Amino Acid Incorporation into Protein by Cell-Free Systems from Rat Skeletal Muscle. V. Effects of Pituitary Growth Hormone on Activity of Ribosomes and Ribonucleic Acid Polymerase in Hypophysectomized Rats*

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ABSTRACT: Administration of growth hormone to hypophysectomized rats increased the yield [μg of ribonucleic acid (RNA)/rat] and activity (transfer of labeled amino acid from s-RNA to protein/mg of RNA) of ribosomes from thigh muscle. Maximal response was observed after five daily injections of the hormone. The yield and activity of ribosomes and the activity of RNA polymerase (aggregate enzyme) were also determined at various times after a single injection of growth hormone. Maximal activity of ribosomes and of RNA polymerase occurred 18 hr after hormone administration; yield of ribosomes increased more slowly and the subsequent rate of decrease was less rapid.

Combined administration of growth hormone and of testosterone propionate gave additive effects in the stimulation of ribosome yield and activity, thus paralleling the known effects of these hormones on weight gains and nitrogen balance in the whole animal. Although it is clear that growth hormone stimulates the synthesis of messenger and ribosomal RNA, the time relationships indicate that these effects cannot account for all of the known physiological effects of this hormone.

The protein anabolic effects of the growth hormone (somatotropin) secreted by the pituitary gland are well established, and a number of attempts have been made to determine the mechanisms by which these effects occur (for reviews, see Knobil and Hotchkiss, 1964; Engel and Kostyo, 1964; Korner, 1965a). Korner (1959, 1961, 1964) studied the effects of hypophysectomy and treatment with growth hormone on protein and nucleic acid synthesis in cell-free systems from rat liver. He concluded that growth hormone stimulated the synthesis of messenger ribonucleic acid (m-RNA) and that the stimulation of protein synthesis was secondary to this effect, although he has recently modified this conclusion (Korner, 1965b,c). It has been suggested that many other hormones act by the same mechanism (Kidson and Kirby, 1964); a review on this effect of steroid hormones has recently been published by Hechter and Halkerston (1965).

It was desirable to extend the elegant and comprehensive studies by Korner to include the observations on the effects of growth hormone on skeletal muscle. The primary objective which prompted our characterization of the amino acid incorporating system in skeletal muscle (Florini, 1962, 1964; Breuer *et al.*, 1964; Florini and Breuer, 1965) was the desire to provide a proper basis for study of the effects of anabolic hormones in this tissue. A report on the effects of testosterone propionate on the activity of ribosomes from muscle of castrate rats has been published (Breuer and Florini, 1965); this paper presents our observations on the effects of growth hormone on protein and ribonucleic acid synthesizing systems from skeletal muscle.

Experimental Section

Materials. Porcine and bovine growth hormones were purchased from the Sigma Chemical Co. and the California Corp. for Biochemical Research. Testosterone propionate was purchased from Mann Research Laboratories, Inc. Materials used in the preparation and assay of ribosomes and of RNA polymerase were obtained as specified in previous papers (Breuer *et al.*, 1964; C. B. Breuer and J. R. Florini, unpublished data).

Methods. Hypophysectomized male rats weighing 50–60 g were purchased from the Charles River Laboratories. Upon receipt, they were placed in individual cages and their body weights were recorded for 2 weeks. Rats gaining >10 g during this period were discarded.

Growth hormone was dissolved in 0.9% saline with the addition of sufficient 0.1 M NaOH to dissolve the protein. The solution was diluted so that the indicated dose was administered in 0.2 ml; dosage was by intraperitoneal injection. In the experiments on time course of hormone effects, all experimental groups received injections of saline at those times at which they did not receive hormone. The repeated injections were administered to minimize stress-induced differences in activity of the RNA and protein synthesizing systems.

For convenience and clarity, the procedure used in

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the preparation of ribosomes will be presented in detail although some of the techniques have been described in general terms in previous papers in this series. Ribosomes were prepared from thigh and gastrocnemius muscles pooled from 12 male rats; three treated and one control group could be included conveniently in each experiment. All operations after removal of the legs from the rats were conducted in a cold room at 4°. Rats were decapitated and the rear legs were skinned and removed by cutting through the pelvic joint. The legs were immediately placed in ice water and stored until all animals involved in the experiment had been sacrificed. Muscle was then cut from the bones and tendons, forced through a meat grinder, weighed (50–70 g), and homogenized in 2 volumes of medium containing 0.25 M sucrose, 0.01 M $MgCl_2$, 0.08 M KCl, and 0.05 M Tris, pH 7.8 for 30 sec at control setting 80 in a VirTis "45" homogenizer in an ice bath. The homogenate was centrifuged for 15 min at 13,000*g* to remove mitochondria, nuclei, myofibrils, and debris. [In experiments in which RNA polymerase was also prepared, nuclei were collected by centrifugation at 700*g* for 15 min prior to the 13,000*g* centrifugation; nuclei were purified and aggregate enzyme was prepared as described by C. B. Breuer and J. R. Florini (unpublished data).]

The 13,000*g* supernate was filtered through a double layer of cheesecloth and then centrifuged 2 hr at 78,000*g* to sediment the microsome fraction. Microsomes from each group were resuspended by 10 up-and-down strokes in a Potter-type homogenizer equipped with a loose-fitting Teflon pestle (A. H. Thomas, Cat. No. 4288-G, size B, clearance was increased to *ca.* 0.020 in. by filing the pestle) in a mixture of 9.75 ml of homogenizing medium and 3.0 ml of 2.5 M KCl in 0.01 M $MgCl_2$. (This and subsequent homogenizations were done with the pestle turning at minimum possible speed and with the homogenizing flask surrounded by ice water.) To the milky suspension in the homogenizer vessel was added 0.75 ml of 10% (w/v) Lubrol WX in 0.01 $MgCl_2$; mixing was achieved by four additional up-and-down strokes with the pestle turning slowly. Finally, 1.5 ml of 10% (w/v) sodium deoxycholate in water was added, and an additional 10 up-and-down strokes were done. Appreciable clarification of the milky microsome suspension occurred upon addition of the detergents, but the final mixture was still slightly opalescent. The detergent-treated microsomes were layered in 5-ml aliquots over 7 ml of 1.0 M sucrose (ion composition of the homogenizing medium) in centrifuge tubes; centrifugation was at 105,000*g* for 2 hr. At the end of this period, the solutions were aspirated off; care was necessary to avoid contamination of the small ribosome pellets with detergents from the upper layer.

The pellets were removed on the tip of a stainless steel spatula, care being taken to avoid contact between the pellet and the sides of the tube. Combined ribosomes from each group were distributed in 3 ml of homogenizing medium by very gentle homogenization in a Kontes all-glass homogenizer; the ribosomes were solubilized by placing the suspension in capped centrifuge tubes on a slowly rotating vertical turntable overnight at 4°. On the following morning, tubes were centrifuged for 5–10 min at *ca.* 3000 *g* to sediment material which had not been dissolved. The white pellet, although rather substantial in appearance, contained relatively little RNA, and was of low purity (ratio of absorbancy at 260/280 mμ was <1.5). The supernate was removed and used for all subsequent analyses of the ribosomes. By this procedure, the muscles from one normal rat yielded ribosomes containing 300–400 μg of RNA with a 260/280-mμ absorbancy ratio between 1.75 and 1.82, corresponding to an RNA/protein >1.25 (Breuer and Florini, 1965); the yield of ribosomes from muscle of hypophysectomized rats was substantially lower (Table I).

Transfer enzymes and [^{3}H]leucyl-s-RNA were prepared and incubations were conducted and analyzed as described by Breuer *et al.* (1964). The preparation of muscle nuclei, isolation of aggregate enzyme, and determination of RNA polymerase activity will be described separately (C. B. Breuer and J. R. Florini, unpublished data). In general, the procedures used were similar to published methods (Weiss, 1960; Gorski, 1964).

Results

Effects of Hypophysectomy and Treatment with Growth Hormone on Activity of Isolated Ribosomes. A series of experiments was done in which the yield of ribosomes from muscle and activity of these ribosomes in the transfer of [^{3}H]leucine from s-RNA to protein was measured after multiple injections of growth hormone. The data from these experiments are presented in Table I. Variations in control values from one experiment to another can probably be attributed to differences in the age of the rats or in the specific activity of the [^{3}H]leucyl-s-RNA preparations used. To facilitate comparisons, all data are presented also as the ratio to the activity or yield from hypophysectomized control rats. Response to treatment with growth hormone gradually decreased after the maximum at 5 days. The effect after eight daily injections was appreciably less, and no stimulation of activity was detected when ribosomes were prepared from rats at the end of a long-term (33 day) growth study. Even after extended treatment with growth hormone, however, the yield of ribosomes per rat was much greater from treated than from control rats. However, after such extended treatment, there were very great differences in size between treated and control animals; the yield of ribosomes expressed on the basis of the wet weight of muscle used was the same in the two groups of rats in expt 4.

Total protein synthesis in muscle is related to both the ribosome content of the tissue and to the activity per ribosome. Consequently, a third quantity was calculated by multiplying the yield by the activity of isolated ribosomes. This was designated the protein synthetic capacity of the tissue; we believe that it presents

TABLE I: Effect of Repeated Injection of Growth Hormone on Activity and Yield of Ribosomes from Rat Skeletal Muscle.

Expt No.	Rats: Operation	Rats: Sex	Rats: Body Wt[a] (g) Initial	Rats: Body Wt[a] (g) Final	Rats: Body Wt[a] (g) Change	Injections: Composition	Injections: No. of Injections	Results: Ribosomes Act.[b] ($\times 10^{-5}$)	Results: Ribosomes Yield[c]	Results: Ratio to Corresponding Hypophysectomized Control: Act.	Results: Ratio to Corresponding Hypophysectomized Control: Yield	Results: Ratio to Corresponding Hypophysectomized Control: Protein Synthetic Capacity
1	Hypophysectomized	Female	83.6 ± 2.6	87.3 ± 2.8	3.6 ± 0.4	Saline	4	2.32	183	...	...	...
	Hypophysectomized	Female	83.4 ± 2.5	92.4 ± 2.9	8.9 ± 0.4	PGH (200 μg)	4	3.23	280	1.39	1.53	2.13
2	Hypophysectomized	Male	73.3 ± 1.5	76.3 ± 1.8	3.0 ± 0.6	Saline	5	1.41	188	...	...	...
	Hypophysectomized	Male	72.5 ± 1.6	80.2 ± 1.9	7.7 ± 0.8	PGH (200 μg)	5	7.85	504	5.57	2.68	14.9
3	Hypophysectomized	Male	65.4 ± 1.3	68.2 ± 1.6	3.2 ± 0.8	Saline	8	1.03	141	...	...	...
	Hypophysectomized	Male	61.2 ± 2.2	79.1 ± 1.7	15.9 ± 1.1	BGH (100 μg)	8	1.13	236	1.26	1.67	2.11
	Intact	Male	72.4 ± 2.5	102.1 ± 3.4	29.7 ± 1.9	Saline	8	1.98	346	1.92	2.45	4.70
4	Hypophysectomized	Male	65.9 ± 1.0	88.6 ± 1.6	21.4 ± 1.2	Saline	33	1.94	113	...	...	...
	Hypophysectomized	Male	66.7 ± 0.6	151.4 ± 3.2	84.4 ± 2.9	BGH (100 μg)	33	1.92	215	0.99	1.90	1.88

[a] Mean ± standard error of body weights of groups of 12 hypophysectomized immature male rats (Charles River strain). [b] Disintegrations per minute incorporated into protein per milligrams of ribosomes (as RNA) incubated. [c] Ribosomes (μg) (as RNA) isolated from the pooled thigh muscles per rat.

TABLE II: Effects of Single Intraperitoneal Injections of Porcine Growth Hormone (100 μg) on Activity of Muscle RNA Polymerase.

Conditions		Ribosomes					RNA Polymerase	
				Ratio to Hypophysectomized Control				
Expt No.	Time after Injection (hr)	Act.[a] ($\times 10^{-5}$)	Yield[b]	Act.	Yield	Protein Synthetic Capacity	Act.[c]	Ratio to Hypophysectomized Control
2	1	5.79	272	1.18	1.05	1.24	120	1.09
3	3	6.09	282	1.24	1.09	1.35	261	1.25
3	12	9.30	238	1.35	1.09	1.21	323	1.84
1	18	2.98	304	1.57	1.28	2.01	230	2.09
3	24	7.90	375	1.14	1.72	1.96	287	1.63
3	36	9.28	280	1.34	1.29	1.73	190	1.08
1	48	2.26	330	1.20	1.39	1.67	80	0.73
1	Control	1.89	238				110	
2	Control	4.90	258				209	
3	Control	6.91	218				176	

[a] Disintegrations per minute incorporated into protein per mg ribosomes (as RNA) incubated. [b] Ribosomes (μg) (as RNA) isolated from the pooled thigh muscles per rat. [c] UMP (μμmoles) incorporate/mg of RNA in the RNA isolated after incubation of the aggregate enzyme under the conditions described by C. B. Breuer and J. R. Florini (unpublished data).

a useful indication of the total effects of hormone administration on protein synthesis in muscle.

Time Course of Response of Ribosomes and RNA Polymerase to Single Injections of Growth Hormone. The rapidity with which various systems respond to hormone treatment is often regarded as indicating whether the responses represent primary or secondary effects of the hormones. Consequently we determined the effects of single injections of growth hormone on ribosomes and RNA polymerase at various times after hormone administration. The results of these experiments are presented in Table II. The activity of the ribosomes and of RNA polymerase reached a peak 18 hr after administration of growth hormone; yield of ribosomes increased more slowly.

Effects of Growth Hormone Administration on Sucrose Gradient Profiles of Isolated Ribosomes. We have previously concluded (Florini and Breuer, 1965) that the factor limiting the activity per ribosome in muscle was the m-RNA content of the preparation, and have shown (C. B. Breuer and J. R. Florini, unpublished data) that administration of testosterone propionate (TP)[1] to castrated rats caused increases in the amount of 260-mμ-absorbing material in the polyribosome region following centrifugation of ribosomes through sucrose gradients. Analysis of ribosomes from muscle of hypophysectomized rats showed similar although less extensive changes upon administration of growth hormone; the results of these analyses are presented

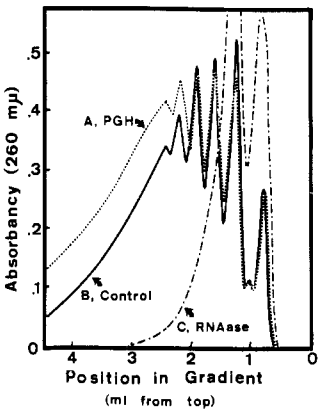

FIGURE 1: Sucrose gradient analyses of ribosomes from muscle of growth-hormone-treated and control hypophysectomized rats. Ribosomes (0.1 ml containing 0.2 mg of RNA) from muscle of growth hormone-treated (100 μg 18 hr before sacrifice) (line A) and control (line B) hypophysectomized rats were centrifuged one hour at 35,000 rpm (SW 39 Rotor, Spinco Model L Ultracentrifuge) through a linear gradient from 15 to 30% sucrose containing 0.01 M $MgCl_2$, 0.08 M KCl, and 0.05 M Tris–HCl, pH 7.6. Equal volumes of control ribosomes and pancreatic ribonuclease (2 mg/ml) were mixed and incubated in an ice bath for 10 min; analysis of 0.1 ml of this mixture gave curve C. Essential identical results were obtained when ribosomes from treated rats were incubated with ribonuclease. Profiles were recorded as described by Breuer *et al.* (1964) using a flow cell with a volume of 0.05 ml and a light path of 1.0 cm.

[1] Abbreviations used: TP, testosterone propionate; BGH and PGH, bovine and porcine growth hormones, respectively.

TABLE III: Effects of Simultaneous Injections of Porcine Growth Hormone and Testosterone Propionate on Activity and Yield of Ribosomes from Rat Skeletal Muscle.[a]

Treatment	Ribosomes		Ratio of Hypophysectomized Control		
	Act.[b] ($\times 10^{-5}$)	Yield[c]	Act.	Yield	Protein Synthetic Capacity
Saline (control)	2.98	185	1.00	1.00	1.00
TP (100 μg)	3.63	181	1.22	0.98	1.20
PGH (100 μg)	4.73	237	1.59	1.28	2.04
TP (100 μg) + PGH (100 μg)	6.49	274	2.18	1.48	3.22

[a] The indicated quantities of hormones were administered by intraperitoneal injection in 0.2 ml of saline 16 hr before the rats (12/group, body weight 70–80 g) were decapitated. Preparation and assay of ribosomes are described under Methods. [b] Disintegrations per minute incorporated into protein per milligram of ribosomes (as RNA) incubated. [c] Ribosomes (μg) (as RNA) per rat isolated from pooled thigh muscles.

in Figure 1. These results clearly demonstrate that treatment with growth hormone caused a small increase in the proportion of heavy material in the ribosome preparations. Treatment with ribonuclease under very mild conditions (curve C) demonstrated that the ultraviolet-absorbing material in the lower portion of the gradient was extremely sensitive to ribonuclease; these are properties of polyribosomes from skeletal muscle (Breuer *et al.*, 1964) as well as from other tissues. Thus we conclude that injection of growth hormone increased the proportion of polyribosomes in the ribosomes prepared from skeletal muscle by our techniques.

Effects of Simultaneous Treatment with Testosterone Propionate and Growth Hormone. Kochakian and Stettner (1948) and Kochakian (1960) have reported that stimulation of body weight gains by testosterone propionate and growth hormone was additive when the hormones were given at doses which caused maximal responses. The results presented in Table III were obtained when single injections of testosterone propionate, growth hormone, or both were given to hypophysectomized rats. The response of the ribosomal system to treatment with both hormones was clearly additive. When ribosomes from the various treated groups were intermixed and assayed by the transfer reaction, the observed activity was within 10% of that calculated by adding the individual activities of the component ribosomes; thus the lower activity of ribosomes from untreated hypophysectomized rats cannot be attributed to the presence of an inhibitor unless that inhibitor remains closely associated with the ribosomes throughout the incubation.

Discussion

An increase in the amount of protein in skeletal muscle is an obvious result of the action of a growth-promoting hormone. There are a number of mechanisms by which this increase may be achieved. Protein synthesis may be stimulated, breakdown may be inhibited, or both may occur simultaneously. Stimulation of protein synthesis may be a result of greater availability of energy or precursors for the synthetic process, or it may be caused by an increase in a limiting component of the synthetic system, as for example the amount of m-RNA available to the ribosomes. We believe that our evidence indicates that the anabolic effects of growth hormone (somatotropin) occur as a result of an increase in protein synthesis, although a decrease in protein degradation as a result of growth hormone treatment has not yet been excluded. To be sure, the effects of growth hormone on energy metabolism and amino acid transport are well established (Knobil and Hotchkiss, 1964; Engel and Kostyo, 1964), but stimulation of RNA and protein synthesis occurs in cell-free preparations in which energy sources and precursors are supplied in large excess (Korner, 1965a; see also Tables I and III).

The parts of the muscle protein synthetic system most likely to be sensitive to stimulation by growth hormone are those which normally limit the process, *i.e.*, ribosomes and m-RNA. We (Florini and Breuer, 1965) have provided evidence that the ribosome content of muscle is relatively low and that the m-RNA content of the ribosome fraction limits its activity in amino acid incorporation into protein. Furthermore, the soluble components required for protein synthesis are present in large excess in skeletal muscle (Florini, 1964). In this paper, evidence has been presented that the ribosome content of muscle (Tables I–III) and the messenger content of isolated ribosomes (inferred from the percentage of polyribosomes as shown in Figure 1) increase following administration of growth hormone. Thus it seems reasonable to suggest that the observed stimulation of RNA synthesis by growth hormone

causes an increase in protein synthesis with a consequent rise in the rate of body weight gain of the treated animal.

The increase in protein synthesis may not be attributable solely to an increase in ribosomal and m-RNA in muscle. To be sure, comparisons of sucrose gradients of ribosomes from growth hormone-treated and control hypophysectomized rats (Figure 1) can be interpreted to indicate that there is an appreciable difference in the m-RNA content of these preparations. However, the difference does not appear to be as great as that observed in our previous studies with testosterone propionate in castrated rats (see Figure 2 in Breuer and Florini, 1965). Evaluation of the quantitative significance of this difference is not-easy. Although it is subjectively obvious that the proportion of polyribosomes in preparations from muscle of growth hormone-treated hypophysectomized rats is greater than in controls, measurement of areas under the curves indicates that the percentage of ultraviolet-absorbing material in the polyribosome region is 83.5 and 79.5%, respectively. This difference certainly does not seem sufficient to account for the 57% greater activity of the ribosomes from hormone-treated rats (Table II, 18 hr). Furthermore, calculations based on areas under various portions of the curve are complicated by the possibility that an unknown amount of the ribosome monomers may be associated with m-RNA and thus be capable of protein synthesis. Korner (1965b) has suggested that growth hormone may control the translation of m-RNA as well as its synthesis; thus, directly or indirectly, it might affect the inherent activity of individual ribosomes in addition to changing the amount of available m-RNA and thus the proportion of polyribosomes. Evidence for various kinds of translational control is accumulating rapidly. Rampersad and Wool (1965) have reported stimulation by insulin of heart muscle ribosomes which could not be attributed to changes in m-RNA content of the preparation. Differences in activity of yeast ribosomes at various points in the growth curve (Lucas *et al.*, 1964) were also unrelated to availability of m-RNA (Dietz *et al.*, 1965). The results of Gross and Cosineau (1963), Monroy *et al.* (1965), Salb and Marcus (1965), Garren *et al.* (1964), Kenney and Albritton (1965), and Gorski and Padnos (1966) can all be interpreted to indicate that translation of existing m-RNA can be controlled in a variety of tissues.

Reports of studies with other tissues led us to expect that growth hormone would stimulate the synthesis of RNA in muscle. Korner (1959, 1961, 1964) demonstrated that treatment of hypophysectomized or normal rats with growth hormone stimulated amino acid incorporation into protein by cell-free preparations from rat liver. The principle conclusion from these studies was that growth-hormone-stimulated protein synthesis as a result of the stimulation of RNA synthesis (Korner, 1965a); direct evidence for the stimulation of RNA synthesis in isolated liver nuclei following injection of growth hormone has recently been presented by Pegg and Korner (1965). Data from other laboratories (Talwar *et al.*, 1962, 1964; Cantarow *et al.*, 1958) indicate that this effect of growth hormone on RNA synthesis can also be detected *in vivo*.

Our studies on hormone effects in muscle have followed the path established by Korner and in general our observations are similar to his. Indeed, Korner (1965b) has reported that hypophysectomy caused changes in cardiac muscle (Earl and Korner, 1965) similar to those reported for liver. In addition, we have measured ribosome yield as well as activity and found that yield also increased upon administration of growth hormone (Tables I and II). The suggestion by Stent (1964), Gros (1965), and Shin and Moldave (1966) that synthesis of messenger may be regulated by ribosome content make this correlation of ribosome yield and activity (Table II) particularly striking. Hormone-induced increases in ribosome content of target tissues have been observed by several groups (Moore and Hamilton, 1964; Greenman and Kenney, 1964; Tata, 1965; Szirmai and Van der Linde, 1965).

Unexpectedly, the changes observed in RNA polymerase activity did not appreciably precede similar increases in ribosome activity (See Table II). In other cases, it was found that increases in RNA synthesis occurred earlier than those in protein synthesis (*e.g.*, see Hamilton, 1964; Noteboom and Gorski, 1963). Our results may be explained by the suggestion that the interval between determinations greatly exceeded the half-life of m-RNA, which according to Manchester (1964) is only 2–3 hr in diaphragm muscle. Considering the relatively long half-life of m-RNA in liver (Munro and Korner, 1964; Staehelin *et al.*, 1963), a tissue in which the turnover of protein is much more rapid than in muscle, it is surprising that muscle m-RNA might have such a short half-life.

Our initial premise that all effects of growth hormone in muscle could be attributed to increased synthesis of m- (and perhaps ribosomal) RNA which in turn directed the synthesis of those enzymes required for the other effects of growth hormone now seems to be a great oversimplification; changes in m-RNA synthesis may even be a secondary effect of growth hormone (Korner, 1965c). The increase in activity of the aggregate enzyme we observed occurred later than the increase in amino acid uptake measured either *in vitro* (Kostyo *et al.*, 1959) or *in vivo* (Riggs and Walker, 1960). However, Kostyo (1964) showed that protein synthesis could be increased in diaphragm muscle under conditions in which there was no increase in amino acid uptake. Apparently these are either separate primary effects of growth hormone or secondary results of a single primary action of the hormone.

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