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Inability of anti-insulin serum to neutralize insulin after the hormone has become bound to muscle

Incubation of isolated frog sartorius muscles with insulin causes a gradual increase in permeability to sugar. After about 3 h of incubation at 19°, permeability reaches a plateau that is proportional to the concentration of insulin in the range of approximately 10 to 500 or 1000 microunits (μU) per ml (ref. 1). Subsequent washing of the muscles with Ringer's solution at 19° causes a slow reversal of the insulin effect, and insulin that has been bound by the tissue is concomitantly degraded under these conditions. On the other hand, neither degradation of bound insulin nor a reversal of the effect of insulin on permeability occurs to an appreciable extent when washing is performed for several hours at 0°. Reversal of the hormonal effect may depend upon removal of insulin from its site of action. Since anti-insulin serum rapidly combines with insulin in solution and effectively neutralizes it[2–4], it was of interest to see whether or not anti-insulin serum would also accelerate reversal of the hormonal effect on permeability.

A question arises as to the ability of antibodies to penetrate into the interstitial space of skeletal muscle. There is histological evidence that γ-globulin can penetrate into the interstitial space of cardiac muscle, at least in patients with rheumatic heart disease[5,6]. Furthermore, HUXLEY[7] has demonstrated that ferritin, a protein with a molecular weight of approximately 750 000, can penetrate into the transverse tubules of muscle cells within a few minutes when frog sartorius muscles are incubated *in vitro*. Therefore, it seems likely that γ-globulin molecules should be able to diffuse through the extracellular space of isolated sartorius muscles and reach the surface of the fibers.

Changes in permeability were measured by observing the initial rate of penetration of sugar into sartorius muscles at 19°. Tritium-labeled 3-*O*-methyl-D-glucose has been shown to be suitable for such measurements because it is not metabolized by frog muscles, and entry into the cells appears to be mediated by the same transport system that facilitates the entry of glucose[8]. For the present experiments, tritium-labeled 3-methylglucose was obtained, purified and counted in the manner described previously[8]. ^{14}C-Labeled mannitol was used for the simultaneous measurement of extracellular space, as in the earlier studies.

Antisera to insulin were obtained (*cf.* ref. 3) by injecting 0.5 mg of beef insulin in Freund's adjuvant into the foot pads of each of six guinea pigs on two occasions, one month apart, and then collecting heart blood. The relative potency of the antisera was tested by their ability to protect [^{131}I]insulin (Abbott Laboratories) from degrada-

tion by a rat-liver extract[9], and the most potent samples were pooled. γ-Globulin was precipitated with ammonium sulfate[10] and the precipitate was resuspended in water and made up to one-tenth of its original volume. This preparation was dialyzed against physiological saline containing 0.005 M phosphate buffer (pH 7.4) until free of sulfate ions and was then stored at $-20°$.

The data of Table I, Experiment A, reveal that when muscles were incubated with frog Ringer's solution (*cf.* ref. 11) containing 200 μU of insulin per ml, the rate of penetration of sugar increased more than 7-fold. Inclusion of anti-insulin globulin

TABLE I

EFFECT OF ANTI-INSULIN GLOBULIN ON THE BIOLOGICAL ACTION OF INSULIN, AS A FUNCTION OF THE ORDER OF ADDITION

Sartorius muscles of winter frogs were used. In Experiment A, an anti-insulin globulin preparation was added to beef insulin in Ringer's solution and left at 19° for 30 min; 2 ml of this mixture were then used per muscle during incubation for 3.5 h at 19°. Either immunoglobulin, or immunoglobulin and insulin, were omitted from media used for other sartorius muscles that were incubated simultaneously. In Experiment B, muscles were exposed to insulin in the absence of antibody for 3.5 h at 19°, excess insulin was removed by washing with 5 ml of Ringer's solution for 2 h at 0°, and then the muscles were washed for another hour at 19° with 2 ml of fresh Ringer's solution. Anti-insulin globulin was added to the 0° and 19° wash fluids for one muscle of each pair. All media and wash fluids in both experiments contained 2 mg of bovine plasma albumin per ml to help stabilize the other proteins. Permeability was measured at the end of the series of procedures described, and is expressed as v, the initial rate of entry of 3-methylglucose, in μmoles per ml of cell water per h. Each value is an average for six muscles, and the standard error of the mean is given.

Experiment	*Incubation*		*Anti-insulin globulin in wash fluids (ml/ml)*	v *(μmoles/ml.h)*
	Insulin (μU/ml)	*Anti-insulin globulin (ml/ml)*		
A	0	0	—	0.48 ± 0.07
	200	0	—	3.71 ± 0.33
	200	$5 \cdot 10^{-5}$	—	0.42 ± 0.03
B	200	0	0	3.55 ± 0.28
	200	0	$5 \cdot 10^{-5}$	3.60 ± 0.31

in the medium together with the insulin prior to addition to the muscle completely prevented this effect of insulin.

Previous studies have shown that the permeability of insulinized muscles to sugar begins to fall within 1 h when the muscles are washed with Ringer's solution at 19° (ref. 1). In Experiment B of Table I, all muscles were first exposed to insulin, then washed at 0° to remove excess extracellular insulin. Next, the muscles were washed for an additional hour at 19° to initiate reversal of the hormonal change in permeability. The main point of interest is that the addition of anti-insulin globulin to all of the wash fluids for one muscle from each frog did not cause a fall in permeability when compared with paired muscles that had been washed with Ringer's solution alone.

Intravenous injection of anti-insulin serum into an animal has been reported to cause hyperglycemia within a few minutes[4]. This phenomenon in undoubtedly

accounted for in large part by neutralization of endogenous circulating insulin. The results of the present experiments suggest that insulin that has already interacted with the muscle cells of the body is not inactivated by this means; enzymatic degradation of insulin may play an important role in reversing the effect of insulin in muscle[12,1,13].

WARDLAW AND WEIDINGER[14] found that anti-insulin serum did cause a small diminution of glycogen deposition during incubation of mouse hemidiaphragms that had previously been exposed to insulin. The experiments were performed by exposing muscles to a supramaximal concentration of insulin (5000 μU/ml) in an ice bath for a brief period of time (5 min), rinsing for 1 or 2 min, and then incubating the muscles at 37° in the presence of glucose, either with or without the addition of antiserum. It is possible that under these conditions residual insulin in the extracellular space acted on the muscle for a while at 37°, and that the antiserum prevented this effect. The present experiments were designed to minimize this complication; sartorius mucles were first exposed to a submaximal concentration of insulin at 19° for a period of time that was sufficient to allow insulin to produce its full effect, and were then washed at 0° for 2 h to remove most of the insulin contained in the extracellular fluid.

The present investigation has demonstrated that an amount of specific immunoglobulin that can neutralize 200 μU of insulin per ml and prevent it from acting on frog sartorius muscle does not abolish the effect of this concentration of insulin if it is applied after the hormone has been allowed to act on the tissue.

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