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Degradation of Insulin by a Particulate Fraction from Adipose Tissue

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The destruction of ^{125}I-labelled insulin by an enzyme system from rat adipose tissue was studied. The system was located in the particulate fraction. Activity was assayed by the amount of ^{125}I-labelled degradation products rendered soluble in trichloroacetic acid. The system was heat-labile, with an alkaline pH optimum. The velocity of the reaction varied directly with the enzyme concentration. Paper chromatography of the degradation products showed six ninhydrin-sensitive areas with radioactivity coinciding with three of them. Albumin inhibited the system; ribonuclease did not. Although only 25% of the total ^{125}I-label was detected by this assay, results with insulin-specific assays suggested that most (80–90%) of the hormone was inactivated. Possible interpretations of these results are discussed. The particulate fractions of other tissues were also studied.

The fate of hormones after they have performed their biochemical function is an area of great interest. For insulin, early work from several laboratories described an efficient coupled enzymic reaction in liver that may function *in vivo* in the destruction of much of the circulating insulin (glutathione–protein disulphide oxidoreductase, EC 1.8.4.2; for references, see Katzen & Stetten, 1962). The fact that some of the molecules do reach the target site of sensitive tissues still presents the problem of how the cells 'turn off' this chemical regulator.

Adipose tissue, as a major insulin-sensitive tissue, might be expected to possess a degradation system for a molecule that causes such profound alterations in its metabolism, and one such system has been described (DiGirolamo, Rudman, Malkin & Garcia, 1965). During a re-examination of their work, a second degradation system was found and forms the basis for this paper.

EXPERIMENTAL

Chemicals. The porcine ^{125}I-labelled insulin was purchased from Abbott Laboratories, North Chicago, Ill., U.S.A., and contained less than one atom of iodine/6000 mol. wt. units (specific radioactivity 50mCi/mg). Before use the radioactive insulin solution was dialysed against four changes of water. This procedure decreased the trichloroacetic acid-soluble radioactivity to 3–4% of the total. Non-radioactive insulin was kindly supplied by Dr O. K. Behrens, Eli Lilly and Co., Indianapolis, Ind., U.S.A. Albumin (fraction V from bovine plasma) was purchased from Armour Laboratories, Chicago, Ill., U.S.A. Crystalline ribonuclease was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Animals. Male rats of the Holtzman strain weighing 250–400g were allowed access *ad libitum* to a standard laboratory chow diet and water for a period of at least 3 weeks before experimentation.

Preparation of resuspended particulate fraction. The animals were killed by decapitation and bled. The epididymal fat pads were quickly excised and placed on chipped ice. A sample of the adipose tissue (4g) was minced and homogenized in 20ml of cold 0.9% NaCl (saline) in a Potter–Elvehjem glass homogenizer. The homogenate was then centrifuged at 24000*g* for 30min in a Sorvall RC2-B automatic centrifuge at 4°C. The solid supernatant floating layer and the soluble fraction were both removed and discarded. The remaining pellet was resuspended in cold saline and adjusted to a total volume of 20ml. This 1:5 resuspended particulate fraction was used in the incubation procedure, unless otherwise stated.

Incubation and assay procedures. The usual incubation media consisted of 5 units (0.2mg) of unlabelled bovine insulin and 100μunits of ^{125}I-labelled insulin in 1ml of 0.1M-glycine buffer containing 0.1M-NaCl and adjusted to pH9.0 with NaOH.

The incubation was carried out in 20ml beakers in a Dubnoff metabolic shaker at 37°C with a shaker speed of 100 oscillations/min. After a 5-min preincubation, 1ml of the resuspended particulate fraction (or saline for controls) was added to each beaker and this was taken to be zero time. At the end of the incubation period, the entire contents of the beaker (2ml) were emptied into a 12ml centrifuge tube. The beaker was then washed once with 1ml of 0.6% albumin solution and the wash added to the

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centrifuge tube. This was immediately followed by 1ml of 20% (w/v) trichloroacetic acid to precipitate any unchanged insulin. The mixture was chilled in ice for 30min and then centrifuged for 25min at 2000*g*.

A blank control and zero-time control were always run along with the experimental beakers. The blank control had 1ml of saline substituted for the resuspended particulate fraction. For a zero-time control, the enzyme–substrate mixture was not incubated, but the albumin and trichloroacetic acid solutions were added immediately after the tissue homogenate.

The amount of radioactivity in the supernatant was measured in a Packard Automatic Gamma Well scintillation counter. After the counts in zero-time control and the background were subtracted from all values, the extent of degradation was expressed as percentage of radioactivity that had become trichloroacetic acid-soluble.

Paper-chromatographic technique. One-dimensional ascending paper chromatograms of the trichloroacetic acid filtrates were developed by using the filter-paper-cylinder technique described by Block, Durrum & Zweig (1958). The tissue-incubation medium was changed to include 0.1M-potassium phosphate buffer, pH7.7, instead of the usual buffer, to eliminate interference from glycine. All other conditions of the incubation were unchanged. The samples (100μl) were spotted on Whatman no. 3MM paper and placed in a chromatographic jar that had been previously equilibrated with the solvent mixture for 24h.

The chromatograms were developed for 19–21h in a solvent system of butan-1-ol–acetic acid–water (4:1:5, by vol.). After development, the solvent front was marked and the chromatograms were dried in a hood and then sprayed with a solution of 0.2% (w/v) ninhydrin in acetone. The ninhydrin colour appeared after the paper was heated for 15min at 80°C. R_F values were calculated for each of the spots. The chromatograms were then cut into strips (2cm wide) and analysed for radioactivity. R_F values were calculated for the radioactive areas.

Insulin assays. Immunoreactive insulin was measured by the double-antibody method described by Hales & Randle (1963), with reagents purchased from Nuclear–Chicago Radiochemical Corp., Des Plaines, Ill., U.S.A. In this method radioactive insulin competes with the insulin in the incubation medium for the anti-insulin serum. The complex is precipitated, and separated by micro-filtration on to cellulose acetate membranes. Samples were prepared for immunoassay by diluting a sample of the incubation mixture 1:10000 with 40mM-sodium phosphate buffer, pH7.4, containing 0.6mM-thiomersalate, 0.1% bovine plasma albumin and 0.9% NaCl. The radioactivity in the insulin–antiserum complex was determined and compared with insulin standards over a range of 0–500μunits/ml.

The net-gas-exchange manometric bioassay (Ball & Merrill, 1961) was also employed. Warburg vessels (capacity 7ml) contained Krebs–Ringer bicarbonate buffer, pH7.4 (Umbreit, Burris & Stauffer, 1959), 4mg of glucose/ml and 2mg of gelatin/ml in a total volume of 2ml. The net CO_2 production by the epididymal fat pad (80–120mg) was measured for 3h as follows: the first hour, as a control period without further additions; the second hour, after 0.4ml of sample was prepared by diluting a sample of the incubation mixture 1:10000 with a solution of 0.9% NaCl containing 0.1% gelatin or a standard insulin solution was tipped in from the side arm (25–200μunits/ml); and the third hour, with excess of crystalline insulin (0.1unit/ml) added. The readings during the last 30min of each period were used for calculating the percentage of maximal insulin response, which has a linear logarithmic relationship with the amount of insulin present.

RESULTS

General Assay Conditions. In their work on an insulin-degrading enzyme system from adipose tissue homogenate, DiGirolamo *et al.* (1965) found significant enzymic activity in the insoluble fatty supernatant fraction, whereas the soluble fraction had none. We reinvestigated this work by using a radioactive assay and found that the pelleted fraction from such a homogenate caused an increase in the amount of trichloroacetic acid-soluble radioactivity over a 2h period, but the soluble fraction caused no appreciable increase in radioactivity (see below). This particulate system was inactivated by exposure to elevated temperatures (90°C for 5min).

Optimum conditions for the degradation were examined. The pH-dependence was studied with buffers covering pH values 6–12. Fig. 1 shows that the pH optimum occurred at pH9.0. The broad shape of the curve is characteristic of other proteolytic enzymes. The proteolytic activity seen in pellets of homogenates from other tissues and due to lysosomal cathepsins probably is not active here, as lysosomes have not been observed in normal adipose cells (Napolitano, 1965).

Experiments involving alteration of the amount of substrate while the concentration of resuspended

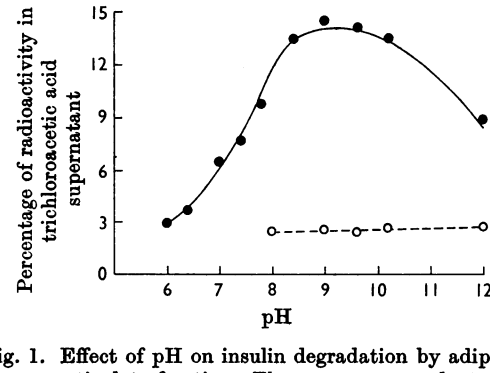

Fig. 1. Effect of pH on insulin degradation by adipose-tissue particulate fraction. The assay was conducted as given in the text. The fraction (1ml) and 1ml of the appropriate 0.1M buffer containing 5units of insulin and 100μunits of ^{125}I-labelled insulin were incubated for 30min at 37°C at the designated pH. 0.1M-Potassium phosphate buffer was used for pH6–8, 0.1M-tris–HCl for pH7–9, and 0.1M-glycine–NaCl for pH9–12. The control flasks (○) contained 1ml of saline in place of the tissue homogenate.

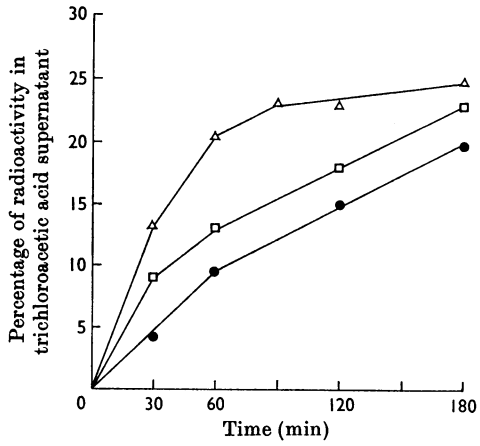

Fig. 2. Appearance of radioactivity in a trichloroacetic acid supernatant caused by the action of resuspended particulate fraction on insulin. Each flask contained 1ml of the fraction and 100μunits of ^{125}I-labelled insulin in 0.1M-glycine–NaCl buffer, pH9.0. The media contained unlabelled insulin: △, 0; □, 1.0; ●, 5.0 units. The assay was conducted as described in the text.

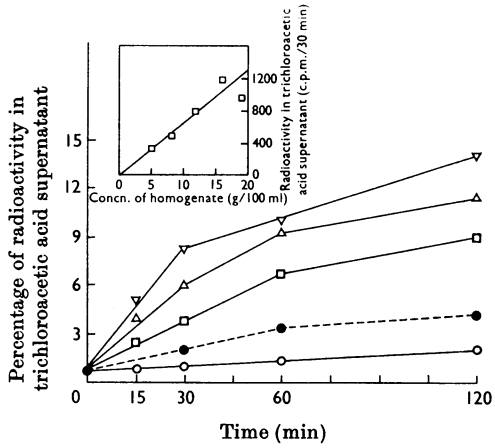

Fig. 3. Relationship between concentration of resuspended particulate fraction and amount of insulin degraded. Incubation media were as described in the text, with the following amounts of fraction added: ▽, 16.0; △, 10.0; □, 4.0g/100ml. ●, Soluble fraction; ○, saline. Inset: relationship between concentration of resuspended particulate fraction and rate of insulin degradation. Conditions were the same as described above except that 10units of unlabelled insulin were used. Incubation was terminated after 30min.

particulate fraction was kept constant (200mg/ml) were performed to determine the amount of insulin necessary to saturate the system. The results are shown in Fig. 2. This graph shows that when increasing amounts of non-radioactive insulin were incubated, the amount of ^{125}I-labelled insulin degraded in the same time-period was decreased. This suggested a competition between the radioactive and non-radioactive insulin molecules for the active site(s) on the enzyme(s). When 5.0 units of insulin were added, degradation appeared to follow linear kinetics for 60min, and thus this concentration of insulin was used for future experiments (except where otherwise noted).

The relationship between the enzyme concentration and reaction velocity was studied in a series of experiments, the results of which are shown in Fig. 3. Although the soluble fraction did not have appreciable insulin-degrading activity, the amount of the hormone destroyed did increase with added amounts of the particulate fraction. The amount of radioactivity appearing in the trichloroacetic acid supernatant during the first 15min of incubation was very small and reproducible results were not obtained. This does not prove that there was not significant enzymic activity; but if there was, this technique was too insensitive to measure it.

The inset of Fig. 3 plots the results of another experiment as enzyme concentration versus velocity co-ordinates. The curve is linear, indicating the usual enzyme–substrate relationship at saturation conditions.

Since no more than 25% of the radioactivity was solubilized, an attempt to increase this percentage was investigated by two methods: (1) resuspending the pellet in the soluble fraction (which might have contained essential activating cofactors), and (2) dialysing the resuspended particulate fraction to remove possible non-protein inhibitors. Neither technique increased the amount of radioactivity detected.

Degradation by other tissues. Since adipose tissue is responsive to insulin, it was decided to investigate the particulate fraction of other tissues for the presence of this enzyme system. In this study, another insulin-sensitive organ (heart) was used as well as a tissue not responsive to insulin (kidney). The resuspended particulate fraction from these tissues was prepared as described for adipose tissue and the appearance of the label in a trichloroacetic acid supernatant was monitored. The results are shown in Fig. 4. This graph demonstrates that: (1) both heart and kidney particulate fractions contain insulin-degrading activity, and (2) the type of degradation is qualitatively different from the adipose-tissue activity, since they converted 65% of the insulin into labelled soluble products, instead of the 25% conversion found with adipose tissue.

Specificity. It was then decided to investigate the selectivity of the enzyme system, by using substrate competition. The effect of various unlabelled

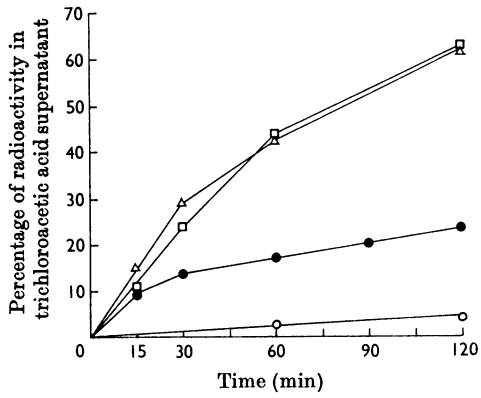

Fig. 4. Insulin-degrading activity of the resuspended particulate fraction from several tissues. The incubation media and procedures are given in the text. The reaction was initiated by the addition of 1ml of 20% (w/v) resuspended particulate fraction from the indicated tissue: □, heart; △, kidney; ●, adipose tissue. ○, Saline.

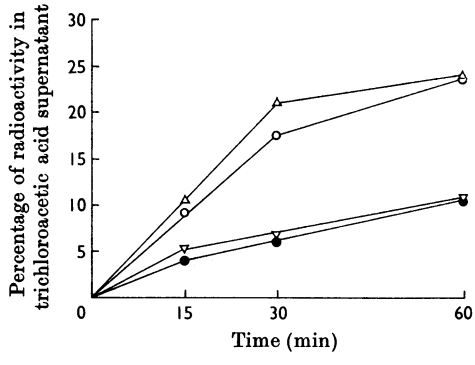

Fig. 5. Influence of various proteins on the adipose tissue insulin-degrading system. Incubation media consisted of 0.1M-glycine–NaCl buffer, pH 9.0, 100μunits of ^{125}I-labelled insulin, 33nmol of each of the indicated proteins (△, no addition; ○, ribonuclease; ▽, albumin; ●, insulin) and adipose-tissue resuspended particulate fraction. Details of the assay are given in the text.

proteins on the extent of degradation of ^{125}I-labelled insulin was measured. The standard incubation was performed with equimolar amounts of one of three proteins (insulin, albumin or ribonuclease) added to compete with the ^{125}I-labelled insulin (the following values for molecular weights were used in determining molar concentrations: insulin, 6000; ribonuclease, 15000; bovine plasma albumin, 69000). The reaction was terminated at various times and the acid-soluble radioactivity was determined. The results are shown in Fig. 5. If the protein competed with the ^{125}I-labelled insulin for the enzyme(s), the amount of ^{125}I radioactivity appearing per unit time should decrease. Insulin competed with the labelled insulin (see also Fig. 2). Albumin also competed, but ribonuclease did not produce a significant decrease suggesting that some degree of selectivity may be involved with the enzyme system and that not all proteins are degraded by it.

Table 1. *R_F values from paper chromatograms of a trichloroacetic acid supernatant from an insulin-degradation study and the relative amount of radioactivity associated with each ninhydrin-sensitive area*

Insulin (10units of unlabelled and 200μunits of ^{125}I-labelled) was incubated with adipose-tissue resuspended particulate fraction as described in the text. The acid-soluble products were subjected to ascending paper chromatography and then sprayed with ninhydrin. The R_F values of the ninhydrin-sensitive areas are given. The relative amount of radioactivity associated with each spot is also given (see also Fig. 6). Controls with resuspended particulate fraction or insulin gave no ninhydrin reaction.

R_F value	Relative amount of radioactivity
0.09	—
0.19	+++
0.23	++
0.37	—
0.76	+
0.82	—

Paper chromatography. An indication of the number of degradation products could be obtained by chromatography of the trichloroacetic acid supernatant. Paper chromatograms of the acid-soluble products were developed as detailed in the Experimental section. After the R_F values of the ninhydrin spots were recorded, the ^{125}I radioactivity was determined. Table 1 shows that six ninhydrin-reactive spots appeared at the indicated R_F values. Three of these spots possessed radioactivity.

A detailed chart of the radioactivity migration is shown in Fig. 6. There were three peaks of radioactivity, corresponding to ninhydrin-sensitive spots with R_F values 0.19, 0.23 and 0.76. The control tube showed a small peak at 0.22. This was probably free iodide, since its R_F value corresponded closely to that obtained under similar conditions by Nakagawa (1965).

Insulin-specific assays. The need for insulin-specific assays resulted in the use of both immunological and biological assays. The radioimmunoassay described by Hales & Randle (1963) was performed in concert with the measurement of trichloroacetic acid-soluble radioactivity. The results of this double experiment are shown in

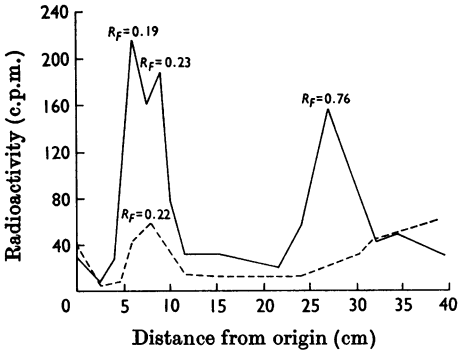

Fig. 6. Radioactivity profile from paper chromatograms of the trichloroacetic acid supernatant after incubation of ^{125}I-labelled insulin with and without resuspended particulate fraction. The incubation media contained 10 units of insulin, 200 μunits of labelled insulin in 0.1M-potassium phosphate buffer, pH 7.7. The reaction was initiated by the addition of the fraction (——) or a saline control (----) and continued for 120 min. The reaction was terminated by addition of albumin and trichloroacetic acid. A sample (100 μl) of the trichloroacetic acid supernatant was subjected to paper chromatography (see also Table 1). The paper was cut into strips (from the origin) and its radioactivity counted.

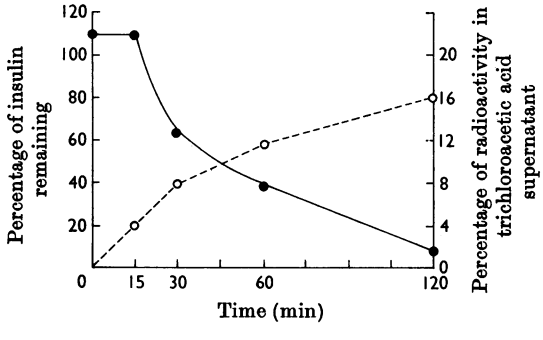

Fig. 7. Simultaneous experiments to measure both the trichloroacetic acid solubility of non-protein ^{125}I products (○) and the radioimmunoassay of insulin-like activity (●). Details of both assays are given in the text.

Fig. 7, which shows that the amount of radioactivity made soluble in the reaction was low, whereas the inactivation of immunoreactive insulin (5 units) was almost complete. As an additional assay, the older and less specific bioassay was employed and similar results were obtained (Fig. 8). Although the fat-pad bioassay results were not as precise as those obtained by immunoassay, the insulin concentrations clearly decreased greatly with increased incubation time.

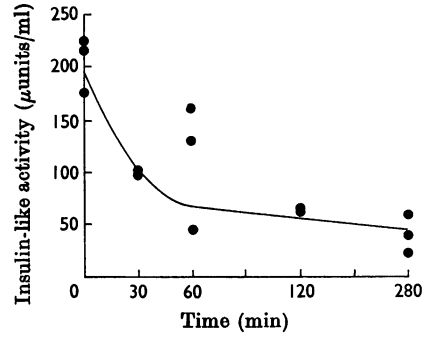

Fig. 8. Time-course of the disappearance of insulin-like activity from an incubation medium containing adipose-tissue resuspended particulate fraction, as measured by the fat-pad bioassay. Because the results showed a lack of precision in some instances, individual points from three experiments were plotted to indicate the scatter.

DISCUSSION

The results given above describe a new enzymic system that degrades insulin. It differs from the two previously described (Tomizawa, 1962*a*; Westman, 1968) in many respects. It appears in the particulate fraction (membrane, nuclei and mitochondria), compared with the fat-soluble adipose-tissue system and the transhydrogenase of liver supernatant. The pH optimum also differs markedly from that found for the other systems. The liver transhydrogenase has a pH optimum of 7.4–8.0 (Tomizawa, 1962*a*) and the fat-soluble adipose-tissue system has an acid pH optimum of 3.0–4.0 (Westman, 1968). The alkaline pH optimum of 9.0 for the present system corresponds to that found for other proteolytic enzymes (trypsin and carboxypeptidase A) for their action on insulin (Carpenter, 1966).

The method of assay chosen to determine degradative activity was a slight modification of that used by Narahara, Tomizawa, Miller & Williams (1955). Although theoretically sound, the technique has the drawback of dependence on the position of the tyrosine moiety to which the iodine is attached in relation to the site of attack by the enzyme. In the case of glutathione–insulin transhydrogenase, the fact that the hormone is split only by the scission of disulphide bonds (Katzen & Stetten, 1962), that the A chain contains most of the label (Springell, 1961) and the A chain is soluble in trichloroacetic acid (Tomizawa, 1962*b*) all lead to a direct measurement of insulin degradation by the use of this assay. In the present work, this assay does not measure insulin degradation stoicheiometrically. It appears that not all of the degradation products are rendered acid-soluble by the enzyme system.

A competitive type of experiment was performed to investigate the effect of substrate concentration on the enzyme system. It showed (Fig. 2) that increasing amounts of unlabelled insulin led to a decrease of acid-soluble radioactivity appearing per unit time. This indicated competition by the additional insulin for the enzyme system.

The fact that an increase in concentration of resuspended particulate fraction yielded a linear increase in activity was additional evidence for the enzymic nature of the insulin destruction, as was the thermolability of the system. The lack of degradative activity in the soluble fraction agreed with previous findings (DiGirolamo *et al.* 1965).

The paper chromatograms showed six ninhydrin spots, of which three had radioactivity associated with them. The ninhydrin spot at R_F 0.23 could be a labelled tyrosine peptide, or an unlabelled peptide or amino acid that had the same R_F as free iodide, or both. The two other radioactive ninhydrin areas (R_F 0.15 and 0.76) probably represent labelled peptide in the former case and either free mono- or di-iodotyrosine in the latter, as both of these labelled amino acids have high R_F values (Smith, 1960).

Kidney has been classically considered as insulin-insensitive, whereas heart muscle is very sensitive, as is skeletal muscle (Krahl, 1961). When the particulate fraction of these tissues was incubated with ^{125}I-labelled insulin, the rate of appearance of radioactive products was entirely different from that of an adipose-tissue preparation. More than 60% of the radioactivity was solubilized by each of the tissues, indicating a different method of degradation. The adipose-tissue system allows only 25% of the radioactivity to become trichloroacetic acid-soluble, indicating that heart and kidney possess systems that cause the release of more of the labelled tyrosine moieties. This degradation might be the result of general proteases found in the lysosomes known to be located in these tissues (de Duve & Wattiaux, 1966), but not found in adipose tissue.

The results of the specific insulin assays were unexpected and, although not definitive, certainly suggest that most of the molecules are being degraded, in contrast with the labelled-insulin assay, where experiments extending as long as 5h never showed acid-soluble radioactivity of more than 24–28%.

There are two possible interpretations of these results: total degradation of 25% of the insulin molecules, or degradation of 100% of the molecules in such a way that only a small portion of the molecule (containing 25% of the radioactivity) was solubilized.

The former interpretation is not likely, based on other observations (Sumner, 1969), that amino acid analysis of the trichloroacetic acid supernatant showed a mole-for-mole correlation between amino acids that occur only once in the molecule and the amount of insulin incubated with the degradation system. The results with insulin-specific assays, presented above, also argue against this explanation.

Assuming that the latter interpretation of 100% degradation is correct, there are two ways to account for the apparently limited amount of degradation of the molecule: (1) a large portion of the hormone (containing the bulk of the radioactivity) is not broken down and is precipitated by the acid; or (2) part of the insulin molecule adsorbs to glass or to fractions of the adipose tissue, or to both, which protects it from further degradation. A corollary phenomenon would be non-specific adsorption of fragments to prevent appearance in the acid supernatant.

The first possibility seems the most likely. It is necessary, though, to consider the second interpretation of the data.

Adsorption to glass is always a problem with very small amounts of insulin, but considering the large concentration used in these experiments, the effect on the results would be negligible. Further, in a report in which specific measures to prevent adsorption to glass were employed, it was also found that only 27% of the radioactivity was made acid-soluble after a 2h incubation of isotopically labelled insulin with adipose tissue (Garratt, Jarrett & Keen, 1966). A report by Crofford (1968) in which no glassware was used (but polyethylene vials and syringes) gave evidence for complete loss of immunoreactive insulin after incubation for 1h with isolated fat cells.

The possibility of adsorption to cell fractions is more difficult to eliminate. The consistency of the results seems to preclude random adsorption of small peptide fragments. The possibility that a large polypeptide fragment is binding to a cell fraction that then removes it from further action by the enzymic system has not been eliminated, however, and is a possibility that must be explored.

Although the physiological importance of this degradation system is at present unknown, it has a similar high capacity for degrading insulin *in vitro* (12.5–25 units/h for 1g of tissue) as the other degradation systems described to date.

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