Insulin Potentiating Action of a Peptide Fraction from Human Urine

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SUMMARY

An impure peptide preparation from human urine has been shown to have similar biological effects in vitro and in vivo to synthetic peptides containing the human growth hormone sequence Leu-Ser-Arg- Leu-Phe-Asp- Asn-Ala (residues 6-13). The urinary preparation potentiates the action of insulin in the isolated rat diaphragm and in rabbits and rats, but the latter animals are far more sensitive to its action. Species specificity is possibly a significant factor in determining dosage in future studies. DIABETES *23*:950-56, December, 1974.

Preliminary reports from these laboratories presented evidence that a peptide-containing fraction isolated from human urine had hypoglycemic activity in intact animals, accelerated the utilization of glucose in isolated tissues, and was capable of reversing Somantin (InG) inhibition of glyceraldehyde 3-phosphate dehydrogenase (G3PD) and other specific enzymes.[1-3]

This communication reports a batch procedure for the isolation and partial purification of the peptide from human urine and its action in vitro and in vivo under conditions identical with those of active synthetic polypeptides related to the amino terminal sequence of human growth hormone (first communication).

METHODS AND MATERIALS

Isolation of the Peptide (U-AcG) from Urine

Urine specimens were collected from normal subjects and kept frozen until processing commenced. A sufficient amount of frozen specimens, corresponding to a three to four day output, was thawed, adjusted to pH 2.1 with hydrochloric acid, filtered through Whatman No. 1 paper to remove insoluble materials, and then ultrafiltered through a Diaflo UM10 membrane (Aminco Corp., U.S.A.) specified to retain proteins of molecular weight greater than 10,000 daltons at 4° and 7 kg./cm.^{2} pressure.

Dowex 50W-X2 resin (200-400 mesh) in the hydrogen form pre-equilibrated with 0.03 M acetic acid was added at 10 gm./liter ultrafiltrate and stirred for three hours. The resin was allowed to settle, the supernatant decanted off and discarded. The resin slurry was then repeatedly washed with 0.03 M acetic acid until the washings showed no absorption at 2,600 Å. The slurry was then suspended in 0.03 M acetic acid and poured into a column with a height diameter ratio of 10:1 and washed with a further 100 ml. of 0.03 M acetic acid. The column was then developed with 0.05 M triethylamine acetate buffer, pH 4.8. As the pH of the eluate changed slowly from 3.4 to 4.3, an irregular but essentially bifurcate peak was eluted (figure 1).

U-AcG activity was only detected in the front part of the peak. This part of the eluate was pooled and concentrated to 10 per cent of the original volume by ultrafiltration at 7 kg./cm.^{2} through a Diaflo UM05 membrane specified to retain proteins of molecular weight greater than 500 daltons. The ultrafiltrate was discarded, and the retentate made up to the original volume with distilled water. This process was repeated three times in order to reduce the buffer components to a minimum and to remove small-molecular-weight contaminants. This washing procedure is absolutely essential, as the presence of excess cations leads to a fall of pH below 3 due to proton release in the next step and, consequently, a failure of fractionation.

The concentrated, washed retentate was adjusted to

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Fractionation of the Urinary Peptide (U-AcG) on Dowex 50W-X2

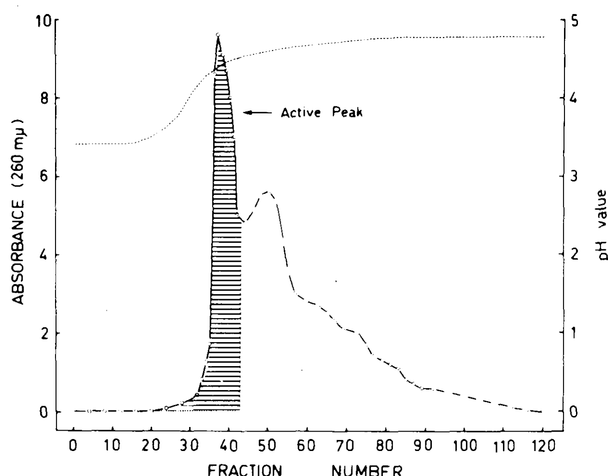


FIG. 1. The column (15 x 1.5 cm.) was eluted at room temperature with 0.05 M triethylamine acetate buffer, pH 4.8. The fractions (8 ml. each) of the front peak were pooled, concentrated and further purified as indicated in the text.

pH 6.8 with 0.5 NaOH and then chromatographed on a column of Amberlite CG50H+, (40 cm. x 1.5 cm.) equilibrated with 1 x 10^{-4} M glycylglycine. The active urinary fraction was eluted from the column during initial development with water being the main part of the peak showing absorption at 2,600 Å.

The eluted peak was pooled, concentrated and washed with water by ultrafiltration as previously described, being finally reduced to minimum handleable volume. The concentrated retentate was then chromatographed by gel filtration on a Sephadex G25 (superfine) column (50 x 3 cm.) equilibrated with 0.1 M ammonium bicarbonate, pH 7.8, and eluted with the same buffer. Two closely related peaks absorbing ultraviolet light at 2,600 A were observed at V_t. The front one of these containing all U-AcG activity was collected, freeze-dried and stored in vacuo. This procedure is summarized in table 1.

Analysis of homogeneity by two-dimensional high-voltage electrophoresis-chromatography has shown that this material consists of one major and several minor ninhydrin staining components, and preliminary chemical studies indicate contamination by urinary pigments and some carbohydrate.

Further purification studies have shown that the biological activity is confined to the peptide fraction, but so far no preparation has been pure enough for structural study, or extensive comparative physical studies.

As it has been found during the course of this and the previously reported study (accompanying paper) that only those preparations which are capable of reversing somantin inhibition of G3PD have hypoglycemic activity, this convenient technic has been used to monitor the purification procedure, although the physiological significance of this test is unknown.

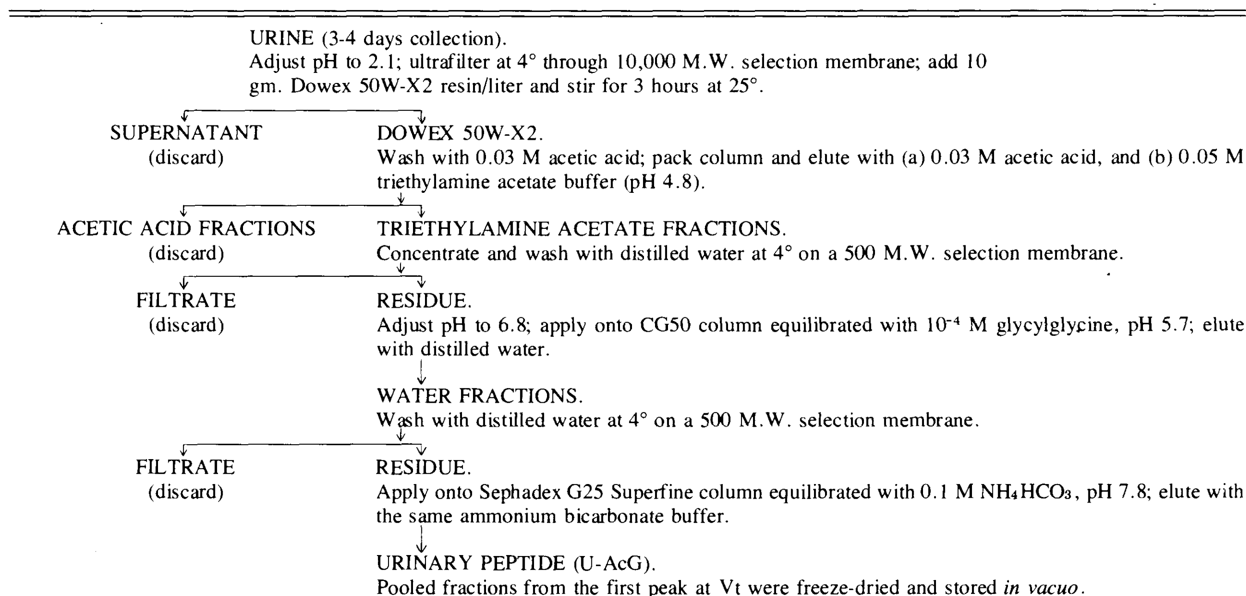
Tests of Biological Activity

a. *Reversal of somantin inhibition of G3PD*

All preparations have been tested in this assay system against somantins derived from ovine growth hormone,

TABLE 1

Procedure for isolation and partial purification of the urinary peptide (U-AcG)


URINE (3-4 days collection).
Adjust pH to 2.1; ultrafilter at 4° through 10,000 M.W. selection membrane; add 10 gm. Dowex 50W-X2 resin/liter and stir for 3 hours at 25°.

SUPERNATANT (discard)	DOWEX 50W-X2. Wash with 0.03 M acetic acid; pack column and elute with (a) 0.03 M acetic acid, and (b) 0.05 M triethylamine acetate buffer (pH 4.8).
ACETIC ACID FRACTIONS (discard)	TRIETHYLAMINE ACETATE FRACTIONS. Concentrate and wash with distilled water at 4° on a 500 M.W. selection membrane.
FILTRATE (discard)	RESIDUE. Adjust pH to 6.8; apply onto CG50 column equilibrated with 10^{-4} M glycylglycine, pH 5.7; elute with distilled water.
	WATER FRACTIONS. Wash with distilled water at 4° on a 500 M.W. selection membrane.
FILTRATE (discard)	RESIDUE. Apply onto Sephadex G25 Superfine column equilibrated with 0.1 M NH_4HCO_3, pH 7.8; elute with the same ammonium bicarbonate buffer.
	URINARY PEPTIDE (U-AcG). Pooled fractions from the first peak at Vt were freeze-dried and stored *in vacuo*.

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human plasma, human urine and the somantin-active synthetic polypeptide representing amino acids 167-191 of the human growth hormone sequence according to the method of Bornstein et al.[4]

b. *Glucose uptake by the isolated rat hemidiaphragm*

Glucose uptake by the isolated rat hemidiaphragm was carried out by the method of Park et al.[5] under the conditions previously described (accompanying paper).

c. *In vivo studies*

Rabbits. Intravenous glucose tolerance tests (IVGTTs) in rabbits of mixed stock (weighing 2.0-3.0 kg.) were carried out as previously described (accompanying paper). Intravenous insulin tolerance tests (IVITTs) were also performed on overnight-fasted animals at a dose of 0.1 unit insulin/kg. body weight, blood glucose concentrations being determined at time intervals as indicated in figure 7 for 150 minutes. Repeat IVGTTs or IVITTs were carried out on the treated animals three days after, without the injection of peptide.

Rats. Male albino rats of Wistar strain, weighing 300-350 gm., were used. The animals were kept in the laboratory for at least seven days and fasted for twenty-four hours prior to experiment. For IVGTTs, rats were anesthetized with a mixture of pentobarbital and phenobarbital (20 mg./kg. and 100 mg./kg. body weight, respectively). The anesthetic was injected intraperitoneally to the animals. After the fasting levels of blood glucose were established, 1 gm./kg. of a 50 per cent glucose solution was administered intravenously through the right femoral vein. When U-AcG was used it was given five minutes later into the left femoral vein. Blood samples for glucose estimation were taken from the tail vein at fifteen minute intervals for the first one and one-half hours and at thirty minute intervals for the next hour. For IVITTs, rats were anesthetized in the same manner. Crystalline insulin (0.1 unit/kg. body weight) was given intravenously immediately after the basal blood glucose level was established. When U-AcG was used it was given intravenously into a different site at the same time. Blood samples for glucose estimations were taken from the tail vein at time intervals as indicated in figure 7.

d. *Toxicity studies*

A formal toxicity trial was carried out at 12 mg. peptide/kg. body weight, and no difference was reported between the tissues of controls and experimental animals by Dr. P. Carbonell, of the Department of Veterinary Pathology, University of Melbourne.

e. *Analytic methods*

All blood glucose estimations were done by the ferricyanide technic as adapted for the Technicon AutoAnalyzer.

All other glucose estimations were done by the glucose oxidase method (Stat Pack, Calbiochem.).

Plasma immunoreactive insulin was determined by the method of Herbert et al.[6]

RESULTS

Enzyme assays

The follow-through of the purification procedure showed that the activity of U-AcG had the same pattern in reversing "somantin" inhibition of G3PD as other "cataglykin" (AcG) preparations from growth hormones and synthetic preparations reported as active (accompanying paper), having no direct effect on the enzyme. This occurred regardless of the source of somantin.

TABLE 2

Reversal by the urinary peptide (U-AcG) of glyceraldehyde 3-phosphate dehydrogenase (GAPD) inhibition by somantin from four different sources

	% Inhibition of GAPD		
Source of Somantin	(a) Somantin	(b) Somantin and U-AcG	%Reversal $\frac{(a)-(b)}{(a)} \times 100\%$
Ovine growth hormone	68.7	34.4	49.9
Human plasma	77.3	29.8	61.4
Human urine	44.5	21.5	51.7
Synthetic	51.2	16.4	68.0

The assays of the enzyme inhibition under each of the eight different conditions were performed in duplicate. Numbers in (a) and (b) represent the mean values of two assays. The concentration of somantin used for each assay was 25 μg/ml. for the reversal of somantin inhibition (b).

Hemidiaphragm studies

U-AcG was found to have no direct effect on the hemidiaphragm of fasted rats in spite of the large concentration used, but potentiated the action of insulin and reversed the inhibitory action of plasma "somantin" in the presence of insulin.

In vivo studies

Rabbits. The hypoglycemic effect of U-AcG was established in rabbits under the conditions of the IVGTT. It was observed that at a dose level of 6 mg./kg. a highly significant depression of blood glucose below the control and fasting levels occurred (figure 2).

A dose response curve was then established over a range of 0.5-12 mg./kg. body weight, and showed a linear dose response relationship to the fall of blood glucose below the fasting level over the range 3-6

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TABLE 3
Effect of the urinary peptide (U-AcG) and plasma somantin on glucose uptake of rat hemidiaphragms

Additional substances in the incubation buffer	Glucose uptake of rat hemidiaphragms (mg./gm. tissue/hr. ± S.E.M.)	Mean Difference	P
Glucose	2.14 ± 0.16 (30)		
		0.08	<0.3500
Glucose and U-AcG	2.22 ± 0.23 (15)		
Glucose and insulin	3.46 ± 0.19 (15)		
		0.77	<0.0025
Glucose, insulin and U-AcG	4.23 ± 0.21 (10)		
Glucose, insulin and plasma somantin	2.35 ± 0.15 (10)		
		0.81	<0.0025
Glucose, insulin, plasma somantin and U-AcG	3.16 ± 0.24 (10)		

The additional substances were present at the following concentration in the medium whenever required: Glucose 2 mg./ml., insulin 500 μU./ml., U-AcG 250 μg./ml., and plasma somantin 100 μg./ml. Figures in parentheses give the number of observations.

mg./kg., this being roughly parallel to that obtained with the active synthetic peptide HGH 1-15 but indicating an activity of about 60 per cent of that peptide (figure 3).

As with the synthetic peptides, depression of the plasma insulin response was only observed at very high doses of U-AcG (12 mg./kg.) despite hypoglycemia being induced by the long-term action of the peptide (figure 4).

Intravenous insulin tolerance tests on rabbits showed that U-AcG potentiated the effect of insulin at the initial test and that sensitivity to insulin continued to increase for at least three days after the injection of the peptide (figure 5).

Rat studies. The hypoglycemic effect of U-AcG was established in rats under the conditions of the IVGTT. It was observed that the rate of fall of blood glucose was greater in the U-AcG-treated animal and that the final level reached was significantly below the controls but, in contradistinction to rabbits, the blood glucose level did not fall below fasting under the conditions of the experiment (figure 6).

Insulin tolerance tests showed that the potentiation of insulin action by U-AcG was much more marked in rats than in rabbits, and occurred at a very much lower dose of the peptide (figure 7).

DISCUSSION

In the first instance, we would point out that when we use the trivial names Cataglykin (AcG) or Somantin (InG) we are not referring to specific structures, but to polypeptides having a certain defined activity in a variety of biological systems.[3]

Although we have so far been unable to purify the urinary peptide (U-AcG) sufficiently for effective physi-

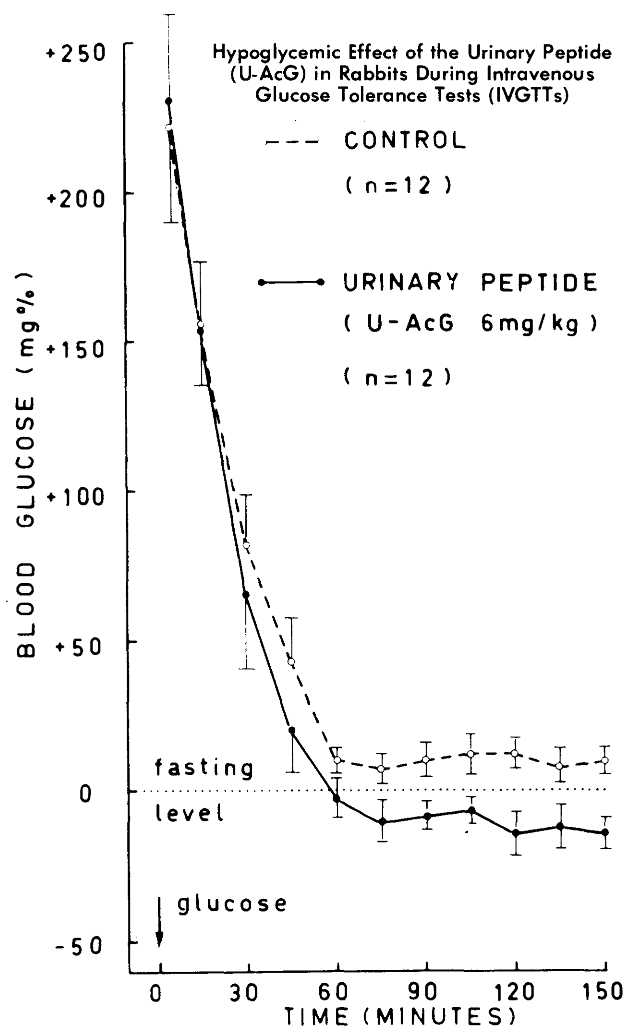

FIG. 2. The changes of blood glucose (mean ± S.D.) between the control (o) and the test (•) groups at each time were statistically evaluated according to Student *t* test: 5 min. (p <0.2500), 15 min. (p <0.4000), 30 min. (p <0.1500), 45 min. (p <0.0025), 60-150 min. (p <0.0005).

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Insulin Potentiating Effect of the Urinary Peptide (U-AcG) at Different Doses in Rabbits During Intravenous Glucose Tolerance Tests (IVGTTs)

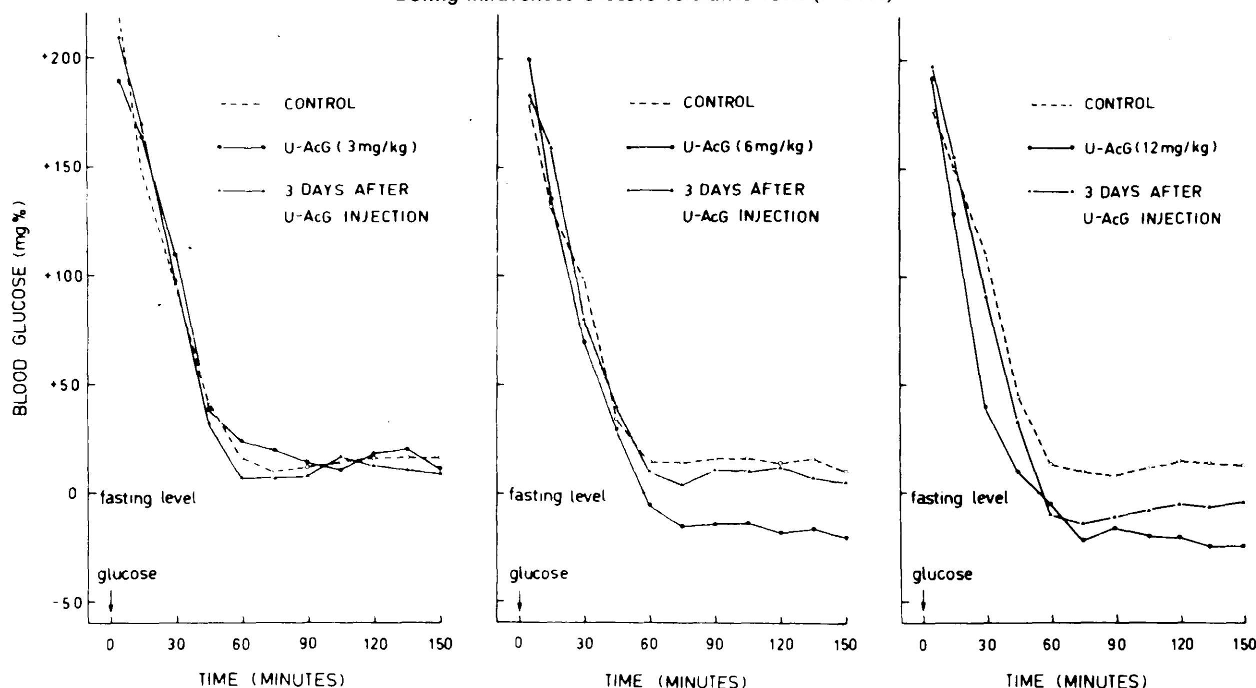

FIG. 3. Each point represents the mean blood glucose values of 5 rabbits. No significant difference observed at 3 mg. U-AcG/kg.; difference between control and 6 mg. U-AcG/kg. significant ($p < 0.05$) in first test from 60 min. to 150 min., but no significant difference 72 hours after treatment. Difference between control and 12 mg. U-AcG/kg. significant ($p < 0.05$) from 30 min. to 150 min. in first test and 60 min.-150 min. 72 hours after treatment.

cal or chemical study, there are some striking resemblances between this preparation and others having the same activity,[3] including the various synthetic peptides containing residues 6-13 of HGH (accompanying paper).

These lie in the similarity of charge, as all so far isolated, whether from HGH, OGH or urine, behave as neutral peptides on carboxylic resins such as IRC 50H+,[2,3] elute from Sephadex G25 at Vt in 0.1 M acetic acid and in the apparently identical pattern of biological activities on in vitro systems[7] and, most importantly, in vivo, as reported in this paper. Unfortunately, due to shortage of materials, it proved impossible to carry out insulin tolerance tests with synthetic peptides.

However, this body of evidence is not sufficient to establish that the urinary peptide is either identical with the other peptides or that it even contains the same minimum *primary* informational sequence. A parallel may be drawn between the data presented in these papers and the fact that although epinephrine, glucagon and corticotrophin have strikingly different structures, they all stimulate lipolysis through the same mechanism, and that although there are extensive amino acid substitutions between bovine and guinea pig insulins,[8] both are active insulins producing hypoglycemia and have the same dimeric tertiary structure, as shown by x-ray crystallography by Hodgkin.[9]

Thus, all that can be said at the present time is that U-AcG and the various growth hormone-derived "cataglykins" and their synthetic analogs trigger the same kind of mechanisms in the systems tested so far.

Again, as with sequences containing HGH 6-13 (first communication), a large dose of U-AcG is required to potentiate insulin in the rabbit and the rat; but the latter animal shows much greater sensitivity in the insulin tolerance test, pointing up the possibility that species specificity is a major problem in determining minimum dosage in these investigations. The fact that U-AcG requires an appreciably larger dose than the synthetic peptide HGH 1-15 may be due to the far greater purity of the synthetic material or to structural differences between them.

The long-term effect of U-AcG in rabbits parallels that observed with synthetic HGH 1-15, but again requires an appreciably higher dose. Again, depression of the IRI response is observed after three days.

In the absence of any evidence of toxic effects, it appears that a dual phenomenon has again been induced, particularly as in the IVITT in rabbits (figure 7) it can be seen that the response to insulin is far larger three days

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Plasma Insulin Responses to Two Different Dosages of the Urinary Peptide (U-AcG) in Rabbits During Intravenous Glucose Tolerance Tests (IVGTTs)

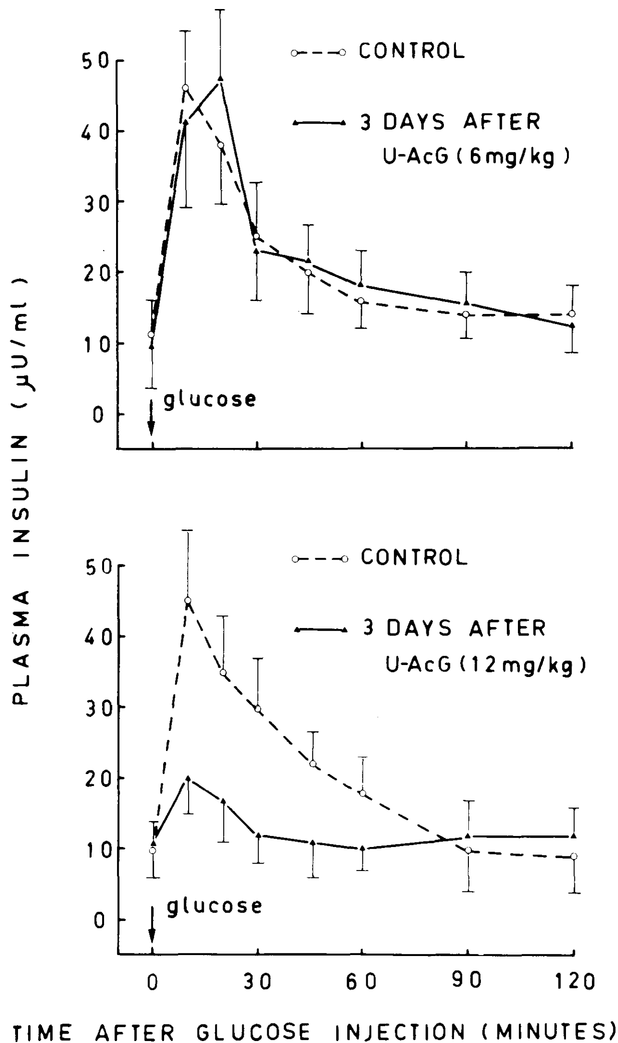

FIG. 4. Statistical analyses of the results (mean ± S.E.M.) show no significant differences at any time between the control IVGTTs and the test IVGTTs of five rabbits (n = 5) if 6 mg. U-AcG/kg. body weight were used. At high dose (12 mg./kg.) of the peptide the plasma insulin levels of the animals (n = 5) are shown to be depressed significantly at: 10 min. ($p < 0.0250$), 20 min. ($p < 0.0750$), 30 min. ($p < 0.0250$), 45 min. ($p < 0.0500$), and 60 min. ($p < 0.1000$).

after the injection of U-AcG than in the initial test.

In conclusion, these results show that a peptide capable of increasing sensitivity to insulin is excreted in the urine and may be isolated and partially purified from that biological fluid. Further, by comparison with other published data, it can be seen that its biological effects in vitro and in vivo are virtually identical with peptides representing certain sequences of the amino terminal end of human growth hormone, and raise the possibility that the deficiency of production of such a peptide may lead to a decrease in the sensitivity to insulin.

Effect of the Urinary Peptide (U-AcG) in Rabbits During Intravenous Insulin Tolerance Tests (IVITTs)

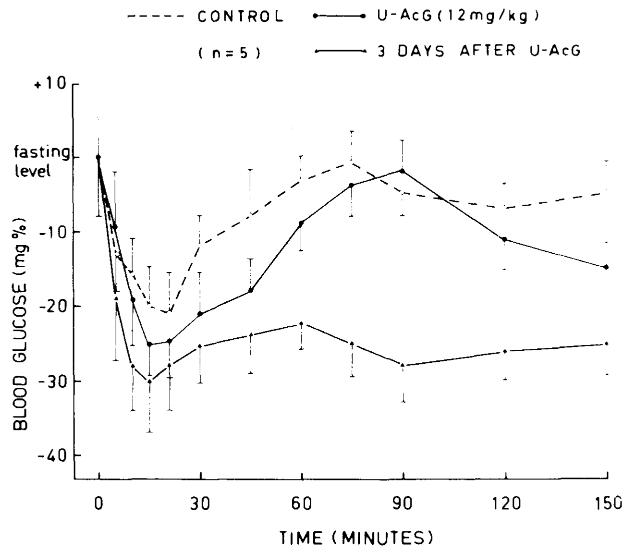

FIG. 5. Each point represents the blood glucose (mean ± S.E.M.) below the fasting levels of a group of five animals during IVITTs. Results show the urinary peptide at high dose (12 mg./kg. body weight) significantly potentiating the insulin action three days after U-AcG injection. The differences between controls and U-AcG-treated are significant ($p < 0.05$) at 30, 45 and 60 min. in the initial test, and from 30-150 min. 72 hours after treatment.

Preliminary studies on the excretion pattern of U-AcG in normal humans and various pathological states indicate that the excretion is low in hypophysectomized patients and during ketoacidosis as compared with normal volunteers (Seiler, Taft, Ng and Bornstein—unpublished data).

Work is at present in progress on the purification of U-AcG with a view to determining its structure, and the studies on the excretion patterns are being extended.

ACKNOWLEDGMENT

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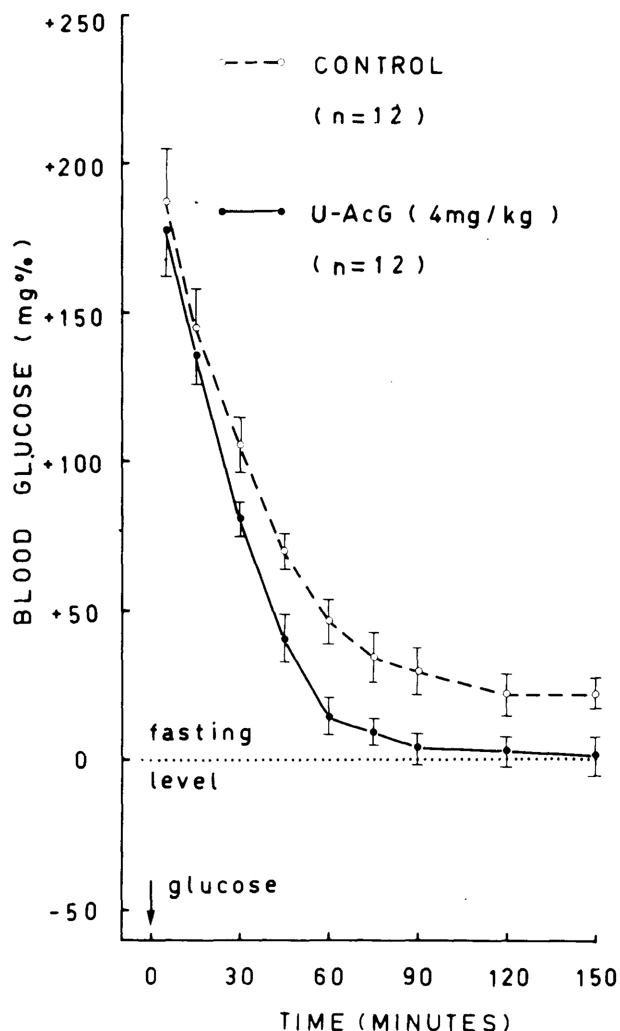

FIG. 6. The changes of blood glucose (mean ± S.D.) between the control (o) and the test (•) groups at each time were statistically evaluated and significantly different: 15 min. ($p < 0.0500$), and 30-150 min. ($p < 0.0005$).

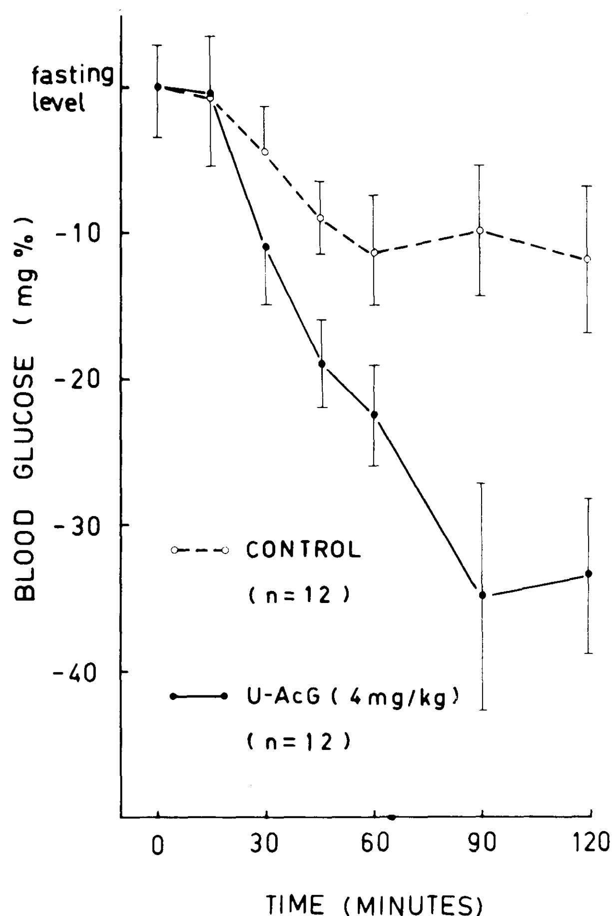

FIG. 7. Each point represents the blood glucose (mean ± S.E.M.) below the fasting level of twelve animals. In comparison with the control (o) and the test (•) groups, the insulin potentiating action of the peptide is shown to be statistically significant ($p < 0.0050$) from 30 min. after the commencement of the experiments. The long-term effect in rats has not been investigated.

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