Growth Hormone and Protein Synthesis

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SUMMARY

The effect of bovine growth hormone (BGH) upon intracellular accumulation and incorporation of L-leucine-1-^{14}C and L-tyrosine-^{14}C (uniformly labeled) into protein was studied *in vitro* in the diaphragm of the hypophysectomized rat. Amino acid uptake apparently was not stimulated at any time up to 90 min, whereas stimulation of incorporation into protein was noted after 40 min. BGH given between 44 and 4 hr before the rats were killed did not cause *in vitro* stimulation of uracil-2-^{14}C incorporation into total nucleic acids of the hypophysectomized rat diaphragm. Actinomycin D partially inhibited L-leucine-1-^{14}C incorporation into protein, and inhibited by 95% the incorporation of adenine-8-^{14}C into nucleic acids. In the presence of actinomycin D, BGH stimulated L-leucine incorporation into protein but did not affect adenine-8-^{14}C incorporation into nucleic acids. Thus, BGH stimulation of amino acid incorporation may not be dependent upon an increase in intracellular amino acid accumulation or a stimulation of RNA synthesis *in vitro*.

INTRODUCTION

Bovine growth hormone (BGH) promotes protein synthesis *in vivo* (1) and *in vitro* (2), but its mechanism of action remains obscure. Several workers (3, 4) have reported that BGH increases intracellular accumulation of α-aminoisobutyric acid (AIB), which is not metabolized, in the rat diaphragm. Other investigators (5, 6) have reported that BGH stimulates accumulation of some amino acids (glycine, alanine, serine, threonine, proline, histidine, tryptophan, glutamine, and asparagine), but does not affect nine others (methionine, valine, tyrosine, phenylalanine, leucine, lysine, arginine, and the two dicarboxylic acids) (7). However, Knobil and Hotchkiss (7) found evidence that some common steps were utilized by AIB and all but two of these amino acids (i.e., not the dicarboxylic amino acids), since they inhibited BGH-stimulated AIB accumulation. It is not known whether BGH stimulation of protein synthesis is dependent upon stimulated accumulation of some amino acids.

Hormonal action in relation to RNA synthesis has been studied recently (8–10), and it has been suggested (11) that BGH may stimulate messenger-RNA production which in turn leads to increased protein synthesis.

The purpose of this investigation was to study the effect of BGH on amino acid accumulation, RNA synthesis, and protein synthesis in rat striated muscle.

MATERIALS AND METHODS

Female rats of the Sprague-Dawley strain, each weighing 70–100 g, were used 2 or more weeks after hypophysectomy. The rats were given 0.25 mg cortisone acetate on the day of operation and again 4 days later. When the animals were killed the sella of each was checked for completeness of hypophysectomy.

Chemicals. Labeled compounds L-leucine-

1-^{14}C (SA 25.4 mC/mmole), L-tyrosine-^{14}C, u.l. (SA 10 mC/mmole), uracil-2-^{14}C (SA 27.8 mC/mmole), and adenine-8-^{14}C (SA 14.7 mC/mmole) were obtained from the New England Nuclear Corporation. Actinomycin D was supplied by Merck, Sharp and Dohme, Montreal. Sodium ribonucleate was obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. The bovine growth hormone used was BGH NIH Lots B-4, B-6, and B-7.

The rats were killed by cervical-cord section. The diaphragm was rapidly excised, leaving rib-cage attachments intact (12) or severing them; the former preparation will be referred to as "intact" diaphragm and the latter as "excised." The diaphragm was hemisected through the midline fibrous tissue, briefly rinsed in ice-cold saline, blotted lightly on filter paper, and placed in incubation flasks (see legend of Fig. 1 for details). Each flask was sealed and gassed with CO_2:O_2 5%:95% for 2 min. After incubation, the hemidiaphragms were rinsed briefly in ice-cold saline and trimmed of fatty tissue. Only intact preparations were used when labeled intracellular amino acid concentration was measured. After incubation the diaphragms were rinsed rapidly in five washes of ice-cold saline. Each diaphragm was blotted, weighed, minced with scissors, and then homogenized with 2 ml ice water and 1 ml cold 15% trichloroacetic acid, using a Teflon homogenizer of the Potter-Elvehjem type. After centrifugation of the homogenate one aliquot of the supernatant fluid was counted in a Packard Tri-Carb liquid-scintillation counter with 10 ml dioxane scintillator (13). An internal standard was used to correct quenching in all samples. In experiments with L-tyrosine an additional 1 ml supernatant fluid was taken after homogenization for determination of tyrosine content by the method of Waalkes and Udenfriend (14).

Protein in the precipitate after homogenization was extracted by a modification of the method of Schneider (15). The precipitate was washed at 5% trichloroacetic acid, heated at 70–80° for 20 min in 5% trichloroacetic acid, and washed successively in 5 ml 10% trichloroacetic acid, 5 ml ethanol:ether 3:1, and 5 ml ether. The final protein precipitate was dried under nitrogen and 1.5 ml 90% formic acid was added; complete solution occurred in 30 min at 60°. Three 0.3-ml aliquots, placed in tared, concentric-ring planchets, were evaporated to dryness under a heat lamp, reweighed, and counted in a Nuclear-Chicago thin-window gas-flow counter, after which the protein was dissolved in 0.1 N NaOH and analyzed by the method of Lowry *et al.* (16). For RNA analysis, the entire experiments were performed in a cold room (4°), using a modification of the technique as described by Hecht and Potter (17) with which trichloroacetic acid solutions are maintained at a 15% w/v concentration (18, 19). The final dry RNA precipitate was dissolved in 2.2 ml 0.1 N NaOH, and 1 ml was taken for counting in dioxane scintillation-counting medium. A second aliquot was taken for determination of RNA ribose, using the orcinol reagent (20); or total RNA was determined by measuring absorption at 260 mμ in a Beckman DU spectrophotometer, using sodium ribonucleate to prepare standard curves.

The inulin space, used as a measure of the extracellular fluid space, was determined by the Schreiner modification (21) of the method of Roe. This was found in preliminary experiments to be 17% of total weight, which agrees with the findings of Kostyo and Engel (4). The total water content of the diaphragm of the hypophysectomized rat, determined after desiccation at 105° for 24 hr, was 75.5% ± 2.4%; calculation of the counts per minute per milliliter of intracellular fluid (ICF) was based on these findings (22).

In experiments in which BGH was injected *in vivo* the hypophysectomized animals were injected subcutaneously with 100 μg BGH (NIH B-7) 4, 12, 20, or 44 hours before sacrifice. Incubation was performed as described above.

RESULTS

The relationship between the intracellular accumulation of labeled amino acid and amino acid incorporation into protein was

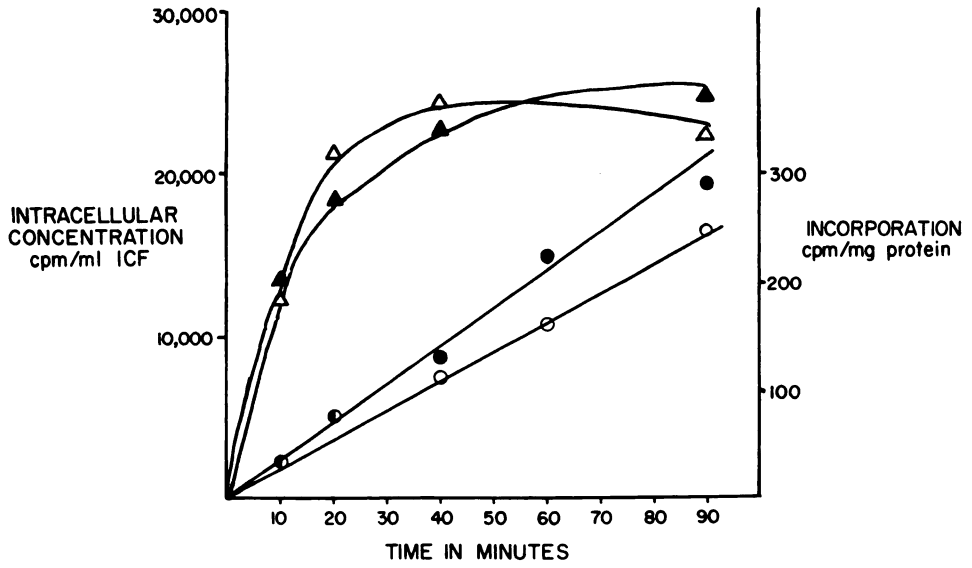

FIG. 1. *Time course of the effect of BGH on intracellular accumulation and incorporation into protein of* L-*leucine-1-*^{14}*C in the "intact" diaphragm preparation*

Medium: Krebs-Ringer bicarbonate solution, pH 7.4, containing 0.1 mM L-leucine and 50,000 cpm/ml. BGH was added at concentration 50 μg/ml in final incubation volume 6 ml. Time of incubation, 60 min; 37°. ○ = incorporation, BGH absent; ● = incorporation, BGH present; △ = intracellular concentration, BGH absent; ▲ = intracellular concentration, BGH present. Each value represents average of 5 or more experiments.

investigated. Figure 1 depicts the accumulation of free intracellular labeled amino acid and incorporation of labeled amino acid into protein at varying time intervals. The accumulation of L-leucine-1-^{14}C was rapid, more than one-half entering within 10 min, and after 40 min stabilizing at a concentration (23,000 cpm/ml ICF) one-half that in the surrounding medium. At no time did BGH stimulate intracellular accumulation of L-leucine-1-^{14}C. Incorporation into protein increased linearly throughout the experiment. BGH stimulation of incorporation became apparent by 40 min ($P < 0.2$) and was statistically significant at 60 min ($P < 0.01$) and 90 min ($P < 0.05$). Because of the difficulty in determining total intracellular leucine concentration, L-tyrosine was used in further studies, as this amino acid can be readily quantitated chemically and, therefore, intracellular specific activity could be determined: if stimulation of incorporation masked stimulated accumulation, the latter would still be detectable, as the specific activity of the free intracellular amino acid would rise. In fact, however, the specific activity of the intracellular tyrosine was not altered by BGH, whereas incorporation

TABLE 1

Effect of BGH upon the free intracellular specific activity and incorporation into protein of L-*tyrosine-*^{14}*C (u.l.)*

L-Tyrosine-^{14}C (u.l.), 0.1 mM and 75,000 cpm/ml concentration, was used in place of L-leucine; otherwise, conditions of incubation were as in Fig. 1. Incubation time, 60 min. Figures in parentheses denote number of experiments.

Sample	Free intracellular tyrosine (cpm/μg tyrosine)	Incorporation (cpm/gm protein)
BGH added	2000 ± 149 (6)	190 ± 5 (6)
Control	1931 ± 180 (6)	157 ± 15 (6)
		$0.1 > P > 0.05$[a]

[a] In each of the six experiments incorporation was stimulated in the presence of BGH, although the degree of this stimulation varied greatly between experiments.

was stimulated after 60 min incubation (Table 1).

When it was established that BGH stimulated protein synthesis, it was decided to investigate whether RNA synthesis, the accepted precursor of protein synthesis, was stimulated. BGH added *in vitro* did not stimulate incorporation of uracil-2-^{14}C into RNA during the 2-hr incubation (Table 2),

TABLE 2

Effect of BGH, added in vitro, on the incorporation of uracil-2-^{14}C into RNA of the hypophysectomized-rat diaphragm

Medium: a total of 1 ml KRB solution, pH 7.4, containing uracil-2-^{14}C (2.0 μC, 0.07 mM) and either BGH (50 μg/ml) in saline or an equal volume of saline. Incubation time, 2 hr.

Sample	Cpm/mg RNA ribose ($\times 10^{-3}$) (average ± SE)	Number of experiments
Control	242 ± 28	18
BGH	231 ± 13	18

although the diaphragm readily incorporated uracil into RNA under these conditions. In another series of experiments BGH was injected *in vivo* and the animals were sacrificed at various times thereafter (Table 3). The diaphragms were removed and were incubated for 2 hr with uracil-2-^{14}C. Stimulation occurred in tissue of animals which had received BGH 12 hr previously, but the difference from control values was not statistically significant.

The apparent lack of relationship between stimulation of protein synthesis and RNA synthesis was investigated further by blocking RNA synthesis and determining whether BGH then stimulated protein synthesis. Paired hemidiaphragms from hypophysectomized rats were incubated for

TABLE 3

Effect of time of BGH injection given in vivo on the in vitro incorporation of uracil-2-^{14}C into RNA of the hypophysectomized-rat diaphragm

Medium: 1 ml KRB, pH 7.4, containing uracil-2-^{14}C (2 μC/ml, 0.07 mM). Time of incubation, 2 hr. BGH injected subcutaneously at a concentration of 100 μg/80–100 g body weight. Numbers in parentheses denote number of experiments.

Time of injection before sacrifice (hr)	Cpm/mg RNA ribose ($\times 10^{-3}$) (average ± SE)
0	230 ± 32 (6)
4	259 ± 65 (5)
12	271 ± 40 (6)
20	255 ± 20 (4)
44	211 ± 20 (5)

3 hr with actinomycin D (Table 4). At the end of the first hour, BGH was added to one of each pair and leucine-1-^{14}C was added to both. The tissue with BGH showed 25% more leucine incorporation, despite the presence of actinomycin, although actinomycin reduced protein synthesis by 35%. The stimulation of leucine incorporation by BGH persisted even when RNA synthesis, as indicated by adenine-

TABLE 4

Effect of BGH on in vitro incorporation of L-leucine-1-^{14}C into diaphragm protein and of adenine-8-^{14}C into RNA of the hypophysectomized rat, with and without actinomycin D

Medium: 3 hemidiaphragms from hypophysectomized rats were incubated for 1 hr in 3.0 ml KRB, pH 7.4, containing 10 μg actinomycin D per milliliter. Subsequent incubation was for 2 hr in 3 ml KRB, pH 7.4, containing actinomycin (10 μg/ml), L-leucine (0.1 μC/ml, 0.1 mM), or adenine-8-^{14}C (3 μC/ml, 0.2 mM), with and without 50 μg BGH (NIH B-7) per milliliter. Control diaphragms were preincubated in KRB, pH 7.4, containing mannitol in concentration identical to that of the actinomycin D diluent. Numbers in parentheses denote number of experiments.

Actinomycin D (10 μg/ml)	Leucine incorporated (μmoles/mg protein, ±SE, $\times 10^{6}$)			Adenine incorporated (μmoles/mg nucleic acid, ±SE, $\times 10^{8}$)		
	Control	BGH	*P*	Control	BGH	*P*
Present	392 ± 38	530 ± 35 (5)	<0.05	6.15 ± 0.51	6.54 ± 0.48 (6)	N.S.
Absent	625	689 (2)	—	205	209 (2)	—

8-^{14}C incorporation, was 95% abolished. Furthermore, BGH did not stimulate RNA synthesis in the presence or absence of actinomycin D.

DISCUSSION

The experiments in which amino acid accumulation and incorporation were measured represent a direct approach to determining the sites of action of growth hormone. The rate of leucine accumulation was very rapid, as has been shown also in studies using the intestine (23) and in those with rat diaphragm (24–26). The data depicted in Fig. 1 indicate that maximal accumulation was achieved equally in the presence and the absence of BGH: this occurred before any BGH stimulation of incorporation was evident and, therefore, the increased incorporation of L-leucine does not appear to be dependent upon any change in total intracellular amino acid content. This finding supports the recent work of Kostyo (27). However, it was not possible to rule out the occurrence of preferential transport of amino acids into a distinct pool not represented by the *total* intracellular pool of free labeled amino acid. Although its presence has been postulated (24, 28), such a pool has not been demonstrated.

It has been suggested that the increased incorporation or catabolism of L-leucine masks BGH-stimulated accumulation (7), but this appears unlikely, since frequent analysis before stimulated synthesis became apparent failed to elicit any differences in the quantity of intracellular labeled amino acid in diaphragms incubated with or without BGH. In addition, if the transport of amino acid into the cell were more rapid in the presence of BGH, the specific activity of the intracellular pool would increase, but this was not demonstrated in our studies with labeled tyrosine. Knobil and Roth (29) were unable to demonstrate BGH-stimulated accumulation of leucine, even when protein synthesis was abolished by puromycin.

It appears that the rapid *in vitro* stimulation of protein synthesis is not mediated by a stimulation of RNA synthesis. Furthermore, inhibition of RNA synthesis by actinomycin D (which blocks DNA-directed RNA synthesis) failed to block the hormone-stimulated protein synthesis. A similar effect in the presence of actinomycin D has been reported recently by others who used BGH (30, 31) and with insulin (32, 33).

It may be postulated, therefore, that a difference exists between short-term *in vitro* protein synthesis and the increased synthesis that occurs after long-term *in vivo* stimulation. In separate studies (K. G. Dawson, unpublished observations) it was noted that skeletal-muscle microsomes from BGH-injected rats were more active in the incorporation of labeled leucine into protein; similar results have been reported by Korner (34), who used microsomes from rat liver. These findings suggest that the RNA of the microsomes from BGH-treated animals has been altered, permitting enhanced protein synthesis, but such studies fail to elucidate the exact point *in vivo* at which BGH initiates this action. Furthermore, similar effects are not apparent in microsomes that have been isolated after *in vitro* exposure of the tissue to BGH. Thus, one of the problems yet to be resolved in the interpretation of this work is whether the stimulation of protein synthesis *in vitro* is a true reflection of the basic physiological process that occurs *in vivo*. Some of the actions of BGH *in vivo*, e.g., the stimulation of RNA synthesis, may involve a time lag (11). Furthermore, the BGH molecule may be altered *in vivo*, although it is more likely that BGH *in vivo* may stimulate production of another material, e.g., sulfation factor (35), which in turn stimulates protein synthesis. Further studies are in progress to help elucidate this point.

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