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Effect of Insulin on Glucose Uptake by Mouse Diaphragm Tissue. (24534)

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The effect of insulin in increasing glucose uptake by isolated rat diaphragm tissue has been employed by various workers(1-7) for estimating minute amounts of insulin. Our preliminary results in determining insulin activity using the rat hemidiaphragm technic were so often variable that we undertook the present study to develop a more reliable test method. We believe the method to be described employing pooled mouse hemidiaphragms is an improvement over the rat diaphragm technic from the standpoint of sensitivity and reproducibility. In developing the method, a systematic study was made of the various factors affecting glucose uptake to assess their effect upon the assay of insulin.

Materials and methods. Swiss-Webster and NIH general purpose male albino mice were used in most of our studies. A few experiments were made with DBA-2 and C-57 strains of mice. No difference in insulin responsiveness which could be attributable to strain differences was found. For each experiment the mice were of same weight ($\pm$ 2 g) and in most experiments were in the 25-30 g weight range. Mice were fasted for 16-24 hours before use. Unless otherwise stated, the medium employed for incubation was a modified Krebs-Ringer-bicarbonate solution, hereafter referred to as KRB solution, having the following composition in millimoles/l: NaCl (95), KCl (4.7), $CaCl_2$ (2.5), KH_2PO_4 (1.2), and $NaHCO_3$ (50). Glucose was added to give a concentration of 1 mg/ml. The insulin used was a glucagon-free, crystalline zinc sample (Eli Lilly & Co.) which assayed 24.6 units/mg. Dilutions of a stock insulin solution (10 units/ml) were made before each experiment with 0.9% saline solution which had been brought to pH 3 with 1 N HCl. A systematic procedure was followed in preparation of the diaphragms. Groups of 5 mice were treated successively in the following manner. As each mouse was killed by decapitation its diaphragm was excised with a pair of iris scissors and placed in ice cold KRB solution (without glucose) which was gassed continuously with a mixture of 95% O_2-5% CO_2.

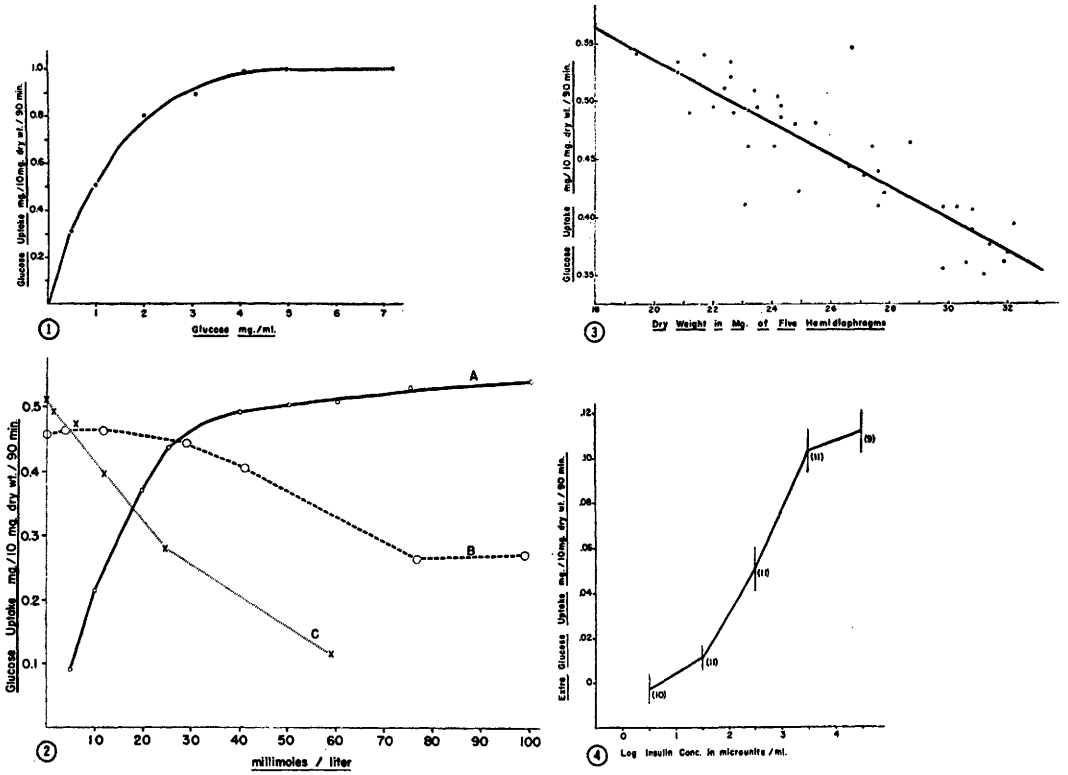

FIG. 1. Effect of glucose concentration on uptake. Each point represents uptake of 5 pooled mouse hemidiaphragms incubated in 3 ml of KRB medium.

FIG. 2. Effect of bicarbonate (A), potassium (B), and magnesium (C) concentration on glucose uptake.

FIG. 3. Effect of diaphragm size on glucose uptake.

FIG. 4. Increase in glucose uptake as a function of insulin concentration. No. of experiments in parentheses; stand. errors as vertical bars.

When all 5 diaphragms had been removed, they were rinsed with fresh medium and each diaphragm was cut in half after careful removal of adipose tissue and the thick posterior portion. The hemidiaphragms were blotted on filter paper and transferred to chilled Warburg vessels containing appropriate incubation medium. Each test vessel contained 5 hemidiaphragms from 5 mice and a second Warburg vessel containing the other hemidiaphragms from the same mice served as a control. For assay of insulin each test vessel contained 3 ml of KRB (1 mg glucose/ml) and 0.10 ml of the appropriate insulin dilution; each control vessel contained 3 ml of KRB glucose solution and 0.10 ml of insulin diluent. The flasks were placed in 37°C bath, gassed with 95% O_2-5% CO_2 for 10 minutes and incubated for 90 minutes at a shaking rate of 130 cycles/min. Approximately 15 minutes were required for preparation of the diaphragms of each group. A single experiment usually consisted of 6 groups (30 mice). Glucose remaining in the medium after 90-minute incubation was determined by the Somogyi modification of Nelson's method(8) using zinc sulfate-barium hydroxide filtrates. The diaphragms from each flask were heated at 110°C for 2 hours to obtain dry weights and glucose uptake calculated as mg glucose taken up from the medium/10 mg dry weight of diaphragm tissue.

Results. Effect of glucose concentration. Fig. 1 shows the effect of varying glucose concentration upon uptake of glucose by pooled mouse hemidiaphragms. Test hemidiaphragm pools were incubated in 3 ml of KRB solutions of varying glucose concentrations and each of the corresponding control pools were incubated in 3 ml of KRB solution containing

1 mg glucose/ml. The uptake was calculated for each glucose concentration as follows: $\frac{t}{c} \times \bar{c}$ = calculated uptake in which t = observed uptake of test, c = observed uptake of corresponding control, and $\bar{c}$ = average uptake of all controls. The uptake was markedly increased as glucose concentration was raised from 0.5 to 2 mg/ml; however, with higher concentrations the uptake leveled off, indicating an approach to maximal rate. Since glucose concentration falls off progressively during incubation, uptake rate progressively decreases and the values shown represent overall average rates for 90-minute incubation period. Under conditions selected for assay of insulin, initial glucose concentration of 1 mg/ml fell to approximately 0.6 mg/ml during incubation. The insulin effect was more reproducible under these conditions than with higher glucose concentrations although the absolute insulin effect was smaller.

Effect of ionic composition. The effect of bicarbonate, potassium, and magnesium concentrations in KRB solution upon basal glucose uptake is shown in Fig. 2. Uptake values were calculated according to the method described for effect of glucose concentration on uptake. The isotonicity of the modified KRB solutions was maintained by adjusting NaCl concentration. The profound effect of bicarbonate concentration (Curve A) on uptake was a pH effect since under a constant CO_2 pressure, the pH of the HCO_3-CO_2 buffer system varies with the HCO_3^- concentration. HCO_3^- concentration range of 5 to 100 millimoles/l corresponds to pH range of 6.2 to 8.2(9). To minimize the effect of any pH change upon uptake during incubation, a 50 millimolar bicarbonate concentration (pH 7.9) was selected for assay of insulin. A significant reduction in uptake rate was effected when potassium concentration exceeded 40 millimolar (Curve B). These results are similar to those of Stadie and Zapp(10) who found that glycogen synthesis by the rat diaphragm was markedly reduced by high potassium concentrations. Due to progressively stronger inhibitory influence of magnesium ion on basal uptake (Curve C), this ion was omitted from the assay medium. Studies on the effect of calcium were limited because Ca CO_3 precipitated from the KRB medium when calcium concentration exceeded 5 millimolar. In the absence of calcium the uptake was increased by an average of about 6% compared to the uptake in KRB medium with calcium. Diaphragms incubated in an isotonic medium having sodium as its only cation constituent (105 millimolar NaCl, 50 millimolar $NaHCO_3$) showed an average 20% lower uptake than diaphragms incubated in KRB medium. Moreover, diaphragms incubated in saline-HCO_3 medium became significantly hydrated and showed total water content of 2-3% more than the average of 78.1% for diaphragms incubated in the KRB medium.

Effect of diaphragm size. It was observed that basal uptake rates of diaphragms from large sized mice were lower than those of smaller sized mice. This inverse relationship between uptake and diaphragm size is shown by Fig. 3. The linear regression line as calculated by the method of least squares is: $Y = -0.135X + 0.805$, where Y is the basal uptake in mg of glucose/10 mg dry wt/90 min and X is the dry weight in mg of 5 pooled hemidiaphragms. The data were obtained from mice weighing 18 to 30 g. Under comparable conditions, the basal uptake of mouse diaphragms was 2½ to 4 times greater than the uptake of diaphragms from rats weighing approximately 100 g. Although this difference might be due to a higher specific metabolic activity of the mouse tissue, the effect of the larger surface exposure for the same weight of tissue must be considered. Rat hemidiaphragms which were cut into smaller pieces consistently showed a higher glucose uptake; for example, when the hemidiaphragms were quartered the uptake was increased by an average of 9%. With mouse hemidiaphragms, on the other hand, similar treatment reduced the uptake by an average of 6%. These results indicated to us that with rat diaphragm tissue the cellular damage caused by mincing was more than compensated by an increase in surface exposure resulting in net increase in uptake. On the other hand, with mouse tissue in which a

thinner dimension favors near maximal uptake rates, the cellular damage produced by mincing overcomes any effect of an increase in surface area.

Effect of insulin. The extra amount of glucose taken up by pooled hemidiaphragms incubated in presence of varying concentrations of insulin is shown by Fig. 4. The extra glucose represents the difference in uptake of insulin-treated hemidiaphragm pool and its corresponding control pool. A linear regression line was obtained between 1.5 and 3.5 log micro-units insulin/ml. The data represent results of 11 consecutive experiments and include all values to illustrate degree of variability and of reliability of the assay under routine conditions ($\lambda = 0.59$). In contrast to our experience with the rat hemidiaphragm technic, where on occasion a hemidiaphragm would not respond to insulin, an insulin response was consistently elicited by the pooled mouse diaphragm technic. It must be emphasized, however, that since the slope of the regression line varied significantly from experiment to experiment, it is necessary to include insulin standards whenever test samples are run. The minimal detectable concentration by this method was approximately 30 microunits of insulin per ml.

Discussion. One of the problems encountered using the rat hemidiaphragm technic has been the variability of individual hemidiaphragms to the action of insulin. Various modifications have been used to minimize this factor such as using pooled hemi-, quarter-, and fifth-diaphragms(3,11,12). Mouse diaphragm tissue because of its smaller size and greater glucose uptake ability is particularly suited for this pooling procedure for, on a weight basis, more than 4 mouse diaphragms can be substituted for a single rat diaphragm.

A further advantage in using mouse diaphragm tissue is its higher surface to mass ratio with a greater proportion of cells in direct contact with the incubation medium.

It is difficult to compare relative sensitivity of mouse diaphragm technic to rat diaphragm technic since the minimum detectable amount reported for the latter method varies over a considerable range(6). Comparative experiments in our laboratory have shown the mouse diaphragm technic to be superior both in terms of sensitivity and reproducibility. Further work is now in progress to apply this method to determination of insulin activity in blood.

Summary. A method for estimating insulin using pooled mouse hemidiaphragms has been developed. A linear log dose-response was obtained between 30-3000 micro-units of insulin/ml. Basal glucose uptake of mouse diaphragm tissue was 2½ to 4 times that of rat diaphragm tissue. Basal uptake was significantly affected by pH of medium, glucose concentration, and diaphragm size. A progressive increase in magnesium or potassium concentration of the medium suppressed the basal glucose uptake.

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