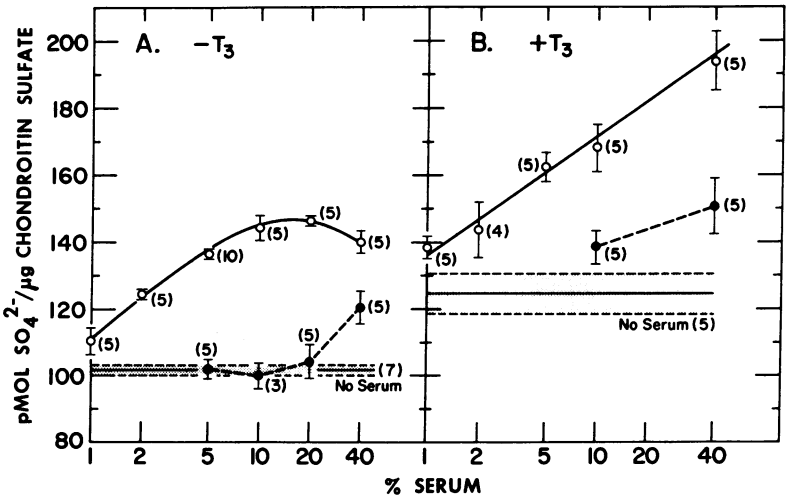

FIG. 1. Stimulation of sulfation by serum from hypothyroid hypophysectomized rats before and after administration of growth hormone. Incorporation of $^{35}SO_4^{2-}$ was determined after precipitation of glycosaminoglycans with alcian blue. ○, Serum from rats treated with GH; ●, serum from untreated rats. (A) No T_3 added to incubation medium; (B) T_3 added *in vitro* at 5×10^{-8} mol/liter of serum. Bars represent SEM for the number of replicate samples indicated. Shaded areas represent: (A) mean ± SEM for seven control incubations; (B) mean ± SEM for five incubations in the presence of 2 nM T_3 without serum.

glycans was measured after digestion of the tissue with papain and precipitation of the glycosaminoglycans with alcian blue (19). In experiments in which incorporation of $^{35}SO_4^{2-}$ and [^{3}H]leucine or [^{3}H]phenylalanine (20–40 µCi/µmol) into whole cartilage was followed simultaneously, the cartilages were extracted with Na_2SO_4, dried, and weighed before determination of radioactivity (20). This procedure removes readily exchangeable amino acids from the cartilage (21); more than 90% of ^{35}S incorporated in such experiments is found in glycosaminoglycans (19). In some experiments, sterna were incubated in medium containing 0.1 mM [^{3}H]uridine (100 µCi/µmol), and incorporation into RNA was determined after extraction with trichloroacetic acid (22).

Chondrocytes were prepared by dissecting sterna free from perichondrial tissue (23) and incubating them at 37° for 1.5–2 hr in Hanks' balanced salt solution containing collagenase (2.0 mg/ml; Worthington) and chondroitin abc lyase (0.2 unit/ml; Miles Laboratories). Binding of ^{125}I-labeled NSILA to chondrocytes was determined in the same way as binding to fibroblasts (7).

RESULTS

Thyroid hormones at near physiological levels stimulate sulfation *in vitro* in chick embryo cartilage. However, the stimulation of sulfation by normal rat serum cannot be explained in terms of its content of thyroid hormones alone (9); hence, it appeared that normal serum might contain other stimulatory factors. Direct evidence for this was obtained by treating hypophysectomized rats, which had been kept in a hypothyroid state by maintenance on a low-iodine diet, with bovine GH (0.5 mg/kg of body weight per day for 5 days). As reported previously (9), pooled serum from untreated rats did not stimulate sulfation in chick embryo sterna; by contrast, pooled serum from GH-treated rats stimulated significantly at all concentrations down to 2% (Fig. 1A). Estimates of thyroxine and T_3 and of the ability of the sera to bind thyroid hormones did not differ significantly between the sera from treated and untreated animals (Table 1); this indicates that differences in thyroid state were not responsible for the different activities of the sera. The difference in the ability of the sera to promote sulfation was maintained when sulfation was assayed in the presence of added T_3 (Fig. 1B). These observations support the existence in serum of a GH-dependent sulfation factor (24) for chick embryo cartilage, whose action is additive to that of thyroid hormones.

Mean levels of NSILA in serum from rats and humans correlate well with the clinical manifestations of GH excess or deficiency (17). In view of the marked effects of NSILA on anabolic processes in chick embryo fibroblasts (5), it seemed reasonable to investigate the action of NSILA on chick embryo cartilage, and in particular to examine the possibility that NSILA is the GH-dependent sulfation factor for this tissue. Levels of NSILA in the sera from GH-treated and untreated hypophysectomized rats were determined as described under *Materials and Methods*. Normal rat serum contains 85 ± 15 µU/ml; as can be seen in Table 1, the serum from GH-treated rats had half this amount, whereas no NSILA could be detected in the serum from the untreated hypophysectomized rats. This provides direct evidence for the GH-dependence of levels of circulating NSILA.

Partially purified NSILA (100 µU/ml) stimulated incorporation of [^{3}H]leucine into cartilage by 40% in the presence or absence of serum from hypophysectomized rats (Table 2). Normal rat serum, as well as serum from GH-treated hy-

Table 1. Thyroid hormone and NSILA content of serum from hypothyroid hypophysectomized rats

Prior treatment of rats	Thyroxine (µg/100 ml)	T_3 (ng/100 ml)	StaT3 index	NSILA (µU/ml)
None	0.1	40	1.37	0*
GH	0.2	35	1.37	42 ± 11†

GH-treated rats received 0.5 mg of bovine GH/kg of body weight per day for 5 days, and were bled on the sixth day. StaT3 index (a measure of thyroid hormone binding capacity) is expressed relative to a value of 1.0 for a sample of pooled normal human serum (Oxford Laboratories).

* Not detectable (< 2 µU/ml).

† Mean ± range of two determinations.

Table 2. Incorporation of [^{3}H]leucine into chick embryo cartilage

Source of serum	[^{3}H]Leucine incorporated (nmol/mg of dry weight)	
	− NSILA	+ NSILA
None	1.14 ± 0.06	1.54 ± 0.03
Hypophysectomized rats, untreated	1.14 ± 0.04	1.64 ± 0.03
Hypophysectomized rats, GH-treated	1.62 ± 0.03	1.58 ± 0.06
Normal rats	1.49 ± 0.10	1.56 ± 0.05

The concentration of serum, where present, was 10% (vol/vol). NSILA was added at 100 μU/ml. Results are expressed as mean ± SEM for five sterna.

pophysectomized rats, stimulated protein synthesis to the same extent as NSILA. When NSILA was added to these sera, no further stimulation occurred. As reported previously (25), T_3 had little or no effect on incorporation of leucine in the presence or absence of serum or NSILA. Identical results were obtained when [^{3}H]phenylalanine was substituted for [^{3}H]leucine.

NSILA alone stimulated incorporation of $^{35}SO_4^{2-}$ into cartilage by 33% (Fig. 2A). Stimulation by 2 nM T_3 was similar, and the action of both hormones together was nearly additive. T_3 added in the presence of nonstimulatory serum from hypothyroid hypophysectomized rats enhanced sulfation no more than when it was added to the incubation medium alone. However, NSILA added in the presence of this serum stimulated sulfation by 105%; and when T_3 was also added, the stimulation over the baseline was 150% (Fig. 2B). This is as much stimulation as can be obtained with normal rat serum supplemented with T_3 (not shown). The concentration of the serum from hypophysectomized rats did not influence the results between 5 and 20%. Since serum contains a specific binding protein for NSILA (18), it seemed possible that NSILA might stimulate sulfation only in the bound form. However, insulin (100 mU/ml) added in the presence of serum from hypophysectomized rats was as efficient a sulfation factor as NSILA (100 μU/ml). Since insulin does not bind to the binding protein for NSILA (18), this suggests that interaction of NSILA with its binding protein probably plays no part in the stimulation of sulfation.

As with chick embryo fibroblasts (5), NSILA is a potent stimulator of incorporation of uridine into RNA of chick embryo sterna. Dose–response relationships for incorporation of uridine and sulfate are shown in Fig. 3. Uridine incorporation was carried out in incubation medium alone, whereas sulfation was performed in medium supplemented with serum from hypophysectomized rats. In both assays a significant stimulation was observed with as little as 3 μU of NSILA/ml, and maximal effects were obtained between 10 and 100 μU/ml. Fig. 3 also shows binding of ^{125}I-labeled NSILA, and displacement by unlabeled NSILA, to chondrocytes from 12-day-old chick embryos. The extent of binding of ^{125}I-labeled NSILA was equal to that observed with chick embryo fibroblasts (7), i.e., 0.5–1% of the label contained in 1 μU of ^{125}I-labeled NSILA was bound by 10^6 cells; and 70% of the total binding could be attributed to specific binding. Half-maximal displacement was obtained with less than 1 μU/ml of unlabeled NSILA; for a similar effect, 3 mU/ml of insulin were required (not shown).

Since impure preparations of NSILA had been used for most of the foregoing experiments, it remained to be established that NSILA itself was responsible for the effects. Table 3 shows that

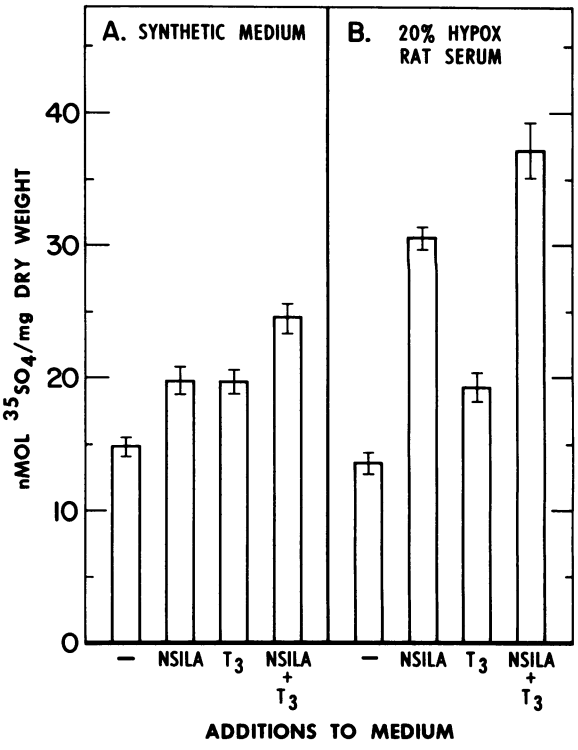

FIG. 2. Effect of NSILA and T_3 on sulfation. Total incorporation of $^{35}SO_4^{2-}$ was determined after cartilages were soaked in unlabeled Na_2SO_4. NSILA was added at 100 μU/ml; T_3 was added at 5×10^{-8} mol/liter of serum, or 2 nM in the absence of serum. Results are expressed as mean ± SEM for five sterna. (A) Synthetic medium alone; (B) synthetic medium supplemented with serum from hypothyroid hypophysectomized rats (20%, vol/vol).

incorporation of uridine and of sulfate were both stimulated by homogeneous NSILA-I or NSILA-II. The dose–response relationship for uridine incorporation was the same as with partially purified NSILA. Three microunits per milliliter of each peptide stimulated significantly, and near maximal effects were obtained with 10 μU/ml, corresponding to 25 ng/ml of peptide.

DISCUSSION

The results presented in this paper demonstrate that NSILA is a potent anabolic factor for chick embryo cartilage, as determined by its effects in assays of protein and RNA synthesis. This agrees with our earlier findings regarding the effects of NSILA on replication, and synthesis of protein, RNA, and DNA, in 12-day chick embryo fibroblasts in secondary culture (5, 26). The sensitivity of chick embryo sternum to partially purified NSILA, and to homogeneous NSILA-I or NSILA-II, is similar to that of the cultured fibroblasts. Our data also suggest that NSILA is responsible for the stimulatory effects of serum on metabolic indices of growth in chick embryo cartilage. Sera that contained NSILA stimulated incorporation of leucine or phenylalanine, and addition of NSILA *in vitro* did not enhance this stimulation (Table 2). Serum from hypophysectomized rats, which had no measurable NSILA, did not stimulate incorporation unless exogenous NSILA was added. The effects of NSILA appear to be mediated through a high-affinity binding site on the chondrocyte membrane, which has characteristics similar to the membrane receptor of the chick embryo fibroblast (7).

In these studies, as in our previous work (9), NSILA was a weak sulfation factor for chick embryo cartilage when added

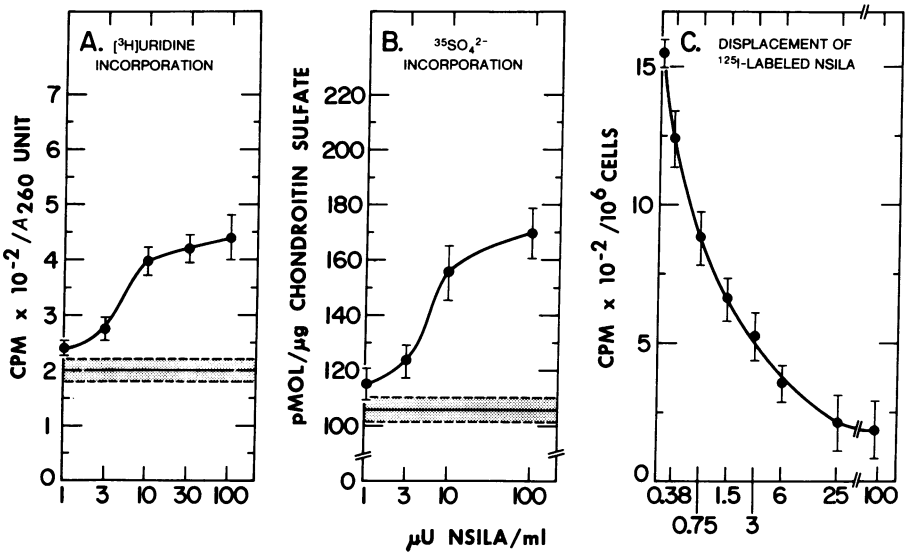

FIG. 3. Stimulation by NSILA of incorporation of uridine and sulfate, and displacement of bound NSILA from chondrocytes. (A) Incorporation of [^{3}H]uridine into RNA of sterna incubated in synthetic medium. (B) Incorporation of $^{35}SO_4^{2-}$ into glycosaminoglycans of sterna incubated in the presence of serum from hypothyroid hypophysectomized rats (10%, vol/vol). (C) Displacement of ^{125}I-labeled NSILA bound to sternal chondrocytes. Results in (A) and (B) are expressed as mean ± SEM for five sterna; results in (C) are mean ± SD for triplicate assays. Shaded areas in (A) and (B) represent mean ± SEM for five incubations without NSILA.

to a synthetic medium. This was true also in the presence of Ham's F-12 medium (27), which contains several trace components of serum and is frequently used to grow chondrocytes in tissue culture. When added to serum from hypothyroid hypophysectomized rats, which by itself exerted no stimulation, NSILA became a potent sulfation factor. When T_3 was added also, the effect on sulfate incorporation was the same as that exerted by normal rat serum supplemented with T_3. These observations indicate that yet another factor(s), in addition to NSILA and thyroid hormones, is involved in the stimulation of sulfation in chick embryo cartilage. At present, the nature of this factor is unknown. The factor is not needed for stimulation of sulfation in rat costal cartilage, in which NSILA alone is virtually as effective as serum (8). One possible role for the factor in the chick embryo system may be to accelerate the rate of secretion of proteoglycan from the cell. In support of this,

Table 3. Stimulation of uridine and sulfate incorporation by homogeneous NSILA

Additions to medium	Concentration (ng/ml)	[^{3}H]Uridine incorporated into RNA (cpm/A_{260} unit)	$^{35}SO_4^{2-}$ incorporated into glycosaminoglycans (pmol/µg of chondroitin sulfate)
—	—	3506 ± 247	92.7 ± 2.9
NSILA-I	7.5	5410 ± 410	—*
	25.0	6611 ± 319	128.7 ± 2.9
NSILA-II	7.5	5671 ± 550	—*
	25.0	7490 ± 330	134.1 ± 5.9

Uridine incorporation was assayed in synthetic medium; sulfate incorporation was assayed in the presence of serum from hypothyroid hypophysectomized rats (10%, vol/vol). Results are expressed as mean ± SEM for five sterna.
* Not determined.

we have found that colchicine markedly reduces the stimulation by NSILA of sulfation, but not of incorporation of leucine; this is compatible with a role for the unidentified factor in microtubule-mediated transport of proteoglycan through the cell (28). However, colchicine is also a potent antagonist to the stimulatory action of T_3 on sulfation (29), which does not appear to require the factor.

Our results have several consequences of immediate significance to research on somatomedins. First, they cast doubt on the specificity of various somatomedin assays, which measure sulfation in cartilage from different sources in an attempt to discriminate between different somatomedins. Under appropriate conditions, NSILA is a potent sulfation factor in assays using chick embryo cartilage, which have been considered to measure somatomedin A (12), as well as in assays using rat costal cartilage (8), which are thought to measure somatomedin C (30). Second, our results call into question the use of assays of sulfation in chick embryo cartilage in the purification of somatomedins. If such an assay were used to follow purification of NSILA, nearly all the biological activity would appear to be lost at the stage at which NSILA peptides are separated from the unidentified serum factor. In this connection, it should be noted that according to the most recent report the purified peptides, somatomedin A_1 and A_2, stimulate sulfation in chick embryo cartilage to the extent of 25% at most, and the logarithmic dose–response curve for those peptides has a slope that is close to zero (12). Third, contrary to earlier conclusions (24), incorporation of sulfate into cartilage is not necessarily correlated with other, more general, anabolic processes. Incorporation of leucine or phenylalanine, and of uridine into RNA, in chick embryo sterna is stimulated by NSILA in the absence of other factors, with little effect on incorporation of sulfate; conversely, incorporation of sulfate is stimulated by T_3 alone (9, 10) with much less effect on incorporation of leucine or uridine (25). Finally, stimulation by serum of sulfation in chick embryo cartilage is a complex phenomenon involving at least three factors. One factor is NSILA or some closely related peptide; this is the only GH-dependent factor. A second, unidentified factor must be present in order for NSILA to act on sulfation; this factor may not be under endocrine control. The

third factor, whose action is additive to that of NSILA, is thyroid hormone. In view of the complexity of this apparently simple system, we suggest that great caution should be exercised in interpreting estimates of somatomedin levels in untreated sera, obtained with sulfation assays based on chick embryo cartilage.

We thank Dr. R. E. Humbel for generous gifts of homogeneous and partially purified NSILA, without which this work could not have been carried out. We are indebted to Dr. W. D. Denckla for making available hypophysectomized rats; and to Dr. R. A. Parker for estimations of triiodothyronine and thyroxine in serum. We also thank Dr. B. L. Horecker and Dr. W. D. Denckla for helpful comments on the manuscript. E.R.F. has been on leave from the University of Zurich, Switzerland; and E.B.-P. is on leave from the Technion Aba Khoushi School of Medicine, Haifa, Israel. This work was supported in part by Grant 3.7180.72 from the Schweizerische Nationalfond.

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