Growth hormone dependent human serum stimulation of thymidine and sulphate incorporation into embryonic chicken cartilage

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Abstract. The embryonic chicken cartilage somatomedin bioassay was modified so that human serum stimulation of simultaneous [3H]methylthymidine and H2[35S]SO4 incorporation could be assessed. The assay consisted of a 6 h pre-incubation of 10 day pelvic rudiments in enriched buffer, followed by a 24 h incubation with buffer and low (0.5, 2 and 5% v/v) serum concentrations. Both labels were present for the final 6 h. Other modifications were shortening of washing, elimination of drying and weighing, and simplification of digestion.

Normal human serum produced a linear log dose-response with these serum concentrations. Potency ratios in patients with GH deficiency were less than those of normal adults for both thymidine 0.39 ± 0.05 (mean ± SEM, n = 16, range 0.22–0.71) vs. 0.90 ± 0.05 (n = 19, 0.62–1.36, P < 0.001) and for sulphate 0.40 ± 0.04 (0.15–0.65) vs. 94 ± 0.05 (0.61–1.29, P < 0.001). Potency ratios for both labels rose following administration of a single dose (0.2 IU/kg im) of hGH to 4 GH deficient children.

The reliability of prediction of GH deficiency, reproducibility, and precision were similar to other Sm bioassays. The major advantages of these modifications were the ability to examine 2 cartilage metabolic processes simultaneously and the small amount of serum (350 μl) necessary for patient assays.

The somatomedins (Sm) are growth hormone (GH) dependent substances which activate general anabolic processes including protein, RNA, and DNA synthesis and sulphate incorporation in cartilage and some other tissues (Daughaday et al. 1972). In a clinical setting Sm are most commonly quantitated by assessing serum stimulation of radioactive sulphate uptake into rat (Salmon & Daughaday 1957), porcine (Van den Brande & Du Caju 1974), or chick embryo cartilage (Hall 1970) in vitro. Direct comparison of human serum stimulation of sulphation and thymidine incorporation has only rarely been reported.

Using hypophysectomized rat costal cartilage segments double-labelled with H2[35S]SO4 and [3H]methylthymidine, Van den Brande et al. (1971) demonstrated that plasma sulphation and thymidine incorporation activities co-purified from acromegalic human serum. Specific GH dependence was not studied. Van den Brande & Du Caju (1973) examined stimulation of SO4 and thymidine incorporation into porcine costal cartilage discs by serum from children with a variety of abnormalities of stature. Sulphation activity was low in GH deficient children and rose following therapy. They state that they obtained similar results with thymidine, but no data were given.
Hall (1970) described GH dependent serum stimulation of SO₄ incorporation into embryonic chicken pelvic rudiments. Despite modification of this 5m assay by several investigators (Schimpff & Donnadieu 1973; Audhya & Gibson 1974) its use for the evaluation of serum stimulation of cartilage thymidine incorporation has not been reported. This may be due to the high basal (unstimulated) incorporation in this rapidly growing embryonic tissue (Van Wyk et al. 1974). We have reported that human serum stimulation of thymidine incorporation into chondrocytes isolated from such rudiments was partially GH dependent (Garland et al. 1976). With the isolated cell method we could not quantitate SO₄ incorporation. We have modified Hall’s method and report the initial results of serum stimulation of simultaneous thymidine and SO₄ incorporation into embryonic chicken pelvic rudiments.

**Methods**

**Incubation procedure**

Pelvic rudiments were removed from 10 day chick embryos and carefully cleaned of adherent tissue on moistened filter paper. During dissection rudiments were placed in sterile Petri dishes containing 10 ml Krebs phosphosaline buffer supplemented with amino acids, dextrose, and penicillin/streptomycin (KPSAAs) as previously described (Garland et al. 1972). After completing dissection, the rudiments and media (approximately 1 rudiment/ml) were transferred to flasks and pre-incubated for 6 h at 37°C in a metabolic shaker.

Assay tubes (12 x 75 mm, glass) were prepared by adding serum to KPSAAs for a total volume of 1 ml. Serum was generally added at 0.5, 2, and 5% (v/v) with 4 replicates at each concentration. An assay routinely consisted of 8 buffer control tubes, 2 standard and 9 patient dose-response curves.

After pre-incubation, a single rudiment was added to each assay tube. All rudiments that were broken or appeared unusually large or small were discarded and the rest were distributed so that the samples for an individual standard or experimental dose-response curve contained pelves removed from 12 different embryos. The tubes were incubated for 18 h at 37°C with shaking. Twenty μl KPSAAs containing (0.3 or 1 μCi) carrier free [35S]O₄ and (0.15 or 1 μCi) [3H]methylthymidine (New England Nuclear) was then added to each tube and the incubation continued for an additional 6 h. Replicate rudiments were then pooled by pouring them into 5 ml disposable Styrex syringe barrels held in a metal rack (4 rudiments per syringe). The rudiments were washed by placing the rack in a bath with running tap water followed by running deionized water (30 min each). Each cartilage was blotted and placed into a 7 ml glass vial containing 250 μl of Soluene 350 (Packard). Digestion was complete in 1 h at 22–25°C. Six ml scintillation fluid (Insta-Fluor, Packard) was added and radioactivity counted.

In several experiments the modified incubation procedure was directly compared to the incubation times described by Hall (1970). Pelvic leaflets from 10 day embryos were dissected and cleaned as above. One of each pair was either pre-incubated and used as described above or added directly to individual assay tubes containing buffer or serum. These tubes were incubated for 1 h at 37°C and then 20 μl KPSAA containing label (0.3 μCi H₂[35S]O₄ and 0.15 μCi [3H]methylthymidine was added and incubation continued for an additional 6 h. The rudiments were processed as described above.

Results for both methods were expressed as DPM ³H and ³⁵S after correction for quenching and crossover. When serum concentrations greater than 5% were used, SO₄ DPM/rudiment were corrected for change in total SO₄ and expressed as μg SO₄/rudiment. Clinical data were plotted as log dose-response curves. Potency ratios were calculated using standard bioassay parallel line analysis (Finney 1978). The index of precision (λ) was calculated for each assay as mean residual square/slope. The means of groups were compared using Student’s t-test.

To examine the time course of unstimulated thymidine and sulphate incorporation, rudiments were removed and cleaned as above. Incubation was done in individual tubes with 1 ml KPSAAs rudiment. H₂[35S]O₄ or [3H]methylthymidine in 20 μl of KPSAAs was added at 0, 6, 21 or 45 h. Double labelling was not done here; one of each pair was incubated with thymidine and its mate with SO₄. After addition of label, incubation was continued for 4 h and the rudiments were washed, digested, and the incorporated label counted.

To examine the importance of various washing procedures, rudiments were removed and cleaned, added to flasks containing KPSAA and [3H]methylthymidine, and incubated for 2 h at 37°C. Groups of rudiments were blotted and processed in one of three ways:

A. Immersed in boiling water for 15 min, soaked overnight in saturated sodium sulphate, washed in tap and then deionized water for 1 h each.
B. Same as A but not heat-killed.
C. Washed in tap and deionized water with no other treatment.

All rudiments were then digested individually and counted as above.

To examine the effect of the longer incubation upon
rudiment weight changes, leaflets were removed and cleaned as above. One member of each pair was then blotted and air dried overnight and weighed. The other member of each pair was added to a flask containing either KPSAs or buffer with 40% normal human serum (1 ml total volume/rudiment). Flasks were incubated for 30 h at 37°C with shaking. Rudiments were removed, rinsed briefly with tap water, blotted, dried overnight and weighed. In one study a group of rudiments was also incubated in 40% GH deficient human serum. For comparison, additional eggs were replaced in the egg incubator (37°C) for 30 h (in ovo) and then dissected, cleaned, dried and weighed.

Sera

Standard curves were determined using a pool of human serum obtained from normal adults arbitrarily assigned a potency of 1.00 U/ml. The pool was kept frozen in aliquots. The same pool was used for all studies. All serum samples were heated (56°C for 30 min) prior to assay (Garland et al. 1976). Serum samples were obtained after an 8–12 h fast from young adults without growth, endocrine, or other medical problems who were taking no medications. Serum was obtained from children (all more than 6 years old) with well documented GH deficiency, either prior to long-term treatment or after discontinuing hGH treatment for at least 4 weeks. All had increased growth velocity during hGH administration. For the studies in Fig. 4 a single dose of human GH (0.2 IU/kg) was given im and samples drawn for Sm at 0, 6, 12, and 24 h. Informed consent was obtained from all individuals and the study was approved by the Institutional Human Investigation Committee. hGH was kindly provided by NIAMDD and the National Pituitary Agency of the University of Maryland.

Results

Technical studies

With 4 h pulse labelling there was a decline in the unstimulated thymidine and sulphate incorporation of paired rudiments to 52 and 31% by 25 h and 35 and 3% by 48 h. The pre-incubation, 18 h incubation with serum, and 6 h incubation with serum plus label were chosen for routine use.

There was no difference whether rudiments were heat killed, soaked overnight, and washed (67, 652 ± 2231 DPM/rudiment, mean ± SEM, n = 10); soaked and washed without prior killing (64, 831 ± 7742, n = 4); or washed only (67, 146 ± 3696, n = 10). Results of several other experiments were similar.

Table 1 compares weight changes after different incubation conditions. Initial weights were consistent from week to week. There was no increase in weight after incubation in buffer or GH deficient human serum. When normal human serum was present, rudiment weight increased compared to either initial weight or to leaflets incubated in buffer, though the increase was smaller than that in those remaining in ovo.

The use of the commercial solubilizer for cartilage digestion was rapid (less than 1 h), simple (no heating or special procedures), and complete (visual assessment).

Serum dose-response

Fig. 1 shows the control serum log dose-responses for consecutive assays. Errors are not plotted, but

<table>
<thead>
<tr>
<th>Table 1.</th>
<th>Rudiment weight changes during 30 h incubation in ovo or in vitro*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Initial weight</td>
<td>0.583 ± 0.032 (13)</td>
</tr>
<tr>
<td>In ovo</td>
<td>1.301 ± 0.087 (8)</td>
</tr>
<tr>
<td>In vitro</td>
<td>1.099 ± 0.092 (10)</td>
</tr>
<tr>
<td>C. Buffer only</td>
<td>0.462 ± 0.057 (6)</td>
</tr>
<tr>
<td>D. NHS</td>
<td>0.795 ± 0.043 (6)a, c</td>
</tr>
<tr>
<td>E. HHS</td>
<td>0.423 ± 0.090 (3)*</td>
</tr>
</tbody>
</table>

* Rudiments were prepared and incubated as described in methods. Results are expressed as mg/rudiment, mean ± SEM (number of rudiments).
A vs. B both P < 0.001. A vs. C all P > 0.05. A vs. D a P < 0.01, b P < 0.02.
C vs. D c P < 0.001, d P < 0.05. D vs. E e P < 0.05.
they were usually less than 10% of the mean. Within an individual assay the shape and slope of the curves for thymidine and for SO₄ incorporation were similar by inspection. The incorporation into unstimulated rudiments (mean ± SEM for these 7 experiments) is plotted at the extreme left and right. In individual assays stimulation by 0.5% serum was always less than buffer for thymidine while it was less than or equal to buffer for SO₄.

The index of precision for the curves in Fig. 1 was: thymidine 0.21 ± 0.03 (mean ± SEM, range 0.10–0.30) and sulphate 0.20 ± 0.02 (0.11–0.34).

Fig. 2 illustrates several of the differences in results between the two incubation schedules: 1. the fall in unstimulated incorporation. In these experiments thymidine and sulphate incorporation fell to 20 ± 4% (mean ± SEM) and 55 ± 4% when labelling from 24–30 h was compared to that from 1–7 h, 2. the lack of significant stimulation of thymidine incorporation by NHS at 7 h, 3. a continued increase in stimulation with serum concentration greater than 5% (30 h), 4. improved linearity and parallelism of NHS and HHS with the longer incubation. The assay was done with the smaller quantities of labels listed in methods, while those in Fig. 1 were done with 1 μCi of each label.

Stimulation of thymidine (a and b) and sulphate (c and d) incorporation by normal (NHS) or GH deficient (HHS) human serum with 7 h total incubation (a and c) and with 30 h total incubation (including the 6 h pre-incubation) (b and d). Incorporation in the presence of buffer alone is plotted to the left of 0.5% serum.
Log dose-response lines for standard serum, serum from a short child with normal GH responses to provocative stimuli, and two children with GH deficiency. Each point is DPM/rudiment, mean and SEM of four replicates. Potency ratio for each is given next to the line.

Clinical studies
Log dose-response curves for 2 children with GH deficiency and a child with short stature but normal GH are compared to the standard curve in Fig. 3. The lines were parallel to the standard with each label, and the response of thymidine was qualitatively and quantitatively similar to that of the sulphate. Thymidine potency ratios for a group of normal adults was 0.90 ± 0.05 (mean ± SEM, n = 19), compared to 0.39 ± 0.05 (n = 16, P < 0.001) in GH deficient children. Sulphate potencies for these groups were similar: normal 0.94 ± 0.05 and GH deficient 0.40 ± 0.04 (P < 0.001). The potencies for individuals in the two groups are

Mean and individual potency ratios for a group of normal adults and a group of GH deficient children.
The rise in potency following a single im injection (0.2 IU/kg) of human GH in four GH deficient children.

given in Fig. 4. Following administration of a single dose of human GH (0.2 IU/kg, im) to several children, both thymidine and sulphate potencies rose, peaking between 12 and 24 h (Fig. 5).

The potency ratios for thymidine and sulphate were very similar in each individual. There was no evidence of dissociation of stimulation as reflected by ratio of thymidine to sulphate potency (T/S) of 0.98 ± 0.03 (mean ± SEM) for normal adults and 0.97 ± 0.03 for GH deficient children. Individual potencies were reproducible from assay to assay as shown in Table 2.

### Table 2.
Inter-assay comparison

<table>
<thead>
<tr>
<th>Patient</th>
<th>Assay No. 1*</th>
<th>Assay No. 2</th>
<th>Assay No. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.00/1.04</td>
<td>1.03/1.16</td>
<td>0.94/0.86</td>
</tr>
<tr>
<td>2</td>
<td>0.75/0.91</td>
<td>0.76/0.92</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.84/1.02</td>
<td>0.86/0.74</td>
<td></td>
</tr>
<tr>
<td>GH deficient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.26/0.33</td>
<td>0.30/0.34</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.26/0.18</td>
<td>0.28/0.23</td>
<td></td>
</tr>
</tbody>
</table>

* thymidine potancy / sulphate potency

**Discussion**

The major modifications of Hall's assay were: 1. use of 10 day rather than 11 or 12 day embryos, 2. use of a pre-incubation and an increase in total incubation time, 3. simplification of the post-incubation washing, weighing, and digestion procedures, 4. labelling with thymidine as well as sulphate.

Herington et al. (1976) reported that with short incubation times (20 h) human serum stimulation of sulphation in chicken embryo rudiments was reduced in GH deficiency, but assay precision was not adequate. With a long incubation period (44 h) serum from a normal subject and that from a GH deficient patient were equipotent. GH dependence and adequate precision were achieved only at an intermediate time (25 h). In our hands the 7 h total incubation frequently yields invalid standard curves (λ > 0.4). It should be noted that this is not a direct comparison with Hall's method since 10 day embryos and the shortened washing procedure without weighing was used. More importantly we could not demonstrate serum stimulation of thymidine incorporation at 7 h. Our errors were smaller when pre-incubation was used, and this 6 h period allowed dissection and tube preparation to be done consecutively.

Our conclusions with respect to drying and weighing were similar to those of Schimpff & Donnadieu (1973). In preliminary studies, calcula-
tion of incorporation as DPM/mg dry weight increased errors and lowered the slope, thus reducing precision. This could have been due, at least in part, to the increase in weight when rudiments were incubated with normal human serum, compared to incubation with buffer or HHS, where no increase could be demonstrated. The problem with weight change is best illustrated with a hypothetical example: a rudiment incubated in buffer alone incorporates 0.5 μg SO$_4$ during the 6 h labelling period. The initial weight was 0.5 mg and does not increase during the 30 h incubation. Thus the incorporation is 0.5 μg/rudiment or 1 μg/mg. If a rudiment with a similar initial weight is incubated with serum and incorporates 1 μg SO$_4$, but the weight increases to 1 mg then incorporation is 1 μg SO$_4$/rudiment or 1 μg/mg. Thus real differences are obscured. With visual selection there were only small variances (3.7–5.4%) in the initial dry weights and there were no significant ($P > 0.5$) differences from week to week. Elimination of prolonged soaking and washing did not appear to alter the results, and this reduced total assay time. The commercial solubilizer acted rapidly and completely without heating.

In previous studies some unheated human sera inhibited thymidine incorporation into chondrocytes isolated from chicken embryo pelvic rudiments; the slopes of those log dose-response curves were negative (Garland et al. 1972). That inhibitory activity was heat labile (56°C × 30 min). With the current method the incorporation using the lowest serum concentration (0.5%) was often less than that with buffer alone. Despite this, the slopes of the log dose-response curves with heated (this study) and unheated (unpublished) human sera were positive and linear between 0.5 and 5%. We do not have an explanation for the 'inhibition' sometimes seen with 0.5% serum.

Our results are similar to those of Van den Brande et al. (1971) using double labelled hyphysectomized rat costal cartilage and also similar to results alluded to by Van den Brande & Du Cajú (1973, 1974) with porcine costal cartilage. Our indices of precision were smaller than those in the former and were slightly greater than those in the latter study. Both activities were low in GH deficiency and rose following hGH administration. This suggests, but does not prove, that the same substance or group of substances enhanced both activities (possibly by coupling of the two processes).

Several chemically distinct substances which stimulate cartilage sulphation (and are presumably GH-dependent) have been isolated from serum (Van Wyk et al. 1974; Hall 1972; Zapf et al 1978). Some partially purified preparations appear to sti-

### Table 3.
Comparison of reported potency ratios of normal and GH deficient subjects for several different somatomedin bioassays.

<table>
<thead>
<tr>
<th>Cartilage</th>
<th>λ</th>
<th>Normals</th>
<th>GH deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± 1 SD</td>
<td>Range</td>
<td>Mean ± 1 SD</td>
</tr>
<tr>
<td>Hyphysectomized rat costal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daughaday et al. (1959)</td>
<td>~ 0.25</td>
<td>0.78 ± 0.49</td>
<td>~ 0.25–2.5</td>
</tr>
<tr>
<td>Daughaday &amp; Parker (1963)</td>
<td>ND</td>
<td>0.86 ± 0.36</td>
<td>0.3–2.5</td>
</tr>
<tr>
<td>Porcine costal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Van den Brande &amp;</td>
<td>0.15</td>
<td>1.00 ± 0.14</td>
<td>~ 0.75–1.35</td>
</tr>
<tr>
<td>Du Cajú (1973)</td>
<td>0.20</td>
<td>0.72 ± 0.13</td>
<td>0.54–0.90</td>
</tr>
<tr>
<td>Phillips et al. (1974)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chick embryo pelvic rudiment</td>
<td>0.20</td>
<td>0.94 ± 0.23</td>
<td>0.54–1.4</td>
</tr>
<tr>
<td>Hall (1972)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current paper</td>
<td>0.20</td>
<td>THY 0.90 ± 0.22</td>
<td>0.62–1.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SO$_4$ 0.94 ± 0.22</td>
<td>0.57–1.29</td>
</tr>
</tbody>
</table>
mulate thymidine incorporation into hypophysectomized rat cartilage (Van den Brande et al. 1971; Van Wyk et al. 1974). Some highly purified somatomedics activate DNA synthesis in certain types of fibroblasts (Zapf et al. 1978; Rechler et al. 1978), but these have not been shown to enhance thymidine incorporation into cartilage. With the data available to date, it is not possible to determine whether the net serum stimulation for either activity was due to coordinate stimulation by a single factor or to some distinct combination of these factors.

One of the most important characteristics of any somatomedin assay is the ability to predict GH dependent factor deficiency. Table 3 compares the mean, SD, and range of potency ratios of normal individuals to those of hypopituitary subjects for several of the reported bioassays. If 2 standard deviations below the normal mean is used as the lower limit of normal, then there are some GH deficient individuals who fall within normal limits in all of the assays except that of Van den Brande & Du Caju (1973, 1974). With our current method 4/16 (thymidine) and 3/16 (sulphate) GH deficient values were 'normal'. The double label procedure does reduce overlap. Both potency ratios were less than 0.7 in only one normal adult (0.65 and 0.61) and both were greater than 0.6 in only one GH deficient child (0.68 and 0.65). It should be noted that the normal and growth hormone deficient groups are heterogeneous and not precisely comparable from report to report. The mean index of precision for the different assays is also given in Table 3. With our current modifications the reliability for prediction of GH dependent factor deficiency and the precision were similar to other reported bioassays. Although the rise in bio-activity following a single dose of GH was not large, our data is consistent with that of others using a single dose of GH (Schimpff & Donnadieu 1973) and also with the rise in Sm A measured by a RRA (Hizuka et al. 1978).

Compared to previous somatomedin bioassays, this modification offers several advantages. The procedure is technically simple and requires less laboratory time and special equipment. Fertile eggs are readily available and may be held at room temperature for at least 1 week prior to incubation without loss of viability. Dissection of leaflets is somewhat easier than rat costal cartilage and the expense is less than that for hypophysectomized rats. The assay can be completed in 48 h. From a clinical standpoint a major advantage of this method is the limited amount (350 µl) of serum necessary for each patient assay. In many instances only a small quantity of serum is available and substantially greater amounts are necessary with other bioassays. In addition, some investigators have suggested that non-Sm perturbations are more likely to occur at high serum concentrations. The ability to monitor serum stimulation of both sulphate and thymidine incorporation is also advantageous. Although there was no evidence of dissociation of stimulation in the current studies, this dissociation is still theoretically possible. We have unpublished data which demonstrate a marked discrepancy in stimulation of incorporation of the two labels by rat serum. The ability to monitor both processes simultaneously should be an advantage in studies of the spectrum of biological activity of purified growth factors.

Radioligand assays have been developed for several of the somatomedins (Hizuka et al. 1978; Hall et al. 1979; D'Ercole et al. 1977; Furlanetto et al. 1977) and insulin-like growth factors (Zapf et al. 1978; Schalch et al. 1978). These require smaller serum specimens than most bioassays. In some instances these give a clearer separation of normal and GH deficient subjects. However, some of the assays may not be specific for a single purified substance (Rechler et al. 1978; Hall et al. 1979), and supplies of the highly purified substances necessary are very limited. Bioassays give less specific information, but they have the advantage of measuring the net serum growth factor activity. Most of the bioassays 'see' some GH dependent and GH independent stimulation and may be affected by circulating inhibitors.

One example of the different information obtained by the radioligand and the bioassays is seen in chronic renal failure. Receptor assayable Sm A is elevated while bioassayable Sm is low (Takano et al. 1979). The discrepancy has been explained by the presence of inhibitors of cartilage metabolism (Takano et al. 1979) and elevation of inorganic sulphate in uraemic serum (Phillips et al. 1978). Thus that RRA gives more precise quantitation of the amount of circulating 'Sm A' while the decreased activity in the bioassay reflects the in vivo growth retardation (possibly due to reduced Sm action) seen in children with chronic renal failure.

There is an unquestioned need for Sm bioassays to study the activity of purified factors and their mechanisms of action. There is also a continued
need for Sm bioassays (sensitive to growth stimulators and inhibitors) in the clinical realm—particularly where the mechanism of growth retardation is not yet well understood.

References


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