UNSPECIFICITY OF THE TIBIA-TEST FOR GROWTH HORMONE ASSAY
Methyl thiocyanate: an interfering substance

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1. Introduction

The growth stimulus of the tibial epiphysial cartilage in hypophysectomized rats caused by the injection of growth hormone has long been used as a sensitive assay for this substance [1, 2]. Later work [3] showed that other hormones like thyroxine, thyrotropin and corticotropin, influenced the results and more recently non-hormone products were added to the list: Zor et al. [4] pointed to various derivatives of aniline and Dikstein et al. [5] to *N*-acetyl-*p*-aminophenol.

In this paper we report the finding of another substance, methyl thiocyanate, present in cyanogen bromide-treated hormones, to which the tibia-test is extremely sensitive.

2. Materials and methods

Bovine growth hormone (BGH) was prepared according to [6]; specific activity: 1.6 USP units/mg. Ovine growth hormone (OGH) was prepared according to [7]; specific activity: 1.6 USP units/mg.

Growth hormone fragments were obtained by reaction of the proteins with cyanogen bromide as described previously [8]. Methyl thiocyanate was synthesized by a standard procedure [9]. *N*-Acetyl-*p*-aminophenol was obtained from Carlo Erba.

2.1. *Tibia-test*

Was performed according to Greenspan et al. [2] on female hypophysectomized rats of the Long-Evans strain.

2.2. *Body weight gain assay*

Was performed according to Kibrick et al. [10] on male hypophysectomized rats of the Long-Evans strain. The animals' weight at the beginning of the assay was 92.0 ± 1.5 g; they were fed on Purina Lab-chow and water ad-libitum and were injected daily during 10 days. The increase in body weight was recorded at the end of this period.

2.3. *Gas chromatography*

A Beckman GCM apparatus was used. The stainless steel column was packed with 15% SE-30 on Chromosorb W 42/60 mesh. Operating temperature: 95°; injector temperature: 145°. Flame ionization detector. Nitrogen gas was used as a carrier.

3. Results and discussion

BGH and OGH are closely related proteins with almost identical primary structure [11, 12]; their reaction with cyanogen bromide gives rise to four polypeptides of identical or very similar structures which can be obtained pure by chromatography on Sephadex [8, 13].

The biological activity in the tibia-test of fragments 1, 2 and 3 [8] is shown in table 1. The values recorded indicate a small, though significant, response of the cartilage to all of them. Nevertheless the fragments were found inactive in the body weight gain assay (table 2).

The rather uniform biological response in the tibia-test of every fragment was suspected as being due to

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Table 1
Tibia-test assay of growth hormone fragments obtained by reaction with cyanogen bromide and of methyl thiocyanate and *N*-acetyl-*p*-aminophenol.

Sample	No. of rats	Total dose nmoles (1)	Tibia width (μ) Average ± S.E. (2)
Solvent	5		109 ± 3
BGH	5	1.5	141 ± 2
		3.0	158 ± 2
		6.0	186 ± 2
OGH	5	1.0	128 ± 2
		2.0	146 ± 2
		4.0	173 ± 2
BGH fragments:			
1	5	15 (0.4)	122 ± 2
2		65 (1.6)	149 ± 2
3		30 (0.8)	160 ± 2
OGH fragments:			
2	5	40 (1.0)	139 ± 2
3		15 (0.4)	136 ± 2
Methyl thiocyanate	5	0.3	122 ± 2
		1.4	144 ± 2
		0.7	136 ± 2
		70.0	131 ± 3
N-acetyl-*p*-aminophenol	5	3,300	162 ± 2
		9,900	160 ± 3

(1) Numbers in parentheses indicate the amount of methyl thiocyanate in the sample.
(2) The significance of the response was measured by the Sukhatme-D statistic [14]. It was, in all cases, $P < 0.001$.

Table 2
Body weight gain assay of growth hormone fragments obtained by reaction with cyanogen bromide and of methyl thiocyanate.

Sample	No. of rats	Daily dose nmoles[1]	Weight increase Average ± S.E.[2]	P[3]
Solvent	8		0.25 ± 2.29	
BGH	8	1	8.98 ± 2.66	<0.05
		2	15.21 ± 2.53	<0.01
BGH fragments:				
1	8	4 (0.1)	0.27 ± 0.92	N.S.
2		20 (0.5)	−0.53 ± 3.91	N.S.
3		55 (1.2)	0.58 ± 4.38	N.S.
Methyl thiocyanate	8	350	1.61 ± 6.22	N.S.

[1] Numbers in parentheses indicate the amount of methyl thiocyanate in the sample.
[2] Measured in grams over 10 days (see Methods).
[3] The significance of the response was measured by the Sukhatme-D statistic [14]. N.S.: non significant.

a contaminant substance common to all samples. The most probable candidate was methyl thiocyanate, a by-product in the reaction of cyanogen bromide with the protein, which may not be separated from the peptides during the chromatography on Sephadex or in the previous lyophilization. This hypothesis was proven to be true, in terms of the presence of a common contaminant in the samples, by submitting them to gas chromatography as described in Methods. The chromatogram from each peptide showed a peak located in the position at which emerged an authentic sample of methyl thiocyanate. Minimum estimates of the contamination could be made by this procedure and the values are shown in table 1.

The response of the rat cartilage to a range of doses of methyl thiocyanate (table 1) indicates that this substance stimulates its proliferation even at very low concentrations. The correlation dose-response is quite different though, to that observed with the growth hormones, but the amounts of methyl thiocyanate present in the fragments may explain the levels of biological activity found. Furthermore the lack of response of the body weight gain assay to methyl thiocyanate (table 2) fits in with the inactivity shown in this test by the polypeptide fragments.

The tibia-test has been reported to be affected by drugs such as analgesics derived from aniline [4] and by *N*-acetyl-*p*-aminophenol [5]. In the latter case the authors indicate the possibility that the drug may act by stimulating an increased production of growth

hormone. That this is not so is shown by the values recorded in table 1 where *N*-acetyl-*p*-aminophenol has a significant effect on the tibia-test carried out with hypophysectomized rats. The peculiar correlation dose-response found for these drugs, as well as for methyl thiocyanate, which shows inhibition at the higher levels, suggests that the underlying mechanisms of action must be very different from those of the somatotrophic hormones. The analgesics derived from aniline, tested by Zor et al. [4], and *N*-acetyl-*p*-aminophenol [5], were active at a total dose of 200 nmole per g of rat. In the present experiments (table 1) *N*-acetyl-*p*-aminophenol was effective at 37 nmole per gram. Surprisingly, the sensitivity of the tibia-test for methyl thiocyanate was several orders of magnitude higher; a clear response started at 0.01 nmole per g of rat. This circumstance makes it necessary critically to evaluate all positive results obtained in the tibia-test, especially when assaying polypeptides prepared by synthetic procedures, or by chemical cleavage of proteins, or of fragments derived from them.

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