Serum Insulin-Like Activity as Measured by Mouse Hemidiaphragm Technic. (25826)

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We described(1) a method for estimating minute amounts of insulin based on its stimulating effect on glucose uptake by isolated mouse diaphragm tissue. In the present study we have applied this technic to serum using stimulation of glucose uptake as an index of "serum insulin" or more precisely of serum insulin-like activity.

Method and materials. The general procedure for preparing diaphragms and measuring glucose uptake previously described(1) was employed except for the following modifications: (a) number of hemi-diaphragms in each pool was reduced from 5 to 4, (b) Mg-free standard Krebs - Ringer - bicarbonate (KRB) solution(2) was employed in incubation medium in place of the previously used high bicarbonate containing modified KRB solution, (c) diaphragms were not chilled during preparation as was done previously but rinsed in buffer solution kept at room temperature. The test incubation medium consisted of 2.0 ml of KRB-glucose (KRBG) solution and 1.0 ml of KRB-dialyzed serum sample. Glucose was dissolved in the KRB solution (1.5 mg/ml) so that initial glucose concentration of the incubation medium was 100 mg%. The control medium was identical to the test medium except for substitution of KRB solution (glucose free) for the serum. Serum insulin-like activity is expressed as net increase in glucose (mg) disappearing from the test medium/10 mg dry wt of diaphragm tissue/90 min incubation over its corresponding control. *Serum samples*: Within 3 hours after a blood sample was taken, serum was separated and dialyzed for a minimum of 3 days against daily changes of fresh KRB solution stirred by a magnetic stirrer in the cold. Blood was obtained by cardiac puncture from Osborne-Mendel rats and Swiss-Webster mice anesthetized with sodium pentobarbital (30 mg/kg). Dog, human, and rabbit blood were obtained without anesthesia. Alloxan-diabetes was produced by injecting alloxan intravenously: 70 mg/kg for rats and 200 mg/kg for rabbits. Rats were maintained by subcutaneous administration of 1 to 2 units of protamine zinc insulin 6 days a week; rabbits were given 4 units. Animals were used no earlier than 2 months following alloxan treatment. Animals were fasted for 24 hours and insulin administration discontinued 48 hours prior to bleeding and only those animals having a blood sugar value over 300 mg% were used in these experiments.

Results. Fig. 1 shows activity of normal rabbit serum to which increasing amounts of insulin were added and then dialyzed according to test procedure. The control to which no insulin was added indicates the endogenous insulin-like activity initially present in serum. Treatment of control serum with $NaSH \cdot 2H_2O$ (25 mg/ml) for 30 minutes at room temperature completely eliminated this activity. Although as little as 0.1 milliunit insulin/ml of serum increased significantly glucose uptake over control serum, comparison of responses to insulin in this serum to that in buffer medium alone (Fig. 2) indicated a possible inhibitory action of normal serum on exogenous insulin.

Other serum protein solutions were studied for their effect on insulin activity (Table I). In these experiments 1 milliunit of insulin in 0.1 ml volume was added to the test incubation medium which consisted of 1.0 ml of the dialyzed protein solution and 2.0 ml of KRBG solution. In each case the corresponding control medium was identical in composition with the test medium except for omission of insulin. Differences in endogenous insulin activity of the various protein solutions were corrected by this procedure. Also, by direct addition of insulin to incubation medium the possibility of loss during the dialysis procedure was eliminated. All protein solutions used had a relatively low endogenous activity, less

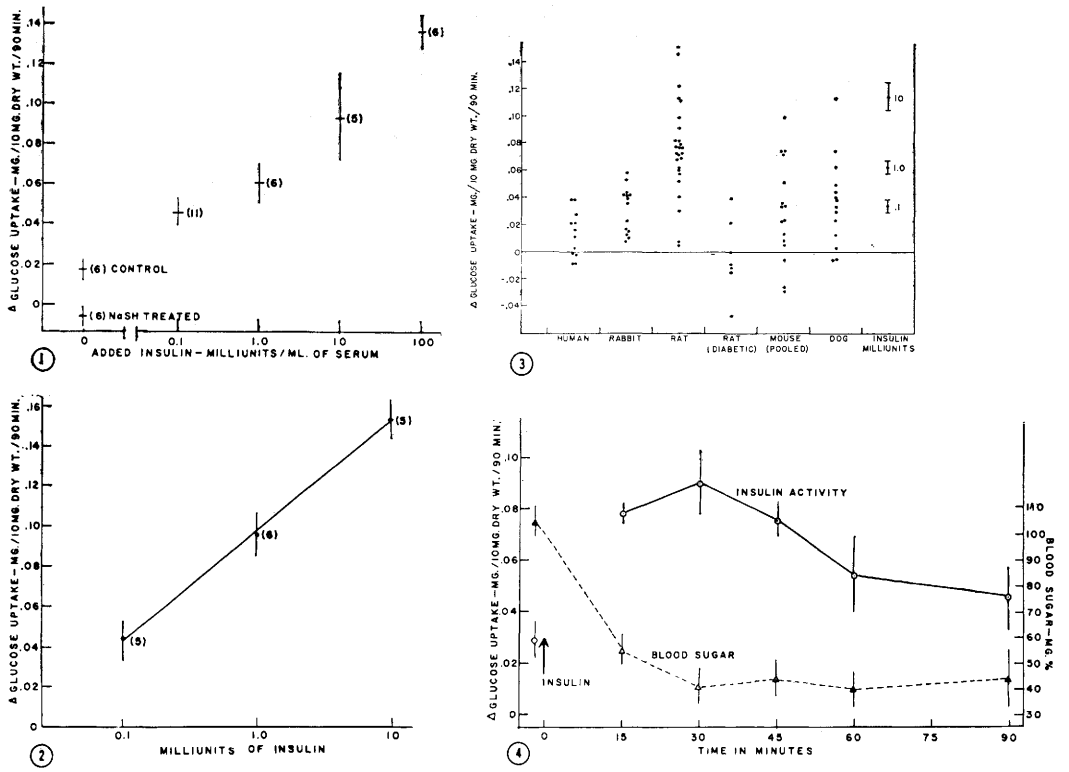

FIG. 1. Mean effect of insulin added to normal rabbit serum. No. of experiments in parentheses; stand. errors as vertical bars.

FIG. 2. Log dose—response of insulin in KRB buffer medium. Milliunits insulin in 3.1/ml total incubation vol.

FIG. 3. Comparison of serum insulin-like activity of various species. See text.

FIG. 4. Serum insulin activity and blood glucose values in rabbits following intrav. inj. of crystalline zinc-insulin, 1 unit/kg. Mean ± stand. error from 5 animals.

than 0.02. Maximum activity of 0.133 effected by 1 milliunit insulin was obtained in the beef serum albumin (BSA) medium (1.6% final conc.). In support of the previously observed inhibitory action of normal serum, activity of insulin in the presence of normal rabbit serum was significantly less than in the KRB medium alone. Although insulin activity measured in the presence of diabetic rabbit serum appeared to be greater than in normal serum, the difference was not statistically significant. Ability of normal rabbit serum to inhibit insulin response was unaffected by diluting the serum as much as 10-fold; ac-

TABLE I. Effect of Serum Proteins on Response to Insulin.

Medium		Glucose uptake, mg/10 mg dry wt/90 min. Control	1 milliunit insulin	Effect	P
(A) Beef serum albumin 1.6%	(9)	.401 ± .007	.535 ± .011	.133 ± .009	A–B <0.025 A–C <0.01 A–D <0.001
(B) Buffer only	(6)	.450 ± .014	.547 ± .013	.096 ± .011	B–C <0.5 B–D <0.05
(C) Rabbit serum, diabetic	(4)	.378 ± .037	.461 ± .032	.084 ± .008	C–D <0.2
(D) Rabbit serum, normal	(8)	.453 ± .018	.517 ± .023	.064 ± .009	

No. of experiments in parentheses. Glucose uptake values = mean ± stand. error. P = level of significance between indicated media.

tivity of 1 milliunit insulin was 0.060 ± .01 in undiluted serum and 0.062 ± .008 in serum diluted 10-fold.

Fig. 3 shows serum insulin-like activity of various species as measured by mouse diaphragm technic. Serum samples were from non-fasted dogs and humans; rabbits, rats, and mice were fasted for 24 hours. Mean activity ± S.E. for each species were: human (0.013 ± .005), rabbit (0.031 ± .004), rat (0.075 ± .007), alloxan-diabetic rat (0.003 ± .010), mouse (0.030 ± .009), and dog (0.036 ± .009). Insulin concentrations (milliunits/ml) corresponding to these mean activities are: human (0.02) rabbit (0.09), rat (1.8), alloxan-diabetic rat (0.0), mouse (0.08), and dog (0.13). These values were calculated from a response curve of insulin standards measured in presence of rabbit serum (Fig. 3, last column). Insulin response from 6 replicate analyses was: 0.114 ± .011 for 10 milliunits, 0.062 ± .005 for 1 milliunit, and 0.033 ± .005 for 0.1 milliunit. Mean activity of normal rats was significantly higher than in any other species studied. Of 7 samples tested from alloxan-diabetic rats, 5 showed no insulin-like activity. Suppression of glucose uptake by a sample of diabetic rat serum below the uptake of its corresponding buffer control appeared to be related to degree of lipemia since the most opaque sample showed the most inhibitory action. In initial attempts to measure insulin-like activity of mouse serum (6 mice/pool), blood was obtained by decapitation. Serum obtained by this procedure consistently showed no significant activity and added insulin (2 milliunits/ml) was not recoverable. These observations may be explained by release from damaged tissue cells of insulin degrading factors(3,4) into blood obtained by decapitation.

Fig. 4 shows mean serum activity and blood sugar level of 5 rabbits before and after intravenous injection of insulin (1 unit/kg). Insulin was injected into the marginal ear vein of one ear and blood samples taken from the opposite ear vein at the indicated times. A significant increase ($p < .005$) in activity from the pre-injection level was noted in all rabbits at 15, 30 and 45 minutes; calculated insulin concentration at these times were 2.2, 3.5, and 1.9 milliunits/ml respectively compared to 0.08 milliunit/ml for initial serum concentration. At 60 and 90 minutes the concentration was calculated to be 0.55 and 0.27 milliunit/ml but these levels were not significantly different from initial pre-injection level.

Discussion. Although most workers employing the diaphragm technic for estimating "serum insulin" use undialyzed serum or plasma, we have employed dialyzed serum because: a) it obviates tedious adjustments in amount of glucose added to incubation medium to compensate for variations in glucose concentration of test serum samples; b) it eliminates dialyzable serum constituents which may possibly affect glucose uptake and; c) it minimizes possible changes effected by serum on pH and buffering capacity of incubation medium which have a marked influence on glucose uptake(1). Dialyzed serum samples have been kept at 4°C for as long as 2 weeks without measurable loss of insulin activity.

Serum insulin concentration is usually calculated by comparison of serum response directly with response of insulin standards which are run in a medium devoid of normal serum proteins. In view of the inhibitory effect which we have found of normal whole serum on insulin response, it is evident that calculations based on such a comparison will give lower values for "serum insulin" than responses corrected for this inhibitory effect of serum. We have attempted to correct for this inhibitory effect of serum by determining response of insulin reference standards in presence of normal serum (rabbit).

Studies are in progress to determine the nature and degree of this inhibitory effect of normal serum: whether it varies from individual to individual and whether there are species differences. Accurate transformation of net glucose uptake effect to equivalent insulin concentration can be made only when this is known. It is apparent that the inhibitory activity probably resides in the non-albumin fractions of whole serum since BSA did not inhibit. The actual enhancing effect of BSA

on insulin response compared to that obtained in buffer medium alone may be due to its protective action in preventing loss of insulin by adsorption or degradation.

Comparison of "serum insulin" levels in normal animals have shown that there is a considerable range among normal individual animals; normal rats, for example, have shown as much as a 1000-fold variation in serum activity. Although some of this scatter may be a reflection of the inherent variability of the bioassay technic employed, results so far lend support to the view that significant changes of "serum insulin" occur under normal physiologic conditions. The most interesting observation in comparing relative activities in the various animal species was the marked difference between human and rat serum insulin-like activity. The order of increasing activity found was: human<mouse, rabbit, dog<<rat. Because of its significantly higher "serum insulin" level, rat serum should be particularly useful in studies to characterize the serum constituent which stimulates glucose uptake and to establish its relationship with "serum insulin."

The general shape of the insulin clearance curve in rabbits (Fig. 4) is similar to that obtained by Scott and co-workers(5) who employed a higher dose (15 units/kg) than the 1 unit/kg dose used in our studies. Although they found a 6-fold maximum increase in insulin concentration over pre-injection level, we have found over a 40-fold increase while employing 1/15 the amount of injected insulin. This difference may be explained in part of the much higher value of 2.5 milliunits/ml they report for normal rabbits before injection compared to 0.08 milliunit/ml which we have found using the mouse diaphragm technic.

Summary. The mouse diaphragm technic has been adapted to measure insulin-like activity of dialyzed serum. Compared to the response effected by insulin in KRB buffer medium alone, normal rabbit serum inhibited while beef serum albumin enhanced insulin response. Mean serum insulin level of adult rats was significantly greater than rabbits, mice, and dogs and much higher than humans. A significant elevation in serum insulin activity which persisted up to 45 minutes following injection, was found in rabbits injected intravenously with 1 unit/kg insulin.

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Immune Electron Microscopy. (25827)

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In the past decade advances in the study of immune reactions of bacterial, viral, and mycotic agents have been made, using immunofluorescent technics. It has become apparent that even more refined technics are desirable because of the low resolution with this technic, to prove that in some systems the reactions are specific and not due to nonspecific uptake of the fluorescent dye, and also to provide a means of studying pathogenesis at a subcellular level. Singer(1) prepared an electron-dense conjugate by coupling ferritin to an antibody, indicating that specificity of the antibody was probably not altered. Using