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INSULIN DEGRADATION

XV. USE OF DIFFERENT ASSAY METHODS FOR THE STUDY OF MECHANISM OF ACTION OF GLUTATHIONE-INSULIN TRANSHYDROGENASE*

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Summary

Insulin degradation by glutathione-insulin transhydrogenase has been studied using three different assay procedures: the measurement of the change in insulin immunoreactivity; the formation of 5% trichloroacetic acid-soluble radioactivity from ^{125}I-labeled insulin and the formation of GSSG via coupling to the oxidation of NADPH with the use of glutathione reductase. The extent of reaction as measured by each assay was different, and the ratios between the assays were not constant with time. Kinetic experiments with the NADPH-coupled assay and the trichloroacetic acid assay yielded similar results: Lineweaver-Burke plots with insulin as variable and GSH as fixed substrate gave a set of straight, intersecting lines, and such plots with GSH as variable and insulin as fixed substrate were parabolic. Apparent K_m values for insulin at 1 mM GSH were found to be quite similar by three assay techniques; however, the V values per unit of enzyme protein varied considerably with different procedures. The results are interpreted as indicating that immunoreactivity is lost after reduction of only one of the disulfide bonds of insulin whereas the two interchain disulfide linkages must be broken to produce the trichloroacetic acid-soluble A chain. The results of the NADPH-coupled assay suggest that all three disulfide bonds of insulin are possible substrates for the enzyme. The trichloroacetic acid precipitation assay seems to be the most practicable technique for general use because of the greater ease in performing large number of samples, precision and sensitivity.

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Introduction

Insulin degradation may be measured in a variety of ways, such as by bioassay [1] or immunoassay of residual insulin [1—6], by immunoassay of A and B chains formed from insulin [7—9], by paper or gel chromatography of the products of degradation [3,5,8—13], by precipitating intact insulin (plus B chain aggregate) with 5% trichloroacetic acid (in which A chain is soluble) [10,13—28], by adsorption of intact insulin to talc, charcoal, or cellulose [29], or by measuring the GSSG formed concommitantly with insulin degradation by glutathione-insulin transhydrogenase (thiol: protein disulfide oxidoreductase, EC 1.8.4.2) via coupling the reduction of GSSG to the oxidation of NADPH with glutathione reductase [1,2,17—19,25,26,30,31]. In practice, only three of these assays have been used routinely: precipitation by trichloroacetic acid, immunoassay of insulin and the glutathione reductase-coupled reaction. However, each of the other assay techniques have been used for specific purposes; for example, both gel chromatography and immunoassay of A chain have been used to demonstrate that glutathione-insulin transhydrogenase provides the obligatory first step in insulin degradation in several tissues [8,9,13].

Glutathione-insulin transhydrogenase catalyzes the cleavage of disulfide bonds of insulin and can utilize a variety of thiol cosubstrates including GSH; the reaction has been found to proceed via a complex random mechanism [32] with uncertain overall stoichiometry. Each of the commonly used assays possibly measures a different facet of the reaction: loss of immunoreactivity might result from breaking any of the three disulfide bonds of insulin, the formation of the trichloroacetic acid-soluble product A chain requires the disruption of both interchain disulfide bridges and the glutathione reductase-coupled spectrophotometric assay might measure the GSSG formed concommitant to reduction of any disulfide bond during insulin degradation. No direct comparison of the three assays has been reported. The present study was undertaken to assess, with the use of purified glutathione-insulin transhydrogenase, the relative merits of the assays.

Results indicate that the trichloroacetic acid precipitation assay is probably the most convenient procedure for routine use. The data suggests the following conclusion about the stoichiometry and mechanism of the reaction catalyzed by glutathione-insulin transhydrogenase: Consistent with the classic observation that the destruction of one disulfide bond results in total loss of hormonal activity [33—35], the cleavage of one disulfide bond of insulin by transhydrogenase probably results in the total destruction of insulin immunoreactivity, the formation of the trichloroacetic acid-soluble product A chain requires the disruption of both interchain disulfide bridges, and in agreement with the report of Katzen et al. [1], all the three disulfide bonds appear to be susceptible to the action glutathione-insulin transhydrogenase.

Methods

Glutathione-insulin transhydrogenase purified from bovine pancreas as previously described [18,36,37] was used in all experiments. ^{125}I-labeled insulin was prepared by the chloramine-T method [38—40], and was purified by chromatography on Sephadex G-50 [23]. The procedure used yields insulin

with an average of 0.6 atom ^{125}I per insulin molecule, with 90% of the label attached to the A chain and 10% to the B chain. Yeast glutathione reductase was obtained from Sigma or Boehringer-Mannheim. Spectrophotometric measurements were done with a Beckman DB-G spectrophotometer coupled to Photovolt logarithmic recorder (1.0 absorbance unit full scale).

The standard incubation buffer was 0.15 M potassium phosphate/25 mM EDTA (pH 7.5). In experiments at insulin concentrations greater than 27 μM, this buffer was diluted 1 : 3 to avoid turbidity; no differences in velocities were observed between these two buffer systems. All incubations were carried out at 37°C.

For measurements by immunoassay or trichloroacetic acid precipitation, the reaction mixture (containing 4 mg bovine serum albumin, 1 μmol GSH and 2 μg of glutathione-insulin transhydrogenase in 0.9 ml of buffer) was preincubated for 5 min at 37°C; the reaction was started by the addition of 0.037—27 nmol of insulin (with a trace amount of ^{125}I-labeled insulin in the case of the trichloroacetic acid precipitation assay) in 0.1 ml of buffer. In the trichloroacetic acid precipitation assay [23,24], the reaction was stopped by the addition of 1.0 ml of 10% trichloroacetic acid, the precipitate was collected by centrifugation for 5 min at 1000 × *g* and washed once with 2 ml of 5% trichloroacetic acid and the radioactivity in both the precipitate and the combined supernatants was measured.

For immunoassay, the reaction was stopped by diluting an aliquot (10—100 μl) of the reaction mixture in a minimum of 37 volumes of diluent (0.1 M Tris(hydroxymethyl)-aminomethane-HCl (Tris) buffer, pH 7.7 at 5°C, with 0.1% bovine serum albumin and 2 mM *N*-ethylmaleimide). Each sample was diluted to a final insulin concentration of 1 nM (assuming no degradation). The immunoassay utilized cellulose powder to separate antibody-bound from free insulin [41]: 0.1 ml of sample (or standard pork insulin) was incubated for 24 h at 5°C with guinea pig anti-insulin serum in 1.25 ml of Tris buffer with 1% albumin, 0.25 ml of 1 nM ^{125}I-insulin was added; after a further 24-h incubation 1.0 ml of cellulose suspension (10 g MN-cellulose + 100 ml Tris buffer with 1% albumin) was added; the cellulose was collected by centrifugation at 1000 × *g* for 5 min and the cellulose-bound radioactivity was determined.

For the spectrophotometric assay [17,19], the standard incubation mixture consisted of 25 μg glutathione reductase, 0.10 mM NADPH, 1 mM GSH, 20 μg glutathione-insulin transhydrogenase and 25 μM insulin in a final volume of 1.40 ml. The reaction mixture without insulin was preincubated at 37°C for 3 min in the thermostated cuvette holder. The reaction was started by the addition of the insulin and the absorbance at 340 nm was recorded for 8 min. It should be noted that in order to maintain the same incubation conditions for the three assay techniques, the procedure for the spectrophotometric assay in the current study differed from the previous studies in temperature and ionic concentration of incubation medium. For concurrent use of the trichloroacetic acid precipitation assay, a tracer amount of ^{125}I-labeled insulin was included in the insulin solution. The reaction mixture was sampled at various times by withdrawing a 50 μl aliquot from the cuvette with an Eppendorf pipet. The aliquot was rapidly diluted in 0.95 ml of phosphate buffer containing 4 mg albumin, and 1 ml of 10% trichloroacetic acid was added to the diluted sample.

Further sample treatment was as noted above.

Statistical evaluation of the kinetic parameters was done by means of nonlinear least-squares curve fitting [32,42,43]. Data obtained by the spectrophotometric assay had a constant variance in velocity and so was fitted directly with the Michaelis-Menten equation ($v = V \cdot S/(S + K_m)$). The variance is not constant with velocity in either the trichloroacetic acid assay or immunoassay, however (see ref. 32 for a more detailed discussion of this point). In the precipitation assay the measured quantity is the percent change in trichloroacetic acid solubility of a constant amount of tracer with varying amount of insulin and in the immunoassay samples at various insulin concentrations are diluted to a constant insulin concentration before assay. Thus in either of these assays the variance should be expected to be constant in v/S (the fractional amount of insulin degraded); this expectation was confirmed experimentally. Therefore the data from these assays were fitted to the transformed equation, ($v/S = V/(S + K_m)$).

Results and Discussion

Initial experiments with the spectrophotometric assay were directed toward defining an optimal assay system. The conditions used were kept as close as possible to those used previously for the trichloroacetic acid assay [23,24,32]. Use of the same buffer system (0.15 M potassium phosphate/25 mM EDTA) was possible except at the higher concentrations of insulin where turbidity occurred; this problem did not occur after three-fold dilution of the buffer. Measured velocities at the lower insulin concentrations were not observably different in the two buffers.

Experiments with varying amount of glutathione reductase indicated that it was desirable to keep the amount of glutathione reductase as low as possible: When the amount of reductase was increased, both the control (transhydrogenase omitted) and the apparent enzymatic rates of NADPH oxidation were increased, but the net enzymatic rate (the difference between the two measurements) remained essentially constant. Thus the only effect of increasing the amount of glutathione reductase beyond 25 μg was to increase the nonspecific oxidation of NADPH. Conversely, when the amount of reductase was decreased to 10 μg, a noticeable lag occurred in the oxidation of NADPH after the addition of GSSG, i.e. the amount of reductase was insufficient to assure the rapid formation of NADP from the GSSG formed by the action of the transhydrogenase.

Albumin has been routinely included in the trichloroacetic acid precipitation assay for three reasons: to prevent possible adsorption of insulin to tube or pipet surfaces, to minimize the effect of non-specific proteases (in crude tissue extracts [12,23]) and to facilitate collection of the protein precipitate. Experiments with varying amounts of albumin have shown that its presence does not affect insulin degradation by glutathione insulin transhydrogenase but that levels in excess of 4 mg/ml can lead to co-precipitation of some of the A chain produced in the reaction [23]. However, it was found that in the spectrophotometric assay, albumin could not be included since addition of it caused oxidation of NADPH in the absence of transhydrogenase or insulin. This effect

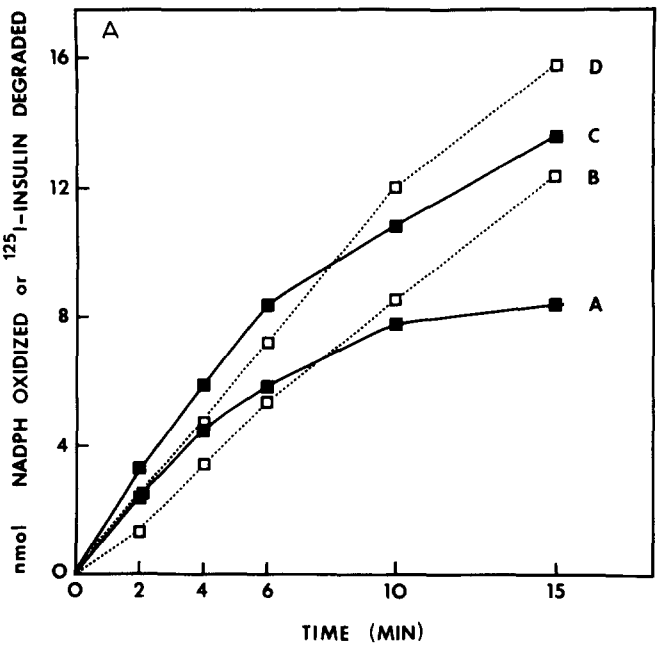
was dependent both on the amount of albumin added and on the concentration of GSH present, and so is probably due to nonenzymatic interchange reaction between GSH and the labile mixed disulfide group which is known to be present in albumin [44]. The loss of GSH occasioned by this reaction is not significant in the nonspectrophotometric assays, however, as the GSSG formed with 1 mM GSH and 4 mg/ml albumin was equivalent to less than 1% of the GSH present.

In all three assay systems, the control rate of reaction (glutathione insulin transhydrogenase omitted) was dependent on the concentrations of both insulin and GSH; thus it was necessary in each case to measure the rate of reaction with and without the transhydrogenase so that the net enzyme-dependent rate could be expressed as the differences between the two. Conversely, the presence or absence of the transhydrogenase had no effect on control reaction rates with insulin omitted (spectrophotometric assay) or GSH omitted (trichloroacetic acid precipitation assay and immunoassay).

The reliable lower limit for the sensitivity of the spectrophotometric assay was found to be about 20 μg of transhydrogenase, since at levels of enzyme lower than this the net enzymatic rate of NADPH oxidation was less than the nonenzymatic control. The actual net changes in absorbance are small, consequently the precision of the assay could probably be improved with the use of a more sensitive spectrophotometer. However, the sensitivity of the assay would still be limited by the rate of the non-enzymatic oxidation of NADPH.

Concurrent assays by the spectrophotometric and by the trichloroacetic acid precipitation technique were performed by repeated sampling from the reaction cuvette. As can be seen in Fig. 1A, these experiments showed that the NADPH oxidation remained linear with time much longer than the reaction measured by precipitation; no slowing of the rate of NADPH oxidation was apparent until more than 40% of the ^{125}I was solubilized. Concurrent immunoassay and NADPH assay (Fig. 1B) confirmed this result, with no nonlinearity being apparent in the NADPH oxidation until less than 20% of the insulin immunoreactivity remained. Thus the ratios between the reaction rates for the three assay procedures were not constant with time; the ratio of NADP to ^{125}I trichloroacetic acid-soluble ranged from 0.8 to 1.5, and the ratio of the oxidation of NADPH to the loss of immunoreactivity ranged from about 0.1 to 1. In contrast, although neither the immunoassay nor the precipitation assay were linear with time after 3 min under the conditions used (Fig. 1C), the ratio between the two assays stayed fairly constant and changed only in the range from 1.6 to 2.0.

There are several possible explanations for these results. One possibility is that there is a delay between the formation of GSSG and its reduction; this could conceivably result either from a slow reaction with glutathione reductase or from a delay in the release of GSSG from glutathione-insulin transhydrogenase. As noted above, however, the amount of the reductase used was found to be sufficient to reduce added GSSG rapidly and quantitatively. No information is available on the rate of release of GSSG from the transhydrogenase; however, if GSSG was indeed released slowly from the enzyme, an initial lag before NADPH oxidation should be apparent, no such lag was evident. Another possible explanation is the incorporation of released A chain into B chain to



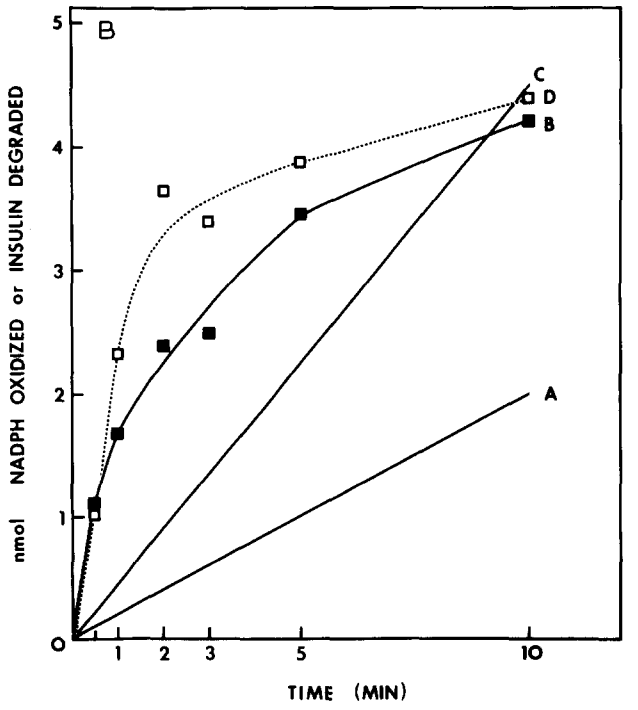
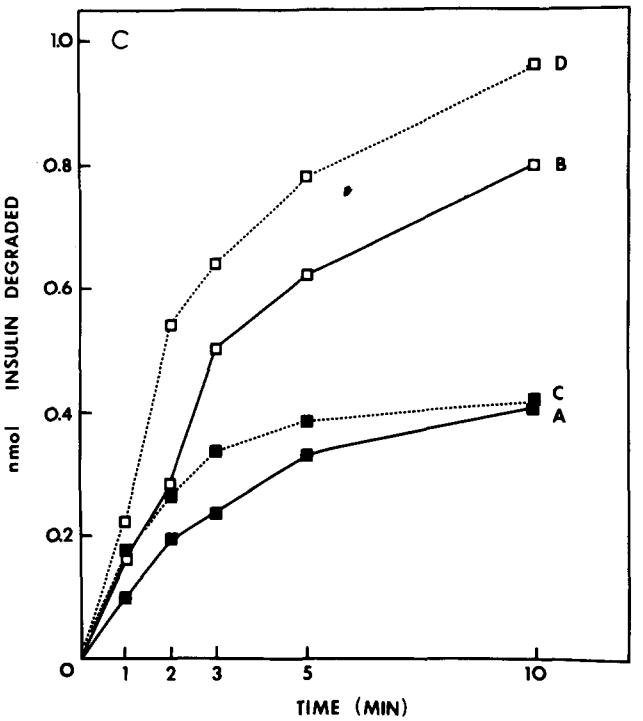


Fig. 1. (A) Concurrent assay of insulin degradation by the spectrophotometric assay and the trichloroacetic acid precipitation assay. The experiments were performed as described in the text with 0.1 mM NADPH, 25 μg glutathione reductase, 20 μg glutathione-insulin transhydrogenase and 1 mM GSH in a total volume of 1.4 ml. The values given are the net enzymatic rates, and either 25 μM insulin (A and B).



or 50 μM insulin (C and D). The measurement was made by the precipitation assay (A and C) and spectrophotometrically (B and D). (B) Concurrent assay of insulin degradation by the spectrophotometric assay and by the immunoassay. The experiment was performed as described in the text with 0.1 mM NADPH, 25 μg glutathione reductase, 1 mM GSH and 5 μM insulin in 1.4 ml of the phosphate buffer. The reaction was carried out with either 10 μg (A and B) or 20 μg (C and D) of glutathione-insulin transhydrogenase; measurement was by the spectrophotometric assay (A and C) and by immunoassay (B and D). Note that the degradation of 5 nmoles would represent 100% of the insulin present. (C) Concurrent assay of insulin degradation by the trichloroacetic acid precipitation assay and by immunoassay. The experiment was performed as described in the text with 4 mg albumin, 1 mM GSH and 1 μM insulin in 1.0 ml of phosphate buffer. Either 5 μg (A and B) or 10 μg (C and D) of glutathione-insulin transhydrogenase was present; measurements were made by the trichloroacetic precipitation assay (A and C) and by the immunoassay (B and D). Note that the degradation of 1 nmol would represent 100% of the insulin present.

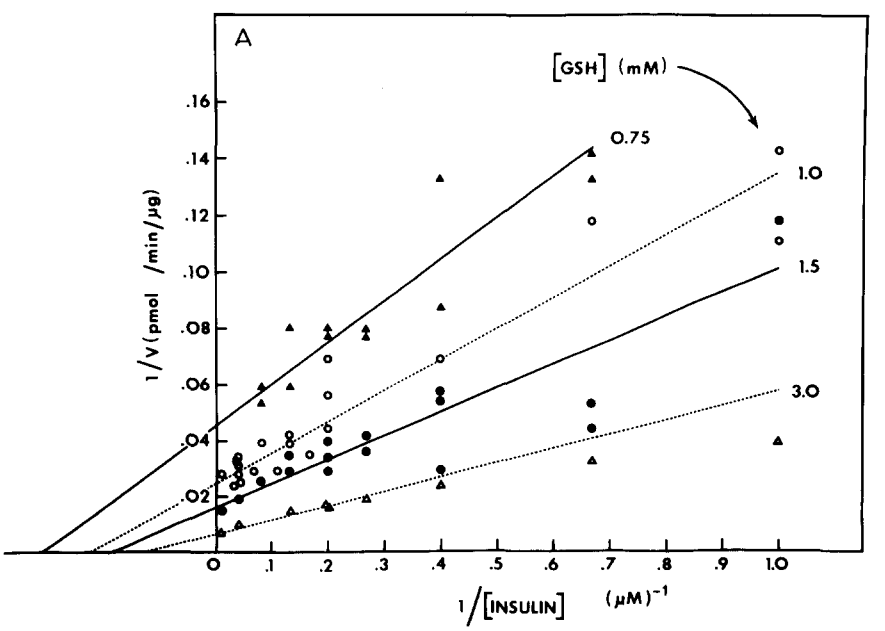
form B chain rich-A chain complex [8,9,12,45]. The most likely explanation depends on the fact that the different assays measure different aspects of the reaction: breaking only one insulin disulfide bond might destroy the immunoreactivity, freeing A chain from the molecule requires splitting of both interchain disulfide bridges, but GSSG might be formed via the attack of glutathione-insulin transhydrogenase on any or all of the three disulfide bonds in insulin [1]. Thus it is possible that total destruction of the insulin immunoreactivity present (by breaking one disulfide per molecule) might represent the utilization of only one third of the substrate for GSSG formation. The ratio of insulin degradation measured by immunoassay to that measured by the trichloroacetic acid precipitation assay is consistent with the expectation of loss of immunoreactivity with reduction of one disulfide bond as opposed to the reduction of two interchain disulfides to free A chain, provided the disulfide bonds are equally accessible to the transhydrogenase. However, these conclu-

sions should be confirmed by additional, more direct evidence, such as by titration of sulfhydryl groups on the insulin molecule at various stages of reaction.

The ratio of NADPH oxidized/^{125}I solubilized is lower than the expected ratio of 2/1 for breaking the interchain disulfides or 3/1 for complete reduction of insulin. Similarly, the ratio of NADPH oxidized to immunoreactivity lost was lower than the expected range of 1 : 1 to 3 : 1. Thus there seems to be poor correlation between the extent of the reaction measured spectrophotometrically and the actual amount of insulin degraded.

Kinetic studies afford another means for comparing the various assays. With the spectrophotometric assay, when each substrate was varied at fixed levels of the other substrate (Figs 2A and 2B), the kinetic patterns were found to be the same as previously observed with the trichloroacetic acid precipitation assay [32]: reciprocal plots with insulin as substrate gave a family of intersecting straight lines; while such plots with GSH as substrate were parabolic. Measurement by the immunoassay technique also gave a linear reciprocal plot with insulin as variable substrate.

The results of kinetic experiments with each of the three assays are given in Table I: the parameter values were derived by nonlinear least-squares fitting of the Michaelis-Menten equation as discussed above. With 1 mM GSH, the apparent K_m values for insulin were found to be very similar with each assay. These values are similar to those previously found with the trichloroacetic acid precipitation assay [23,32], but are different from the previously reported values (21—64 μM) for the spectrophotometric assay [18,19,22]; these differences with the spectrophotometric assay are probably related to the different



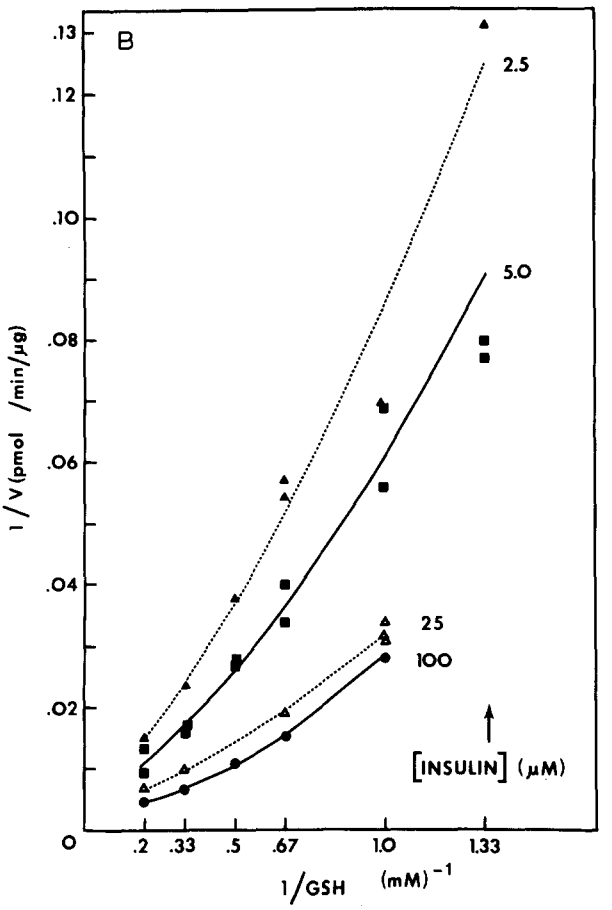


Fig. 2. Kinetic studies of insulin degradation with the spectrophotometric assay. The experiments were performed as described in the text, with 0.1 mM NADPH, 25 μg glutathione reductase, 20 μg glutathione-insulin transhydrogenase, 1.0—100 μM insulin and 0.75—5.0 mM GSH in 1.4 ml of phosphate buffer. The lines shown were derived by nonlinear least-squares fitting as described in the text. A. Reciprocal ($1/v$ versus $1/S$) plot with insulin as variable substrate. GSH (mM) was present at different fixed levels as indicated. The lines are from fits to the Michaelis-Menten equation ($v = V \cdot S/S,(S + K_m)$). B. Reciprocal plot with GSH as variable substrate. Insulin (μM) was present at different fixed levels as indicated. The lines were derived by fitting the reciprocally parabolic equation, $v = V/(1 + b_1/S + b_2/S^2)$.

TABLE I

KINETIC CONSTANTS OF GLUTATHIONE-INSULIN TRANSHYDROGENASE

Each assay was performed as described in the text; the reaction was carried out with 1 mM GSH and 2 μg transhydrogenase (precipitation assay and immunoassay) or 20 μg transhydrogenase (spectrophotometric assay). The parameter values were derived by nonlinear least-squares fitting of the Michaelis-Menten equation. The maximum velocity is given in pmol per min per μg transhydrogenase, the K_m for insulin is μM. The error figures given are estimates of the standard errors obtained from the fitting procedure.

Assay method	V	K_m
Immunoassay	216 ± 52	6.2 ± 1.7
Trichloroacetic acid precipitation	80 ± 4	7.4 ± 0.4
Spectrophotometric	41 ± 2	4.5 ± 1.0

experimental conditions employed in the current study from those used in the previous studies. In view of the well-known dependence of the apparent K_m values on the precise conditions studied, these differences in the apparent K_m values are not surprising for an enzyme involving a complex random mechanism. The values for the apparent V per μg of enzyme, however, were significantly different. The ratio of the V values from the precipitation assay and the immunoassay is consistent with the ratios of the two assays found in the concurrent time studies; thus, the rate of loss of immunoreactivity (i.e. inactivation) proceeds faster than the rate of reduction and supports the possibility that the splitting of one disulfide bond results in total inactivation of insulin [33—35]. The relative V found by the spectrophotometric assay was however lower than expected. This difference is probably because the experiments were performed under non-steady state kinetic conditions (i.e. due to the use of relatively high concentration of enzyme in this assay procedure); especially at the lower insulin concentrations, this amount of enzyme might not be rate-limiting.

The precisions of the three assay systems were quite different. Both the trichloroacetic acid precipitation assay and the immunoassay were found to have an approximately constant coefficient of variation in (v), while the spectrophotometric assay gave a constant variance in (v); these differences are consistent with the differences in the assay techniques. The coefficients of variation for the entire range of insulin levels used calculated from the residuals after fitting to the Michaelis-Menten equation were ±3.8% for the precipitation assay and ±33.1% for the immunoassay. The standard deviation calculated similarly for the NADPH assay was 3.9 pmol/min/μg of transhydrogenase (equivalent to a coefficient of variation of 18% at 5 μM insulin).

These results indicate that for most purposes the trichloroacetic acid precipitation assay is better than the two other assays: the precipitation assay is much less time-consuming than the other assays for large number of samples, it is at least 10 times more sensitive than the spectrophotometric assay, and it yields much better precision than either of the other two assays. While the immunoassay should be nearly twice as sensitive as the precipitation assay (judging from the ratio of measured insulin degradation in the concurrent assays) this increased sensitivity is in practice not achievable due to the much worse precision of the immunoassay for the following reasons: by immunoassay residual insulin is measured, i.e. one is measuring, by difference, the small change in a large quantity of insulin. The trichloroacetic acid precipitation assay measures the product of insulin degradation (A chain) directly. Since an immunoassay technique requires a greater number of steps than the trichloroacetic acid precipitation assay, it is expectable that the simpler assay technique will be more precise.

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