Insulin-Like Activity of Crystalline Glucagon as Measured with Rat Epididymal Fat Pad Preparation. (25706)

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Crystalline preparations of glucagon were reported by Staub *et al.*(1) to contain approximately 0.05 unit/mg insulin activity. This was measured by the mouse convulsion assay. Review of literature on glucagon by Behrens and Bromer(2) revealed conflicting data on insulin-like activity of glucagon. Some workers found high concentrations of glucagon inhibited response of insulin. Others reported low concentrations of hormone augmented uptake of glucose by muscle, an insulin-like effect. Two reports, relating to insulin content of both amorphous and crystalline glucagon, have appeared recently. McNaught(3) used slices of lactating rat mammary gland to study effect of crystalline glucagon, lot No. 258-234B-43, prepared in this laboratory. Responses measured were increase in gas output and augmented uptake of glucose. The order of insulin-like activity for this sample, estimated by both responses, was 1.08%. Randle(4) likewise reported that measurable amounts of insulin-like activity were found in 3 lots of glucagon tested by isolated rat diaphragm method. The amorphous sample contained equivalent of 1.2% insulin, whereas remaining lots, including crystalline, lot No. 258-234B-54-2, were far less active. Both McNaught and Randle found that pretreatment of glucagon with cysteine completely eliminated the insulin-like response. During a study of rat epididymal fat pad preparation of Martin *et al.*(5), our preliminary tests indicated that crystalline glucagon increased oxidation of glucose in the same fashion as insulin. This was shown by its augmentation of $C^{14}O_2$ output when glucose-1-C^{14} was the labeled substrate. Quantitative data comparing the effects of crystalline glucagon, crystalline glucagon (cysteine-treated), and insulin, using a modification of the Martin method as reported here. Observations on the action of these hormones on glucose uptake of the rat epididymal fat pad are also included.

Material and methods. Crystalline glucagon lot Nos. 258-234B-43 and 258-234B-54-2, reported by McNaught and Randle, respectively, and lot No. 258-234B-167-1 were compared with insulin. Lot No. 258-234B-167-1 was tested both before and after treatment with cysteine. Inactivation of insulin content of glucagon with cysteine consisted of incubation of a 1% solution of crystalline glucagon with 1% cysteine at pH 8.7 overnight at 10-15°C. This was followed by dialysis against water, lyophilization, and recrystallization of glucagon from a pH 9 water solution at 4°C. Approximately 50% glucagon was recovered. No glucagon activity was lost after cysteine treatment. *Crystalline insulin* (25.3 units/mg), trypsin-treated and low in glucagon, was used as reference standard. Separate fresh stock solutions of insulin and glucagon were made up daily using Krebs bicarbonate buffer. N HCl was added to insulin solutions and N NaOH added to glucagon solutions to dissolve the hormones. Care was taken to maintain pH of stock solutions of glucagon at pH 9 or less. The fresh stock solutions were diluted to desired concentrations with Krebs buffer at pH 7.6. Final concentration of glucose in all incubations was 2 mg/ml. To minimize the loss of insulin on glass surfaces, a concentration of 100 μg/ml of crystalline egg albumin was maintained in each dilution made from hormone stock solutions. Tissue incubations with labeled glucose-1-C^{14}, 0.4 μC/flask and varying amounts of hormone were made in 15 ml Warburg flasks in a Dubnoff metabolic shaker at 37°C for 4 hours. Volume of incubation medium was 2 ml. The flasks were gassed 5 minutes initially with a 95% O_2 plus 5% CO_2 gas mixture. The mode of trapping $C^{14}O_2$ in Hyamine solution was essentially the same as that described by Siperstein and Fagan(6). Radioactivity in the Hyamine solution was counted to a precision of 5% or greater in a toluene scintillation mixture containing 0.05%

2,5 diphenyloxazole and 0.001% 1,4-bis-2(5 phenyloxazole) benzene. The counter employed was a liquid scintillation spectrometer (Packard 314X). Addition of an internal standard was added to correct for variation in quenching from sample to sample. The efficiency of transfer of $C^{14}O_2$ into the Hyamine solution was determined experimentally to be of the order of 94%. Radioactivity found as $C^{14}O_2$ was expressed as counts/min/g of tissue wet weight. An initial study of the $C^{14}O_2$ response of tissue to insulin indicated that within the range of 1 to 1,000 μunits/ml, the response varied essentially linearly with the log of concentrations of insulin. Similarly, with crystalline glucagon, a usable range was found between 10 and 100 μg/ml. For quantitative work, a 6 x 6 assay design was used to compare insulin, glucagon, and cysteine-treated glucagon at each of 2 concentrations. Each rat contributed 6 pieces of tissue, thus there were 36 pieces in all (Table I). With glucose uptake experiments, which were made separately, the procedure was altered as follows: volume of incubation medium was 0.8 ml and incubations were made in 10 ml beakers under an atmosphere of 95% O_2 plus 5% CO_2, which was maintained throughout the 4 hours. Glucose concentrations at the end of incubation time were determined in the remaining fluid with an AutoAnalyzer (Technicon), and uptake of glucose was expressed in terms of mg/g wet weight of tissue/4 hours. Male albino rats, non-fasted, weighing 230 to 300 g, were used in both studies. The animals were sacrificed by decapitation with a small guillotine. The epididymal fat tissue was removed, and each pad was cut into 2 or 3 pieces. Each animal thus furnished 4 or 6 pieces of tissue. Minimal handling of tissue and avoidance of chilling were essential for good responses to the various agents. With glucose uptake experiments, a study of the relationship between concentration of agents and response was not attempted. Only one concentration/sample was used. Each animal furnished 4 pieces of tissue, 3 of which were given separate treatments and one was utilized as a control (Table II).

Results. The data on comparison of insulin and the 2 glucagon samples are found in Table I. An analysis of variance of these data showed that the differences due to animals, samples, and concentrations were all greater than that expected due to chance. The calculated constants relating concentration to response for insulin and glucagon are not significantly different. These were respectively 144.8 and 124.4. A combined slope constant 134.54 $\pm$ S.E. 34.46 of the data from both glucagon and insulin was, therefore, used to calculate relative insulin-like activity of glucagon on a weight basis. This calculated value indicated that 1 mg of this sample of glucagon, lot No. 258-234B-167-1, was equivalent to 0.134 μg of insulin. The 95% confidence limits were 0.058 to 0.306 μg of insulin. Therefore, this glucagon sample contains as a best estimate 0.013% of insulin-like potency.

The concentration-response data for cysteine-treated glucagon showed a slope constant of 31.75 $\pm$ 26.85. Disregarding this difference in slope constant between glucagon and cysteine-treated glucagon, the best estimate of insulin-like activity in this sample was calculated to be 8.45% of the untreated

TABLE I. Comparison of Insulin, Glucagon, and Cysteine-Treated Glucagon on $C^{14}O_2$ Response of Rat Fat Pad in 6 Rats. Glucose-1-C^{14} was the substrate.

	Treatment					
	I_{10}	I_{100}	G_{10}	G_{100}	GT_{10}	GT_{100}
	93	427	218	514	132	225
	55	191	170	279	114	123
	55	244	125	200	106	127
	44	119	96	149	63	92
	118	169	164	284	174	212
	75	158	153	186	112	113
Means	73.3	218	154.3	278	116.8	148.5

Numerical values represent $\frac{\text{epm} \times 10^3}{\text{g tissue/4 hr}}$.

I_{10} = insulin, 10 μu/ml; I_{100} = insulin, 100 μu/ml; G_{10} = glucagon, 10 μg/ml, lot No. 258-234B-167-1; G_{100} = glucagon, 100 μg/ml, lot No. 258-234B-167-1; GT_{10} = cysteine-treated glucagon, 10 μg/ml, lot No. 258-234B-177-1; GT_{100} = cysteine-treated glucagon, 100 μg/ml, lot No. 258-234B-177-1.

Mean value of $\frac{\text{epm} \times 10^3}{\text{g tissues}}$ of untreated 14 control tissues = 37.56 $\pm$ S.E. 2.21.

Composition of Krebs bicarbonate buffer: K^+ 5 mM, Ca^{++} 0.99 mM, Mg^{++} 0.53 mM, Na^+ 146 mM, Cl^- 114 mM, HCO_3^- 40 mM.

TABLE II. Effect of Insulin, Glucagon, and Cysteine-treated Glucagon on Glucose Uptake of Rat Epididymal Fat Pad.

Sample	No. of tissues tested	Concentration, μu ml	Mean glucose uptake ± S.E., mg/g/4 hr	t*	p†
Control	4		1.274 ± .145		
Glucagon 258-234B-167-1	4	100	7.94 ± .557	29.2	<.001
Glucagon, cysteine treated 258-234B-177-1	4	100	7.42 ± 1.04	6.15	<.001
Insulin	4	100	6.38 ± 1.34	4.37	<.01

* = t value of significance treated *vs* control.
† = probability of difference arising by chance.

sample, or approximately 0.001% insulin by wt.

Two additional lots of crystalline glucagon were compared with insulin in a similar manner. The results of these comparisons are: Lot No. 258-234B-54-2 = 0.0074% insulin; 95% confidence limits 0.0021-0.0258%; Lot No. 258-234B-43 = 0.08% insulin; 95% confidence limits 0.0235-0.285%.

The data on the glucose uptake experiments are found in Table II. All samples, including glucagon preparations, caused a significant uptake of glucose at concentrations used.

Discussion. Our results differ only quantitatively from those described by Randle and McNaught. In most instances the insulin-like activity of crystalline glucagon, as determined by rat epididymal fat pad method, were lower than those reported with other tissues. The test of the cysteine-treated glucagon did not produce a reliable estimate of activity. We are unable to state that all insulin has been removed from this sample by cysteine treatment. The fact that the slope of concentration-$C^{14}O_2$ response curve has been altered and that this insulin-inactivated sample still caused a significant uptake of glucose, could be used as arguments for an effect of glucagon itself on fat tissue, although the effect is very small in comparison with insulin. A more direct solution to the problem would involve studies similar to those described by Winegrad and co-workers(7), who found that bovine growth hormone increased glucose oxidation but failed to stimulate fat synthesis.

Summary. A small amount of insulin-like activity has been found in crystalline preparations of glucagon with rat epididymal fat pad preparation. Treatment of glucagon with cysteine lowers this insulin-like activity to a small fraction of initial activity.

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