Insulin Action upon Insulin-Insensitive Tissue Following Impaired Degradation of the Hormone.* (32229)

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That insulin acts to facilitate the entry of certain sugars into cells has been amply demonstrated since the initial contributions of Levine and associates(1). According to this criterion tissues have been classified as "insulin sensitive" (*i.e.*, striated muscle, cardiac muscle, fibroblasts and adipose tissue) or "insulin-insensitive" (*i.e.*, red blood cells, intestinal mucosa, brain, liver and kidney). In the present communication we present evidence that one of these tissues, kidney, under appropriate conditions may be demonstrated to possess insulin sensitivity.

Since liver and kidney inactivate insulin by an "insulinase" system, which involves reductive cleavage followed by proteolysis, inhibition of the system might allow the demonstration of biologic activity of the hormone.

Methods. Incubation under hypothermia. Fresh rat kidney slices were immersed in bicarbonate buffer containing .017 M glucose and 100 μU/ml of pork ^{131}I insulin (300 mc/mg). Identical flasks were incubated at 37°C and at 20°C and at appropriate time intervals 0.1 ml aliquots of incubation solution were removed and precipitated with 10% trichloroacetic acid. Radioactivity of supernatant and precipitate was determined in a well-type scintillation counter. The lowered incubation temperature resulted in marked curtailment in the rate of enzymatic degradation, in the first 10 minutes, of the labelled hormone (Fig. 1). Kidney slices obtained from another animal were placed alternately into bicarbonate buffer containing .017 M glucose solution alone or with addition of 1,000 μU of insulin/ml. All flasks were gassed through sealed stoppers with 95% O_2—5% CO_2 for 10 minutes and incubated in a Dubnoff metabolic shaker at 37°C and 20°C for 90 minutes with moderate agitation.

The tissue was removed and dried, residual glucose concentration determined by autoanalyzer utilizing a modification of the ferricyanide reduction method of Hoffman(2) and glucose uptake calculated as mg per 10 mg dry weight of tissue per 2 ml of incubation medium. Whereas incubation at 37°C failed to show an effect of insulin, those tissues exposed to insulin at 20°C demonstrated a clear-cut enhancement of net glucose utilization during 90-minute incubation (Fig. 2). Comparable unmasking of insulin activity on blood glucose levels was observed by Kestens in dog liver perfusions at low temperature(3).

Insulin degradation. Attention was then directed toward modifying the insulin degrading mechanism at normal body temperature. The reductive cleavage of the disulfide bonds of insulin by "insulinase" depends upon reduced glutathione (GSH)(4,5), and the insulin reducing enzyme component of the "insulinase" system has been renamed "glutathione-insulin-transhydrogenase"(6). Recent evidence has suggested, in fact, that GSH causes reduction to the split products of insulin by reduction of the transhydrogenase enzyme itself, the reduced enzyme being regenerated during the course of disulfide-interchange. Although the transhydrogenase enzyme has been most extensively studied in liver, and appears to be present in greatest amounts in that tissue, a surprisingly high degree of sulfhydryl dependent activity has been recovered in the kidney slice as well as homogenate(7,8). Owing to similarities of insulin degradation by liver and kidney tissue, it may be presumed that the mechanism of degradation as well as dependence upon the presence of reduced glutathione are comparable.

Intravenous administration of alloxan into rats causes a prompt diminution in the concentration of blood(9-11) and liver(12) GSH. Since a linear relationship exists between rate of enzymatic degradation of insulin and concentration of GSH *in vitro*(6), the possibility

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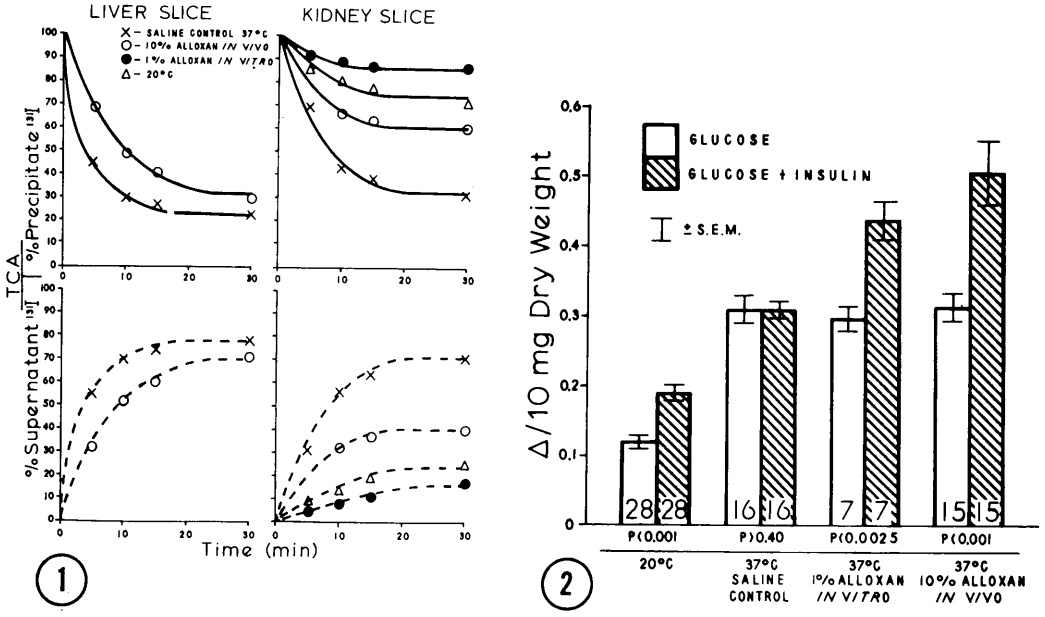

FIG. 1. (Left) Rate of ^{131}I insulin degradation by rat liver and kidney slices. Saline (×) or alloxan (open circles) was administered intravenously 10 min prior to sacrifice. Alternatively, kidney slices from untreated animals were pre-incubated in alloxan for 7 min. (closed circles) at 37°C. All tissue was subsequently placed into bicarbonate buffer containing 200 μU ^{131}I pork insulin for incubation at 37°C. Comparable incubations were performed with kidney slices at 20°C (triangles). Figures are expressed as per cent dpm from initial radioactivity following precipitation with 10% trichloroacetic acid. Each curve represents the mean of 4 individual experiments.

FIG. 2. (Right) Glucose utilization by paired rat kidney slices in presence and absence of insulin. Initial glucose concentration .017 M; insulin concentration 1,000 μU/ml.

exists that a reduction in concentration of GSH *in vivo* as provoked by alloxan may result in retarded insulin degradation.

Alloxan administration in vivo. Consequently, following ether anesthesia, male Wistar rats were injected intravenously with either 10 g% alloxan dissolved in 0.9% NaCl (20 mg/100 g rat weight) or a comparable volume of normal saline alone. After 10 minutes the animals were sacrificed and kidney or liver slices incubated at 37°C in bicarbonate buffer containing .017 M glucose and 200 μU ^{131}I insulin as previously described. A 10-minute delay following alloxan administration was selected since Bhattacharya *et al* have previously reported greatest reduction of GSH in blood, liver and pancreas at that time (12). As seen in Fig. 1, the prior administration of alloxan *in vivo* results in a marked reduction in initial rate of insulin degradation by both liver and kidney.

Alloxan administration in vitro. Since preincubation of diaphragm tissue with alloxan has been noted to cause continuing reoxidation of glutathione(13), kidney slices were next subjected to comparable *in vitro* conditions. After 7 minutes of incubation at 37°C in 1% alloxan in 0.9% NaCl, freshly prepared rat kidney slices were rinsed and placed into bicarbonate buffer containing .017 M glucose and 100 μU/ml pork ^{131}I insulin as previously described. Fig. 1 demonstrates profound inhibition of insulin degradation after *in vitro* alloxanization. Preliminary determination of free sulfhydryl groups in kidney homogenates derived from alloxan treated animals has revealed a reduction of 55 mg% which correlates well with the ^{131}I insulin data presented. Hence it may be concluded that alloxan administration *in vivo* or *in vitro* results in a retarded rate of insulin degradation, probably by continuing reoxidation of glutathione.

Glucose utilization. Of far greater signifi-

TABLE I. Correlation Between Glucose Uptake and Weight of Kidney Slice in Presence and Absence of Insulin. All animals were injected intravenously with 10% alloxan 10 min prior to sacrifice and samples incubated for 90 min at 37°C in .017M glucose alone or with 1,000 μU of insulin/ml. Note lack of significance between tissue weight of the 2 groups ($p > .20$) with highly significant difference in glucose uptake ($p < .001$).

Rat #	Glucose uptake* in absence of insulin	Dry wt of tissue (mg)	Glucose uptake* in presence of insulin	Dry wt of tissue (mg)
2	.342	15.8	.443	19.9
4	.270	20.7	.421	17.6
5	.282	18.4	.404	16.9
7	.280	19.2	.439	17.8
11	.340	10.0	.501	13.3
12	.364	13.7	.468	20.5
Mean	.313	16.3	.446	17.6
P			<.001	>.20

* Uptake is expressed as Δ mg/10 mg dry wt of tissue/2 ml incubation medium.

cance to us were the results of metabolic studies. Either 10 minutes after the intravenous injection of 10% alloxan or 7 minutes after the incubation of rat kidney slices in 1% alloxan, paired slices obtained from the same animal were placed into .017 M glucose solution alone or in the presence of 1,000 μU of insulin/ml respectively. All flasks were gassed and incubated for 90 minutes at 37°C as noted previously, and glucose disappearance from the medium correlated with tissue weight. As seen in Fig. 2, alloxan pre-treatment either *in vivo* or *in vitro* results in the appearance of an insulin effect upon glucose utilization by the rat kidney slice, which is absent in control tissues pre-treated with saline. Hyperglycemia was not a factor, since the alloxan-treated animals had normal blood glucose values at time of sacrifice. As noted in Table I tissue slices obtained from the same animal in every case had a greater glucose uptake in the presence of insulin, regardless of tissue weight, thus obviating the error of proportionately greater uptake by smaller amounts of tissue. Although the ferricyanide reduction method of Hoffman as modified for the autoanalyzer records alloxan as a reducing substance, only trace amounts of alloxan could have remained on the washed tissue, and a comparable insulin effect was obtained when a number of samples were analyzed by a glucose oxidase procedure (C.F. Boehringer & Soehne GMBH, Mannheim, Germany) as well as the autoanalyzer.

The normal rat kidney slice incubated at 37°C *in vitro* fails to demonstrate insulin enhancement of glucose uptake above control values. To determine whether total degradation of all insulin contained in the medium might account for this observation, the following experiment was performed. After 15 minutes incubation of normal rat kidney slices at 37°C in either glucose buffer alone or glucose buffer containing 100 mU of insulin/ml, each kidney slice was removed and replaced, in each incubation vessel, by a single rat hemidiaphragm. Residual glucose in the medium was determined initially and after each 15-minute period of tissue incubation. Whereas no statistically significant difference in glucose uptake was noted between kidney slices incubated in glucose alone and those incubated in glucose and insulin (202.1 $\pm$ 18.8 μg/10 mg and 241.8 $\pm$ 16.4 μg/10 mg respectively) (mean $\pm$ S. E. M.); diaphragms incubated in the identical media showed a highly significant insulin effect (90.1 $\pm$ 10.8 μg/10 mg and 161.6 $\pm$ 22.2 μg/10 mg respectively) ($p < .01$).

Discussion. Prior investigators have failed to observe a clearly demonstrable direct metabolic effect of insulin upon the kidney. Kidney slices removed from normal rats made profoundly hypoglycemic by injection of insulin showed enhanced glucose utilization (14). However, no *in vitro* effect of insulin was demonstrated(14-16). In the present study, under conditions of altered hormonal degradation, we have been able to demonstrate a direct insulin action upon rat kidney slices.

When the capacity of the kidney to degrade insulin was not altered by either hypothermia or injection of alloxan, the kidney slices failed to show an *in vitro* effect of insulin. Since, after such incubation, the media still contained insulin which was active on diaphragm, it is obvious that the lack of effect was not due to total inactivation of insulin in the medium.

The results suggested at least two possibilities. One is that the action of insulin

observed under conditions of depressed hormonal degradation is intracellular, and is not seen under normal conditions because of rapid destruction of the hormone. A similar mechanism has been proposed for insulin action upon glucose utilization by liver(3). Another possibility is that the insulinase system of the kidney, like that of liver and pancreatic beta cells, involves the splitting of the molecule into A and B chains, and that one or both chains serve metabolic functions generally attributed to insulin (*e.g.*, protein synthesis, fatty acid esterification, K^+ flux, etc.), while other effects require the presence of the intact hormone. In any case the general concept of insulin sensitivity would require reevaluation.

Summary. Retardation of the rate of insulin degradation results in a highly significant insulin effect upon glucose utilization by the rat kidney. Insulin inactivation may be inhibited by lowered incubation temperature or the *in vivo* and *in vitro* administration of alloxan. These observations suggest that under appropriate conditions, tissues heretofore believed to be insulin-insensitive may be shown to respond to insulin.

1. Levine, R., Goldstein, M. S., Recent Prog. Hormone Res., 1955, v11, 343.
2. Hoffman, W. S., J. Biol. Chem., 1937, v120, 51.
3. Kestens, P. J., La Perfusion du foie isolé. 1964, Editions Arscia, S. A., Bruxelles.
4. Mirsky, I. A., Recent Prog. Hormone Res., 1952, v7, 437.
5. Tomizawa, H. H., Nutley, M. L., Narahara, H. T., Williams, R. H., J. Am. Chem. Soc., 1954, v76, 3357.
6. Katzen, H. M., Stetten, D., Jr., Diabetes, 1962, v11, 271.
7. Rall, T. W., Lehninger, A. L., J. Biol. Chem., 1952, v194, 119.
8. Williams, R. H., Hay, J. S., Tjaden, M. B., Ann. N.Y. Acad. Sci., 1959, v74, 513.
9. Lazarow, A., Physiol. Rev., 1949, v29, 48.
10. Brückman, G., Wertheimer, E., J. Biol. Chem., 1947, v168, 241.
11. Collin-Williams, J., Renold, A. E., Marble, A., Endocrinology, 1950, v46, 1.
12. Bhattacharya, S. K., Robson, J. S., Stewart, C. P., Biochem. J., 1956, v62, 12.
13. Haft, D. E., Mirsky, I. A., J. Pharm. Exp. Ther., 1952, v104, 340.
14. Teng, C. T., Arch. Biochem., 1955, v57, 61.
15. Flinn, R. B., Leboeuf, B., Cahill, G. F., Jr., Am. J. Physiol., 1961, v200, 508.
16. Dzúrik, R., Krajči-Lázary, B., Niederland, T. R., J. Physiol (London), 1963, v168, 782.

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Reduced Immune Potential of Aged Mice: Significance of Morphologic Changes in Lymphatic Tissue.* (32230)

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A number of investigations have suggested that immune competence decreases during aging(1-7). Recent studies of the immunological potential of aged (*i.e.*, ~2.5 to 3-year-old) mice by Makinodan and Peterson(5,6) have shown (a) that primary antibody-forming potential decreases with age and (b) that this decline is mainly due to a reduction in the number of potential antibody-forming cells. These results were obtained both (a) from intact animals and (b) by the *in vivo* culture method in which a given number of spleen cells from donors of different ages are cultured together with the test antigen in heavily X-irradiated young adult (*i.e.*, 12- to 14-week-old) isologous recipients.

Any study of the immune reaction in lymphatic tissue of aged animals is complicated by a number of specific and unspecific pathologic variables associated with aging.

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