

Inactivation of adrenocorticotropin, α - and β -melanocyte-stimulating hormones, vasopressin, and pituitary fraction H by adipose tissue

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SUMMARY Rat adipose tissue homogenate contains a peptidase or group of peptidases which abolish the adipokinetic activity of ACTH, α -MSH, β -MSH, vasopressin, and pituitary fraction H. The peptidase system is attached to an insoluble component in the homogenate. Homogenized rabbit adipose tissue inactivates only α -MSH, and homogenized guinea pig adipose tissue does not reduce the adipokinetic activity of any of the above hypophyseal peptides. Operation of the peptidase system in surviving slices of rat adipose tissue is suggested by these characteristics of this tissue: (a) rapid disappearance of adipokinetic activity from peptide-containing incubation medium; (b) absence of response to β -MSH, vasopressin, or fraction H; (c) cessation of response to ACTH within 1 hr after exposure to the peptide is terminated. In contrast, slices of adipose tissue from the rabbit or guinea pig do not cause the disappearance of a detectable amount of activity from the medium; these slices are responsive to β -MSH, vasopressin, and fraction H; their response to ACTH continues for at least 2 hr after exposure to the hormone is terminated. Differences between species in responsiveness of their adipose tissue to hypophyseal peptides appear to be related to differences in peptidase content of the fat cell.

AT LEAST EIGHT naturally occurring peptides have the capacity to stimulate the production and release of free fatty acids (FFA) by mammalian adipose tissue: the hypophyseal peptides ACTH,¹ TSH, arginine vasopressin, α -MSH, β -MSH, "fraction H" ("peptide II") and "peptide I," and the pancreatic peptide glucagon (1-6).

This effect may be demonstrated either in living animals by measuring the increase in FFA in the blood following injection of the peptide (4, 5, 7), or in slices of adipose tissue incubated in buffer solutions containing suitable amounts of the peptide (1-6). Marked differences in responsiveness to adipokinetic peptides are shown by the adipose tissue of different species (4-6). For example, the adipose tissue of the rabbit is highly sensitive to β -MSH, "fraction H," and "peptide I," while that of the rat exhibits little or no reaction to these hormones (5, 6). The adipose tissue of rabbit, guinea pig, hamster, rat, pig, and dog each shows a different pattern in its responsiveness to the series of adipokinetic peptides (6).

Enzymes capable of inactivating hypophyseal peptide hormones have been demonstrated in mammalian liver, kidney, muscle, brain, and placenta (8-12). If similar enzyme systems are present in adipose tissue, differences in their concentration or in their substrate specificity might account for the differences between species in the responsiveness of adipose tissue to adipokinetic peptides.

MATERIALS AND METHODS

Animals. Male rabbits of mixed breed, weighing 3.0-4.5 kg, fed Purina rabbit chow pellets ad lib.

Male albino guinea pigs, weighing 750-1000 g, fed Purina guinea pig chow pellets ad lib.

Male Wistar rats, weighing 300-350 g, fed Purina laboratory chow checkers ad lib.

Hormones. Oxycellulose purified ACTH, 100 units/mg (Wilson Laboratories).

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¹ The following abbreviations are employed: ACTH, adrenocorticotropin; TSH, thyroid-stimulating hormone; α - and β -MSH, α - and β -melanocyte-stimulating hormones.

Synthetic α -MSH (Dr. K. Hofmann, University of Pittsburgh).

Bovine β -MSH, 1×10^9 units/g (Dr. J. D. Fisher, Armour Pharmaceutical Co.).

Vasopressin (a mixture of arginine vasopressin and lysine vasopressin prepared from a pool of bovine and ovine posterior pituitary lobes), 64 pressor and 4 oxytocic units/mg (Dr. F. C. Armstrong, Parke Davis and Co.).

Porcine pituitary fraction H, prepared by the method described previously (13).

Methods. Perirenal adipose tissue from the rabbit, guinea pig, or rat, obtained immediately after death from intravenous or intraperitoneal pentothal, was homogenized in Krebs-Ringer phosphate or other aqueous solution for 20 sec with a motor-driven Teflon pestle (Tri-R Instruments, Jamaica, N.Y.). Suitable amounts of ACTH, α -MSH, β -MSH, vasopressin, or fraction H were incubated with the homogenized adipose tissue for 1 hr at 37° in air. The incubation mixture (or the fat-free infranatant solution obtained after centrifugation at 0° for 10 min at 3500 rpm) was then tested for adipokinetic activity. Activity was assayed either in the intact rabbit (7) or in slices of perirenal adipose tissue from the rabbit, guinea pig, hamster, or rat (6).

RESULTS

Effect of Homogenates of Rabbit and Rat Adipose Tissue upon the Adipokinetic Activity of Fraction H in the Intact Rabbit

Porcine fraction H possesses adipokinetic activity for rabbit adipose tissue but not for rat adipose tissue (6). One-half-gram samples of perirenal adipose tissue from the rabbit and from the rat were homogenized in 10 ml 0.9% NaCl. Samples of fraction H (0.75 mg) were added to these homogenates and to 10 ml 0.9% NaCl, and the mixtures incubated for 1 hr at 25° . The effect of each mixture upon the serum FFA concentration of the intact rabbit was then tested. The results are given in Table 1. No loss of adipokinetic activity was apparent after incubation of fraction H with homogenized rabbit adipose tissue; incubation with homogenized rat adipose tissue abolished this activity.

Applicability of In Vitro Assay Techniques to Measurement of Adipokinetic Activity in Incubation Mixtures of Peptides with Adipose Tissue Homogenates

When slices of adipose tissue from the rabbit, guinea pig, or hamster are incubated in Krebs-Ringer phosphate (KRP) medium containing an adipokinetic peptide, the concentration of intracellular FFA after 2 hr shows a reproducible sigmoid relationship to the logarithm of the

concentration of peptide added to the medium (6). Free fatty acids are not discharged into the medium in this albumin-free system. The dose-response relationship for rabbit adipose tissue slices exposed to various concentrations of ACTH was found to be unaltered by the presence, in the albumin-free medium, of homogenized rabbit or guinea pig adipose tissue (30 mg tissue per ml). Free fatty acids did not appear in the medium despite the presence therein of adipose tissue lipases and triglyceride; this is attributed to the absence of a fatty-acid acceptor.

The albumin-containing assay system is preferable to the albumin-free system for measuring adipokinetic activity upon isolated rat adipose tissue (6). Addition of albumin to homogenates of adipose tissue, however, permits lipolysis to proceed in the homogenate (14). This complication can be prevented by removal of the triglyceride before the addition of albumin. The effectiveness of this procedure was shown in the following experiment. Various amounts of ACTH (0.03 to 3 μ g/ml) were added to a homogenate of rabbit adipose tissue in KRP (30 mg tissue per ml) and the triglyceride was removed by centrifugation at 0° . To the fat-free infranatant solutions, bovine plasma albumin was added to a concentration of 4 g/100 ml. Slices of rat adipose tissue were then incubated in these solutions, and in a corresponding series of solutions prepared by adding ACTH directly to KRP-albumin solution. The relationship between the rate of discharge of FFA by the slices into the medium, and the concentration of ACTH, was the same for both series of media.

The above observations indicated that the previously described *in vitro* techniques (6) could be used to measure the adipokinetic activity of peptides dissolved in suspensions of homogenized adipose tissue.

Effect of Homogenates of Rabbit and of Rat Adipose Tissue upon the Adipokinetic Activity of Fraction H for Isolated Rabbit Adipose Tissue

Fraction H was added to KRP, to a homogenate of rabbit adipose tissue in KRP, and to a homogenate of rat adipose tissue in KRP. The final concentrations in the mixtures were 3 μ g/ml fraction H and 30 mg/ml homogenized adipose tissue. Four 2-ml aliquots from each of the three mixtures were incubated in individual flasks for 1 hr at 37° . A slice of rabbit adipose tissue (100–150 mg) was then placed in each flask and the incubation continued for an additional 2 hr, after which the concentration of FFA in the slices was measured. Table 2 shows that the adipokinetic activity of fraction H upon rabbit adipose tissue persisted after incubation of this material with homogenized rabbit adipose tissue, but disappeared after incubation with

TABLE 1 EFFECT OF INCUBATING FRACTION H WITH HOMOGENIZED ADIPOSE TISSUE UPON ITS ADIPOKINETIC ACTIVITY IN THE INTACT RABBIT

Material Injected (Subcutaneously)	Serum FFA Level (μ Eq/liter) of Rabbit 90 min after Injection
10 ml 0.9% NaCl	220 \pm 25*
0.75 mg fraction H incubated in 10 ml 0.9% NaCl	2700 \pm 178
0.75 mg fraction H incubated with 500 mg homogenized rabbit adipose tissue in 10 ml 0.9% NaCl	2520 \pm 296
0.75 mg fraction H incubated with 500 mg homogenized rat adipose tissue in 10 ml 0.9% NaCl	430 \pm 25

* Mean \pm standard error (4 observations).

homogenized rat adipose tissue. Table 2 shows also that a homogenate of guinea pig adipose tissue did not inactivate fraction H.

This experiment, utilizing the in vitro assay for adipokinetic activity, confirmed the findings with the in vivo assay (Table 1). The more convenient in vitro procedure was employed in the remainder of the study.

Effects of Homogenates of Rabbit, Guinea Pig, and Rat Adipose Tissue upon the Adipokinetic Activity of ACTH, α -MSH, β -MSH, Vasopressin and Fraction H on Isolated Rabbit Adipose Tissue

The experimental design was the same as that just described. The data (Table 2) show that the homogenate of rat adipose tissue abolished the adipokinetic activity of all five hypophyseal peptides for isolated rabbit adipose tissue. Homogenized adipose tissue from the rabbit abolished the activity of α -MSH, but did not affect that of ACTH, β -MSH, vasopressin, or fraction H. Guinea

TABLE 2 EFFECT OF HOMOGENIZED ADIPOSE TISSUE FROM RABBIT, GUINEA PIG, OR RAT UPON THE ADIPOKINETIC ACTIVITIES OF ACTH, α -MSH, β -MSH, VASOPRESSIN, AND FRACTION H FOR ISOLATED RABBIT ADIPOSE TISSUE*

Peptide	Homogenate			
	None	Rabbit	Guinea Pig	Rat
	<i>μEq FFA per g rabbit adipose tissue</i>			
None	1.0 \pm 0.2†	1.4 \pm 0.4	1.5 \pm 0.5	1.4 \pm 0.3
ACTH	12.0 \pm 0.9	14.9 \pm 1.1	13.9 \pm 0.4	2.1 \pm 0.4
α -MSH	9.3 \pm 1.1	2.1 \pm 0.2	8.5 \pm 1.2	1.0 \pm 0.1
β -MSH	11.5 \pm 0.6	13.1 \pm 1.6	12.0 \pm 0.7	1.9 \pm 0.4
Vasopressin	9.5 \pm 1.2	9.8 \pm 1.0	10.1 \pm 0.8	1.6 \pm 0.2
Fraction H	8.2 \pm 0.7	10.5 \pm 0.7	10.1 \pm 0.8	1.5 \pm 0.1

* 60 mg homogenized adipose tissue was incubated for 1 hr at 37° with 6 μ g of the peptide preparation in 2 ml KRP. Slices of rabbit adipose tissue were then added to each flask and after an additional 2-hr incubation, the concentration of FFA in each slice was measured. The values in the table represent these concentrations.

† Mean \pm standard error (4 observations).

TABLE 3 DETERMINATION OF MINIMAL AMOUNT OF HOMOGENIZED RAT ADIPOSE TISSUE THAT ABOLISHES THE ADIPOKINETIC ACTIVITY OF 6 μ g ACTH FOR ISOLATED RABBIT ADIPOSE TISSUE*

ACTH	Homogenized Adipose Tissue (mg)				
	0	2	6	20	60
	<i>μEq FFA per g rabbit adipose tissue</i>				
None	0.4 \pm 0.1†	0.5 \pm 0.3	0.7 \pm 0.2	1.2 \pm 0.4	0.8 \pm 0.2
6 μ g	7.0 \pm 1.0	8.2 \pm 0.5	6.8 \pm 1.4	1.1 \pm 0.2	0.9 \pm 0.2

* Different amounts of homogenized rat adipose tissue were incubated for 1 hr at 37° with 6 μ g ACTH in 2 ml KRP. Slices of rabbit adipose tissue were then introduced into each flask and after an additional 2-hr incubation, the concentration of FFA in the slices was measured. The values in the table represent these concentrations.

† Mean \pm standard error (4 observations).

pig adipose tissue homogenate did not reduce the activity of any of the five peptides.

In those instances where inactivation was effected by 60 mg of homogenate, the minimal amount of homogenate required for inactivation was then determined. Table 3 shows a representative experiment in which the minimal concentration of homogenized rat adipose tissue required to inactivate 6 μ g ACTH was found to be between 6 and 20 mg. The results of analogous experiments with the other adipokinetic peptides under study are given in Table 4. Each value in this table was confirmed in two to six identical but separate experiments.

Effects of Homogenized Rabbit and Rat Adipose Tissue upon the Adipokinetic Activity of ACTH and of Vasopressin for Isolated Adipose Tissue from the Guinea Pig, Hamster, and Rat

The adipokinetic activity of ACTH and of vasopressin for guinea pig adipose tissue persisted after incubation of these hormones with homogenized rabbit adipose tissue, but disappeared after incubation with homogenized rat adipose tissue (Table 5). Similarly, the adipokinetic activity of ACTH for hamster or rat adipose tissue slices persisted during incubation of ACTH with homogenized rabbit adipose tissue, but disappeared during incubation with homogenized rat adipose tissue (Tables 5 and 6).

TABLE 4 MINIMAL AMOUNT OF HOMOGENIZED ADIPOSE TISSUE (MG) FROM THE RABBIT, GUINEA PIG, OR RAT THAT ABOLISHES THE ADIPOKINETIC ACTIVITY OF 6 μ g ACTH, α -MSH, β -MSH, VASOPRESSIN, OR FRACTION H UNDER THE CONDITIONS DESCRIBED IN TABLE 3

Source of Adipose Tissue	Peptide				
	ACTH	α -MSH	β -MSH	Vasopressin	Fraction H
Rabbit	NI*	60	NI	NI	NI
Guinea pig	NI	NI	NI	NI	NI
Rat	20	20	60	20	60

* No inactivation by 60 mg homogenized adipose tissue.

TABLE 5 EFFECT OF HOMOGENIZED ADIPOSE TISSUE FROM THE RABBIT OR RAT UPON THE ADIPOKINETIC ACTIVITIES OF ACTH AND OF VASOPRESSIN FOR ISOLATED ADIPOSE TISSUE OF THE GUINEA PIG AND HAMSTER*

Peptide (6 µg)	Type of Adipose Tissue Used to Assay Adipokinetic Activity of Peptide-Homogenate Mixture	Homogenate (60 mg)		
		None	Rabbit	Rat
<i>µEq FFA per g tissue in assay slices</i>				
None	Guinea pig	1.0 ± 0.5†	1.2 ± 0.4	1.4 ± 0.6
ACTH	“ “	8.6 ± 0.7	7.2 ± 0.4	1.4 ± 0.3
Vasopressin	“ “	5.9 ± 0.4	5.1 ± 0.8	1.9 ± 0.9
None	Hamster	1.4 ± 0.2	1.5 ± 0.4	1.3 ± 0.3
ACTH	“	10.9 ± 1.1	12.5 ± 1.4	1.6 ± 0.2

* Conditions as described in Table 2, with the single difference that slices from the guinea pig or from the hamster were used, in place of those from the rabbit, to assay the adipokinetic activity of the peptide-homogenate incubation mixture.

† Mean ± standard error (4 observations).

Comparison of the Response to ACTH by Isolated Rabbit Adipose Tissue and by Isolated Rat Adipose Tissue

The capacity of homogenates of rabbit adipose tissue, of guinea pig adipose tissue, and of rat adipose tissue to inactivate each of the five adipokinetic peptides under study is, in general, inversely related to the capacity of the tissue to respond to these peptides (Table 7). Two exceptions to this generalization, however, were apparent at this point in the investigation: (a) rat adipose tissue is responsive to ACTH, despite its capacity to inactivate this hormone; and (b) rabbit adipose tissue likewise responds to, but possesses the capacity to inactivate, α -MSH. Certain differences were now observed between the response to ACTH by isolated rat adipose tissue, and by isolated rabbit adipose tissue, which are relevant to exception (a) noted above.²

(a) *Effect of Incubating Slices of Rabbit or Rat Adipose Tissue in ACTH-Containing Medium upon the Adipokinetic Activity of the Medium.* When slices of rabbit adipose tissue were incubated for 1 hr in KRP + ACTH (1 µg/ml), the amount of adipokinetic activity that disappeared from the medium was so small that it could not be detected by the method employed (Table 8). In contrast, incubation of rat adipose tissue slices under the same conditions led to complete disappearance of adipokinetic activity from the medium. Also shown in Table 8 are the results of an analogous experiment with vasopressin. Again activity persisted in the medium after incubation with rabbit slices, but disappeared following incubation with rat slices. Table 8 shows also that slices of guinea pig adipose tissue (the homogenate of which lacks the ca-

² Sufficient supply of α -MSH was not available to perform analogous experiments with this peptide.

TABLE 6 EFFECT OF HOMOGENIZED ADIPOSE TISSUE FROM THE RABBIT OR RAT UPON THE ADIPOKINETIC ACTIVITY OF ACTH FOR ISOLATED ADIPOSE TISSUE OF THE RAT*

ACTH	Homogenate (60 mg)		
	None	Rabbit	Rat
<i>µEq FFA released per g rat adipose tissue in 3 hr</i>			
None	2.2 ± 0.4†	2.4 ± 0.6	2.6 ± 0.5
6 µg	12.6 ± 1.6	14.7 ± 1.0	2.4 ± 0.2

* The complete system contained homogenized adipose tissue (60 mg/2 ml) and ACTH (6 µg/2 ml) in KRP medium. After incubation for 1 hr at 37°, the incubation mixtures were centrifuged at 0°. To the fat-free infranatant solutions, plasma albumin (4 g/100 ml) was added and pH adjusted to 7.40. Rat adipose tissue slices were placed in 2-ml aliquots of these solutions and after incubation for 2 hr at 37° measurement was made of the amount of FFA released by these slices into the medium.

† Mean ± standard error (4 observations).

TABLE 7 CORRELATION OF (A) CAPACITY OF ADIPOSE TISSUE SLICES FROM DIFFERENT SPECIES TO RESPOND TO ADIPOKINETIC PEPTIDES, AND (B) CAPACITY OF HOMOGENATES OF THESE TISSUES TO INACTIVATE THE PEPTIDES*

Source of Adipose Tissue	Peptide				Fraction H
	ACTH	α -MSH	β -MSH	Vasopressin	
Rabbit	+ / 0	+ / +	+ / 0	+ / 0	+ / 0
Guinea pig	+ / 0	+ / 0	+ / 0	+ / 0	+ / 0
Rat	+ / +	0 / +	0 / +	0 / +	0 / +

* Left-hand symbol represents capacity to respond; right-hand symbol represents capacity to inactivate. Thus, for rabbit adipose tissue vis-a-vis ACTH, + / 0 signifies capacity to respond and lack of capacity to inactivate. Information on responsiveness, from reference 6; on capacity to inactivate, from Table 2.

capacity to inactivate ACTH or vasopressin) did not cause the disappearance of a detectable amount of the activity of either of these peptides from the medium.

(b) *Duration of the Response by Isolated Rabbit Adipose Tissue and by Isolated Rat Adipose Tissue to a 10-min Exposure to ACTH.* When slices of rabbit adipose tissue were incubated in KRP-albumin medium containing 10 µg/ml ACTH, FFA were released at an hourly rate of 4–7 µEq FFA/g of tissue during a 2-hr incubation (Fig. 1, Curve I). This rate was 8–14 × greater than that of slices incubated in KRP-albumin (Curve II). Rabbit slices incubated in the KRP-albumin-ACTH medium for only 10 min, and then transferred to KRP-albumin to incubate for an additional 1 hr and 50 min (Curve III), released FFA at a rate initially equal to, and subsequently greater than, that of slices continuously exposed to ACTH (Curve I). The analogous experiment with rat adipose tissue slices (also shown in Fig. 1) showed that the response to ACTH ceased within 1 hr after the slices were transferred from KRP-albumin-ACTH medium to KRP-albumin. Similar findings concerning the relative briefness of the lipolytic response of rat adi-

TABLE 8 EFFECT OF INCUBATING SLICES OF RABBIT, GUINEA PIG, OR RAT ADIPOSE TISSUE IN KRP MEDIUM CONTAINING ACTH OR VASOPRESSIN UPON THE ADIPOKINETIC ACTIVITY OF THE MEDIUM FOR RABBIT ADIPOSE TISSUE*

Peptide	Type of Adipose Tissue Slice			
	None	Rabbit	Guinea Pig	Rat
	<i>E_μg FFA per g rabbit adipose tissue</i>			
None	1.3 ± 0.4†	0.9 ± 0.2	1.2 ± 0.4	1.0 ± 0.4
ACTH	6.7 ± 0.9	6.2 ± 0.4	6.5 ± 0.9	1.0 ± 0.4
Vasopressin	9.6 ± 2.3	9.4 ± 1.2	7.3 ± 0.9	1.3 ± 0.3

* Two slices of rabbit, guinea pig, or rat adipose tissue (total weight 250 mg) were incubated 1 hr in 2 ml KRP containing 2 μg ACTH or vasopressin. These slices were then removed, and each medium was tested for residual adipokinetic activity by incubating in it a slice of rabbit adipose tissue for 2 hr. The values in the table represent the concentration of FFA in these tissue slices at the end of the 2-hr incubation.

† Mean ± standard error (4 observations).

pose tissue slices to ACTH have been reported by Vaughan and Steinberg, who concluded that the peptide underwent rapid inactivation by this tissue (15).

Properties of the Component in Homogenized Rat Adipose Tissue Which Inactivates ACTH and Vasopressin

(a) *Location of the Inactivating Component in the Homogenate.* A homogenate of rat adipose tissue in KRP (30 mg/ml) was spun at 100,000 × *g* for 3 hr at 0°. The bulky, triglyceride-rich, solid upper layer ("cake"), the transparent intermediate liquid layer, and the sedimented "pellet" were collected. "Cake" and "pellet" were dispersed in KRP. The three fractions of the homogenate ("cake," "liquid," and "pellet") were then tested for their capacity to inactivate ACTH. Cake inactivated ACTH completely, pellet partially, and liquid not at all (Table 9). Subsequent experiments showed that when the homogenate was centrifuged at 1000 × *g* for 10 min at 0°, all the inactivating capacity of the homogenate could be recovered in the cake. The infranatant aqueous solution and sedimented pellet prepared under these conditions were devoid of inactivator.

(b) *Solubility Properties of the Inactivator.* Aliquots of a homogenate of rat adipose tissue in KRP were centrifuged at 1,000 × *g* for 10 min at 0°. The resulting cakes were shaken at 25° for 2 min with 50 ml KRP, ethanol, acetone, isopropyl alcohol, or heptane. The insoluble residues remaining after these various types of extraction were then collected by centrifugation at 0°, resuspended in KRP, and tested for their capacity to inactivate ACTH. Inactivating capacity remained in the insoluble fraction after extraction with KRP, isopropyl alcohol, or heptane, but disappeared after extraction with ethanol or acetone. The materials extracted by ethanol or acetone were recovered by evaporation of the extracts under air at 25°,

resuspended in KRP and tested for capacity to inactivate ACTH; they were found to lack inactivating capacity.

Additional experiments showed that the inactivating capacity of the insoluble fraction of the homogenate was not diminished by 3 extractions with heptane followed by 3 extractions with KRP. This procedure removes the major proportion of the triglyceride, as well as of the water-soluble components present in the cake, and provides a partially purified preparation of the inactivating system.

(c) *Inactivation of ACTH at 37° and at 0°.* The cake of rat adipose tissue homogenate (30 mg/ml in KRP) was prepared as described above, extracted three times with heptane and twice with KRP, and finally resuspended in the original volume of KRP. To this suspension was added ACTH to a concentration of 3 μg/ml. One-half of this mixture was then incubated at 37°, and the remainder at 0°. Periodically an aliquot of each mixture was removed, centrifuged at 0° for 10 min (1000 × *g*), and the resulting fat-free infranatant solution was collected. Two-milliliter aliquots of these filtrates were assayed for adipokinetic activity upon rabbit adipose tissue slices in the usual manner. Inactivation of ACTH by the heptane-extracted cake proceeded rapidly at 37°, reaching completion at 30 min (Fig. 2). Little or no inactivation occurred at 0°.

(d) *Effect of Temperature and Dehydration upon the Inactivator in Rat Adipose Tissue.* Exposure of the homogenate to 100° for 1.5 min abolished the capacity to inactivate ACTH or vasopressin. When rat adipose tissue was stored at -20° for 24 hr, thawed, and homogenized, the resulting homogenate showed a 50-75% reduction in capacity to inactivate ACTH. When adipose tissue was lyophilized and then homogenized in KRP, the resulting homogenate had no capacity to inactivate ACTH.

(e) *Chromatographic and Titrimetric Observations on the Interaction between the Cake of Rat Adipose Tissue Homogenate and ACTH.* The cakes of rat adipose tissue homogenate (300 mg/ml), and of rabbit adipose tissue homogenate

TABLE 9 EFFECT OF THREE FRACTIONS OF HOMOGENIZED RAT ADIPOSE TISSUE UPON THE ADIPOKINETIC ACTIVITY OF ACTH FOR ISOLATED RABBIT ADIPOSE TISSUE*

Peptide	Fraction of Homogenate			
	None	Cake	Liquid	Pellet
	<i>μEq FFA per g rabbit adipose tissue</i>			
None	2.2 ± 0.5†	1.8 ± 0.3	2.2 ± 0.5	2.0 ± 0.4
ACTH	10.9 ± 0.8	1.7 ± 0.2	9.5 ± 1.0	4.4 ± 0.7

* An amount of fraction derived from 60 mg homogenized adipose tissue was incubated with 6 μg ACTH in 2 ml KRP for 1 hr at 37°. Slices of rabbit adipose tissue were then added to each flask and after an additional 2-hr incubation, the concentration of FFA in the slices was measured. The values in the table represent these concentrations.

† Mean ± standard error (4 observations).

(300 mg/ml), were prepared in the usual way, extracted twice with KRP, and resuspended in the original volume of KRP. These suspensions were then incubated with ACTH (1 mg/ml) for 2 hr at 37°. The fat-free aqueous infranatant solutions were then prepared by centrifugation of the mixtures at 0°. These solutions (IV and V in

Fig. 3), together with appropriate control solutions (I, II, III in Fig. 3), were then subjected to descending paper chromatography (16) in butanol-acetic acid-H₂O 12:3:5. ACTH incubated for 2 hr in KRP (III) remained at the origin in this system, as did ACTH incubated in the presence of rabbit adipose tissue cake

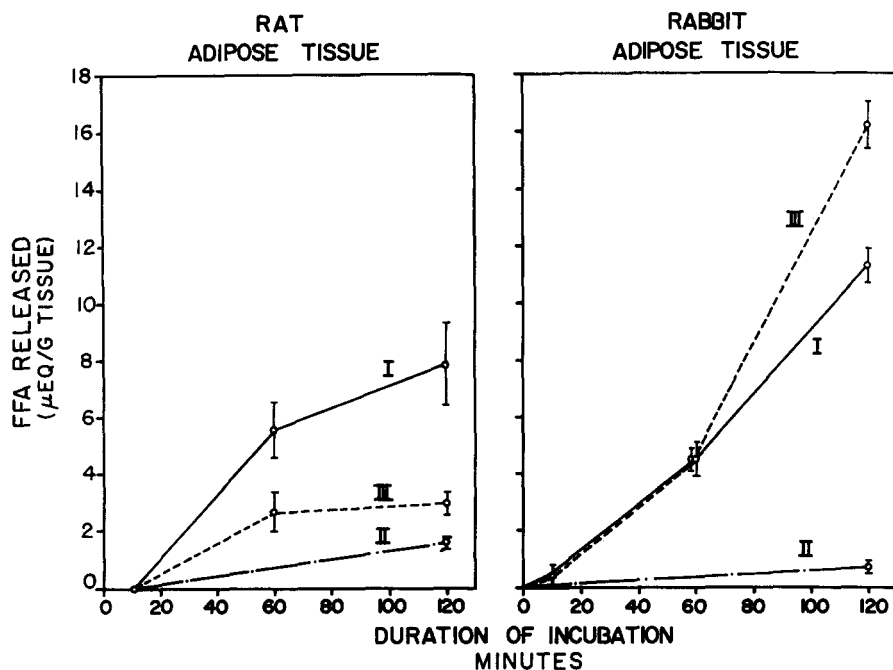


FIG. 1. Response of slices of adipose tissue from the rabbit and from the rat to limited or continuous exposure to ACTH in KRP-albumin medium. Curve I: slices incubated for 2 hr in 2 ml KRP-albumin containing 10 μg/ml ACTH. Curve II: slices incubated for 2 hr in 2 ml KRP-albumin. Curve III: slices incubated for 10 min in 2 ml KRP-albumin containing 10 μg/ml ACTH, then transferred to KRP-albumin. Vertical axis: μEq FFA released into medium per g tissue. Each point represents mean of 4 values. Standard error of mean is shown.

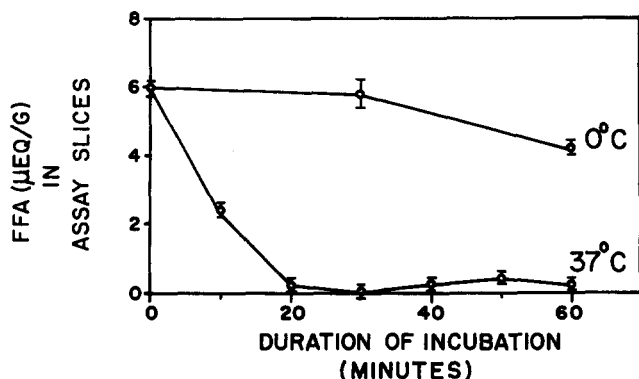


FIG. 2. Inactivation of ACTH by heptane-extracted cake of rat adipose tissue at 37° and at 0°. Conditions and methods as described in text. Vertical axis: concentration of FFA (μEq/g tissue) in the slices of rabbit adipose tissue used to assay infranatant solutions obtained from incubation mixtures after various periods of incubation. Each point represents the mean of 4 values. Standard error of mean is shown.

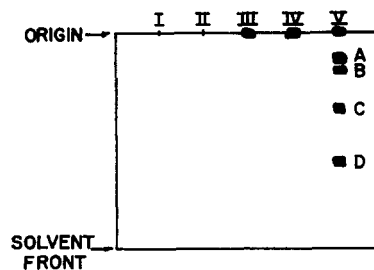


FIG. 3. Chromatograms of incubated mixtures of ACTH plus washed cake of rabbit or of rat adipose tissue and of appropriate control mixtures. Incubated mixtures: I, rabbit cake in KRP; II, rat cake in KRP; III, ACTH in KRP; IV, rabbit cake with ACTH in KRP; V, rat cake with ACTH in KRP. The complete system contained cake derived from 500 mg adipose tissue, and 5 mg ACTH, in 5 ml KRP. After incubation for 2 hr at 37°, the fat-free infranatant solutions were collected and 60 μl aliquots were subjected to descending chromatography for 18 hr on Whatman No. 1 paper in butanol-acetic acid-water 12:3:5. Four new ninhydrin-reactive bands were detected in incubation mixture V, with the following R_F values: A, 0.12; B, 0.18; C, 0.38; D, 0.60.

(IV). No other ninhydrin-reactive components were visible in these two samples. In contrast, incubation of ACTH with rat adipose tissue cake (V) generated four new ninhydrin-reactive components with R_F 0.12, 0.18, 0.36, and 0.60.

When the above experiment was conducted in a medium of 0.9% NaCl, instead of KRP, the chromatographic findings were the same as shown in Fig. 3. Titration of the fat-free filtrates of the incubation mixtures in 90% ethanol (17) showed the formation, under the influence of rat adipose tissue cake, of five free amino groups per molecule of ACTH during the 2-hr incubation (Fig. 4). Free amino groups were not generated when ACTH was incubated with the cake of rabbit adipose tissue.

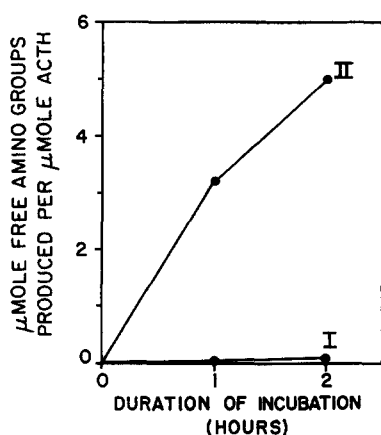


FIG. 4. Appearance of free amino groups during incubation of ACTH with cake of rabbit and of rat adipose tissue. The cake derived from 1000 mg adipose tissue was incubated at 37° with 10 mg ACTH in 10 ml 0.9% NaCl. Periodically an aliquot of the incubation mixture was removed, centrifuged at 0° for 10 min, and the fat-free aqueous infranatant solution titrated in 90% ethanol for free amino groups (17). Vertical axis: μ mole of free amino groups produced per μ mole ACTH, assuming 1 mg of the oxycellulose purified ACTH preparation (100 units/mg) to contain 0.146 μ mole ACTH. Curve I: rabbit cake + ACTH. Curve II: rat cake + ACTH.

DISCUSSION

Nature of the Inactivator. The inactivating factor of rat adipose tissue has these properties: (a) It abolishes the adipokinetic activity of the hypophyseal peptides ACTH, α - and β -MSH, vasopressin, and fraction H. (b) The capacity for inactivation is abolished by heating at 100° for 1.5 min. It is reduced by freezing and thawing, and abolished by lyophilizing. (c) The inactivator is insoluble in H_2O , the major proportion of inactivator being found, after centrifugation of the homogenate at $100,000 \times g$, in the triglyceride-rich supernatant cake and the minor proportion in the sedimented pellet. Unlike the lipid components of the cake, the inactivator

is not extracted by heptane or isopropyl alcohol, nor is its inactivating capacity reduced by exposure to these solvents. (d) The rate of inactivation is more than $100 \times$ greater at 37° than at 0° . (e) The disappearance of the adipokinetic activity of ACTH, under the influence of the inactivator, is associated with the appearance of four ninhydrin-reactive components not present in untreated ACTH and with the appearance of at least five free amino groups per molecule of ACTH.

These observations indicate that the inactivating factor is a peptidase (or group of peptidases), which is attached to an insoluble component of the fat cell. In order to determine the precise cytologic location of the inactivating enzyme, a method must first be devised for separating the various subcellular particles and lipid droplets that are present in the fat cell. The present data do not establish whether a single peptidase in rat adipose tissue is responsible for the inactivation of ACTH, α -MSH, β -MSH, vasopressin, and fraction H, or whether several peptidases are involved. Inactivation by rabbit adipose tissue homogenate of α -MSH alone, among these five adipokinetic peptides, suggests that either more than one peptidase is present in the rat's fat cell, or the substrate specificity of the peptidase in the rat's fat cell differs markedly from that in the rabbit's. A related problem is whether the peptidase(s) of rat and rabbit adipose tissue act specifically upon adipokinetic peptides or whether, like the "cathepsins" of other organs, they catalyze the hydrolysis of a wide variety of peptides.

Enzyme systems have been demonstrated in liver, kidney, bladder, muscle, and brain which abolish the adrenotropic activity of ACTH, the melanotropic activity of β -MSH, or the pressor and antidiuretic activities of vasopressin (8-12). The possible relationship between the inactivating peptidase system of adipose tissue and these inactivating enzymes in other organs remains to be determined.

Relation of the Inactivating Enzyme to the Responsiveness of Adipose Tissue to Adipokinetic Peptides. Table 7 correlates (a) the capacity of slices of rabbit, guinea pig, and rat adipose tissue to respond to ACTH, α -MSH, β -MSH, vasopressin, or fraction H, with (b) the capacity of homogenates of these three types of adipose tissue to abolish the adipokinetic activity of these five peptides. In 13 of the 15 instances, capacity of the homogenate to inactivate is inversely related to capacity of the tissue to respond. In two instances, responsiveness occurs despite the presence of inactivator, namely, rat adipose tissue vis-a-vis ACTH, and rabbit adipose tissue vis-a-vis α -MSH. Further examination in the former instance revealed that the response to ACTH of rat adipose tissue (which contains inactivating enzyme for ACTH) differs from that of rabbit adipose tissue (which lacks this enzyme) in that rat adipose tissue exhibits a comparatively

brief response to a limited exposure to ACTH, and inactivates a comparatively large amount of ACTH during continuous exposure to the hormone. Additional experiments are necessary to determine whether these characteristics of the response of rat adipose tissue to ACTH, which may be explained by the presence of an inactivating enzyme for ACTH, are also features of the response of rabbit adipose tissue to α -MSH.

The above correlations suggest that the inactivating enzyme system influences the responsiveness of adipose tissue to adipokinetic peptides and that variations in the amount of this enzyme system in the fat cell are among the factors responsible for species differences in this responsiveness. The present data do not exclude the possibility of other species-variable determinants of responsiveness, such as structure of the cellular "receptor," permeability of the cell membrane, or presence of "activating" peptidases (18). Possible species differences in these functions of the fat cell must be considered in investigations in this field.

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REFERENCES

1. White, J. E., and F. L. Engel. *J. Clin. Invest.* **37**: 1556, 1958.
2. Freinkel, N. *J. Clin. Invest.* **40**: 476, 1961.
3. Vaughan, M. *J. Biol. Chem.* **235**: 3049, 1960.
4. Raben, M. S., R. Landolt, F. A. Smith, K. Hofmann, and H. Yajima. *Nature* **189**: 681, 1961.
5. Friesen, H., R. J. Barrett, and E. B. Astwood. *Endocrinology* **70**: 579, 1962.
6. Rudman, D., S. J. Brown, and M. F. Malkin. *Endocrinology* **72**: 527, 1963.
7. DiGirolamo, M., D. Rudman, M. B. Reid, and F. Seidman. *Endocrinology* **68**: 457, 1961.
8. Geschwind, I. I., and C. H. Li. *Endocrinology* **50**: 226, 1952.
9. Dicker, S. E., and A. L. Greenbaum. *J. Physiol.* **132**: 199, 1956.
10. Birnie, J. H. *Endocrinology* **52**: 33, 1953.
11. Shizume, K., and M. Irie. *Endocrinology* **61**: 506, 1957.
12. Hooper, K. C., and D. C. Jessup. *J. Physiol.* **146**: 539, 1959.
13. Rudman, D., R. L. Hirsch, F. E. Kendall, F. Seidman, and S. J. Brown. *Recent Progr. Hormone Res.* **18**: 89, 1962.
14. Vaughan, M. In *Fat as a Tissue*, edited by K. Rodahl, New York, McGraw-Hill Co. Inc., in press.
15. Vaughan, M., and D. Steinberg. *J. Lipid Res.* **4**: 193, 1963.
16. Li, C. H., J. S. Dixon, and D. Chung. *J. Am. Chem. Soc.* **80**: 2587, 1958.
17. Davis, N. C., and E. L. Smith. *Methods Biochem. Anal.*, **2**: 215, 1955.
18. Papkoff, H. *Endocrinology* **70**: 754, 1962.