A Re-evaluation of in Vitro Methods for Insulin Bio-assay

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Most of the information about the concentration of insulin in plasma is based upon bio-assays performed with in vitro systems. Herein, the unknown sample of plasma is incubated with an insulin-responsive tissue and the resultant change in glucose metabolism is measured. A value for the insulin-content of the plasma is assigned on the basis of the dose-response relationships which obtain in the assay system when simple saline incubation media are supplemented with known amounts of insulin.

In the light of recent developments, these methods require re-examination. First, it has been shown in many laboratories that proteolytic mechanisms for insulin degradation are present in many tissues, and that the hypoglycemic effectiveness of insulin is destroyed by proteolysis.[1-8] Such proteolysis occurring in the conventional bio-assay systems might introduce error into the observed dose-response relationships. Secondly, work in this laboratory has demonstrated that normal fasting plasma contains protein factors which may act as non-competitive inhibitors of insulin degradation.[9] If plasma were to modify insulin proteolysis during bio-assay, the validity of comparing unknown plasma samples with known aqueous standards might be subject to error.

The present studies were initiated to assess these possibilities in the four most widely quoted in vitro systems for the bio-assay of insulin—i.e., the rat diaphragm methods of Randle,[10,11] Groen and Willebrands[12,13] and and Vallance-Owen and Hurlock,[14] and the rat adipose tissue method of Martin, Renold and Dagenais,[15] in which the epididymal fat pad is employed as the insulin-responsive tissue. The salient details of these four bio-assay procedures are summarized in figure 1.

	RANDLE	GROEN	VALLANCE-OWEN	MARTIN, et al
Tissue	HD (6)	HD (4)	HD (1)	FAT PAD
Medium	G & G	G & G	G & G	KRB
Volume (mL.)	1	2	2	2
Gas (O_2 % : CO_2 %)	93 : 7	93 : 7	95 : 5	95 : 5
Incubation (min.)	180	90	90	120
Plasma (mL.)	0.25	0.3	1.9	1.9
Normal Human Plasma (milliunits/mL.) *FED **FASTING	9 - 22*	0.1-3.0**	.031-.082**	.05 - .35**

HD = Hemidiaphragm G & G = Gey & Gey KRB = Krebs-Ringer-Bicarbonate

FIG. 1. Systems for the in vitro bio-assay of insulin.

MATERIALS AND METHODS

Tissues were prepared exactly as outlined in the individual assay procedures.[10-15] Thus, for studies with the Randle system, six hemidiaphragms were "washed for exactly five minutes at room temperature in buffer-glucose (glucose concentration 2.5 mg. per ml.),"[10] and subsequently incubated in a 1 ml. volume of the Gey and Gey[16] balanced salt solution. The Groen and Vallance-Owen systems were reproduced by immersing the four and the one hemidiaphragms which are used in these respective procedures in glucose-free, chilled, balanced saline preparatory to incubation in 2 ml. volumes of Gey and Gey media. For all of the rat diaphragm studies, male animals weighing 100 to 130 gm. were fasted for eighteen and twenty-four hours. Wet weights of single hemidiaphragms from these animals averaged 106.1 ± 23.1* mg. in eleven estimations.

Epididymal fat pads were obtained from fed animals weighing 190 to 220 gm. Portions of adipose tissue were cut directly into 2 ml. volumes of Krebs-Ringer-bicarbonate medium contained within tared vessels. Filled flasks were reweighed to assess the weight of the fat pads by difference. In some instances, weights were estimated directly on a torsion balance.

Rats were Sprague-Dawley descendants secured from the Charles River Breeding Laboratories, Brookline, Massachusetts, and maintained in this laboratory on

*Mean ± Standard Deviation.

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Purina pellets. Tissues were obtained within three minutes following sacrifice of the animals by a blow on the head and exsanguination.

Incubation was performed with a Dubnoff metabolic agitator (shaking rate: ninety-two cycles per minute). Suspending media were equilibrated at 38° C. and timed incubation at this temperature was initiated by the introduction of the tissues into the reaction vessels. The details of incubation intrinsic to the individual assay procedures were uniformly modified as follows:

1. All media were supplemented to a standard glucose content of 3 mg./ml. (except where indicated in the "Results" section).

2. A mixture of 95 per cent oxygen—5 per cent carbon dioxide was used as the gas phase for all systems.

3. Tracer quantities of I^{131}-insulin (v.i.) were used to document the metabolic fate of the insulin in the individual assay procedures.

4. All suspending media were supplemented with 6 mg./ml. human serum albumin (HSA) to reduce adsorption to glassware[17] and spontaneous degradation of dilute insulin solutions. Control studies have demonstrated that these methodological artifacts are more effectively eliminated by HSA than by the 100 mg. per cent gelatin that has been recommended.[15,18] The 6 mg./ml. HSA does not modify insulin degradation.

I^{131}-insulin for the present studies was obtained from Abbott Laboratories, Oak Ridge, Tennessee. Upon receipt in this laboratory, the labeled insulin was diluted with phosphate-buffered mixtures of HSA and saline (pH 7.4) and dialyzed as outlined elsewhere.[19] As judged by chromatographic, zone electrophoretic, and immunologic criteria, the labeled insulin retained molecular homogeneity. No preparation was employed for more than ten days following receipt in this laboratory. Under these conditions, measurable reduction in the hypoglycemic potency of the I^{131}-insulin could not be demonstrated.[19] Labeled insulin was adjusted to the desired specific activity for individual experiments with crystalline "glucagon-free" insulin provided by Dr. O. K. Behrens, Lilly Research Laboratories, Indianapolis, Indiana.

Degradation of insulin during incubation for ten and ninety minutes in the various systems was assessed on the basis of the changes in the solubility characteristics of the radioactivity in trichloracetic acid (TCA). Abundant work has indicated that this constitutes a valid index of the proteolysis and biological inactivation of insulin in vitro.[2-5,8,19] In all experiments, the tissues were removed at the end of incubation and suspending media were quantitatively decanted into 13 × 100 mm. glass test tubes containing 2 ml. of chilled 10 per cent TCA. The radioactivity in the media was partitioned into TCA-soluble and TCA-insoluble fractions by centrifugation. The insoluble residues were resuspended for washing with 2 ml. of 10 per cent TCA and repeat centrifugation was performed. TCA-supernatant fractions were pooled in 13 × 100 mm. tubes and the washed sediments were dissolved in a comparable volume of 30 per cent KOH. Control media which had been incubated without tissues were processed similarly. Diaphragms were briefly blotted and boiled in 1 ml. of water for thirty seconds. The water and boiled diaphragms were transferred to Potter-Elvehjem glass homogenizers for homogenization with 3 ml. of 10 per cent TCA. Thereafter, the contents of the homogenizers were quantitatively poured into 13 × 100 mm. tubes and fractionated into TCA-soluble and TCA-insoluble moieties as above. Separate processing of tissues and media was instituted with the diaphragm to minimize variable coprecipitation artifact. Moreover, because of the different amounts of tissue and medium in the three diaphragm procedures, total insulin degradation was assessed by combining the results of these analyses. Thus, after appropriate correction for control vessels, the sum of the TCA-soluble (nonprecipitable) I^{131} in the media and diaphragms was expressed as a percentage of the total I^{131} which was recovered from both sources. On the other hand, in the epididymal fat pad system, the contribution of tissue radioactivity was small and relatively constant. In twenty-two observations, with tissue weights ranging from 85 to 679 mg., the fat pad contained only 1.98 ± 0.98* per cent of the total radioactivity. Regardless of its nature, this amount of radioactivity could not appreciably influence estimates of insulin degradation. Thus, breakdown of insulin in the epididymal fat pad system was assessed solely on the basis of the changes in the TCA-solubility of the radioactivity within the media.

Radioactive assay of the TCA-soluble and TCA-insoluble fractions was performed by placing the 13 × 100 mm. tubes directly in a Nancy Wood well-type scintillation counter with a sensitivity of 1.0×10^6 cpm per uc. I^{131} and a background of 125 cpm. Sufficient counts were observed to reduce the probable error of the measurements to less than ±3 per cent.

Tests for the significance of differences between means and correlation coefficients were based upon the appropriate modifications of the "t" test of "Student."

RESULTS

1. Degradation of insulin by diaphragm systems in media containing 0.1 milliunit of insulin per ml.: Results are illustrated in figure 2. Values for the percentage

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of the total insulin degraded in the Vallance-Owen, Groen, and Randle procedures during ten minutes of incubation were 5.5 ± 4.0* per cent, 14.8 ± 6.0* per cent, and 24.2 ± 8.3* per cent respectively. During ninety minutes of incubation, the respective values were 43.3 ± 16.3* per cent, 70.2 ± 7.5* per cent, and 72.9 ± 7.2* per cent. The differences between the three methods were statistically significant for the ten-minute interval ($p<0.05$). At ninety minutes, the difference between insulin degradation in the Groen and Randle systems was not significant ($p>0.1$);† however, the Vallance-Owen procedure still differed significantly from the other two methods ($p<0.01$). Thus, the proportional relationship between tissue mass (i.e., the number of suspended hemidiaphragms/ml. of medium) and percentile insulin degradation was most clearly demonstrated at the ten-minute interval (figure 3). At this time, insulin degradation was still relatively linear and asymptotic values had not yet been achieved.

To assess whether insulin degradation in the rat diaphragm systems is conditioned by extracellular events, the experiments pictured on the right side of figure 3 were performed. Hemidiaphragms were incubated without insulin as per Vallance-Owen, Groen, and Randle. Two milliliters of medium and twelve hemidiaphragms were employed for the Randle procedure. After ninety minutes of incubation, 1 ml. of medium was removed from the diaphragm vessels and combined with 1 ml. of fresh, insulin-containing medium to yield 2 ml. cell-free systems containing 0.1 milliunit insulin per ml. The contribution of the diaphragms to these systems was expressed in terms of "hemidiaphragm equivalents"—i.e., the number of diaphragms which had been leached per milliliter of final reaction mixture. Insulin degradation occurred in the cell-free preparations and was proportional to the number of diaphragms which had been leached (figure 3, right). In paired comparisons, during ten and ninety minutes of exposure to insulin, the systems containing the leached extracts exhibited greater insulin-degrading capacities than their counterparts containing intact hemidiaphragms (figure 3). While these studies were in progress, Narahara and Williams reported a similar leaching phenomenon.[20]

2. Degradation of insulin by rat epididymal fat pad in media containing 0.1 milliunit of insulin per ml.: Correlation of insulin degradation with tissue mass is

†Proteolysis of I^{131}-insulin at low concentrations of insulin is not a linear function in any insulin-degrading system and asymptotic values exceeding 70-85 per cent have not been reported.[2-8] Thus, the ninety-minute values in Groen and Randle systems represent essentially complete insulin degradation.

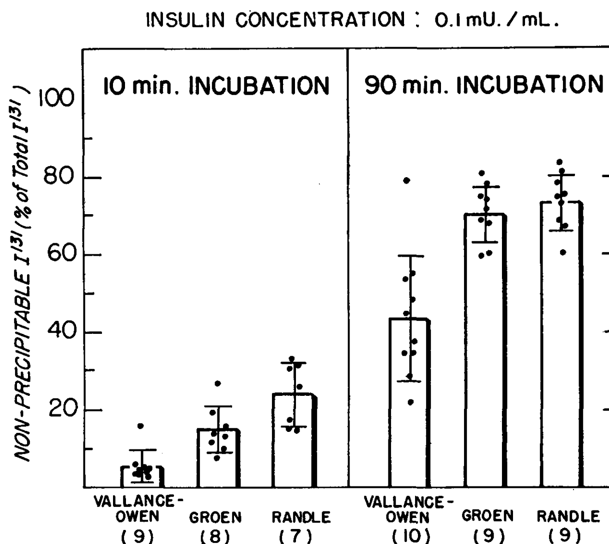

FIG. 2. The degradation of insulin in rat diaphragm bio-assay systems: Mean values for the percentage of total insulin that was degraded are indicated by the heights of the bar graphs and standard deviations by the joined transverse lines. Each point denotes an individual experiment; the bracketed numbers indicate the total number of experiments that were performed.

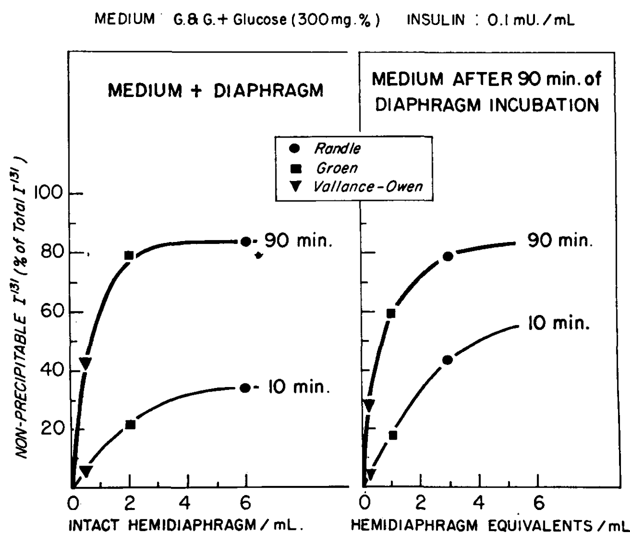

FIG. 3. Release of insulin degrading activity into the medium during incubation of rat diaphragms: Results obtained during the standard ten- and ninety-minute incubation of rat hemidiaphragms with insulin are shown on the left. Insulin degradation during ten- and ninety-minute incubation of cell-free systems with insulin is illustrated on the right. Cell-free systems had been prepared by leaching varying quantities of rat hemidiaphragms for ninety minutes. "Hemidiaphragms equivalents" refers to the number of hemidiaphragms which had been leached per milliliter of final reaction mixture. For details, see text.

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illustrated in the thirteen experiments pictured in figure 4. In each of these studies, three differently weighing portions of adipose tissue from a single rat were incubated separately. Results were similar in both Gey and Gey and Krebs-Ringer-bicarbonate media (figure 4). For any single animal, insulin degradation was proportional to the weight of the incubated tissue. When equally weighing tissues from different animals were compared, considerable variability in insulin degradation was observed. Nonetheless, pooled values from forty-nine observations with tissues from twenty animals yielded a correlation coefficient of 0.650 ($p<0.01$) between tissue mass and insulin degradation during ninety minutes of incubation. Thus, the results obtained with the Martin, Renold and Dagenais procedure in which 80-200 mg. of tissue are recommended have been subdivided on the basis of weight:

During ninety minutes of incubation, 13.9 ± 7.9* per cent of the total radioactivity was rendered TCA-soluble in fifteen observations with 60-139 mg. pieces of adipose tissue. Degradation values during comparable intervals with 140 to 219 mg. pieces of fat pad were 19.2 ± 6.8* per cent in ten observations. The difference between the two weight groups was significant ($p<0.01$). In nineteen experiments conducted with

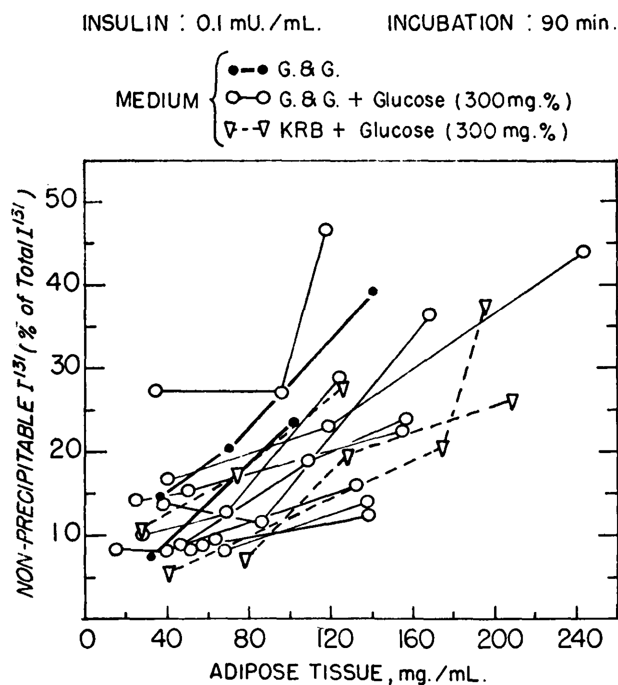

FIG. 4. Insulin degradation in the rat epididymal fat pad system: Final reaction mixture contained 2 ml. of the suspending medium denoted by the open and closed symbols. Results obtained with differently weighing portions of adipose tissue from individual animals are joined by lines.

tissues in the 80-200 mg. weight range recommended by Martin, insulin degradation at ninety minutes averaged 14.6 ± 7.9* per cent. This value was significantly less than that observed with any of the rat diaphragm methods ($p<0.01$). Values for the ninety-minute interval have been presented because of the essentially negligible insulin degradation by adipose tissue during ten minutes of incubation (table 1).

To assess whether extracellular events participate in the catabolism of insulin in the fat pad system, leaching studies were performed (figure 5). Three differently weighing portions of right epididymal adipose tissue from three rats were incubated in media containing 0.1 milliunit of insulin per ml. and insulin degradation was measured at the end of ninety minutes of incubation (figure 5, left). Comparable portions of the left epididymal fat pads from these animals were incubated simultaneously without insulin. Thereafter, 1 ml. aliquots were removed from the latter systems and combined with 1 ml. of fresh medium containing sufficient insulin to yield a final concentration of 0.1 milliunit/ml. in the cell-free reaction mixtures. As shown on the right in figure 5, the cell-free systems effected negligible degradation of insulin. Thus, release of insulin-degrading enzymes into the suspending medium by adipose tissue could not be demonstrated with the leaching technic.

3. The effects of insulin concentration and normal human plasma on the degradation of insulin in in vitro bio-assay systems: The experiments presented above were all conducted at a single concentration of insulin (0.1 milliunit/ml.) that is well within the quoted range for normal fasting human plasma. To assess the effects of increased insulin concentrations, paired comparisons were conducted in which the insulin in the aqueous suspending media was augmented one hundredfold (table 1). At these insulin concentrations of 10.0 milliunits/ml., the percentage of the total insulin that was degraded was unaltered in the Randle system and only slightly reduced in the Groen preparation. On the other hand, the proportional breakdown of insulin was diminished in the Vallance-Owen and Martin preparations (table 1). In some of these experiments, the effects of plasma were evaluated concurrently. Specimens of plasma were obtained from fasting, normal laboratory personnel and added, as recommended in the individual assay procedures[10-15] (figure 1) to suspending media to yield mixtures containing a final concentration of 0.1 milliunit I^{131}-insulin/ml. In all systems, the presence of plasma was accompanied by a reduction in the percentage of I^{131}-insulin that was degraded (table 1). It does not appear likely that this phenomenon can be attrib-

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TABLE 1

The effects of insulin concentration and normal, fasting human plasma upon insulin degradation during bio-assay in vitro

Assay system	Suspending medium*		Nonprecipitable I^{131} (Per cent of total I^{131}) Incubation: 10 minutes		Incubation: 90 minutes	
	Insulin (mU/ml.)	Plasma (ml./ml.)	N†	Per cent	N†	Per cent
Randle	0.1	0	3	26.6	3	82.2
	10.0	0		29.5		83.0
	0.1	0.25		21.6		76.3
Groen	0.1	0	3	18.4	3	75.3
	10.0	0		14.3		66.1
	0.1	0.16		9.3		61.4
Vallance-Owen	0.1	0	4	7.8	4	44.7
	10.0	0		4.7		36.7
	0.1	0.95		0.7		22.5
Martin et al.	0.1	0	4	1.2	11	13.1
	10.0	0		1.3		10.2
	0.1	0.95		0.2	(5)	5.9

* Suspending medium supplemented with insulin and plasma. Concentration of insulin expressed in terms of milliunits added per milliliter of final reaction mixture (mU/ml.). Plasma added as outlined in the individual assay procedure. Concentration of plasma expressed in terms of milliliter plasma per milliliter of final suspending medium (ml./ml.).

† N denotes the number of experiments in which the three situations were compared simultaneously. Per cent indicates the average percentage of the total insulin that was degraded in these experiments, after appropriate correction for TCA-soluble radioactivity in control vessels incubated without tissues for comparable intervals. Degradation in Martin et al. system expressed as percentage degraded per 100 mg. of fat pad. As denoted by the parentheses, the effects of plasma in the Martin system were assessed in only five of the eleven experiments.

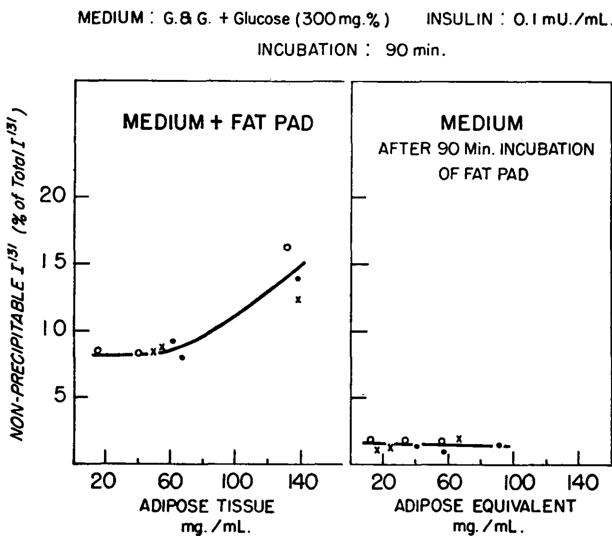

FIG. 5. Release of insulin degrading activity into the medium during incubation of rat epididymal fat pads: The symbols refer to individual values obtained with differently weighing portions of adipose tissue from three rats; mean values are indicated by the solid line. Results obtained during the standard ninety-minute incubation of insulin with adipose tissue are shown on the left. Insulin degradation during ninety-minute incubation of cell-free systems with insulin is illustrated on the right. Cell-free systems had been obtained by leaching adipose tissue from the three animals for ninety minutes. "Adipose equivalent" refers to the amount of adipose tissue which had been leached per milliliter of final reaction mixture. Weights are expressed on the basis of initial wet weight. For details, see text.

uted to the endogenous insulin of the plasma since in every paired experiment, a greater proportion of the total insulin was degraded in simple aqueous media even at insulin concentrations of 10.0 milliunits/ml. (table 1). The effects of plasma were least pronounced in the Randle and Groen systems where intrinsic insulin degrading capacities are great and only 0.25 or 0.16 ml. of plasma are added per milliliter of medium. The effects of plasma were most pronounced in the Vallance-Owen and the Martin systems where intrinsic insulin-degrading capacity is low and 0.95 ml. of plasma are added per milliliter of final suspending solution.

DISCUSSION

The present studies have demonstrated the occurrence of appreciable degradation of insulin in four of the major systems for the in vitro bio-assay of insulin. The data prompt certain methodological and theoretical considerations.

It has been shown that insulin proteolysis is greatest in the systems in which cut hemidiaphragms are employed and that the magnitude of insulin breakdown is conditioned by the number of suspended hemidiaphragms (Randle > Groen >> Vallance-Owen). Therefore, attempts to increase the biological sensitivity of these procedures by increasing the mass of insulin-responsive tissue entail the attendant risk of augmented insulin degradation. In the diaphragm systems, degradatory phenomena may be facilitated by the release of

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proteolytic enzymes from cut muscle fibers. Such leaching is minimal with adipose tissue, and here, insulin degradation may be determined by rates of cellular penetration as well as intracellular proteolytic activity. Nonetheless, in the fat pad as in the hemidiaphragm, a relationship exists between tissue weight and insulin breakdown. Thus, for standardized bio-assay, it may be desirable to limit mass to a narrower range than the 80 to 200 mg. of adipose tissue[15] that has been proposed.

Under the conditions recommended for the individual bio-assays, the system of Martin and others effected the least degradation of insulin. Such comparison of bio-assay procedures was the intent of the present studies. It should be noted, however, that the manifestly low level of insulinolytic activity in adipose tissue may be more apparent than real because of the dilution of tissue weight by triglycerides. Thus, absolute comparison of the insulin degrading potential of adipose tissue and other structures should be on a nitrogen basis.

Intrinsic to all bio-assay procedures are the assumptions that (a) the assay portion of the dose-response curve reflects a constant relationship and (b) the unknown specimen does not contain factors which may alter the standard dose-response relationships. The present data provide relevant information in both areas.

First, it has been demonstrated that a smaller percentage of the total insulin in aqueous media is degraded as the concentration of insulin is increased from 0.1 to 10.0 milliunits per ml. As might be anticipated, the effects were inversely related to the insulin degrading capacities of the individual systems (Martin > Vallance-Owen > Groen > Randle). The fact that the proportional breakdown of insulin is affected by the concentration of insulin would suggest that a constant correction factor cannot be employed to correct aqueous bio-assay standards for insulin degradation over this one hundredfold range of concentration. Whether such correction would be justified over narrower bio-assay ranges cannot be determined from present data.

Secondly, it has been demonstrated that normal, fasting plasma inhibits insulin degradation in all of the bio-assay systems. In the least, this observation indicates that unknown specimens of plasma and known aqueous standards are not completely comparable in any of the systems. Could correction be made for the effects of plasma? Although the presence of plasma theoretically should result in a closer approximation of the true and observed dose-response relationships, such phenomena would have to be examined at multiple concentrations of insulin. Moreover, the factors in normal plasma which inhibit insulin degrading enzymes are relatively specific and largely concentrated in Cohn Fractions III and IV-1.[9] Hence, a constant potential for inhibition need not obtain in abnormal plasmas. The present findings that plasma caused least change in the Randle system (where the medium is supplemented with 25 per cent plasma), and was most inhibitory in the Vallance-Owen and Martin preparations (where the medium is supplemented with 95 per cent plasma) only support the prediction that the effects of plasma upon insulin proteolysis in vitro will be conditioned by (a) the insulin-degrading capacity of the assay tissue, and (b) the quantity of plasma employed in the assay.*

Within the accepted bio-assay range for normal plasma insulin, the contributions of degradation would not be very pronounced. For example, even with 50 per cent degradation, a true value of 0.10 milliunit/ml. would be reduced to only 0.05 milliunit/ml. However, the apparent "smallness" of this difference does not detract from its significance but rather attests to the relative crudeness of bio-assay, and the relatively large range of "permissible" error. The present studies were designed to focus upon one possible source of this variability with the hope that future inquiry may elucidate many other factors which must be contributory also.

It should be mentioned that all of the above extrapolations from the available data contain the tacit implication of a prolonged stoicheiometric relationship between unaltered insulin and insulin action. This need not be the case. The possibility cannot be excluded that insulin in vitro initiates an immediate change in the assimilitative capacities of responsive tissues, and that the magnitude and duration of this effect are conditioned by the initial concentration of insulin rather than by its subsequent fate. However, this possibility seems less likely on the basis of recent data. Narahara and Williams[20] have demonstrated a potentiation of the effects of small quantities of insulin in the rat diaphragm system by enriching suspending media with protein hormones which retard insulin degradation. Moreover, work in this laboratory has demonstrated that the response of surviving rat adipose tissue to suboptimal doses of insulin is reduced when the system is immediately supplemented with soluble insulin-degrading enzymes which do not, per se, appreciably alter the metabolism of adipose tissue.[22] Nevertheless, whether the *continuing* availability of extracellular insulin is necessary for *sustained* insulin action during *prolonged* incubation has still to

**These phenomena presumably bear little relevance to an-*other aspect of plasma dilution described by Randle;[21] namely, an apparent enhancement of insulin-like activity by serial dilution of plasma.

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be answered. In any event, for the moment, it would appear that the present experimental findings and implications merit consideration and reservation in interpreting the quantitative results of in vitro bio-assay.

SUMMARY

I^{131}-insulin has been employed to assess the magnitude of insulin degradation during in vitro bio-assay performed according to the rat diaphragm procedures of (a) Randle, (b) Groen and Willebrands, (c) Vallance-Owen and Hurlock, and (d) the rat adipose tissue method of Martin, Renold, and Dagenais. Appreciable degradation of insulin occurs in all systems. Degradation is greatest in the cut muscle preparations and proteolytic enzymes are leached into the suspending medium. Insulin breakdown is least in the adipose tissue preparation and leaching phenomena are minimal. In all systems, the proportional degradation of insulin is conditioned by the mass of incubated tissue and by the extracellular concentration of insulin. Moreover, in all systems, the degradation of insulin is significantly inhibited by factors present in normal plasma. Theoretical implications of these findings are discussed.

SUMMARIO IN INTERLINGUA

Re-Evalutation del Methodos de Bio-Essayage pro Insulina in Vitro

Insulina marcate con I^{131} esseva usate pro evalutar le magnitude del degradation de insulina durante le bio-essayage in vitro effectuate con le technicas a diaphragma de ratto de (a) Randle, (b) Groen e Willebrands, (c) Vallance-Owen e Hurlock, e (d) con le technica a histo adipose de ratta de Martin, Renold, e Dagenais. Appreciabile grados de degradation de insulina occurre in omne le systemas mentionate. Le degradation es le plus marcate in le preparatos de musculo secate ab que enzymas proteolytic es extrahite a in le medio de suspension. Le degradation de insulina es le minus marcate in le preparatos de histo adipose ab que le extraction es minimal. In omne le systemas le degradation proportional de insulina es conditionate per le massa de histo incubate e per le concentration extracellular de insulina. In plus, in omne le systemas il occurre un inhibition del degradation de insulina como effecto de factores que es normalmente presente in le plasma.

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