Antagonism to Insulin Action on the Perfused Hind Limb of the Rat by a Reduced Insulin B Chain-Albumin Complex

Richard J. Mahler, M.D., Olga Szabo, B.S., and Juan C. Penhos, M.D., New York

SUMMARY

A muscle perfusion technic, employing the hind limb of the rat, has been developed. Significant enhancement of glucose utilization by this tissue is seen with 500 μU./ml. of insulin. Maximal glucose uptake is seen at an insulin concentration of 1,000 μU./ml., although a significantly earlier insulin effect is noted when 10,000 μU./ml. is used.

The presence of 7.5 μg./ml. of a reduced B chain-albumin complex in the perfusion system causes highly significant inhibition of action of 1,000 μU./ml. of insulin. Neither equivalent concentrations of sulfonated B chain nor equimolar concentrations of a sulfhydryl donor, 2-mercaptoethanol, are capable of causing comparable insulin antagonism. DIABETES *17*:1-7, January, 1968.

The role of a circulating insulin antagonist has presented an attractive hypothesis concerning the etiology of diabetes mellitus.[1] One such anti-insulin substance has been found to reside with the albumin fraction of the serum proteins and hence has been termed the synalbumin insulin antagonist.[2] Although controversy exists concerning the nature and demonstrability of this factor,[3,4] the postulate that there may be a small polypeptide capable of inhibiting insulin action has stimulated further investigation in this area.

Ensinck, Coombs, Williams and Vallance-Owen have recently reported that the polypeptide B chain component of insulin,* when enzymatically reduced, interacts with serum albumin.[6] Furthermore, the albumin-bound B chain complex has been demonstrated to be capable of causing antagonism toward insulin action upon rat hemidiaphragm in vitro.[7]

Since the apposition of various proteins directly to diaphragm tissue may result in alterations of glucose utilization related to protein effect per se,[8,9] we have sought a more physiologic means of testing the hypothesis of reduced B chain antagonism toward insulin action upon muscle. For this reason, a perfusion system of the isolated hind limb of the rat has been developed.

MATERIALS AND METHODS

Perfusion apparatus

The perfusion apparatus, with minor modifications, is that described previously for the perfusion of an isolated rat liver.[10] The entire apparatus is contained within a wooden box (2.5 × 0.5 × 0.5m.) with a perfusion pump (peristaltic pump—Aminco) [1] protruding from one side (figure 1). The apparatus consists of the following components: a blood filter (Saftilene—Cutter) [2]; a small glass chamber [3] which serves as the apex of a 100 cm. column of blood. Excess blood is directed via this chamber through a bypass [4] of plastic tubing (Tygon tubing surgical formulation 5221, 1/4″ × 1/6″ into a glass aeration chamber [9]. A glass door [6] connected by surgical tape to the perfusion chamber [5] facilitates introduction of the muscle preparation. The chamber is covered with a round, three-holed plastic disc. Through the center hole is directed a Tygon plastic tube [7] (S50HL 1/8″ × 1/32″) connected above to the small glass chamber [3] and below to a needle (BD Yale Luer-Lok TLNR No. 18, 1/2″) which is connected via a cannula into the aorta. The remaining two holes allow the passage of wires from the muscle preparation within the chamber to an outside support.

The inferior portion of the perfusion chamber communicates through a rubber stopper [16] with an aeration chamber [9]. A mixture of 95% O_2—5% CO_2

*For the purpose of brevity and in compliance with convention,[5] the term B chain refers throughout this manuscript to the phenylalanyl NH_2-terminal chain of insulin.

From the New York Medical College. Dr. R. J. Mahler is Assistant Professor in Medicine, New York Medical College and is the recipient of a Research and Development Award from the American Diabetes Association. Dr. Juan C. Penhos' current address is George Washington University, Washington, D.C.

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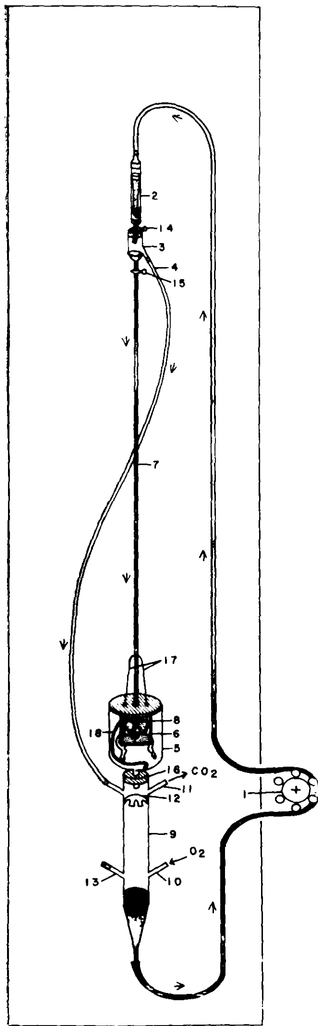

FIG. 1.
Muscle perfusion apparatus: for details see under Methods.

enters the aeration chamber through a glass portal [10] located slightly above the reservoir of perfusion solution, and an exit for CO_2 is provided in the superior portion of the aeration chamber [11]. A perforated convex glass diaphragm [12] directs a stream of perfusate from the perfusion chamber along the glass walls of the aeration chamber. An additional glass portal is provided above the perfusing medium [13] to facilitate sampling from the pool. The peristaltic pump [1] recirculates the perfusate from the reservoir to the filter [2]. By means of a thermoregulator the temperature in the box is maintained at 37° C. ± 0.5 (Bimetal thermoregulator No. 545 and Supersensitive Relay No. 506 A, the American Instrument Co., Inc., Silver Spring, Md.). A hair dryer and a regular heater bulb are located in the floor of the box to circulate warm air as a source of heat, respectively.

Surgery

Male Wistar rats weighing 350-450 gm. were fasted for eighteen hours prior to surgery. Under sodium amytal anesthesia (5 mg./100 g.i.p.) a midline abdominal incision was made from the xiphoid to the pubis. "Functional" evisceration of the animal was performed.[11] The

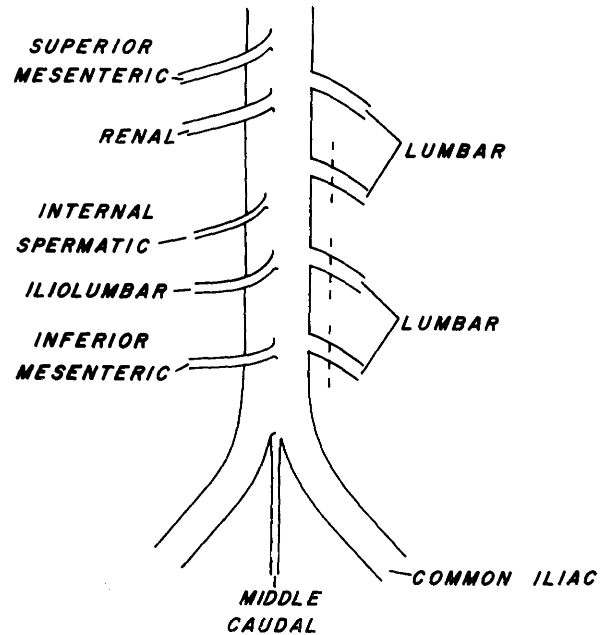

FIG. 2. Lumbar arterial system of the rat: for details see under Methods.

testicles and epididymal fat pads were removed after placement of a ligature around the vas deferens. After the hypogastric vessels were ligated bilaterally, the bladder, prostate gland, penis and rectum were removed.

The connective tissue between the aorta and inferior vena cava was carefully dissected by means of a blunt curved forceps. Other vessels ligated bilaterally in the following order were: iliolumbar, adrenal, renal and lumbar. The latter vessels were found posterior to the aorta and vena cava, between the renal vessels and the iliac bifurcation (figure 2). Ligatures to support the arterial and the venous cannulas were then placed beneath the origin of the spermatic arteries and below the origin of the iliolumbar vessels (figure 3). Further ligatures were placed around the aorta and vena cava, above the origin of the renal vessels.

Following placement of all ligatures, the skin was removed from the inferior half of the animal taking care to keep the exposed muscle covered with gauze dressings soaked in warm saline. The tail was crushed

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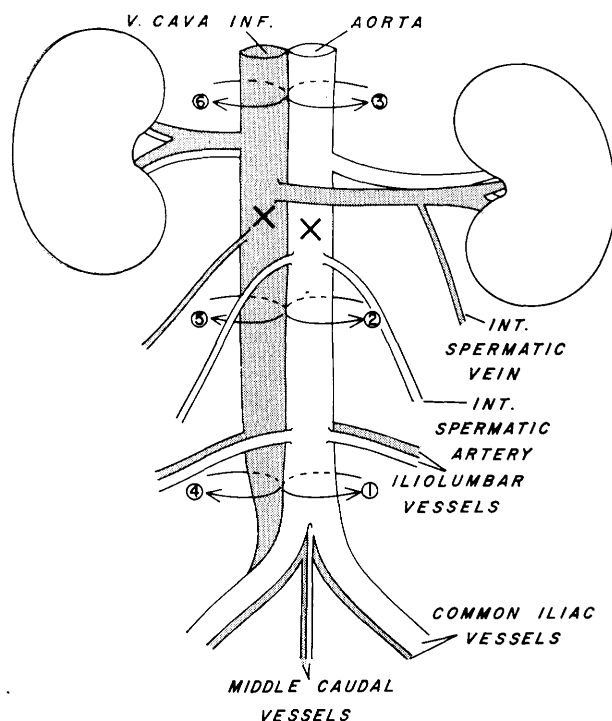

FIG. 3. Placement of aorta and vena cava ligatures: for details see under Methods.

near its origin with a pliers, tied strongly with No. 1 thread and the distal portion removed. By this procedure, the middle caudal artery lying within the vertebral bone becomes simultaneously ligated.

The aorta was then tied above the origin of the renal vessels and a small incision made above the origin of the internal spermatic with a sharp iridectomy scissor. A polyethylene cannula attached to a No. 18 needle was inserted into the aorta and the needle connected to a 20 ml. syringe containing heparinized rat blood (see below). The cannula was then tied in place and blood delivered to determine the viability of the vessel. Care was taken to avoid the entrance of air into the aorta by filling the cannula with blood prior to insertion.

The inferior vena cava was next tied above the origin of the renal vessels, and an incision made at the same level as the aortic incision. A similar cannula 15 cm. long, previously washed with heparin, was inserted. Adequate cannulation was confirmed by the immediate return of venous blood from the cannula.

The rat was hemisected just below the level of the kidneys, utilizing a bone cutter to sever the vertebral column. A No. 1 thread [17] was quickly tied onto each side of the lumbar zone to provide suspension of the hind limb within the perfusion chamber (figure 1).

Sequence of manipulation

One-hundred milliters of a heparin solution (5 mg. per cent in 0.9 per cent saline) was introduced via the perfusion chamber into the reservoir and the peristaltic pump started. Sufficient wetting of the entire apparatus occurred within ten minutes, at which time the solution was drained from the system. With the pump turned off, 100 ml. of whole rat blood collected as described below was carefully introduced via the perfusion chamber. The pump was then started and the flow rate adjusted sufficiently to initiate the circulation. The O_2-CO_2 mixture was then introduced into the aeration chamber through portal (figure 1 [10]). The Hoffman clamp [14] under filter [2] was adjusted so as to maintain a blood level of 2 cm. in the filter in order to avoid bubbling or foam formation. The clamp [15] below the small chamber [3] was then closed, limiting blood flow to the bypass. A 20 cm. length of polyethylene tubing was then connected to the needle [8] and passed through opening [16] in order to be brought into contact with the glass diaphragm [12]. This procedure was performed in order to avoid streaming of blood from the needle when clamp [15] is opened.

Following completion of surgery, the aortic cannula was severed 6 cm. above its entrance into the aorta and, keeping the end of the cannula firmly closed between thumb and index finger, the muscle preparation was brought to the perfusion chamber. Tubing [7] was then clamped, the polyethylene tubing added to the needle [8] was removed and the aortic cannula re-attached to the same needle. The clamp was opened immediately and blood flow was established. The thread on each side of the lumbar zone [17] was passed through the holes of the cork and tied to an outer support following proper positioning of the muscle within the perfusion chamber.

The cannula inserted into the inferior vena cava [18] was carefully curved and its distal end placed against the wall of the inferior portion of the chamber [5]. An average flow of 5 ml. per minute from the venous cannula [18] provides a good index that the preparation is supplied with an adequate flux of blood. Samples were taken from the inferior vena cava cannula at specified time intervals as detailed in Results. All muscle preparations were perfused for seventy minutes.

Preparation of the perfusate

Male Wistar rats of 350-450 gm. weight were fasted overnight and injected with sodium amytal (5 mg./ 100 gm. weight) intraperitoneally. Blood was collected

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from the abdominal aorta with a 20 ml. syringe and No. 18 needle previously moistened with heparin. The volume of blood obtained varied from 10 to 17 ml. per rat, and the total required volume was pooled in a heparin washed beaker chilled in ice and filtered. The perfusion volume was 100 ml. of whole blood to which 100 mg. of glucose was added.

Preparation of insulin and derivatives

Bovine crystalline insulin, Lot 795372 was supplied by Eli Lilly Co., through the courtesy of Dr. Ronald E. Chance. Five milligram aliquots were solubilized once weekly in 0.6 per cent acetic acid to yield a final concentration of 50 U. per milliliter. Subsequent dilutions were performed in Gey and Gey buffer[12] to a final concentration of 1 U., 0.1 U. or 0.05 U. per milliliter and 1 ml. aliquots added to 100 ml. of perfusate at zero time to yield a final insulin concentration of 10,000, 1,000 or 500 μU./ml., respectively.

Sulfonated B chain (Lot 488-527 B-65-2) prepared according to the procedure of Dixon and Wardlaw[13] was kindly provided by Dr. Ronald E. Chance of the Lilly Research Laboratories. Seven hundred and fifty (750) μg. of $BSSO_3$* were solubilized in a centrifuge tube containing 50 μl. 2.7M formic-acid-8 M urea. One milliliter of 0.1 M "tris" buffer *p*H 7.4 containing 12.5 mg. of normal human albumin (Cohn Fr. V. Mann Research Laboratories) was added to the B chain solution and incubated at 37° C. for sixty minutes with moderate agitation. The sulfonated B chain-albumin solution was then added to the perfusate as indicated in Results.

Reduced B chain was prepared as follows: A 6.6 mg. aliquot of $BSSO_3$ was dissolved, in a Thunberg tube, in 0.68 ml. of a solution containing 500 μmoles of thioglycollic acid, 30 μmoles versene and 40 mmoles of urea. The *p*H was adjusted to 5 with methylamine and the solution left overnight at 4° C. under nitrogen.[7] The addition of 25 ml. of acetone resulted in an opalescent solution which was concentrated to a small volume in a vacuum pump with the temperature kept below 40° C. To the residue were added 0.5 ml. of 2.7M formic-acid-8 M urea and 2.0 ml. of 5 per cent albumin in 0.1 M tris buffer *p*H 7.5. Following incubation at 37° C. for one hour, the small molecular components were removed by two passages through a 20 × 1 cm. column of Sephadex G-15. The amber colored eluate was lyophilized and stored in vacuo. As indicated in Results, 12.5 mg. albumin containing 0.75 mg. BSH* were dissolved in 1 ml. of 0.1 M tris buffer *p*H 7.5 and added to the perfusate.

*Throughout the manuscript $BSSO_3$ refers to the sulfonated B chain of insulin.

Preparation of 2-mercaptoethanol

As detailed in Results, 29 mg. of 2-mercaptoethanol (Eastman Kodak) were dissolved in 1 l. of distilled water and 1 ml. of this solution was added to the perfusate.

RESULTS

The hind limb preparation was placed into the chamber, and a ten-minute equilibration period was allowed at the start of each perfusion. All additions to the perfusate and sampling from the venous return were started after equilibration. In the presence of glucose alone, the disappearance rate of glucose was 0.56 mg./min./100 gm. and was constant throughout the perfusion period. When appropriate control experiments were performed in which the hind limb was removed at the end of the equilibration period, glucose utilization by the red blood cell mass equalled the total utilization when the limb was perfused. Hence, in agreement with the observations of Andres, Cader and Zierler, the resting muscle appears to utilize minimal amounts of glucose.[14]

TABLE 1

Perfusate glucose concentration before and after perfusion of hind limb*

	Zero minutes		Fifty minutes	
	Average	Range	Average	Range
No additions	114	92-136	89	48-110
500 μU./ml. insulin	121	113-134	65	55-76
1,000 μU./ml. insulin	121	110-140	32	25-40
10,000 μU./ml. insulin	128	100-152	30	25-59
7.5 μg./ml. BSH	111	101-130	91	76-115
1,000 μU./ml. insulin +7.5 μg./ml. BSH	123	102-133	75	76-98
1,000 μU./ml. insulin +7.5 μg./ml. $BSSO_3$	122	111-144	35	25-44
1,000 μU./ml. insulin +29 μg./100 ml. 2-mercaptoethanol	126	111-155	42	25-80

*All figures are expressed as milligrams per 100 ml. and represent the average of four experiments with its corresponding range.

In view of significant glycolysis by the red blood cell, all subsequent calculations of utilization have been derived after accounting for glucose utilization by blood alone. Thus without insulin, utilization of glucose by muscle is close to zero milligram. With the addition

*Throughout the manuscript BSH refers to the reduced B chain of insulin.

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of 500 μU./ml. of insulin at the start of the perfusion, significant enhancement of glucose uptake above basal values is seen after thirty minutes, with an average disappearance rate of 0.44 mg./min./100 gm. (figure 4).

In the presence of an added 1,000 μU. of insulin per milliliter, the glucose disappearance rate increased to 0.76 mg./min./100 gm. of perfused tissue. An insulin effect was first noted at fifteen minutes, and maximal glucose utilization occurred anywhere from fifteen to thirty minutes (figure 5). 1,000 μU. of insulin added to the perfusate in the absence of muscle tissue failed to cause enhanced glucose utilization by the red blood cell.

Finally, with 10,000 μU. of insulin per milliliter, significant enhancement is noted after five minutes, although the net glucose utilization of 0.79 mg./min./100 gm. is not significantly different from that seen in the presence of 1,000 μU. of insulin/ml. (figure 4).

In the next series of experiments, 12.5 mg. of normal human albumin containing 0.75 mg. of BSH dissolved in 1 ml. of 0.1 M tris buffer *p*H 7.5 was added to the glucose perfusate in the absence of insulin. Neither enhancement nor impairment of basal glucose utilization was observed (figure 5). Since optimal insulin sensitivity in this system is seen with 1,000 μU. of insulin per milliliter, it was decided to test the effect of BSH in the presence of this insulin concentration. Immedi-

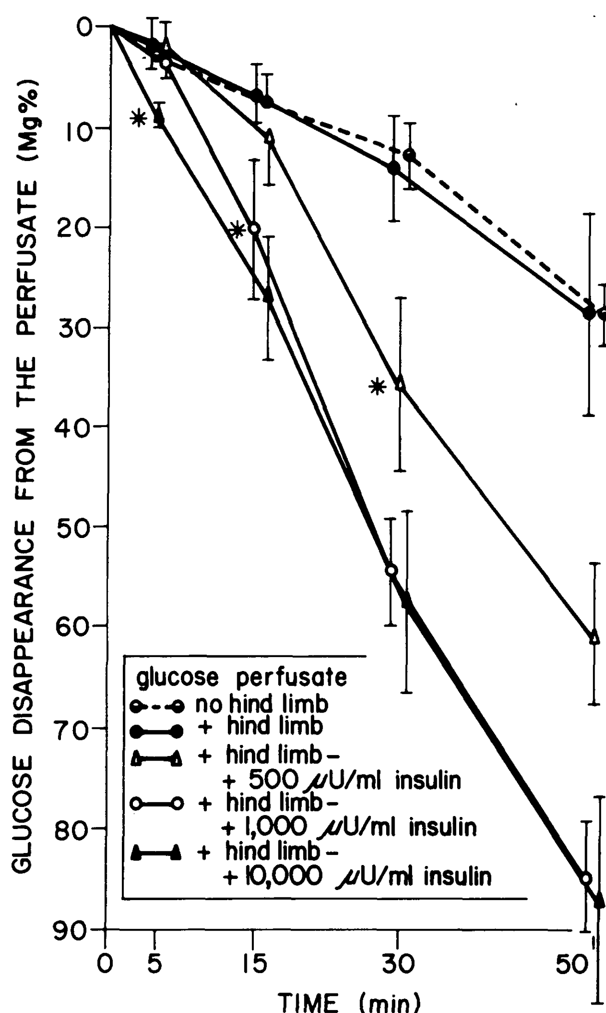

FIG. 4. All insulin additions to the perfusate pool were made at 0 time; all glucose samples were collected from the venous effluent at the indicated times. Each point represents the mean of four individual experiments. * $p < 0.01$ as compared to hind limb alone at the corresponding time.

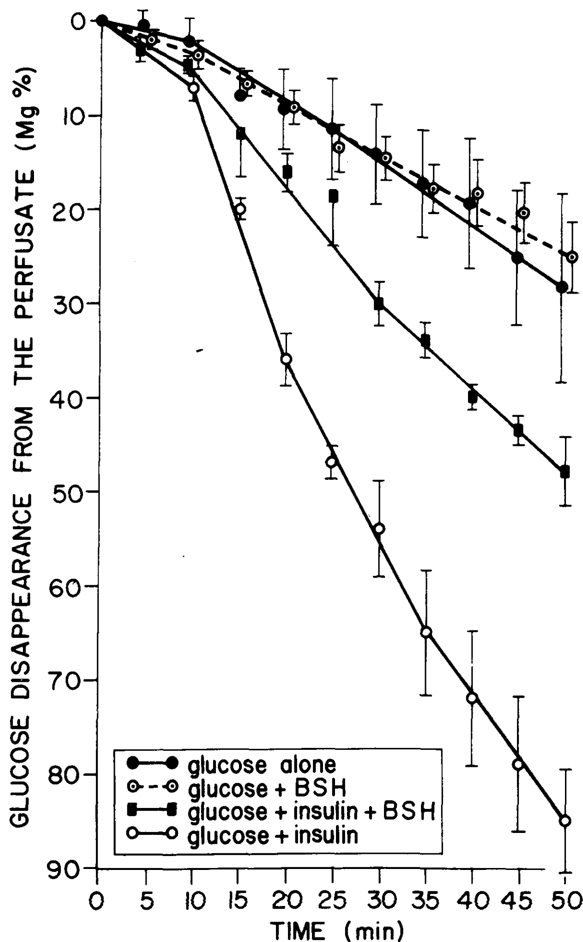

FIG. 5. 0.1 U. of insulin and 0.75 mg. of BSH were added to the perfusate as described in Methods. Each point represents the mean of four individual experiments ± S.E.M.

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ately following the addition of 0.1 U. of insulin to the perfusate, 1 ml. of tris buffer containing 12.5 mg. of albumin and 0.75 mg. of BSH was added. Significant impairment of insulin action was seen in ten minutes ($p < .01$) which continued, in a highly significant manner, throughout the perfusion (figure 5). There was, however, significant residual insulin enhancement of glucose uptake above basal values ($p < .001$). Therefore, although antagonism toward 1,000 μU./ml. of perfusate insulin clearly exists in this system, the antagonism appears to be incomplete.

Since prior information has demonstrated the sulfonated B chain to be devoid of antagonistic activity toward insulin action in vitro,[7] comparable experiments have been performed upon the perfused hind limb. Immediately following the addition of 0.1 U. of insulin, 12.5 mg. of human albumin containing 0.75 mg. of $BSSO_3$, dissolved in 1 ml. of 0.1 M tris buffer *p*H 7.5 was added to the perfusion. As recorded in figure 6, no insulin antagonism was demonstrated.

Finally, since the presence of reduced sulfhydryl groups, in adequate concentration, has been reported to be capable of causing the reductive cleavage of the insulin molecule and thereby inhibition of insulin action,[15] the following experiment was performed. Immediately following the addition of 0.1 U. of insulin, 29 μg. of 2-mercaptoethanol was added to the perfusate, thus providing free SH groups in a concentration equimolar to that of the SH groups of the reduced B chain (0.375×10^{-3} mM per total volume). It is apparent in figure 6 that this concentration of 2-mercaptoethanol is not sufficient to cause insulin antagonism. Hence, it appears unlikely that the insulin antagonism caused by the reduced B chain is due solely to its content of free SH.

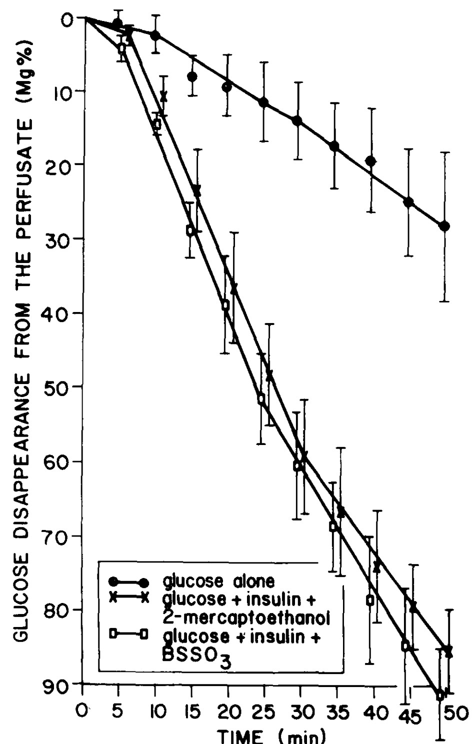

FIG. 6. 0.1 U. insulin and either 0.75 mg. of $BSSO_3$ or 29 μg. of 2-mercaptoethanol were added to the perfusate as described in Methods. Each point represents the mean of four individual experiments ± S.E.M.

DISCUSSION

The present perfusion system has been selected in this study of insulin antagonism for two reasons. One, the sensitivity and consistency of the responses to graded doses of insulin; and two, the fact that the muscle tissue is bathed physiologically, i.e., through its vascular bed. Present modification of earlier rat muscle perfusion technics[16] are: extreme care in ligating vertebral arterial and venous channels so that the spinal column is not included in the perfusion; and the cannulation of both aorta and vena cava to insure the source of venous effluent. Instillation of bromphenol blue into the aorta has insured that retroperitoneal adipose tissue is not perfused by the present technic. In addition, the measurement of plasma nonesterified fatty acid prior to and following perfusion does not show significant change (unpublished results).

The degree of insulin enhancement of glucose uptake by muscle cells is seen, in this system as in others, to be related to insulin dose.[17] There is also a significantly earlier effect on disappearance of glucose as insulin concentration is raised above a level which produced a maximal total uptake in fifty minutes (figure 4). These observations suggest that the time necessary for insulin action appears to be inversely related to insulin dose.

When the BSH-albumin complex is added to the perfusion system at concentrations of 7.5 μg. of B chain per milliliter, highly significant antagonism toward 1,000 μU. of insulin per milliliter results. Although it may be considered that this B chain concentration represents a two hundredfold increase over that of insulin, these calculations assume quantitative reduction, solubilization and binding of B chain to albumin, which would seem unlikely. Since, in the absence of added insulin, the reduced B chain-albumin complex fails to influence

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basal glucose transport, the effects of this preparation appear to be dependent upon the presence of insulin. In the experiments reported, the BSH-albumin complex has been added to the perfusate pool within one minute following the addition of insulin. In two experiments not described, when reduced B chain-albumin was added prior to the addition of insulin, no insulin antagonism was seen (unpublished observation). Hence, it would appear that the reduced B chain-albumin complex instead of acting as a competitive inhibitor at insulin receptor sites on the tissue, may cause chemical alteration of the insulin molecule. A similar temporal relationship between reduced B chain-albumin antagonism and insulin action has been noted by Fenichel, Bechmann and Alburn.[18]

That the B chain must be in the reduced state in order to exert activity is supported by a lack of insulin antagonism with the sulfonated B chain complexed with albumin in this system as well as in vitro systems.[6] Whether the available sulfhydryl groups per se are the cause of insulin antagonism is seemingly answered by lack of inhibitory activity with equimolar concentrations of a sulfhydryl donor, 2-mercaptoethanol. Of course, it may be considered that the reduced B chain may be more active in the reductive cleavage of insulin. In any event, the present data along with those of Fenichel et al. are consistent with insulin antagonism by a reduced B chain-albumin complex mediated via a specific chemical alteration of the insulin molecule.[18]

Finally, although this work demonstrates the presence of insulin antagonism upon perfused muscle, whether this interaction applies to in vivo situations or exerts any physiologic role in glucose homeostasis is an important question that, at this time, remains unanswered.

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