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Human Pituitary Growth Hormone*

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The early studies of CROWE[1], EVANS and LONG[2], and SMITH[3] showed that the growth of an animal is influenced by the anterior lobe of its pituitary gland. The existence of a hormone responsible for this activity was finally proved by LI et al.[4,5] in 1944 by the isolation of the growth hormone (GH, somatotropin) from bovine pituitaries in a highly pure form. During the past twenty years, the pituitary growth hormone has been extensively studied. In the last five years, human pituitary growth hormone (HGH) in particular has been the subject of much interest, since its clinical effectiveness in man has been so amply demonstrated. In this article, we will focus our attention on the chemistry and experimental biology of GH, with special reference to HGH, but our intent does not cover the clinical aspects of the human hormone.

Bioassay. The two methods that are used the most frequently for the bioassay of growth hormone are the tibia test, and the assay based on the increment of body weight[6]. The latter method measures the increase in body weight in hypophysectomized rats following the administration of GH. The tibia test is based on the increase in the width of the proximal epiphyseal cartilage of the tibia of hypophysectomized rats and is the most sensitive biological test available at present.

As performed in our laboratory, female Long-Evans rats 26–28 days of age are hypophysectomized and are used for the bioassay 12–14 days after the operation. The GH solution (in water) is administered intraperitoneally (usually in the amount of 0.5 ml) once daily for 4 days. On the fifth day, which is 24 h after the final injection, the animals are sacrificed, a tibia is dissected free of tissue, and the bone is split at the proximal end in the mid-saggital plane. The bone halves can then be stained with 2% silver nitrate. The uncalcified epiphyseal cartilage plane, which does not stain and thus remains white, is measured under low-power microscope with a calibrated micrometer eyepiece. Ten individual readings are made across the epiphysis. The response is shown to be in a straight line relationship with the logarithm of the dose. It is of interest that the slopes obtained with GH from different species are relatively constant (Table I). This suggests the usefulness of the tibia test for comparison of the growth-promoting potency of various GH preparations.

Methods of Isolation. The first homogeneous growth hormone was isolated from ox pituitaries by Li et al.[4,5] in 1944. Their method of isolation depended essentially upon variations in pH and salt concentration. A few years later, WILHELMI et al. isolated the same hormone from ox pituitaries by a serial fractionation with ethanol[7], the results of which confirmed the earlier studies.

Failures in treating human dwarfism[8] with purified bovine growth hormone (BGH) stimulated the interest of many investigators in the field of hormone study to search for the growth-promoting material of pituitary

Table I. Assay of various growth hormones by the tibia test

Growth hormone	Response[a]			Slope	Index of precision
	20 μg	60 μg	120 μg		(λ)
Bovine	232 ± 6(4)	250 ± 2(4)	288 ± 6(4)	69.6	0.152
Cetacean[b]	220 ± 4(5)	250 ± 2(5)	268 ± 4(5)	62.6	0.130
Simian	210 ± 4(4)	242 ± 5(6)	261 ± 3(5)	65.7	0.147
Human	206 ± 7(5)	237 ± 6(4)	254 ± 7(5)	61.9	0.240

[a] Mean tibia width (micra) ± standard error. Number of rats in parentheses. [b] Humpback whale.

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[1] S. J. CROWE and J. HOMANS, Bull. Johns Hopkins Hosp. *21*, 127 (1910).
[2] H. M. EVANS and J. A. LONG, Anat. Record. *21*, 61 (1921).
[3] P. E. SMITH, Amer. J. Anat. *45*, 205 (1930).
[4] C. H. LI and H. M. EVANS, Science *99*, 183 (1944).
[5] C. H. LI, H. M. EVANS, and M. E. SIMPSON, J. biol. Chem. *159*, 353 (1945).
[6] H. PAPKOFF and C. H. LI, in *Methods in Hormone Research*, vol. II (Ed. R. I. DORFMAN, Academic Press, New York 1962), p. 671.
[7] A. E. WILHELMI, J. B. FISHMAN, and J. A. RUSSEL, J. biol. Chem. *176*, 737 (1948).
[8] L. L. BENNETT, H. WEINBERGER, R. ESCAMILLA, S. MARGEN, C. H. LI, and H. M. EVANS, J. clin. Endocrin. Metabol. *10*, 492 (1950).

origin that would be biologically active in human subjects. In 1956, LI and PAPKOFF[9,10] succeeded in isolating this hormone from human pituitaries. The method they used involved calcium oxide extraction, ammonium sulfate fractionation, chromatography on a cation exchange resin column with Amberlite IRC-50 (XE-97), and isoelectric precipitation with ethanol. The hormone prepared by this procedure was shown to be homogeneous by the criteria of electrophoresis, ultracentrifugation, and NH_2-terminal group analysis. Owing to difficulties in obtaining a uniform source for human pituitaries, the material prepared by the above method could not be secured with enough consistency for structural investigations. To meet this requirement, a modified method was recently developed[11]; the products prepared by this modified procedure, although they were from different batches of glands, were proven to be homogeneous and consistently reproducible in their chemical compositions, by the criteria of countercurrent distribution, amino acid analysis, and the peptide patterns of their enzymatic digests. Table II presents the protocol of the modified procedure, which involves eight steps and gives an average yield of 70 mg of HGH from 50 fresh pituitaries. It should be emphasized that the product is particularly suitable for chemical studies.

Preparations of human growth hormone have also been obtained by other methods[12–15] involving chromatography on DEAE-cellulose and gel filtration on Sephadex. A quite different method was employed by RABEN in the preparation of growth hormone from pig[16] and human[17] pituitaries; this method depended upon extraction of glands with glacial acetic acid at 70°C, removal of adrenocorticotropin by oxycellulose absorption, and precipitation of growth hormone by ethanol. Since bovine growth hormone is known to be unstable in acidic media[18], materials prepared by the glacial acetic acid procedure are likely to be denatured.

Growth hormone concentrates from horse, sheep, and fish pituitaries have been prepared by WILHELMI[19] by a procedure similar to that employed for the isolation of bovine growth hormone[7]. He also attempted to separate all the hormones from a single crude extract of human pituitaries by fractional extraction. This procedure, which was worked out by ELLIS[20] for bovine, ovine, and porcine pituitaries, failed when applied to human pituitaries[21].

Besides bovine[4,5,22] and human[9–11] growth hormones, sheep[23], whale[24], monkey[9,10], and pig[25] growth hormones have been isolated in a homogeneous form in this laboratory. The method used for the isolation of the hormones from pituitaries of these species was essentially the same as the original method employed for the isolation of human growth hormone[9,10]. For the isolation of pig growth hormone two additional steps were introduced: countercurrent distribution in a sec-BuOH/0.4% dichloroacetic acid solvent system and gel filtration on a Sephadex G-50 column[25]. For a number of years, attempts made by various workers to prepare pig growth hormone failed to yield a homogeneous preparation[16,19,26]. The material prepared by us[25], however, was shown to be homogeneous by the criteria mentioned above.

Table II. Protocol for the isolation of HGH by the modified procedure[a]

Fraction	Procedure	Weight g
	Fresh glands (50 pituitaries)	25
	Extracted with pH 7 saline solution; fractionated with $(NH_4)_2SO_4$	
	↓	
B	1.9 *M* $(NH_4)_2SO_4$ precipitate, dialyzed and lyophilized	1.5
	↓	
	Extracted with 0.45 *M* $(NH_4)_2SO_4$ in pH 5.1 phosphate buffer	
	↓	
C	Soluble fraction submitted to chromatography on IRC-50 resin column	0.5
	↓	
D	Water eluate (active peak), dialyzed and lyophilized	0.15
	↓	
	pH and ethanol fractionation	
	↓	
E	25% (v/v) ethanol precipitate	0.09
	↓	
S	Exclusion chromatography on Sephadex G-50 using 0.1 *M* acetic acid on eluent	0.07

[a] See reference [11].

[9] C. H. LI and H. PAPKOFF, Science *124*, 1293 (1956).
[10] C. H. LI, Fed. Proc. *16*, 775 (1957).
[11] C. H. LI, W. K. LIU, and J. S. DIXON, Arch. Biochem. Biophys. Suppl. *1*, 327 (1962).
[12] U. J. LEWIS and N. C. BRINK, J. Amer. chem. Soc. *83*, 4429 (1958).
[13] A. L. C. WALLACE and K. A. FERGUSON, J. Endocrinol. *23*, 285 (1961).
[14] R. A. REISFELD, B. G. HALLOWS, D. E. WILLIAMS, N. G. BRINK, and S. L. STEELMAN, Nature *197*, 1206 (1963).
[15] P. ROOS, H. R. FEVOLD, and C. A. GEMZELL, Biochim. biophys. Acta *74*, 525 (1963).
[16] M. S. RABEN, Proc. Soc. exp. Biol. Med. *93*, 339 (1956).
[17] M. S. RABEN, Science *125*, 883 (1957).
[18] C. H. LI and H. PAPKOFF, J. biol. Chem. *204*, 391 (1953).
[19] A. E. WILHELMI, in *The Hypophyseal Growth Hormone, Nature and Action* (Eds. R. W. SMITH, O. H. GAEBLER, and C. N. H. LONG, Blakiston Division, McGraw-Hill Inc., New York 1955), p. 59.
[20] S. ELLIS, Endocrinol. *69*, 554 (1961).
[21] A. E. WILHELMI, Ciba Foundation Colloquium on Endocrinology *13*, 25 (1960).
[22] C. H. LI, J. biol. Chem. *211*, 555 (1954).
[23] H. PAPKOFF and C. H. LI, Biochim. biophys. Acta *29*, 145 (1958).
[24] H. PAPKOFF and C. H. LI, J. biol. Chem. *231*, 367 (1958).
[25] H. PAPKOFF, C. H. LI, and W. K. LIU, Arch. Biochem. Biophys. *96*, 216 (1962).
[26] J. H. OTTAWAY, Biochem. J. *72*, 22P (1959).

Physicochemical Properties. It has been known for a long time that preparations of the same protein hormone isolated from different species may differ one from another. Some physicochemical data on the six growth hormones isolated are summarized[27,28] in Table III. This Table discloses some special points of interest. First, the molecular weights of the various growth hormones vary from 25,000 to almost double that value, 48,000, with that of the primate hormone being the lowest, and that of the bovine and ovine hormones being the highest. The isoelectric points follow the same order as their molecular weights. Generally, it can be said that the growth hormones with the lower molecular weights are more acidic than those with the higher molecular weights. If the specific optical rotation is taken as an index of the extent of helical structure within the protein molecule, human and bovine growth hormones are the highest in this respect, with pig, sheep, whale, and monkey growth hormones in decreasing order. From the end-group data, it can be seen that pig, whale, monkey, and human growth hormones, each having only one COOH- and NH_2-terminal residue, consist of a single peptide chain with phenylalanine at both termini. On the other hand, bovine and ovine growth hormones have two NH_2-terminal residues and one COOH-terminal residue, suggesting a branched structure for them[29]. The difference in the number of –S–S– linkages may indicate that the tertiary structures of various growth hormones differ.

Biological Behavior. Studies on the biological responses[6] of various experimental animals to growth hormones of different species are listed in Table IV. It is of interest to note that the rat can respond to various mammalian growth hormones, whereas the guinea-pig is unresponsive to them[30]. Fish respond to bovine growth hormone[31], but its own hormone is not active

[27] C. H. Li, First Int. Congr. Endocrin. Copenhagen (1960), p. 75.
[28] C. H. Li, in *Survey of Biological Progress* (Academic Press Inc., New York 1962), vol. 4, p. 93.
[29] C. H. Li, in *Advances in Protein Chemistry* (Academic Press, New York 1956), vol. 11, p. 101.
[30] M. L. Mitchell, R. Guillemin, and H. Selye, Endocrin. *54*, 111 (1954).
[31] G. E. Pickford, Endocrinology *55*, 589 (1954).

Table III. Some physiochemical characteristics of pituitary growth hormone from various species

Properties[a]	Beef	Sheep	Pig	Whale (humpback)	Monkey (Macacus)	Human
Molecular weight	45,000	48,000	41,000	40,000	25,000	21,500
Isoelectric point, pH	6.8	6.8	6.3	6.2	5.5	4.9
$[\alpha]^{D}_{25°}$	−35.6°	−49.4°	−47.4°	−52.1°	−55.0°	−38.7°
Number of -S-S-bridges	4	5	3	3	4	2
N-terminal sequence	Phe-Thr-Ala... Ala. Phe. Ala...	Phe... Ala...	Phe...	Phe...	Phe...	Phe. Pro.Thr...
C-terminal sequence	...Ala. Phe. Phe	...Ala. Leu. Phe	...Ala. Phe. Phe	...Leu. Ala. Phe	...Ala. Gly. Phe	...Gly. Leu. Phe

[a] $[\alpha]^{D}_{25°}$ determined in 0.1 *M* acetic acid.

Table IV. Body-growth responses[a] of different animals to growth hormones from various species

Experimental animal	Pituitary growth hormone							
	Ox	Sheep	Human	Monkey	Pig	Whale	Horse	Fish
Human	–	–	+	+	?	?		
Monkey	–		+	+	–			
Sheep	+							
Goat	+							
Ox	+							
Rat	+	+	+	+	+	+	+	–
Mouse	+		+	+				
Guinea-pig	–		–	–	–			
Dog	+		+	+	+			
Cat	+							
Tadpole	+							
Fish	+							+

[a] – represents no response; + a definitive response; ? response doubtful or not yet established.

in mammals[19]. Furthermore, the primates can respond only to primate growth hormones, but not to any of the other animal growth hormones[32,33].

Although all mammalian growth hormones are active in the rat, their capacities for eliciting body-weight gain in hypophysectomized rats vary. It was found that the non-primate growth hormones (bovine, ovine, porcine, and whale) elicited a continuous increase of body-weight in the rat, apparently for an unlimited period of time, whereas primate growth hormones exerted this effect for only 10 days. After 10 days, those animals that received primate growth hormone become resistant to the hormone[34], although they could still respond to the other non-primate growth hormones injected into them later (see Figure 1).

The *in vitro* growth-stimulating effect of HGH on human cells was first reported by MOON et al.[35]. It was noted that HGH caused an increase in the nuclear multiplication of human liver cells in tissue culture and that the increase was proportional to the hormonal concentration and length of incubation time (see Figure 2). The relative specificity of the response of the cells to HGH was indicated by the failure of other proteins, including bovine somatotropin, to produce comparable changes in the rate of growth. Moreover, the effect of HGH on nuclear multiplication was abolished by the antibody to the hormone. In this connection, it should be noted[36] that BGH has been shown to regulate the rate of protein biosynthesis in the rat liver *in vivo*.

In addition to the marked influences of GH on protein metabolism, it is known that the hormone plays an important role in both fat and carbohydrate metabolic processes. One of us[37] in 1956 proposed that pituitary growth hormone is a metabolic hormone as well as a biological synergist. For example, BGH enhances the effect of interstitial cell-stimulating hormone (ICSH) on the weights of the ventral prostates[38] in hypophysectomized rats. There have been some indications[39] that the administration of BGH results in an increase in the antibody level of adult rats that have been immunized with *P. pestis*. Recent studies[40,41] with HGH have clearly demonstrated that the hormone possesses intrinsic prolactin-like activities. HGH promotes pigeon crop-sac growth when administered by either local or systemic procedures, and induces localized milk secretion when injected with prednisolone into hypophysectomized-ovariectomized-adrenalectomized rats. Other investigators[42,43] have also reported that both lactogenic and growth-promoting activities are found in preparations of HGH. It is generally agreed that HGH does not possess prolactin-like activities to the same degree as the ovine lactogenic hormone, but only to the extent of 20% or less of the latter.

Immunochemistry. A comparative immunological study of growth hormones from various species by means of several different procedures, including precipitin ring and agar gel diffusion tests with rabbit antiserum, anaphylatic shock experiments in guinea-pigs, and antihormone tests in hypophysectomized rats, was first reported by HAYASHIDA and LI[44]. Their results showed that growth hormones form species-specific

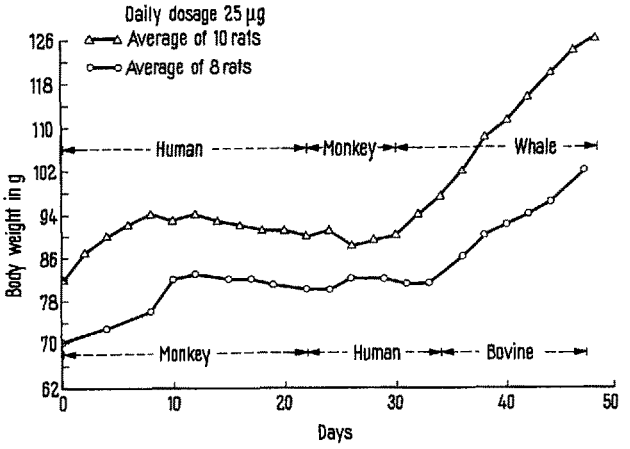

Fig. 1. Curves representing average body-weight gain in hypophysectomized female rats for 47-48 days. Animals were hypophysectomized at 28 days of age and injections begun 14 days later with human, monkey, bovine, or whale growth hormone. Taken from ref. [34].

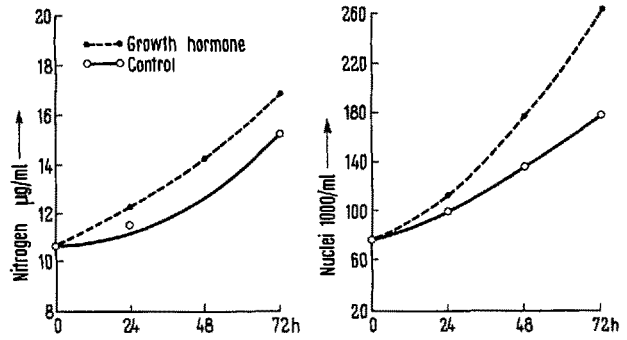

Fig. 2. Effect of HGH on rate of protein synthesis and nuclear multiplication at varying periods of time. Taken from ref. [35].

[32] E. KNOBIL and R. O. GREEP, in *Recent Progress in Hormone Research* (Ed. G. PINCUS, Academic Press, New York 1959), vol. 15, p. 1.
[33] C. H. LI, Laboratory Investigations *8*, 574 (1959).
[34] C. H. LI, H. PAPKOFF, and C. W. JORDAN, Proc. exp. Biol. Med. Med. *100*, 44 (1959).
[35] H. D. MOON, V. L. JENTOFT, and C. H. LI, Endocrinol. *70*, 31 (1962).
[36] A. KORNER, Biochem. J. *81*, 292 (1961).
[37] C. H. LI, Science *123*, 617 (1956).
[38] A. J. LOSTROH, P. G. SQUIRE, and C. H. LI, Endocrinol. *62*, 833 (1958).
[39] T. HAYASHIDA and C. H. LI, J. exp. Med. *105*, 93 (1957).
[40] W. R. LYONS, C. H. LI, and R. E. JOHNSON, Proc. End. Soc. Meeting, New York (June 1961), p. 4.
[41] C. H. LI, J. gen. Physiol. *45*, 169 (1962).
[42] A. CHADWICK, S. J. FOLLEY, and C. A. GEMZELL, Lancet *1961*, 241.
[43] A. E. WILHELMI, Canadian J. Biochem. Physiol. *39*, 1659 (1961).
[44] T. HAYASHIDA and C. H. LI, Science *128*, 1276 (1958); Endocrinol. *65*, 944 (1959).

antibodies. This phenomenon was also observed by other workers[45–47]. The antiserum to BGH reacted only with ovine growth hormone, but not with growth hormones from whale, pig, human, and monkey glands (Figure 3). On the other hand, the antiserum to HGH reacted only with monkey growth hormone, but not with growth hormones from other species (Figure 4). Moreover, guinea-pig antiserum to porcine growth hormone has been shown[48] to cross-react with the whale hormone by quantitative precipitin tests (Figure 5) and immunoelectrophoretic procedures. From these results, it may be inferred that the antigenic structures of human and monkey growth hormone, bovine and ovine growth hormone, pig and whale growth hormone, might be quite similar. Even though the precipitin curves of human and monkey growth hormones, as well as those of bovine and ovine growth hormones, were about the same, their equivalence points and their ratios of antibody to antigen differed[49,50]. This suggested that there are subtle differences in the structures of these molecules. Furthermore, it was found that HGH induced antibody formation in the rat[51], which might account for the fact that the effect of HGH on the increment of body weight in hypophysectomized rats ceases after 10–12 days[34].

For the past few years, a great deal of effort has been centered on the assay of HGH by immunological means[45,52]. The usefulness of any immuno-assay method depends upon the basic premise that the immunologically active centers and the biologically active sites in the molecule are identical. From our limited experiences, we have come to conclude that they may not be identical. It has been shown[53], for example, that HGH whose growth-promoting activity has been abolished by performic-acid oxidation can still react immunologically with rabbit antisera to the native hormone.

Limited Digestion with Proteolytic Enzymes. Whether or not the integrity of the entire protein molecule is essential for its biological activity is a problem that has interested many workers. Growth hormone from various species has been subjected to limited digestion with various proteolytic enzymes. The results suggest that the whole molecule is not essential for the biological activity[54–58].

It has been reported that various growth hormones could be partially digested with chymotrypsin without loss of activity. The extent to which digestion could be

[45] C. H. Read and G. T. Bryan, Ciba Foundation Colloq. on Endocrinol. *13*, 68 (1960).
[46] M. M. Grumbach, S. L. Kaplan, and S. Solomon, Nature *185*, 170 (1960).
[47] J. Fishman, E. E. McCarry, and J. C. Beck, Proc. Soc. exp. Biol. Med. *102*, 446 (1959).
[48] A. Trenkle and C. H. Li, Gen. comp. Endocrinol., in press.
[49] C. H. Li, N. R. Moudgal, and H. Papkoff, J. biol. Chem. *235*, 1038 (1960).
[50] N. R. Moudgal and C. H. Li, Arch. Biochem. and Biophys. *93*, 122 (1961).
[51] N. R. Moudgal and C. H. Li, Endocrinology *68*, 704 (1961).
[52] F. S. Greenspan, J. A. Cofell, W. Lew, and C. T. Peng, J. lab. clin. Med. *59*, 520 (1962). – R. D. Utiger, M. L. Parker, and W. H. Daughady, J. clin. Invest. *41*, 254 (1962).
[53] A. Trenkle, C. H. Li, K. Sadri, and H. Robertson, Arch. Biochem. Biophys. *99*, 288 (1962).
[54] C. H. Li, H. Papkoff, P. Fönss-Bech, and P. G. Condliffe, J. biol. Chem. *218*, 41 (1956).
[55] C. H. Li, in *Perspectives in Biology* (Eds. C. F. Cori, V. G. Foglia, L. F. Leloir, and S. Ochoa, Elsevier Amsterdam 1963), p. 24.
[56] C. H. Li, in *Symposium on Protein Structure* (Ed. A. Neuberger, Methuen, London 1959), p. 302.
[57] C. H. Li, H. Papkoff, and T. Hayashida, Arch. Biochem. Biophys. *85*, 97 (1959).
[58] C. H. Li, J. gen. Physiol. *45*, Suppl. 1, 169 (1962).

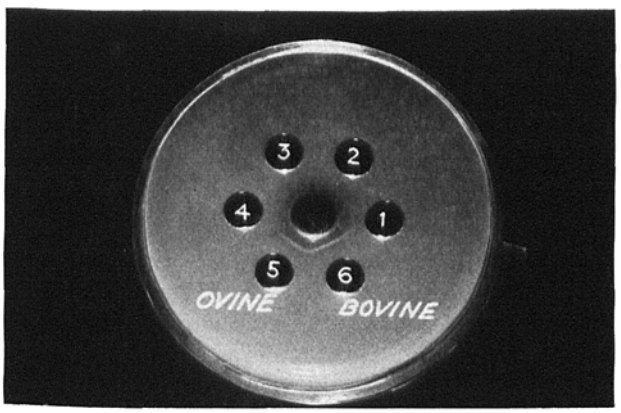

Fig. 3. The interaction of antiserum to bovine somatotropins (BGH) with growth hormone from various species, as determined by the Ouchterlony test. Central well contains antiserum to BGH; wells 1–6 contain 10 μg each of human, monkey, porcine, whale, ovine and bovine somatotropins, respectively. Taken from ref. [44].

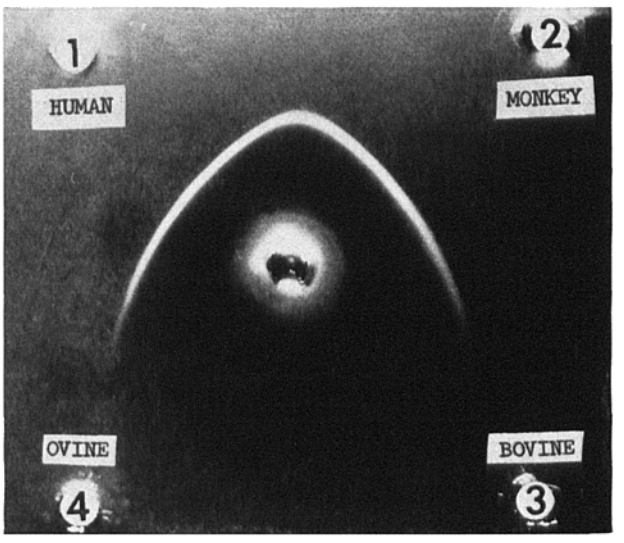

Fig. 4. The interaction of antiserum to HGH with growth hormone from various species, as determined by the Ouchterlony test. Central well contains antiserum to HGH; wells 1–4 contain 2 μg HGH, 2 μg monkey growth hormone, 5 μg BGH and 5 μg ovine growth hormone, respectively. Taken from ref. [49].

carried out while full activity was still retained differed with the different species: 25% for bovine, ovine, whale, and pig; 20% for monkey; and 10% for human growth hormone. The rates of the digestion also differed. Human growth hormone appears to be the most resistant to the action of the enzyme, whereas bovine growth hormone is the most susceptible[10, 54, 56]. Further studies on the 'core' isolated from a chymotryptic digest of bovine growth hormone showed that it differs from the native hormone in electrophoretic and immunological characteristics, as well as in NH_2-terminal residues[57].

Bovine growth hormone could be digested by trypsin to an extent of 30% without loss of activity[29], whereas pig growth hormone lost 75% of its activity when it was digested up to a similar extent[25].

Pepsin was also used in these studies, and it is surprising to note[58] that HGH retained its activity up to 40% digestion with this enzyme (Table V). On the other hand, pig growth hormone completely lost its activity after digestion up to 22% and bovine growth hormone also is rapidly inactivated by this enzyme[25, 29].

A preliminary study by DIXON and LI[59] showed that when HGH was treated with leucineaminopeptidase, about 50 amino acid residues were removed from the NH_2-terminus. Under these conditions, the hormone still retained its activity. Studies on the COOH-terminus of growth hormones from all six species showed that they can be digested by carboxypeptidase to release the terminal phenylalanine residue without affecting their biological activity[25, 60, 61].

Some Data on the Primary Structure of HGH[62]. In spite of the numerous studies that have been made on the biological actions of growth hormones, little has been done with respect to elucidation of their primary structures. Because of the importance of HGH for clinical and experimental studies, and because it has been firmly established that the HGH molecule plays a dual biological role – i.e. growth promotion and lactogenic activity, two functions that are separate in pituitaries of non-primate origin – we felt that it would be of particular value to gain an understanding of its chemical structure. Furthermore, this molecule seemed a good subject for structural studies because it has a lower molecular weight than either the bovine or the

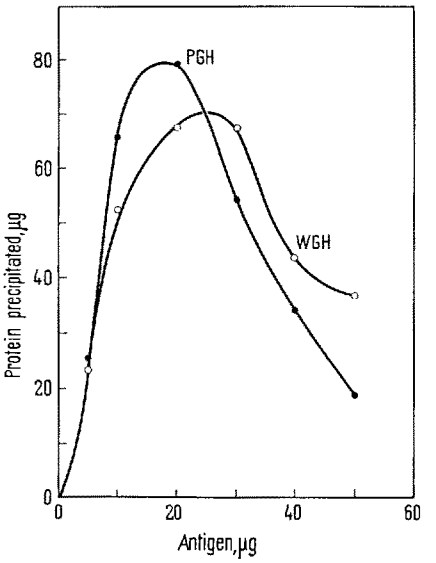

Fig. 5. Precipitin curves showing reaction between porcine growth hormone and its homologous guinea-pig antibody, and cross reaction between whale growth hormone and guinea-pig antibody to porcine growth hormone; 0.1 ml of guinea-pig antiserum to porcine growth hormone per tube. Taken from ref. [48].

[59] J. S. DIXON and C. H. LI, Fed. Proc. *21*, 199 (1962).
[60] J. I. HARRIS, C. H. LI, P. G. CONDLIFFE, and N. G. PON, J. biol. Chem. *209*, 133 (1954).
[61] C. H. LI, A. J. PARCELLS, and H. PAPKOFF, J. biol. Chem. *233*, 1143 (1958).
[62] The authors wish to thank Dr. J. S. DIXON for helpful suggestions during the course of these studies.

Table V. Action of pepsin on HGH[a]

Time of digestion[b]	Non-protein nitrogen	Bioassay				
		Tibia test		Crop-sac stimulation[c]		
		No. of rats	Tibia width[d]	Dose	No. of pigeons	Response
min	%		µ	µg		
0	0	6	251 ± 2	4	7	1+ (4), 2+ (3)
30	15.5	5	258 ± 3			
60	38.9	5	238 ± 4	4	5	1+ (2), 2+ (3)
120	64.0	5	166 ± 3	10	3	all negative

[a] Taken from reference [58]. [b] Enzyme/hormone (w/w) = 1/150, 0.01 *M* HCl at 25°C. [c] – No stimulation; 1+ moderate stimulation; 2+ good stimulation; 3+ marked stimulation. [d] A total dose of 40 µg in 4 days; mean ± standard error.

Table VI. The amino acid composition of human growth hormone[a]

Amino acid	Grams of amino acid residue per 100 g of protein			Average	N as % of total N	Calculated number of residues for molecular weight of 29,000	Number of residues to nearest integer
	Time of hydrolysis (h)						
	22 h	22 h	72 h				
Lysine	4.77	5.29	4.84	4.97	6.76	12.3	12
Histidine	1.59	1.81	1.62	1.67	3.30	3.6	4
Amide-NH_3				1.57[b]	8.00		27[g]
Arginine	6.90	7.40	7.07	7.12	15.55	13.6	14
Aspartic acid	11.41	10.73	10.51	10.88	8.21	27.4	27
Threonine	4.77	4.53	4.24	4.84[c]	4.09	13.9	14
Serine	6.63	6.65	5.46	7.23[c]	7.07	24.0	24
Glutamic acid	16.71	16.16	15.76	16.21	10.81	36.3	36
Proline	3.98	3.78	4.04	3.93	3.83	11.8	12
Glycine	2.55	2.42	2.42	2.46	3.85	12.5	13
Alanine	2.65	2.42	2.42	2.50	3.11	10.2	10
Half-cystein[d]	1.86	1.91	1.62	1.80	1.62	5.6	6
Valine	3.18	2.87	3.09	3.09[e]	2.66	9.0	9
Methionine	1.86	1.81	1.81	1.83	1.12	4.0	4
Isoleucine	3.18	3.02	3.34	3.34[e]	2.53	8.7	9
Leucine	11.94	11.79	12.15	12.15[e]	9.24	31.1	31
Tyrosine	5.57	5.59	5.25	5.47	2.87	9.7	10
Phenylalanine	8.22	8.16	8.08	8.15	4.80	16.0	16
Tryptophan				0.64[f]	0.53		1
Total				99.85	99.95		252

[a] Values reported are for the anhydrous, ash-free protein. The analysis was carried out in the Beckman Amino Acid Analyzer Model 120 according to the procedure of SPACKMAN et al.[63]. [b] Calculated on the basis of 27 moles per 29,000 g of protein from the independent amide determination. [c] Values for threonine and serine, which decompose during hydrolysis are extrapolated by the method of HIRS et al.[64]. [d] Also determined as cysteic acid with performic acid oxidized HGH. [e] Values after 72 h of hydrolysis are taken as the true values for valine isoleucine, and leucine. [f] Calculated on the basis of 1 mole per 29,000 g of protein as determined by the spectrophotometric method[65]. [g] Not included in total.

ovine hormone, and consists of a single polypeptide chain[10, 56].

On the basis of a molecular weight[66] of 29,000, the empirical formula (252 amino acid residues) is as follows (see Table VI): Lys_{12} His_4 Arg_{14} Asp_{27} Thr_{14} Ser_{24} Glu_{36} Pro_{12} Gly_{13} Ala_{10} CyS_6 Val_9 Met_4 $Ileu_9$ Leu_{31} Tyr_{10} Phe_{16} Try_1. The nitrogen content of HGH as obtained in lyophilized form[11] was determined by the micro-Dumas method in the Coleman Nitrogen Analyzer, to give a value of $14.23 \pm 0.2\%$. Its amide content was also determined by a colorimetric method[67]; an average value of 27.2 ± 1.1 moles of ammonia per mole of HGH was obtained.

In order to acquire some information about the chemical structure of the HGH molecule, the technique of paper chromatography and electrophoresis, to give a peptide pattern in two dimensions, was employed to separate the peptide mixtures resulting from proteolytic digests of the hormone. When peptide patterns obtained from digestion of HGH by pepsin, chymotrypsin and trypsin are compared, it is evident that the tryptic digest gives the simplest pattern and contains comparably fewer ninhydrin-positive spots (see Figure 6). Moreover, trypsin has a greater specificity than the other two proteolytic enzymes and gives rise to peptides with amino acid residues at their COOH-termini; hence, the tryptic digest was employed for the first exploration of the primary structure of HGH.

For a typical run, 100 mg of HGH were dissolved in 20 ml of 0.2*N* ammonium acetate buffer of pH 8.5; 0.5 mg of trypsin was added to this solution and the digestion was carried out at 37°C for 24 h. At the end of the digestion, the solution was lyophilized several times until most of the ammonium acetate was removed. The dried material was then dissolved in 0.1*N* acetic acid (ca. 5 to 6 ml). The insoluble material, which represents about 15% of the total digest, was removed by centrifugation. The peptide pattern of this insoluble fraction indicated that it corresponds to the peptide spots OA, OB, and OC (see Figure 6c). Amino acid analysis showed that this fraction contains all the amino acid residues but that they are present in a different ratio than in the HGH molecule. This

[63] D. H. SPACKMAN, W. H. STEIN, and S. MOORE, Anal. Chem. *30*, 1190 (1958).
[64] C. H. W. HIRS, W. H. STEIN, S. MOORE, J. biol. Chem. *211*, 941 (1954).
[65] G. H. BEAVEN and E. R. HOLIDAY, in *Advances in Protein Chemistry* (Academic Press, New York 1952), vol. 7, p. 319.
[66] P. G. SQUIRE and K. O. PEDERSEN, J. Amer. chem. Soc. *83*, 476 (1961).
[67] W. E. STONE, Proc. Soc. exp. Biol. Med. *93* 589 (1956).

suggests that the insoluble fraction does not represent undigested growth hormone, but rather some of the large fragments from the tryptic digest. At the present time, our attention will be concentrated only on the soluble fraction. The sum of the amino acid residues from both the insoluble and soluble fractions of the tryptic digest is equivalent to the composition of human growth hormone.

For convenience, the peptides from the tryptic digest of HGH are designated according to their positions in the pattern as shown in Figure 6c. Besides the ninhydrin color test for the peptides, specific color tests were also used to locate the tryptophan-containing, histidine-containing, tyrosine-containing, and arginine-containing peptides. A summary of these results was given in Table VII. It is of interest to note that, besides a spot (OC) derived from the insoluble material, there is only one spot (4C') which gave a positive reaction to the Ehrlich reagent. The HGH molecule contains 14 residues of arginine and, as shown in Table VII, a total of 15 spots reacted with the Sackaguchi reagent. It may therefore be concluded that the two-dimensional paper chromatographic-electrophoretic procedure is an effective method for complete resolution of all peptides which occur in the tryptic digest of HGH.

The anion exchanger, DEAE-cellulose, was found to be useful in separating the tryptic digest into three fractions (D-I, D-II, D-IV); the soluble fraction was applied to the cellulose in a column which has been equilibrated with 0.01 *N* ammonium acetate buffer of pH 8.1. The column was developed with the same buffer; D-I, which emerged after the first hold-up volume of the column, contains the peptides that have the fastest mobilities in electrophoresis in the direction of

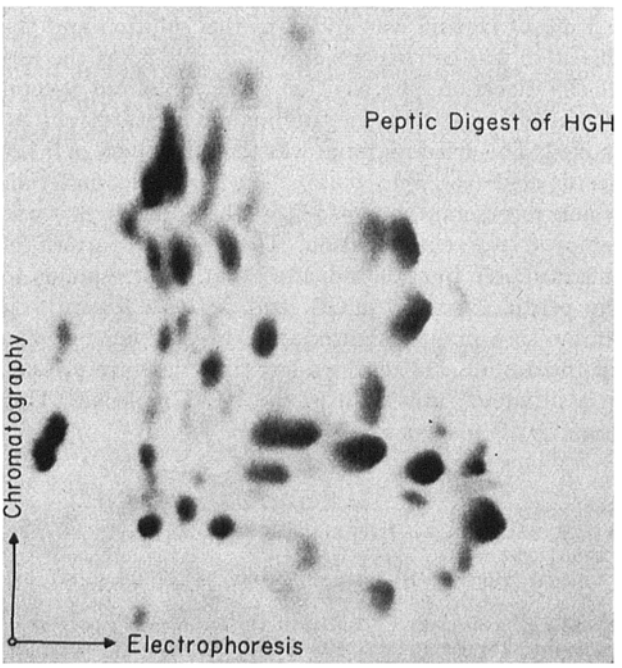

Fig. 6a

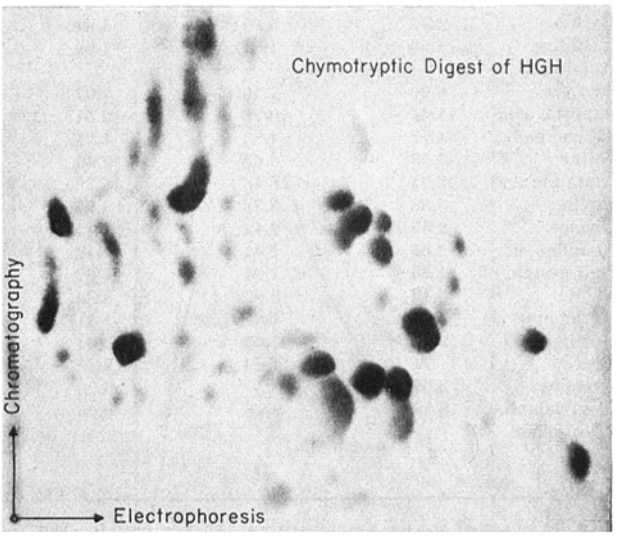

Fig. 6b

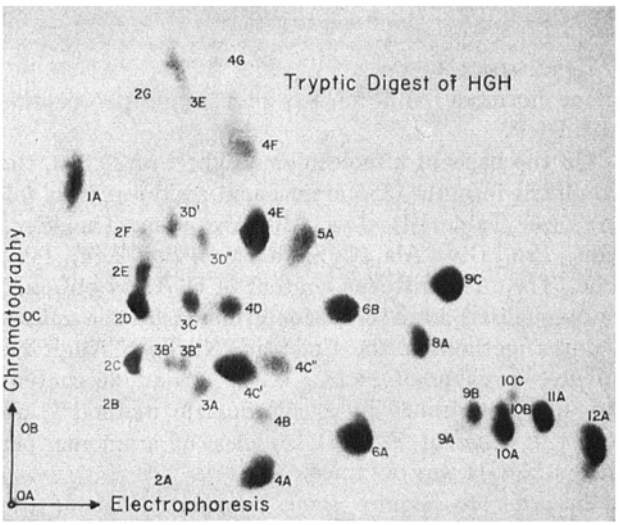

Fig. 6c

Fig. 6. Peptide patterns of enzymatic digests of HGH; chromatography in solvent system, *n*-butanol/acetic acid/water (4/1/5) and electrophoresis in pH 3.7 pyridine-acetic acid buffer for 105 min at 2000 volts according to the procedure of KATZ et al.[68]: (A), pepsin/HGH, 1/100 (w/w), 37°C for 24 h in 0.01 *M* HCl; (B), chymotrypsin/HGH, 1/100 (w/w), 37°C for 24 h in 0.2 *M* ammonia acetate buffer of pH 8.5; (C) trypsin/HGH, 1/200 (w/w), 37°C for 24 h in 0.2 *M* ammonia acetate buffer of pH 8.5.

[68] A. M. KATZ, W. J. DREYER, and C. B. ANFINSEN, J. biol. Chem. *234*, 2897 (1959).

the cathode. Their behavior in both column chromatography and electrophoresis suggests that they are basic peptides which are not held up by the anionic exchange cellulose and which have fast electrophoretic mobilities towards the cathode. Following D-I, D-II emerged with the same developer; this fraction contains eight peptides which were found to be located in the center part of the peptide pattern obtained by two-dimensional paper chromatography-electrophoresis, and are considered to be the neutral peptides. D-IV is a large fraction which did not emerge until the developer was changed to the 0.5 *N* ammonium acetate buffer of pH 6.7. As revealed from its two-dimensional paper chromatographic-electrophoretic pattern, this fraction contains the remainder of the peptides in the tryptic digest. From their behavior in electrophoresis, they are acidic peptides.

Table VII. Color tests on tryptic digest of human growth hormone

Peptide no.[a]	Color reaction		
	Ehrlich	Pauli	Sackiguchi
12A		+[b]	+
10B			+
10C			+
9A			+
9B			+
9C			+
8A			+
5A			+
4B			+
4C′	+		+
4C″		+[b]	
4F		+[c]	
3A		+[b]	
3B′		+[c]	
3B″		+[c]	
3E		+[c]	
2C		+[c]	+
2D			+
2F		+[c]	
2G		+[c]	
OA		+[b]	+
OB			+
OC	+		+

[a] Peptides are designated according to Figure 6c. [b] Positive for histidine. [c] Positive for tyrosine.

Table VIII. Amino acid sequences of some peptides resulting from tryptic digestion of HGH

Peptides[a]	Sequence
T-12A	Ala-His-Arg
T-11A	Lysine
T-10A	Ser-Lys
T-10B	Gly-Arg
T-10C	Arginine
T-9A	Asp-Arg
T-9B	Ser-Arg
T-9C	Leu-Arg
T-8A	Met-Gly-Arg
T-6A	Glu(NH$_2$)-Glu-Lys
T-6B	Thr-Gly-Glu(NH$_2$)-Ileu-Phe-Lys
T-5A	Asp-Leu-Glu-Leu-Leu-Arg
T-4A	Glu(NH$_2$)-Glu-Thr-Glu
T-4E	Asp-Leu-Leu-Lys

[a] See Figure 6c.

Further separation of these three fractions were carried out by high-voltage paper electrophoresis at pH 3.7 and 2,000 volts, followed by paper chromatography in a solvent system consisting of *n*-BuOH/pyridine/acetic acid/water (30/20/6/24, v/v). The amino acid sequences of twelve purified peptides were deduced by the usual methods of terminal analysis, including quantitative amino acid analysis[63] and the Edman stepwise degradation procedure[69]. In addition, lysine and arginine were found as free amino acids in the digest (see Table VIII). These data represent results obtained from the first attempts to elucidate the internal primary structure of HGH.

Concluding Remarks. From animal experiments with bovine growth hormone, it is clear that the hormone exerts a variety of biological functions other than its well-known growth-promoting activity. In man, HGH has been shown to be the most potent anabolic agent of all drugs or hormones that have been tested in recent years. Because of the limited supplies of human pituitaries, other aspects of the biological usefulness of HGH in health and disease have not been explored.

Limited hydrolysis with pepsin indicated that the integrity of the HGH molecule is not essential for its hormonal activity. It is likely that a portion of the 252-amino acid chain is responsible for the biological functions of HGH. We have described above initial studies which were undertaken in this laboratory on the internal structure of the HGH molecule. If the complete primary structure of HGH were known, it might be possible to synthesize an amino-acid sequence that exhibits all the biological properties of the hormone. It is hoped that this goal will soon be realized and that a synthetic product with HGH activities shall be available for extensive clinical and experimental investigations[70].

[69] P. EDMAN, Acta chem. scand. *4*, 283 (1950).

[70] Added to the proof, January 24, 1964: The molecular weight of the HGH monomer has recently been determined by sedimentation equilibrium studies (C. H. LI and B. STARMAN, Biochem. biophys. Acta, in press), and was found to be 21,500. On the basis of this molecular weight, the empirical formula (187 residues) of HGH as obtained from its amino acid composition (see Table VI) becomes: $Lys_9\ His_5\ Arg_{10}\ Asp_{20}\ Thr_{10}\ Ser_{18}\ Glu_{27}\ Pro_9\ Gly_9\ Ala_8\ CyS_4\ Val_7\ Met_3\ Ileu_7\ Leu_{23}\ Tyr_7\ Phe_{12}\ Try_1\ (\text{-}NH_2)_{20}$.

Zusammenfassung. Aus Tierexperimenten mit Wachstumshormonen aus Rindern wird klar, dass das Hormon eine Anzahl anderer biologischer Funktionen ausübt als seine wachstumsfördernde Tätigkeit. Im Menschen hat sich das Wachstumshormon als stärkstes anabolisches Agens der bisher untersuchten Arzneimittel oder Hormone erwiesen. Infolge der limitierten Hypophysenausscheidung blieben andere Merkmale der biologischen Förderung der Wachstumshormone für den gesunden wie auch den kranken Menschen unerforscht.

Beschränkte Hydrolyse mit Pepsin ergab, dass die Integrität des Wachstumshormonmoleküls nicht Voraussetzung für sein harmonisches Wirken ist. Hingegen ist es wahrscheinlich, dass ein Teil der 252 Aminosäurekette die biologischen Funktionen der Wachstumshormone bedingt. Die Kenntnis der vollständigen Primärstruktur des Wachstumsmoleküls würde die künstliche Herstellung einer entsprechenden Aminosäurefolge mit den biologischen Eigenschaften des Hormons ermöglichen.

Mineralhaushalt und hormonale Aktivität im Winterschlaf

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Seit den Untersuchungen von WYSS[1] am Siebenschläfer ist bekannt, dass der Winterschlaf der Säugetiere eine besondere Form der Thermoregulation, eine Art «Sollwertverstellung des Temperaturreglers» darstellt, die während des normalen Ruheschlafes ausgelöst wird. Bei Körpertemperaturen von wenigen Graden über Null wird der neue «Sollwert» bei Belastung durch äussere Kälte festgehalten; eine Überlastung wirkt als Weckreiz. Der Mechanismus der Umschaltung der Thermoregulation ist bis heute nicht geklärt, jedoch besteht Einvernehmen über die Teilnahme hormonaler und nervöser Mechanismen. Gleichgültig, ob man der Theorie der hormonalen oder der Theorie der nervösen Genese des Winterschlafs den Vorzug gibt, ist zu erwarten, dass primäre Umstellungen im endokrinen System oder sekundäre über eine Veränderung im neurovegetativen Gleichgewicht auch im Mineralhaushalt zum Ausdruck kommen sollten. Die Untersuchung der Elektrolyte, im engeren Sinne des Natriums, Kaliums, Calciums und Magnesiums, kann daher – kombiniert mit Befunden über den Kohlenhydrat-, Säure/Basen- und Wasserhaushalt, histologische und histochemische Drüsenuntersuchungen usf. – gewisse Aufschlüsse über die endokrine Aktivität im Winterschlaf geben.

Eine allgemeine Schwierigkeit solcher Untersuchungen besteht in der Weckempfindlichkeit, die bisher keine kontinuierliche Verfolgung von humoralen Veränderungen während des Schlafzustandes am Einzeltier zugelassen hat. Man ist daher zur Prüfung ihrer Abhängigkeit von der Körpertemperatur, des Kriteriums der Schlaftiefe im Winterschlaf, auf die Zusammenstellung von Einzelergebnissen möglichst vieler Tiere mit unterschiedlicher Körpertemperatur angewiesen[2–4]. – Bei der tabellarischen Darstellung der Daten des Schrifttums über die Blutelektrolyte wurden alle mg%-Angaben in Milliäquivalent pro Liter (mval/l) umgerechnet. Einzelergebnisse von Versuchsserien wurden, soweit das statthaft erschien, zusammengefasst und daraus Mittelwert und Standardabweichung berechnet.

I. *Der Natrium- und Kaliumhaushalt.* Natrium-, Kalium- und Wasserstoffionen sind über mehrere Regulationssysteme miteinander verknüpft. Austauschprozesse an Zellmembranen der Körpergewebe, speziell der Niere und der Magendrüsen, betreffen meist alle drei Ionenarten. Bei Sichtung der in Tabellen I und II dargestellten Ergebnisse über das *Serum-Natrium* bzw. – *NaCl* ergibt sich, dass der Natriumgehalt durch den Winterschlaf kaum verändert wird. Allenfalls kann ein geringer Anstieg, der nach eigenen Befunden am Hamster[4] sein Maximum bei 20° Körpertemperatur haben würde, abgelesen werden. Im tiefsten Winterschlaf liegen wieder die Messwerte des Wachzustandes vor (Figur 1). Der erhöhte Natriumspiegel, der bei einer Gruppe wacher Goldhamster[5] und Hamster[4] festgestellt worden ist, kann durch Nahrungsaufnahme[5] oder durch die Gonadenentwicklung während des Nachwinters[4] bedingt sein. Bekanntlich vermögen Sexualhormone die renale Natriumretention zu er-

[1] O. A. M. WYSS, Pflügers Arch. ges. Physiol. *229*, 599 (1932).
[2] R. S. PERSON, Trudy instit. morphol. zhivotnich im. Severtsova *6*, 173 (1952).
[3] M. L. RIEDESEL und G. E. FOLK, Amer. Naturalist *92*, 307 (1958).
[4] P. RATHS, Z. Biol. *113*, 173 (1962).
[5] A. DENYES und J. HASSETT, Bull. Mus. Comp. Zool. Harvard Univ. *124*, 437 (1960).