Actions of Insulin-Potentiating Peptides on Glycogen Synthesis

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SUMMARY

The action of urinary and synthetic AcG (acceleratory factor from growth hormone) peptides was studied in vitro and in vivo. Both peptides were inactive alone and active only in the presence of insulin to enhance glucose uptake, glycogen synthesis, and glycogen synthase conversion to the active *I* form in vitro and in vivo. Responses were dependent on both peptide and insulin concentrations in a dose-dependent manner. No response was obtained with glucose alone, but the presence of glucose did enhance the response of insulin alone or insulin in the presence of peptide. It is concluded that both AcG peptides enhance either the effective concentration or the activity of insulin at its site of action. DIABETES *25*:413-19, May, 1976.

Bornstein and Hyde isolated a peptide from human pituitary glands[1] and from human growth hormone[2] that accelerated glucose uptake by isolated rat hemidiaphragms, which they termed "AcG" (*Ac*celeratory factor from *g*rowth hormone). Structural studies[3] indicated that AcG was a fragment of the amino terminal sequence of growth hormone. Peptides with similar structures were then synthesized and shown to have similar physical properties and in vitro biologic activities as those derived from growth hormone.[4] Studies in vivo demonstrated that the synthetic peptide corresponding to the first 15 residues of the amino terminal sequence of human growth hormone had an insulin-potentiating action. The synthetic pentadecapeptide (S-AcG) produced hypoglycemia in laboratory animals without any stimulation of release of immunoreactive insulin. The mechanism of action suggested at that time was one related to increased glucose transport and oxidation or to enhanced glycogenesis or to both.[5]

A peptide with similar biologic effects as the synthetic pentadecapeptide (S-AcG) was then isolated from human urine.[6] Insulin tolerance tests on rabbits with the urinary peptide (U-AcG) showed an increased sensitivity to the action of insulin.

In the present study, experiments were undertaken to examine the effects of the synthetic peptide (S-AcG) and the urinary peptide (U-AcG) on glucose uptake and on glycogen synthesis. Since it has been well established that insulin stimulates glycogen synthesis by increasing the glucose-6-phosphate independent (*I*) form of glycogen synthase,[7] we considered it of interest to determine whether the peptides directly potentiated the action of insulin to effect glycogen synthase conversion (shift from *D* to *I* form). The findings of the present study are consistent with the previous observations[1,5,6] and show for the first time that both peptides stimulate glucose uptake, glycogen synthesis, and enhanced glycogen synthase conversion only in the presence of insulin. The peptides alone were without effect. The activating effect of the peptides in the presence of insulin did not depend on the presence of glucose under our experimental conditions but was nevertheless further stimulated in its presence.

METHODS AND MATERIALS

Male Wistar rats (120-150 gm.) were kept in our animal house under a synchronized feeding program for at least two weeks before use. The program of feeding the animals ad libitum on Purina laboratory

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Accepted for publication January 16, 1976.

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chow only from 7:30 A.M. to 4:30 P.M. appeared to reduce considerably the variation of the experimental results.

Following decapitation, the hemidiaphragms were immediately dissected out and preincubated in small plastic beakers containing Krebs-Ringer bicarbonate buffer (2 ml.) with or without the peptides in a metabolic shaker at 37° under constant gassing with 95 per cent O_2-5 per cent CO_2. After 30 minutes of preincubation, the hemidiaphragms were transferred to a second set of beakers containing the same buffer, together with the additions as indicated in various experiments (insulin, peptide, etc.) and incubated for a further period of 30 minutes. Glucose was added at a concentration of 5 mM unless otherwise indicated. The experiments were terminated by rapidly freezing the tissues in liquid nitrogen. The frozen tissues were then powdered in a percussion mortar prior to ^{14}C-glycogen estimations and glycogen synthase assays. Aliquots of the incubation medium were removed after incubation for ^{14}C-glucose analysis. ^{14}C-glycogen and glycogen synthase were assayed in duplicate, glucose uptake in triplicate.

For the studies of the effect of the peptides in vivo on glycogen synthesis in rat skeletal muscle, rats were injected intramuscularly with 0.2 ml. of S-AcG (6 mg./kg.) or U-AcG (7.5 mg./kg.) 60 minutes before the experiment. To the control animals, the same volume of saline was injected under the same experimental conditions. All injections were made into the muscular region of either hind leg of the animals.

In the studies in vivo, 60 minutes after the peptide injection, hemidiaphragms were dissected out and either immediately frozen in liquid nitrogen and powdered for glycogen synthase assays or preincubated in buffer for 30 minutes and then incubated in the presence or the absence of insulin as described above. Glycogen synthase activity and glucose uptake were measured with the same procedures as for the studies in vitro.

Powdered diaphragms were extracted with 10 volumes (w/v 1:10) of 100 mM KF-10 mM EDTA pH 7.8 and assayed for glycogen synthase according to Thomas et al.[8] Analyses of ^{14}C-glucose uptake and determination of ^{14}C-glycogen content of rat hemidiaphragms were performed by methods described by Miller and Larner.[9]

The effect of U-AcG on plasma glucose responses in rats was studied by performing an intravenous glucose tolerance test on the animals as described in a previous paper[5] after prior (60-minute) intramuscular injection of U-AcG.

Plasma glucose was estimated by the ferricyanide procedure[1] as adapted for use in the Technicon AutoAnalyzer.

The U-AcG was isolated from urine of normal human volunteers as reported by Ng et al.[6]

The S-AcG (HGH 1-15) was synthesized by the solid-phase technic as described by Stewart and Young[11] and was the generous gift of Prof. J. Bornstein, Department of Biochemistry, Monash University School of Medicine, Melbourne, Australia. Crystalline insulin, specific activity 25 U./mg. was a gift from NOVO Research Institute, Copenhagen, Denmark. UDP-glucose (U^{14}C) was prepared from glucose (U^{14}C) according to the method of Thomas et al.[8]

All experimental data are expressed as mean ± standard deviation. The significance of differences between means was established by Student's *t*-test.

RESULTS

It is well established that glucose uptake, glucose imcorporation into glycogen, and glycogen synthase activation in rat skeletal muscle are stimulated by insulin.[7,9,12] Under our experimental conditions, 2 mU./ml. (an intermediate but not maximal dose; see figures 3 and 4) of insulin produced a significant increase over the control values in all the above three parameters as indicated in figures 1 and 2 (compare basal values in figure legends with corresponding values in figures 1 and 2). For example, this amounted to a 59 per cent stimulation in the case of glucose uptake, a 94 per cent stimulation in the case of glycogen synthesis, and a 31 per cent stimulation of per cent *I* form of glycogen synthase (calculated as percentages over basal values). The data of the S-AcG and U-AcG dose-response curves illustrated in these figures demonstrate that the synthetic and the urinary peptides enhance insulin action on glucose uptake in vitro, on glycogen synthesis, as well as on glycogen synthase conversion from *D* to *I* form in a dose-dependent manner, which is statistically significant. In the absence of insulin, neither S-AcG (100 μg./ml.) nor U-AcG (240 μg./ml.) had any observable effect on any of the three parameters studied (see also table 1, figures 3 and 4). In the case of glycogen synthase conversion, for example, in the presence of 2 mU./ml. of insulin, S-AcG at a concentration of 200 μg./ml. further increased per cent *I* form glycogen synthase from 46.7 ± 2.4 to 52.2 ± 2.6 and U-AcG at 360 μg./ml. further increased per cent *I* form of the en-

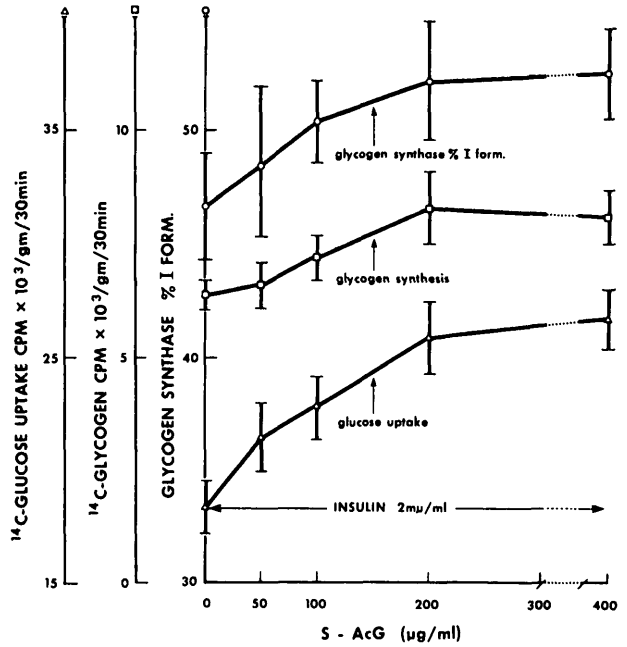

FIG. 1. Dose response curve of S-AcG action in presence of insulin on diaphragm in vitro. Three animals were used per group in each experiment, and two separate experiments were performed. Incubation of diaphragms and analysis of glucose uptake, ^{14}C-glycogen synthesis, and per cent glycogen synthase *I* form were carried out as described in Methods and Materials. Peptide was added during both 30-minute incubation periods. Glucose and insulin were added to all flasks during the second incuabtion period only. Values for basal glucose uptake were as follows: minus insulin minus S-AcG, 11.5 ± 1.6 cpm/gm. tissue/30 min. × 10^3; minus insulin plus 100 μg./ml. S-AcG, 10.8 ± 0.9 cpm/gm. tissue/30 min. × 10^3. Values for basal ^{14}C-glycogen synthesis were as follows: minus insulin minus S-AcG, 3.3 ± 0.3 cpm/gm. tissue/30 min. × 10^3; minus insulin plus 100 μg./ml. S-AcG, 3.4 ± 0.2 cpm/gm. tissue/30 min. × 10^3. Values for basal glycogen synthase per cent *I* form were as follows: minus insulin minus S-AcG, 35.8 ± 1.2 (cpm/gm. tissue/30 min. × 10^3); minus insulin plus 100 μg./ml. S-AcG, 37.2 ± 1.7. To convert glucose uptake to mg. glucose/gm. tissue/30 min., divide by 10.

zyme from 45.3 ± 1.7 to 50.5 ± 2.0. Double the concentration of the synthetic or urinary peptides—i.e. 400 μg./ml. (S-AcG), or 720 μg./ml. (U-AcG) activated the enzyme system to a minor extent (U-AcG) or not further at all (S-AcG). The findings are consistent with the previous observation at the physiologic level in the whole animal that an increased dose of peptide over the maximal dose did not induce any further decrease of plasma glucose concentration.[5,6]

When insulin concentration was varied and S-AcG and U-AcG were held constant at concentrations of 100 μg./ml. and 240 μg./ml., respectively, again the three parameters of glucose uptake, glycogen synthesis, and per cent *I* form of glycogen synthase were increased in a dose-dependent manner (figures 3 and 4). S-AcG at a concentration of 100 μg./ml. produced a further 7.3-10.4 per cent absolute increase of per cent synthase *I* form (figure 3-A) and U-AcG (240 μg./ml.) a further absolute 8.4-14.3 per cent increase (figure 4-A). No effect of either peptide was observed in the absence of insulin on any of the three indices studied. Under these conditions, as insulin concentrations were increased, the stimulatory effect of S-AcG on glucose uptake (figure 3-C) was brought to the same level as that observed in the presence of insulin alone. A similar trend, although not as marked, was noted with U-AcG (figure 4-C). By contrast, the effects of S-AcG and U-AcG on ^{14}C-glycogen synthesis and on glycogen synthase conversion were observed to increase at an insulin concentration of 8 mU./ml. and above (figures 3-A and B and 4-A and B).

From the evidence so far obtained, the effect of both S-AcG and U-AcG on glucose uptake, glycogen synthesis, and glycogen synthase conversion in vitro was clearly insulin-dependent. However, it was not

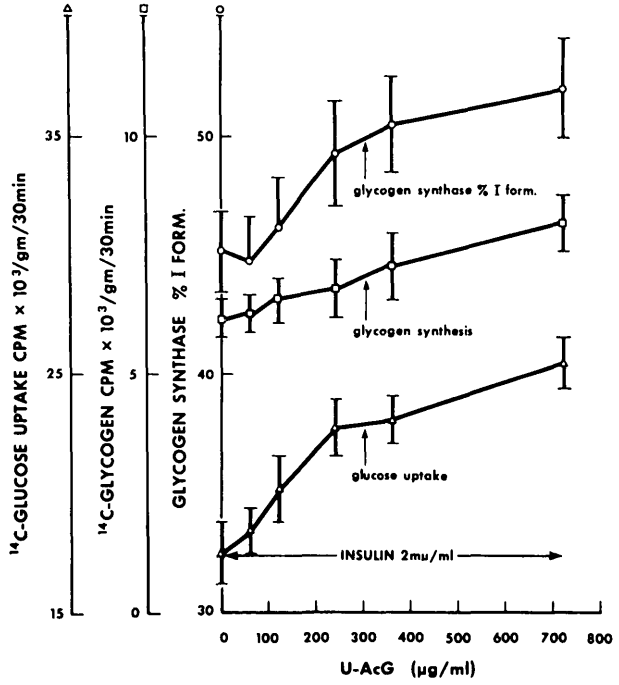

FIG. 2. Dose-response curve for U-AcG action in presence of insulin on diaphragm in vitro. Same number of animals, experiments, and conditions as figure 1. Additions were as described in figure 1. Values for basal glucose uptake were as follows: minus insulin minus U-AcG, 10.6 ± 0.8 cpm/gm. tissue/30 min. × 10^3; minus insulin plus 240 μg./ml. U-AcG, 11.2 ± 1.2 cpm/gm. tissue/30 × 10^3. Values for basal ^{14}C-glycogen synthesis were as follows: minus insulin minus U-AcG, 3.4 ± 0.4 cpm/gm. tissue/30 min. × 10^3; minus insulin plus 240 μg./ml. U-AcG, 3.3 ± 0.3 cpm/gm. tissue/30 min. × 10^3. Values for basal glycogen synthase per cent *I* form were as follows: minus insulin minus U-AcG, 34.3 ± 1.3; minus insulin plus 240 μg./ml. U-AcG, 36.4 ± 1.7.

TABLE 1

Effect of glucose, insulin, and peptides on glycogen synthase conversion in rat hemidiaphragms in vitro

Experiment*	Substances added to incubation buffer				Synthase per cent *I* form (mean ± S.D.)
	Glucose (900 μg./ml.)	Insulin (2 mU./ml.)	S-AcG (100 μg./ml.)	U-AcG (240 μg./ml.)	
A	−	−	−	−	34.2 ± 1.5
B	+	−	−	−	33.5 ± 1.8
C	−	+	−	−	38.9 ± 2.2
D	+	+	−	−	45.0 ± 1.5
E	−	−	+	−	34.9 ± 1.0
F	+	−	+	−	35.5 ± 2.4
G	−	+	+	−	45.5 ± 2.6
H	+	+	+	−	49.1 ± 1.3
I	−	−	−	+	34.8 ± 2.3
J	+	−	−	+	34.9 ± 2.1
K	−	+	−	+	43.4 ± 1.7
L	+	+	−	+	49.3 ± 2.2

*Three animals were used per group in each experiment, and at least two to nine separate experiments were performed. Methods for diaphragm incubation and enzyme assay were as described under Methods and Materials. Peptide when added was present during both 30-minute incubation periods. Insulin and glucose when added were present during the second incubation period only. A vs. C, p value <0.02; C vs. D, p value <0.009; E vs. G, p value <0.008; G vs. H, p value <0.09, N.S.; I vs. K, p value <0.06; K vs. L, p value <0.05.

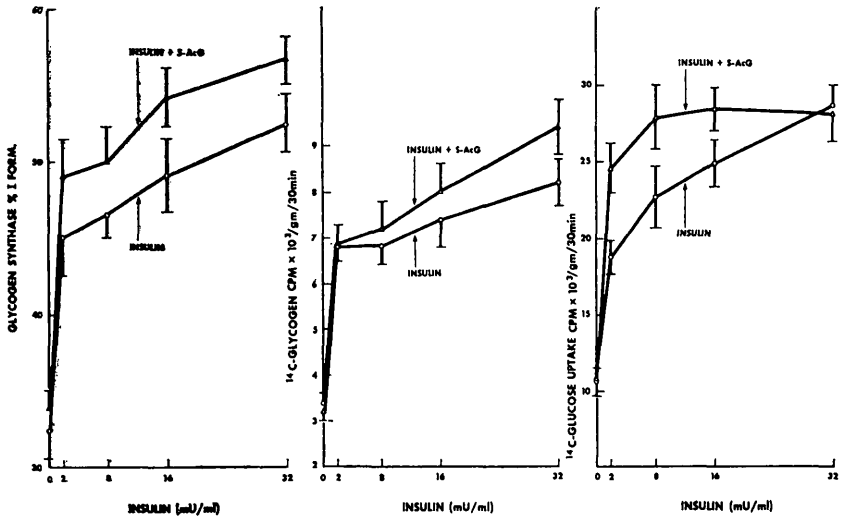

FIGURE 3

Insulin dose-response curve in presence of S-AcG on diaphragm in vitro. Two hemidiaphragms were incubated per flask per point in each experiment. Two separate experiments were done. Other conditions as in figures 1 and 2. Peptide, when present, was added during both 30-minute incubation periods. Glucose and insulin were added to all flasks during the second incubation period only.

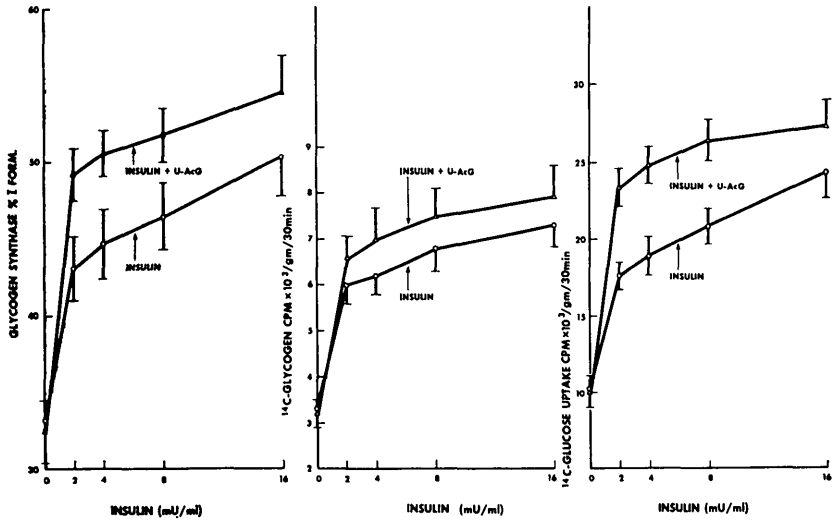

FIGURE 4

Insulin dose-response curve in presence of U-AcG on diaphragm in vitro. Same number of hemidiaphragms, experiments and conditions as in figure 3. Additions were as described in figure 3.

TABLE 2

Insulin-potentiating effect in vitro of peptides injected in vivo on glucose uptake and glycogen synthase conversion in rat hemidiaphragms*

Treatment in vivo	Glucose uptake (cpm/gm. tissue/30 min.) Insulin in vitro			Glycogen synthase (per cent *I* form) Insulin in vitro		
	(0 mU./ml.) (mean ± S.D.)	(2 mU./ml.) (mean ± S.D.)	P value	(0 mU./ml.) (mean ± S.D.)	(2 mU./ml.) (mean ± S.D.)	P value
Saline injection	10.8 ± 1.0 ($\times 10^3$)	17.3 ± 1.2 ($\times 10^3$)	<0.001	33.5 ± 2.0	46.2 ± 1.5	<0.001
S-AcG injection	12.1 ± 1.2 ($\times 10^3$)	20.5 ± 1.3 ($\times 10^3$)	<0.001	47.5 ± 2.8†	53.4 ± 2.9§	<0.035
U-AcG injection	11.9 ± 1.1 ($\times 10^3$)	21.9 ± 1.3 ($\times 10^3$)	<0.001	49.2 ± 3.1‡	56.0 ± 2.4¶	<0.013

*Three animals were used per group in each experiment, and two separate experiments were performed. Methods for injection of peptides, diaphragm incubation, glucose-uptake analysis, and enzyme assays were as described under Materials and Methods. Insulin and glucose were added during the second incubation only.

†Significantly different from 33.5 ± 2.0, P <0.001.
‡Significantly different from 33.5 ± 2.0, P <0.001.
§Significantly different from 46.2 ± 15, P <0.004.
¶Significantly different from 46.2 ± 15, P <0.001.

clear whether glucose was necessary for observing the effects on synthase conversion. Further experiments were undertaken to elucidate the role of glucose, and the findings are shown in table 1. As can be seen, glucose alone (without insulin) in a concentration of 900 μg./ml. was ineffective in promoting the conversion of glycogen synthase from *D* to *I* form (compare A and B). Insulin alone increased per cent *I* form from 34.2 ± 1.5 to 38.9 ± 2.2 (compare A and C). However, glucose in the presence of insulin produced a further stimulation from 38.9 ± 2.2 to 45.0 ± 1.5 per cent *I* (compare C and D).

Similar results were observed with the S-AcG and U-AcG. Neither S-AcG nor U-AcG alone (without insulin) had any effect (compare A and E, and A and I). Glucose together with S-AcG or U-AcG, in the absence of insulin, again was ineffective (compare E and F, and I and J). However, peptides together with insulin were stimulatory (compare E and G, and K and I). Addition of glucose to the insulin-peptide combination caused further stimulation (compare K and L).† Glucose alone thus appeared to play no demonstrable role in the activation of this enzyme system, in keeping with previous results in muscle.[13] However, in the presence of insulin, as well as in the presence of insulin plus peptide, glucose did further enhance the effect of insulin on synthase activation.

Experiments were next designed to determine if S-AcG or U-AcG elicited similar effects in vivo. One hour after intramuscular injection of the urinary peptide (7.5 mg./kg.) a hypoglycemic effect was observed in rats during glucose tolerance tests that was similar to the effect previously observed after intravenous injection of the peptide.[6] When hemidiaphragms were removed from treated rats, immediately frozen in liquid nitrogen, powdered, extracted, and assayed for glycogen synthase, the per cent *I* form of synthase of U-AcG-treated animals increased from 24.4 ± 0.9 to 31.4 ± 1.3 over controls injected with saline. S-AcG exhibited a similar effect in vivo and increased the per cent *I* form of synthase to 29.9 ± 1.2. Both effects were statistically significant at a P value <0.001. When the isolated hemidiaphragms from treated rats were incubated under the identical experimental conditions as the initial studies in vitro, the effects in vivo of the two peptides as well as the insulin-potentiating effect of both peptides on glycogen synthase conversion in vitro was also evident, as shown in table 2. For example, for the effect in vivo compare 47.5 ± 2.8 and 49.2 ± 3.1 per cent *I* form for the animals injected with peptides vs. 33.5 ± 2.0 per cent *I* form for the controls. For the effect of insulin in vitro compare 33.5 ± 2.0 with 46.2 ± 1.5; 47.5 ± 2.8 with 53.4 ± 2.9; and 49.2 ± 3.1 with 56.0 ± 2.4 per cent *I* form without and with insulin respectively. The insulin-potentiating effect in vitro on glucose uptake by hemidiaphragms from treated animals was also clearly demonstrated. For example, compare 10.8 ± 1.0 with 17.3 ± 1.2; 12.1 ± 1.2 with 20.5 ± 1.3; and 11.9 ± 1.1 with 21.9 ± 1.3. However, no effect in vivo on glucose uptake was demonstrated under these experimental conditions, again in keeping with a dissociation in the control by insulin of glucose uptake and glycogen synthesis.

†Although with U-AcG the effect was statistically significant, in the case of S-AcG this effect was not statistically significant because of the small numbers of experiments.

DISCUSSION

Bornstein et al.[14] carried out detailed studies on the sites of action of a number of natural and synthetic peptides on the various glycolytic, tricarboxylic-acid cycle, and fatty-acid-synthesizing enzymes and showed that the synthetic peptide HGH 1-15 (S-AcG) and the urinary peptide (U-AcG) had no action in vitro on any of the enzymes tested. This study indicates that both S-AcG and U-AcG in the presence of insulin promoted glycogen synthase activation and provides the first evidence that the peptides enhanced the insulin-mediated glycogen synthase shift (from *D* to *I* form) in vitro and in vivo. This action is independent of glucose, although the presence of glucose does stimulate the effect with insulin in the absence as well as the presence of the synthetic or urinary peptides.

In view of the present findings, the hypoglycemic effect of both S-AcG and U-AcG observed in studies in vivo previously reported can be explained at least in part by the increase of glycogen synthesis in skeletal muscle (perhaps also in other insulin-sensitive tissues) through the activation of glycogen synthase by the peptides.

Data presented in this paper also confirm and extend the observations that the actions of both S-AcG and U-AcG in vitro and in vivo are dependent on the presence of insulin and that both peptides trigger the same kind of regulatory mechanism in the systems studied. With the other published data, evidence is still insufficient to establish that the urinary peptide is either identical with the synthetic material or that it even contains the same minimum primary informational sequence. The conclusive evidence to establish the identity of the urinary peptide can be obtained only from structural study of the molecule, and such studies are now in progress.

It is well documented that the mechanism of insulin action on the glycogen-synthesizing system is to effect the activation of glycogen synthase (shift from *D* to *I* form) as well as the inactivation of synthase kinase inactivation (shift from *I* to *D* form).[15] However, preliminary data from this laboratory‡ indicate that both the S-AcG and U-AcG appeared to have no direct effect in vitro on the activity of the purified protein kinase system.

Therefore, a possible explanation of the data obtained is that the peptides stabilize the insulin molecule so that its effective concentration at its biologic site of action is increased or, alternatively, that they enhance the action of the insulin present at a given concentration. No decision between these two alternatives can be made from the present studies. What is clear, however, is that insulin is required for their action and glucose is not. The fact that glucose is not required is in keeping with previous observations in muscle and in liver as well.[13,16] Even though it is not required, it is stimulatory in the system, suggesting that it may play an accessory role, perhaps an intracellular one.

This present work documents a second peptide that is able to modify the insulin-mediated glycogen-synthase cascade. The other is the higher-molecular-weight pituitary diabetogenic peptide.[9] In contrast to the present peptides studied, the pituitary diabetogenic peptide was able to block the action of insulin.

‡Unpublished experiments of F. Ng, R. Hazen, and J. Larner.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service Grant 1 P17 AM17042-01, the University of Virginia Diabetes-Endocrinology Research Center; American Diabetes Association, Inc., and Eli Lilly, Indianapolis, Indiana.

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