Degradation of Insulin *in vitro* by Liver and Epididymal Adipose Tissue from Obese–Hyperglycaemic Mice

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The insulin-degrading activity of liver supernatants and epididymal adipose-tissue homogenates from genetically obese–hyperglycaemic mice (*obob*) and their lean litter mates was studied by measurement of radioactive trichloroacetic acid-soluble degradation products of the insulin molecule. Optimum assay conditions for the decomposition of the hormone were devised. The properties of the degrading activity suggested the presence of enzymic insulin destruction in both the liver and epididymal adipose tissue. There was no difference in insulin degradation in liver samples from obese and lean mice when the results were related to the protein content of the supernatants. The epididymal adipose-tissue homogenates from obese mice displayed about eightfold higher degrading activity per unit of protein than did homogenates from lean animals. The physiological significance of this finding is discussed in the light of the increased fat depots, hyperphagia, raised serum insulin concentrations and increased insulin tolerance previously recorded in this strain of mice.

Insulin is rapidly inactivated in the intact organism (Elgee, Williams & Lee, 1954; Weisberg, Friedman & Levine, 1949) and enzymic cleavage of insulin has been demonstrated in various tissues, e.g. liver (Mirsky, 1953) and fat (DiGirolamo, Rudman, Malkin & Garcia, 1965). In the light of these observations it has been suggested that an increased insulin destruction may contribute to the relative deficiency of this hormone that is characteristic of the diabetes syndrome (Mirsky, 1957; Sabo, 1964; Tomizawa & Varandani, 1965).

The existence of several strains of laboratory animals that display spontaneous diabetes makes it possible to study the significance of insulin degradation for the development of diabetes mellitus experimentally. In the present investigation mice with a recessive trait for obesity have been used in a study of the insulin-degrading activity *in vitro* of liver and epididymal adipose tissue. The obese mice are characterized by, among other things, hyperglycaemia, a high concentration of circulating insulin and a marked insulin tolerance (Mayer, Bates & Dickie, 1951; Westman, 1968), whereas their lean litter mates show no apparent abnormalities of carbohydrate metabolism.

MATERIALS AND METHODS

A total of 24 male obese–hyperglycaemic mice (gene symbol *obob*) belonging to a strain originally obtained from R. B. Jackson Memorial Laboratory, Bar Harbor, Maine, U.S.A., and 13 of their lean litter mates were used. The animals were allowed free access to water and a commercially available mouse chow (AB Ewos, Södertälje, Sweden). At the age of 5 months they were killed by decapitation and exsanguinated and the liver and epididymal fat pads were quickly excised and treated as follows.

(1) The insulin-degrading activity of the liver samples was determined mainly as described by Mirsky, Perisutti & Dixon (1954). The whole organ was immediately placed in ice-cold 0·1 M-potassium phosphate buffer, pH 6·2. A piece of the organ was taken for determination of the water content and another one was homogenized in the same cool buffer for 10 sec. in a glass homogenizer with a Teflon pestle. The homogenate (100–400 mg./ml.) was subsequently centrifuged at 900 *g* at 7° for 10 min. Samples of the supernatant (200 μl. each) were used for the determination of the insulin-degrading activity by incubation with 200 μl. of medium of the following composition: 0·1 M-potassium phosphate buffer, pH 6·2, containing bovine serum albumin (fraction V; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) (2%, w/v), ^{125}I-labelled bovine insulin (approx. 50 mc/mg.; The Radiochemical Centre, Amersham, Bucks.) (0·25 mμg./ml.) and unlabelled bovine insulin (ten-times recrystallized; Novo Industri A/S, Copenhagen, Denmark) (400 μg./ml.) as carrier. Before use the radioactive material was dialysed overnight in Visking 18/32 seamless cellulose tubing (Union Carbide Corp., Chicago, Ill., U.S.A.) against distilled water. Duplicate samples were incubated for 10 min. in glass tubes at 37° in a water thermostat with constant shaking (average shaking rate 110 cyc./min.), the reaction being stopped by the addition of 400 μl. of 10% (w/v) trichloroacetic acid. In each experiment blank values were obtained by arresting the reaction at zero time and the tubes were then centrifuged at 1100 *g* for 15 min. The

amounts of trichloroacetic acid-soluble radioactive substances in the supernatant fraction were assayed in a liquid-scintillation counter. The insulin-degrading activity was calculated as the difference between the sample and blank values, the error for the single determination being ± 12%.

Protein determinations were performed on triplicate samples of the liver supernatant as described by Lowry, Rosebrough, Farr & Randall (1951), with guinea-pig serum of known protein concentration as standard. The error for the single determination was calculated as ± 5%.

(2) The adipose-tissue assay was performed basically as described by DiGirolamo et al. (1965). The fat pads were rapidly chilled in ice-cold 0·1 M-sodium acetate buffer, pH 3·2, before determination of the wet weight. This buffer was also used for the homogenization procedure, which was performed as described above. For the subsequent determination of the insulin-degrading activity 200 μl. samples of the adipose-tissue homogenate (100–200 mg./ml.) were incubated in duplicate with 200 μl. of 0·1 M-sodium acetate buffer, pH 3·2, containing the same additives as used for the assay with liver supernatants. The protein concentrations of the homogenates were determined as indicated above.

For the determination of optimum assay conditions 16 of the obese mice were used. The effect of pH on the insulin degradation was evaluated in four mice by homogenization of the tissues with 0·1 M-sodium acetate buffer in the pH range 2·0–5·0, 0·1 M-potassium phosphate buffer for pH 6·0–8·0 and 0·1 M-tris hydrochloride–tris buffer at pH 8·5. The incubation media were composed of the same buffer as used for the homogenization and with the same additives as described above, the time of incubation being 10 min. The effect of different concentrations of carrier insulin (100–800 μg./ml.) was studied in six mice at pH 6·2 for liver and pH 3·1 for adipose tissue. The influence of varying the incubation times on the insulin-degrading activity and the effect of serial dilutions of the liver supernatant or adipose-tissue homogenate was separately studied in groups of three mice each.

The heat-lability of the insulin-degrading activity was estimated by preincubation of the liver supernatant and fat-pad homogenates at 60° for 10 min. or at 100° for 2 min. After being cooled the samples were added to the incubation media and the assays performed as described above.

For the comparative study of the insulin-degrading activity in the liver and epididymal adipose tissue eight obese and 13 lean mice were used. The homogenization and incubation procedures were carried out as described above but the liver supernatants were incubated for 20 min. All assays were run in duplicates and at five levels of tissue concentration obtained by serial dilutions.

RESULTS

Optimum assay conditions. The insulin-degrading activity was determined over the pH range 2·0–8·5. The values obtained for the liver and epididymal adipose tissue from obese mice are shown in Fig. 1. For the liver the highest activity was at about pH 6, whereas for the adipose tissue the pH optimum was as low as 3–4. As shown in Fig. 2 there was a linear relationship between insulin degradation and incubation time throughout the
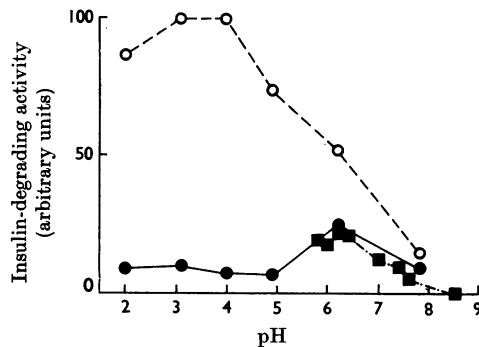

Fig. 1. Degradation of insulin at different pH values by the liver supernatants (● and ■) and epididymal adipose-tissue homogenates (○) expressed in arbitrary units. For the liver extracts the results from two experiments are given.
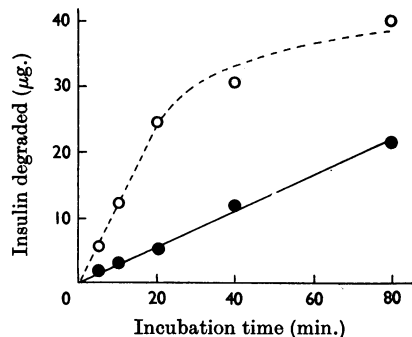

Fig. 2. Relation between incubation time and degradation of insulin by liver supernatants (●), measured at pH 6·2, and by epididymal adipose-tissue homogenates (○), measured at pH 3·1.
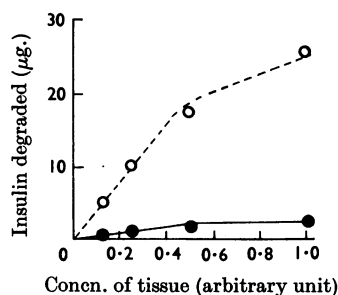

Fig. 3. Relation between tissue concentration (arbitrary units) and degradation of insulin by liver supernatants (●), measured at pH 6·2, and by epididymal adipose-tissue homogenates (○), measured at pH 3·1.

experiment for the liver and for up to 20 min. for the adipose tissue.

The effect of different concentrations of liver supernatant and adipose-tissue homogenate on the rate of insulin degradation is shown in Fig. 3. There
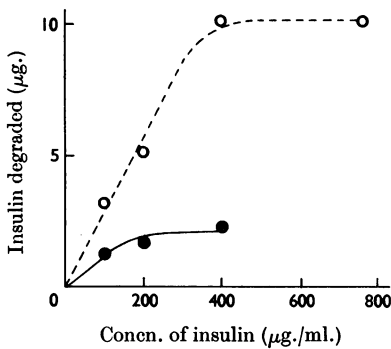
Fig. 4. Relation between concentration of insulin in the incubation media and the amount of insulin degraded by liver supernatants (●), measured at pH 6·2, and by epididymal adipose-tissue homogenates (○), measured at pH 3·1. The incubation time was 10 min.

Table 1. *Body weight, liver weight, weight of the two epididymal fat pads and water content of the liver*

Values are given as means ± S.E.M., with the numbers of animals in parentheses.

Animals	Body wt. (g.)	Wet wt. of liver (mg.)	Water content of liver (% of wet wt.)	Wet wt. of epididymal fat pads (mg.)
Obese	52·9 ± 1·3 (8)	3318 ± 189 (8)	52·7 ± 2·1 (5)	1173 ± 147 (8)
Lean	29·2 ± 1·1 (13)	1125 ± 54 (13)	62·1 ± 0·6 (10)	794 ± 97 (13)

was a linear increase of substrate degradation in the range 1–3 μg. of insulin degraded for the liver samples, whereas the corresponding value for adipose tissue was 4–20 μg. The relation between insulin-degrading activity and substrate concentration is illustrated in Fig. 4. For the liver samples an insulin concentration in the medium of 200 μg./ml. seemed to be enough for maximal insulin cleavage, whereas for the adipose-tissue homogenates the highest activity was reached with about 400 μg./ml. The insulin-degrading activity was found to be thermolabile in the liver as well as in the adipose tissue. Heating of the samples at 60° resulted in a more than 90% loss of the activity, whereas after preincubation at 100° the degrading activity was completely destroyed.

Comparisons between obese and lean mice. The total weights of the livers and epididymal fat pads were higher in the obese mice than in their lean litter mates (Table 1). Moreover, the livers of the obese mice contained significantly less water

Table 2. *Degradation of insulin by liver supernatants and epididymal adipose-tissue homogenates*

Values are given as means ± S.E.M., with the numbers of animals in parentheses.

Animals	Degradation of insulin (mg./g. of protein/hr.)	
	Liver	Epididymal fat pad
Obese	5·8 ± 1·0 (8)	341·3 ± 106·8 (8)
Lean	5·4 ± 0·6 (13)	39·4 ± 12·5 (8)

($t = 5·57$, $P < 0·001$). When expressed per unit wet weight the protein content of the liver supernatant was, however, the same in the obese and the lean mice. Neither was the protein content of the adipose-tissue homogenates of the obese mice, $2·3 ± 0·6\%$ of the wet weight, significantly different from that of the lean ones, $1·3 ± 0·2\%$ ($t = 2·03$, $P > 0·05$).

The insulin-degrading activities of liver supernatants and adipose-tissue homogenates are shown in Table 2. When the values were expressed per unit of protein there was no difference in the degradation by liver supernatants between the two groups of mice. For the adipose-tissue homogenates from four of the lean mice the insulin-degrading activity was below the sensitivity of the method even at the highest tissue concentration used. It seemed reasonable to exclude these data, although this resulted in fewer degrees of freedom for the applied t test and a decrease in the difference between the two groups. Despite this the adipose-tissue homogenates from the obese mice showed, however, more than eight times higher insulin degradation per unit of protein as compared with the remaining lean litter mates ($t = 2·81$, $P < 0·02$).

DISCUSSION

Previous studies with radioactive trace insulin have shown that the degradation of the insulin molecule *in vitro* by liver is paralleled by an increased concentration of radioactivity in the non-protein fraction (Mirsky *et al.* 1954). In addition, differential centrifugation of liver homogenates has demonstrated that the insulin-degrading activity is localized in the water-soluble fraction of the cytoplasm (Mirsky, 1953; Beyer, 1955). Also, adipose tissue was found to split insulin into trichloroacetic acid-soluble fragments (Rudman, Garcia, DiGirolamo & Shank, 1966). In the adipose-tissue homogenates, however, decomposition of the insulin molecule occurred only in the fat-containing fraction, whereas the water-soluble subnatant

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appeared completely inactive in this respect (DiGirolamo et al. 1965).

The present study demonstrates that liver and epididymal adipose tissue from both the obese and the lean mice decompose insulin in vitro into trichloroacetic acid-soluble fragments. Enzymic properties of the insulin-degrading system are suggested by the pH–activity curves, the effect of different incubation times, the relations between homogenate and substrate concentrations and the thermal lability. With the liver supernatants the highest activity was obtained at about pH 6. This was approximately the same as recorded in earlier investigations, in which the pH optimum was reported to vary between 6·5 and 9·5 (Mirsky, 1953). With the adipose-tissue homogenates, on the other hand, optimum degrading activity was found at the considerably lower pH region 3–4. An optimum at about pH 4 for the proteolytic breakdown of insulin has been reported for calf pancreas slices (Schucher, 1965). The different characteristics of the insulin-degrading activities in the liver and epididymal adipose tissue of mice suggest that these tissues decompose the insulin molecule by different enzyme systems.

An insulin-degrading enzyme from liver has previously been purified and used for the study of reaction kinetics. The enzyme catalysed the cleavage of the interchain disulphide bridges of insulin in the presence of a thiol such as glutathione and was designated as glutathione–insulin trans-hydrogenase (Tomizawa, 1962; Katzen & Stetten, 1962). The enzymic degradation of the hormone resulted in separation of the A and B chains, the trichloroacetic acid-soluble product of this reaction being the A chain (Varandani, 1966). As compared with liver, adipose tissue displayed a different mode of destroying the insulin molecule, which was split into several trichloroacetic acid-soluble fragments through the action of proteolytic enzymes (Rudman et al. 1966).

In the present study the most obvious difference between the two groups of animals was the very high insulin degradation in vitro by the epididymal adipose tissue of the obese mice as compared with that of their lean litter mates. The insulin-degrading activity of the epididymal fat pads observed in vitro would be of even greater significance if it were assumed to represent the adipose tissue in vivo, since the total body fat of obese mice constitutes as much as 90% of their overweight (Bates, Nauss, Hagman & Mayer, 1955). The low values for the water content of the livers from the obese mice reflect the fatty infiltration (cf. Hellman, Larsson & Westman, 1962). Despite this there was no difference in the degrading activity per unit wet weight between the two groups of mice.

Previous studies indicate that the feeding pattern exerts a marked influence on the insulin-degrading activity. Starvation was thus reported to be associated with a decrease of insulin degradation by rat liver supernatants (Mirsky, Perisutti & Diengott, 1957), whereas re-feeding with a high-carbohydrate diet increased the degradation above normal (Broh-Kahn, Simkin & Mirsky, 1950). Fed gold-thioglucose obese mice, in which the obesity is due to hyperphagia, have a raised serum insulin concentration (Stauffacher, Lambert, Vecchio & Renold, 1967) as well as an increased insulin-inactivating capacity in the livers (Larsson, 1959). Also, homogenates of livers from mice belonging to the markedly insulin-tolerant KL strain show a high rate of insulin degradation (Beyer, 1955). The pronounced insulin-degrading activity in the epididymal adipose tissue from obese–hyper-glycaemic mice is of particular interest in this connexion. Against this background the possibility that the hyperphagia or hyperinsulinism or both of the obese–hyperglycaemic mice may be of importance for the high insulin-degrading activity must be considered. It is so far obscure, however, whether the marked insulin destruction in vitro is of any significance for the conspicuous insulin tolerance of the obese–hyperglycaemic mice (Mayer et al. 1951; Westman, 1968). It seems important to analyse this question further by comparing the insulin destruction in vivo of the obese–hyper-glycaemic mice and of their lean litter mates.

REFERENCES

Bates, M. W., Nauss, S. F., Hagman, N. C. & Mayer, J. (1955). *Amer. J. Physiol.* **180**, 301.

Beyer, R. E. (1955). *Acta endocr., Kbh.,* **19**, 309.

Broh-Kahn, R. H., Simkin, B. & Mirsky, I. A. (1950). *Arch. Biochem.* **27**, 174.

Elgee, N. J., Williams, R. H. & Lee, N. D. (1954). *J. clin. Invest.* **35**, 1952.

DiGirolamo, M., Rudman, D., Malkin, M. F. & Garcia, L. A. (1965). *Diabetes,* **14**, 87.

Hellman, B., Larsson, S. & Westman, S. (1962). *Acta endocr., Kbh.,* **39**, 457.

Katzen, H. M. & Stetten, D. (1962). *Diabetes,* **11**, 271.

Larsson, S. (1959). *Acta physiol. scand.* **46**, 159.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Mayer, J., Bates, M. W. & Dickie, M. M. (1951). *Science,* **113**, 746.

Mirsky, A. (1953). *Ciba Found. Colloq.: Endocrinology,* vol. 6, p. 263.

Mirsky, A. (1957). *Recent Progr. Hormone Res.* **13**, 429.

Mirsky, I. A., Perisutti, G. & Diengott, D. (1957). *Endocrinology,* **60**, 303.

Mirsky, I. A., Perisutti, G. & Dixon, F. J. (1954). *Proc. Soc. exp. Biol., N.Y.*, **86**, 228.

Rudman, D., Garcia, L. A., DiGirolamo, M. & Shank, P. W. (1966). *Endocrinology,* **78**, 169.

Sabo, I. (1964). *Endocrinologie,* **46**, 111.

Schucher, R. (1965). *Canad. J. Biochem.* **43**, 1143.

Stauffacher, W., Lambert, A. E., Vecchio, D. & Renold, A. E. (1967). *Diabetologia,* **3**, 230.

Tomizawa, H. H. (1962). *J. biol. Chem.* **237**, 428.

Tomizawa, H. H. & Varandani, P. T. (1965). *J. biol. Chem.* **240**, 3191.

Varandani, P. T. (1966). *Biochim. biophys. Acta,* **118**, 198.

Weisberg, H. F., Friedman, A. & Levine, R. (1949). *Amer. J. Physiol.* **158**, 332.

Westman, S. (1968). *Proc. 6th Congr. int. Diabet. Fed.* (in the Press).