sensitized animals. 2) A polypeptide substance most probably related to bradykinin was present in a higher amount during the first weeks and began to disappear between 4 to 6 weeks. 3) There was a closer correlation between the appearance of this substance and testicular inflammation than with the development of antispermatic antibodies.

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Immunoassay of Human Insulin and Growth Hormone Simultaneously Using I-131 and I-125 Tracers.* (31451)

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The successful application of the two-antibody technique for assay of insulin(1) or growth hormone(2) has prompted an attempt at simultaneous immunoassay of these 2 hormones. The interrelationship of these 2 hormones in metabolic disorders has long been of interest(3,4,5).

Materials and methods. Each of 12 guinea pigs was injected subcutaneously with 1 mg of human growth hormone† (HGH) on days 0, 7, 14, 21, and 28. The HGH was prepared in a solution of 0.15 *M* NaCl, phosphate buffer (pH 7.4); the final concentration was 1 mg/ml. Equal volumes of HGH solution and lanolin-oil were mixed to a thick emulsion (6). After the 5 injections, each animal was bled *via* cardiac puncture on days 35 and 49, *i.e.*, at 7 and 21 days after the fifth injection. Three of the 12 guinea pigs responded with anti-human growth hormone serum (AHGHS-GP) suitable for immunoassay.

The anti-insulin guinea pig serum (AIS-GP) used in these experiments was prepared as previously described(7).

The I-125-HGH was prepared commercially.‡ I-131-insulin, porcine, was obtained from the same commercial source. The specific activity of each radioisotope labeled hormone was in excess of 100 mc/mg.

Anti-guinea pig serum (AGPS-R) was obtained from rabbits as previously described (7).

For immunoassay standards of HGH, the Wilhelmi preparation (NIH-HS-612B) was used. This was the same material sent to Abbott for labeling with I-125.

The insulin standards were a human insulin preparation (lot 515-734B-33) supplied by Dr. Mary Root of Eli Lilly Co. This lot of insulin had 23.4 units of activity per milligram.

The diluent used consisted of 1% bovine

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‡ Abbott Laboratories, North Chicago, Ill. This human growth hormone was kindly supplied by Dr. A. E. Wilhelmi, Emory Univ., Atlanta, Ga.

TABLE I. Specificity of Insulin and Growth Hormone Immunoassay Reactions. (Each value listed is the mean ± one standard deviation for 4 determinations.)

Unlabeled hormone	HGH-125 plus AHGHS-GP		Insulin-I-131 plus AIS-GP	
	I-125	Δ	I-131	Δ
	cpm ppt		cpm ppt	
0	29,331 ± 744	—	13,818 ± 271	—
128 μU insulin	28,738 ± 657	nil	2,284 ± 126	11,534
128 mμg HGH	12,245 ± 454	17,086	13,992 ± 527	nil

serum albumin in a borate buffer containing 0.01 *M* EDTA, final pH 7.5 ± 0.2. This is designated as 1% BSA.

The reactions were carried out as follows. *First antibody reaction:* (a) 1.0 ml 1% BSA, or standard, or assay sample was mixed with (b) 0.5 ml 1% BSA containing 0.5 mμg HGH-I-125 and 5 μU Insulin-I-131, and (c) 0.5 ml 1% BSA containing AIS-GP, 1:100,000; AHGHS-GP, 1:10,000; and normal guinea pig serum, 1:1,000. This reaction mixture was placed at 4°C for 24 hours. *Second antibody reaction:* a sufficient amount of AGPS-R was added to ensure maximum precipitation in 16 hours (in this case 0.3 ml of a 1:10 dilution of high titer AGPS-R was used). This reaction mixture was placed at 4°C for 20 hours. Then the tubes were centrifuged at 2000 rpm for 15 minutes. The resultant supernatants were decanted and discarded. The precipitates were counted in a Packard 410 autogamma counter with dual channels. One channel was adjusted to a window setting of 25 to 73 KEV for reading I-125. The second channel was adjusted to a window setting of 230-800 KEV for reading I-131. A small portion of I-131 activity was registered in the 25-73 KEV channel; therefore a correction factor had to be applied to readings from this channel. It was found that with I-131 alone, the counts in the 25-73 KEV channel were 4.6 ± 0.4% of the counts in the 230-800 KEV channel. To obtain net I-125 counts, the gross I-125 counts were reduced by the background counts and by 4.6% of the I-131 counts on the 230-800 KEV channel.

Results. In order to check cross-reactivity of insulin or growth hormone with antisera to growth hormone or insulin, 128 microunits of insulin standard in 1 ml was mixed with 0.5 ml 1:10,000 AHGHS-GP and 0.5 ml HGH-I-125 (0.5 mμg) and reacted for 24 hours. Likewise, 128 mμg of HGH in 1 ml was mixed with 0.5 ml 1:100,000 AIS-GP and 0.5 ml Insulin-I-131 (5 μU). These determinations were carried out in quadruplicate. The second antibody reaction was carried out as explained above. The results of the counts of radioactivity in the precipitates of these reaction tubes are shown in Table I. Unlabeled insulin did not compete with HGH-I-125 tracer for AHGHS-GP, nor did unlabeled growth hormone compete with Insulin-I-131 tracer for AIS-GP.

Results obtained with insulin and growth hormone standards by the simultaneous procedure were compared with results obtained by individual assay of each hormone (Table II). There was a decrease in quantity of I-125 precipitated with increasing concentrations of unlabeled growth hormone in the range of 1 to 128 mμg. Likewise, there was a decrease in the quantity of I-131 precipitated with increasing concentrations of unlabeled insulin over the range of 1 to 128 microunits. From these data, assay curves were drawn(2), and hormone values for human serum samples were obtained (Table III). The results obtained by simultaneous assay were equivalent to those obtained by assay of each hormone separately.

Discussion. The results reported here indicate that simultaneous two-antibody immunoassay of insulin and growth hormone can be an effective tool for clinical diagnosis and biomedical research. This is a one-tube reaction of relative simplicity. The success obtained here suggests the use of simultaneous assay of other protein hormones by utilizing multiple radiotracers emitting at different energy levels.

Summary. I-125 growth hormone and I-131-insulin were utilized in a simultaneous two-antibody immunoassay procedure. Results obtained by simultaneous assay were

TABLE II. Comparison of Insulin Assay and Growth Hormone Assay with Simultaneous Assay of These Two Hormones. (Each value listed is the mean ± one standard deviation for 4 determinations.)

Unlabeled hormone*	Insulin alone		Simultaneous				HGH alone	
			Insulin		HGH			
	I-131	Δ	I-131	Δ	I-125	Δ	I-125	Δ
	cpm ppt							
0	32,391 ±155	—	33,509 ±393	—	40,474 ±1150	—	41,869 ±1570	—
1	31,267 ±552	1,124	34,076 ±339	—	36,498 ±544	3,976	38,118 ±1090	3,751
2	30,882 ±134	1,509	32,489 ±252	1,020	32,603 ±223	7,871	35,202 ±1490	6,667
4	29,085 ±412	3,306	31,438 ±613	2,071	29,333 ±632	11,141	31,234 ±1450	10,635
8	25,424 ±203	6,967	26,772 ±386	6,737	21,776 ±383	18,698	23,567 ±641	18,302
16	19,697 ±174	12,694	20,796 ±331	12,713	16,402 ±554	24,072	17,833 ±767	24,036
32	13,491 ±253	18,900	14,472 ±133	19,037	12,457 ±276	28,017	13,106 ±675	28,763
64	8,786 ±45	23,605	9,947 ±604	23,562	9,675 ±251	30,799	10,019 ±745	31,850
128	—	—	6,057 ±199	27,452	7,140 ±305	33,334	7,901 ±471	33,968

* μU insulin or mμg HGH.

TABLE III. Values of Insulin and Growth Hormone Obtained by Simultaneous Immunoassay of Human Serum Compared with Values Obtained by Assay of Each Hormone Separately.

Patient	Insulin alone		Simultaneous				HGH alone	
			Insulin		HGH			
	cpm	μU/ml	cpm	μU/ml	cpm	mμg/ml	cpm	mμg/ml
W.C.	16,869	44	17,670	44	16,554	36	17,037	34
1-11-65	17,176	42	20,593	32	15,456	40	16,100	38
F.R.	16,688	44	17,377	46	32,876	4	35,808	4
5-12-66	16,420	46	16,876	48	33,058	4	34,564	4
T.C.	21,189	28	23,577	24	14,830	44	14,922	46
5-17-66	21,881	26	21,929	28	12,939	58	16,199	38
M.B.	8,535	>128	8,446	166	33,593	4	36,014	4
5-12-66	8,713	>128	8,975	150	35,556	3	37,253	2
F.R.	29,907	10	29,293	10	32,892	4	37,601	2
4-29-66	27,763	12	29,343	10	33,038	4	37,234	2
0 T.C.	11,188	86	12,478	82	31,774	6	35,800	4
4-17-66	—	—	12,182	86	31,381	6	—	—
#1 T.C.	13,181	66	12,475	82	25,016	12	29,291	10
4-16-66	—	—	13,293	74	29,100	8	—	—
#2 T.C.	22,054	26	19,801	36	23,156	14	32,501	6
4-17-66	—	—	22,878	26	26,335	10	—	—
#1 M.B.	8,807	128	9,254	141	34,553	4	37,150	2
5-12-66	—	—	9,130	146	34,729	3	35,727	4
#3 M.B.	8,094	>128	8,380	166	38,168	2	35,769	4
5-12-66	7,945	>128	8,516	164	34,503	4	35,170	4

equivalent to those obtained by assay of each hormone separately.

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Transmission of WEE Virus to Snakes by Infected *Culex tarsalis* Mosquitoes.* (31452)

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Previous studies have shown that *Culex tarsalis* mosquitoes will readily feed on snakes (Thamnophis spp) under laboratory conditions (Gebhardt *et al*(1); Thomas(2), and that a WEE virus titer in snakes as low as 2.7×10^2 PFU (plaque forming units) per ml of blood will infect 31.2% of the mosquitoes feeding (Gebhardt *et al*), which is equivalent to at least 1.0 PFU per infectious blood meal.† The present report deals with the transmission of WEE virus to snakes by infected *C. tarsalis* mosquitoes.

Materials and methods. Baby chicks were infected by subcutaneous injection of 2×10^4 PFU of chick cell cultured WEE virus. Mosquitoes *(C. tarsalis)*‡ were fed on these baby chicks, the virus allowed to maturate for 14 days, then 12 snakes (Thamnophis spp) were exposed to these infected vectors. After taking a blood meal from the snakes, the mosquito's head and thorax was removed and triturated with measured amounts of ground glass in complete Melnick's culture medium. A total of 2 ml of culture fluid was added during the grinding. During the trituration process the samples were frozen and thawed several times in acetone-dry ice. These samples were stored at −27 to −30°C until tested for virus. Snakes were bled from the heart at various times after the mosquito had taken a blood meal and the bloods diluted 1:10 with complete Melnick's culture medium and stored at the above temperatures until tested for virus. All snakes were about the same size, thus approximately the same age. Snakes were maintained at room temperature, 72-76°F. Virus assays were made on monolayer cultured chick embryo cells, using 60 × 10 mm Falcon plastic petri dishes. Melnick's culture fluid with phenol red to which was added 5 g/liter of lactalbumin hydrolysate and 5% calf serum, with 100 units of penicillin and 100 μg streptomycin per ml was used. Plaque forming units (PFU) were determined after incubating the inoculated monolayers at 37°C under 5% CO_2 for 30-36 hours with an agar overlay. Plaques were counted after adding neutral red and incubating for 12 to 18 hours at room temperature.

Results. These results (Table I) clearly show that not all snakes are equally susceptible to WEE virus injected by an infected mosquito during a blood meal. The results

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† The female mosquitoes from our colony average 3 mg of blood per meal.

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