主論文

INSULIN-STIMULATING PROTEIN FROM HUMAN PLASMA: CHEMICAL CHARACTERISTICS AND BIOLOGICAL ACTIVITY

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Naokatu Arakaki

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Naokatu Arakaki

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CURRICULUM VITAE

氏名	新垣尚捷　（Naokatu Arakaki）
生年月日	１９５３年　１２月３日
本籍地	大阪府吹田市佐井寺２丁目２４－５
現住所	徳島県徳島市八万町大坪２３２－１大坪住宅４－１６

略歴

１９７７年３月	徳島大学薬学部製薬化学科卒業
１９７７年４月	徳島大学大学院薬学研究科製薬化学専攻入学
１９７９年３月	同修了
１９７９年４月	徳島大学薬学部微生物薬品化学教室（谷勇　教授）研究生
１９８０年３月	同修了
１９８０年４月	大阪大学大学院理学研究科生物化学専攻博士課程入学 大阪大学蛋白質研究所酵素反応学部門（堀尾武一　教授）に在籍
１９８１年９月	同中退
１９８１年１０月	徳島大学歯学部口腔生化学教室（竹田義朗　教授）文部教官助手、現在に至る（１９８７年４月）。

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ABBREVIATIONS

H-ISP, human insulin-stimulating protein; Cm-H-ISP, carboxymethylated H-ISP; apo A-II, apolipoprotein A-II; HPLC, high-performance liquid chromatography; FAB, fast-atom-bombardment mass spectrometry; SDS, sodium dodecyl sulfate; CPase Y, carboxypeptidase Y; PCA, pyrrolidone carboxylic acid; pI, isoelectric point; TPCK, p-toluenesulfonyl-L-phenylalanine chloromethyl ketone; TLCK, p-toluenesulfonyl-L-lysine chloromethyl ketone; p-APMSF, (p-amidinophenyl) methanesulfonyl fluoride

SUMMARY

1. A protein that potentiates the action of insulin *in vitro* was purified from human plasma by a procedure involving Sephadex G-100 column chromatography and reversed-phase HPLC. The purified material gave a single band with an apparent molecular weight of 16,000 on SDS-polyacrylamide gel electrophoresis. When reduced with 2-mercaptoethanol and then carboxymethylated, it also gave only a single band, but with a lower molecular weight than that of the unreduced material. By gel filtration chromatography on Sephadex G-75 in 6 M guanidine hydrochloride, the molecular weight of the unreduced material was estimated as 17,000 and that of the reduced material as 9,000, indicating that it was composed of two polypeptide chains of equal or nearly equal molecular weight connected by one or more disulfied bonds. The modified subunit tended to inhibit rather than stimulate insulin activity.

2. A distinctive feature of the amino acid composition of this protein (H-ISP) was the absence of histidine, arginine, and tryptophan. The N-terminal amino acid residue of H-ISP was pyrrolidone carboxylic acid. The molecular weight, subunit composition, the characteristic amino acid composition and the N-terminal amino acid residue of H-ISP are very similar to those of human plasma apolipoprotein A-II (apo A-II), one of the two major apolipoprotein components of human plasma high density lipoproteins. The isoelectric point of H-ISP was estimated to be 4.91, which is identical with that of the major apo A-II

isoform.

3. H-ISP did not itself have insulin-like activity in increasing CO_2 liberation from labeled glucose, lipid synthesis, and 2-deoxyglucose uptake by isolated rat adipocytes, but it potentiated the action of insulin in these parameters. It had no appreciable affect on the binding or degradation of ^{125}I-labeled insulin by adipocytes. Like H-ISP, apo A-II isolated from human plasma also had no insulin-like activity by itself, but stimulated the effect of insulin on CO_2 production from labeled glucose in isolated rat adipocytes. From these results, it is concluded that H-ISP is identical with the major apo A-II isoform.

4. Incubation of isolated adipocytes with H-ISP resulted in marked increase in the activity of pyruvate dehydrogenase in a dose-dependent manner in the absence of added insulin. H-ISP also stimulated pyruvate dehydrogenase activity in a subcellular system consisting of plasma membranes and mitochondria from rat adipocytes. The effect of H-ISP on pyruvate dehydrogenase activity could be produced by treatment of the isolated mitochondrial fraction alone. Sodium fluoride, an inhibitor of phosphatase, blocked the action of H-ISP almost completely, suggesting that the stimulatory effect of H-ISP on pyruvate dehydrogenase activity is at least partly due to its activation of pyruvate dehydrogenase phosphatase.

5. We studied whether the insulin-potentiating action of H-ISP is related to a modification of the insulin receptor kinase.

In a solubilized rat adipocyte receptor system, H-ISP caused dose-dependent inhibition of the stimulation by insulin of phosphorylation of the 95,000 dalton subunit of insulin receptor. During the phosphorylation reaction, no phosphorylated forms of the protein could be detected. H-ISP had no effect on dephosphorylation of the phosphorylated β subunit of the insulin receptor. These results strongly suggest that the inhibition of phosphorylation by H-ISP is due not to either simple substrate competition or activation of phosphoprotein phosphatase, but to specific inhibition of tyrosine-specific protein kinase.

INTRODUCTION

Since the discovery of insulin over 60 years ago, many investigators have devoted considerable effort in attempting to unravel the mechanism of action of this hormone. This is not only the result of the utility of this hormone as a model system for peptide hormone action, but also a reflection of the importance of insulin in human biology and medicine (1,2). Whereas deficiencies of most peptide hormones lead to relatively mild or moderate disease states, total lack of insulin is incompatible with life. In addition, diabetes mellitus and other disorders of carbohydrate metabolism characterized by abnormalities in insulin secretion or insulin action are among common metabolic diseases. Thus, a clear biochemical understanding of the mechanism of insulin action is of utmost importance.

It is well accepted that the action of insulin is greatly influenced by alterations in sensitivity or responsiveness of target tissues to the hormone (1,2). However, little is known about the mechanisms of these phenomena. In 1929, Glaser and Halpern (3) reported that yeast extract contains a factor(s) that potentiates the hypoglycemic action of insulin *in vivo*. This interesting observation was, however, not followed up and hence the nature of the factor and even its existence are still unknown. It has also been reported from different laboratories that a pentapeptide from the carboxyl-terminal of the insulin B-chain (B22-26) (4,5), a hexapeptide from the amino-terminal

sequence of human growth hormone (hGH 8-16) (6) potentiate the action of insulin *in vitro* when added at rather high concentrations. Recently, we tried to purify the insulin-stimulating factor from brewer's yeast and clarify its action. During this study, we incidentally found that limited proteolysis of bovine serum albumin with trypsin produced an insulin-stimulating peptide that was functionally similar to that in yeast extract and we determined the structure of this peptide (7-9). These findings suggest the presence of a substance(s) that controls the action of insulin *in vivo*. Furthermore, Arner *et al*. (10) and Livingston *et al*. (11) demonstrated a marked increase in the insulin sensitivity of human and rat fat cells after carbohydrate challenge and suggested the existence of some physiological process causing rapid increase in the effect of insulin.

An insulin-stimulating peptide (ISP) isolated from a tryptic digest of bovine serum albumin has the following characteristics: 1) ISP is a two-chain polypeptide consisting 71 amino acid residues (calculated Mr=8,496), corresponding essentially to residues 115-143 and 144-184(185) of bovine serum albumin connected to each other by a disulfied bond. The linkage of the subunits through a disulfied bond is essential for manifestation of its action. 2) ISP potentiates the effects of insulin on glucose transport and glucose metabolism in isolated rat adipocytes. It shifts the concentration-response curve of insulin-stimulated D-[1-^{14}C]glucose oxidation, 2-deoxyglucose

transport, and lipid synthesis from D-[U-^{14}C]glucose to lower insulin concentrations, but it has little effect alone. It also increases the maximal response of these parameters to insulin. 3) ISP has no appreciable effect on specific binding of insulin to adipocytes, and effectively protects insulin from degradation. These findings indicate that ISP may be useful in studies on the mechanisms of insulin action including both the sensitivities and responsiveness of target cells to the hormone. Subsequently, we found that a protein with the ability to potentiate the action of insulin in vitro is present in human plasma and we isolated this protein from human plasma (12,13). Like ISP, this protein (H-ISP) did not itself have insulin-like activity, but enhanced the action of insulin on fatty acid synthesis from glucose in rat adipose tissue explants and on CO_2 production from glucose in isolated rat adipocytes. The present paper reports further characterization of this protein, including its primary structure.

The events that follow the binding of insulin to its receptor on the plasma membrane of target cells and that mediate the intracellular effects of the hormone have long been the subject of speculation and investigation but remain largely unknown. A major hindrance to solving this problem has been the lack of an adequate in vitro model system for studying the mechanism of insulin action. Recently however, it has been demonstrated that a simplified subcellular system consisting of plasma membranes and mitochondrial fractions from rat adipocytes

could be used as a model system for studying the action of insulin (14-16). A number of recent studies have indicated that the interaction of insulin with its receptor on the plasma membrane of target cells results in the generation of a "putative" insulin mediator(s) that modulates the activities of various insulin-sensitive enzymes. These enzymes include pyruvate dehydrogenase (14-17), cAMP-dependent protein kinase (18), glycogen synthase (18), acety-CoA carboxylase (19), adenylate cyclase (20), low-Km phosphodiesterase (21,22) and Ca^{++}, Mg^{++} ATPase (23). However, the exact chemical nature and biological significance of the mediator(s) are poorly understood. In the present study, we showed that an insulin-stimulating protein from human plasma potentiates the activity of pyruvate dehydrogenase in intact cells and in subcellular systems in the absence of added insulin.

Insulin receptor is an integral membrane glycoprotein composed of two α subunits (Mr 135,000) and two β subunits (Mr 95,000) linked by disulfied bonds (24,25). Covalent cross-linking of ^{125}I-insulin to the receptor suggests that the α subunit is the insulin-binding subunit (24-26). The β subunit appears to be a transmembrane protein which contains a binding site for ATP (27). Recent <u>in vitro</u> studies have demonstrated that insulin binding to the receptor stimulates tyrosine autophosphorylation of the β subunit of this receptor both in intact cells and in solubilized receptor preparations (28-30) and that the insulin receptor itself functions as a tyrosine-specific

protein kinase (29,30). It is believed that insulin-stimulated autophosphorylation of the receptor kinase is the first postbinding event in insulin signal transmission across the plasma membrane.

In the present study, the first part deals with 1) purification of an insulin-stimulating protein from human plasma, 2) amino acid sequence of this protein. In the second part, biological activities of this protein, including the mechanism of action of this protein, were investigated. We also studied the effect of this protein on the phosphorylation of rat adipocyte insulin receptor to determine whether the potentiation of insulin-stimulated glucose metabolism by this protein is correlated with alteration of the tyrosine protein kinase activity of the insulin receptor.

MATERIALS AND METHODS

Materials

The following materials were purchased; crystalline porcine insulin (25.9 U/mg), bovine albumin essentially free from fatty acid globulin, thiamine pyrophosphate, β-NAD, CoA, aprotinin, and pyruvic acid (sodium salt) from Sigma Chemical; collagenase Type 1 (*Clostridium histolyticum*) and TPCK-treated trypsin from Cooper Biomedical; ampholine, pH 4-6, from LKB; ATP (disodium salt), dithiothreitol, and pyrrolidonecarboxylate peptidase from Boehringer Mannheim GmbH; carboxypeptidase Y from Oriental Yeast Co. (Tokyo); *Staphyolcoccus aureus* V8 protease and wheat germ agglutinin coupled agarose from Miles Laboratories; aminopeptidase M from Pierce Chemical Co,; sodium fluoride, *p*-APMSF, and Triton X-100 from Wako Pure Chemical Industries, Ltd. (Osaka); N-acetyl-D-glucosamine and TLCK from Nakarai Chemicals, Ltd. (Kyoto); leupeptin from the Peptide Institute, Inc. (Osaka); Pansorbin from Carbiochem; D-[1-^{14}C]glucose, D-[U-^{14}C]glucose, and 2-deoxy-D-[1-^{3}H]glucose from the Radiochemical Centre; [γ-^{32}P]ATP from ICN; [1-^{14}C]pyruvic acid and porcine ^{125}I-insulin (2.2 Ci/μmol, receptor grade) from New England Nuclear. Human apo A-II was prepared by Mr. Tsutomu Uenoyama, Department of Research and Development, Otsuka Pharmaceutical Factory, Inc., Tokushima, by the methods of Burstein *et al.* (31) and Scanu *et al.* (32). All other reagents were standard products of the highest grade available commercially.

Amino acid analysis

For determination of the amino acid composition of H-ISP, samples were hydrolyzed in 4 M methanesulfonic acid at 110°C for 24, 48 and 72 h in evacuated sealed tubes (33). For determination of the amino acid compositions of proteolytic peptides of H-ISP, about 3-6 nmoles of peptides were hydrolyzed under the same conditions but for 24 h. The half-cystine content was determined as S-carboxymethylcysteine (34). All analyses were carried out in a Hitachi 835 amino acid analyzer.

Modification reactions

Reduction and carboxymethylation of H-ISP was carried out essentially as described by Crestfield et al. (35). H-ISP (2 mg) was reduced with 2-mercaptoethanol (60 µmol) in 3 ml of 0.36 M Tris-HCl/8 M urea/0.2 % Na_2EDTA, pH 8.0, for 4 h at 25°C. and subsequently S-carboxymethylated with iodoacetic acid (58 µmol) for 15 min at 25°C. The reaction mixture was applied directly to a Sephadex G-75 column (2.2 X 45 cm) that had been equilibrated with 0.5 N acetic acid and wrapped in aluminium foil. The S-carboxymethylated H-ISP was purified by chromatography on a Hi-Pore RP-304 reversed-phase HPLC column.

Edman-degradation

Reduced and carboxymethylated H-ISP (Cm-H-ISP) was degraded manually by the Edman method (36) and the resulting 3-phenyl-2-thiohydantoin derivatives of amino acids were analyzed as described by Shimonishi et al. (37).

Enzymatic digestion

Cm-H-ISP (about 25 nmol) was divided into two portions. One portion was digested with TPCK-treated trypsin in 0.5 % ammonium bicarbonate (pH 8.0) at 37°C for 4 h at a substrate:enzyme ratio of 50:1 to 100:1. One quarter of the digest was used for FAB mass spectrometry and the remainder was separated by HPLC. Another portion of Cm-H-ISP was treated with V8 enzyme in 0.5 % ammonium bicarbonate (pH 8.0) at 37°C for 16 h at a substrate:enzyme ratio of 50:1. Two-fifths of the digest was used for mass spectrometry, and three-fifths for HPLC. Carboxypeptidase Y and aminopeptidase M digestions were carried out in 0.1 M pyridinium acetate at 37°C and pH 6.0 for 1.5 h and at 37°C and pH 7.0 for 1 h, respectively. Pyrrolidonecarboxylate peptidase digestion was done as described by Tsuru et al (38).

High-performance liquid chromatography (HPLC)

The HPLC apparatus consisted as a Shimadzu HPLC apparatus (Kyoto) fitted with a solvent programmer (GRE-2B) and a data processor chromatopac C-RIA. The tryptic or V8 peptides dissolved in 5 % acetonitrile containing 0.1 % trifluoroacetic acid (pH 2.35) were injected into a reversed-phase column (4 X 250 mm) packed with YMC-ODS S-5 (Yamamura Chemical Laboratory Co., Kyoto) equilibrated with the same solvent. Materials were developed with the same solvent for 5 min and then with a linear-gradient of 5-75 % acetonitrile in 0.1 % trifluoroacetic acid at room temperature for 70 min at a flow rate of 1 ml/min.

For purification of H-ISP, a Waters ALC/GPC model 206D liquid

chromatography system was used with an autogradient set (M-680 autogradient control and two M510 pumps).

Fast atom bombardment (FAB) mass spectrometry

FAB mass spectra were recorded with a Jeol double-focusing mass spectrometer HX100 equipped with an FAB ion source and a data processor (Jeol JMA-3100 mass data analysis system), as described by Takao et al. (39). Typical experimental conditions were carried out with a xenon atom beam source at 7-KeV accelating potential. Mass assignment was made using a mixture of CsI and KI as a mass reference. A sample solution containing 1-10 μg of peptides was loaded on a stainless steel plate and mixed with glycerol and α -thioglycerol on the plate.

Polyacrylamide gel electrophoresis

The modified Laemmli system of Ito et al. (40) was used, except that the final acrylamide/bisacrylamide concentrations were 20.86/0.096 %. Samples were dissolved in Laemmli's sample buffer (41) and boiled for 3 min in the presence or absence of 2-mercaptoethanol before application to the gels. The size of gel slab was 150 (height) X 135 (width) X 1 (thickness) mm.

Electrophoresis was carried out at room temperature and at 15 mA for 5 h. The gel was subjected to staining overnight with 0.25 % Coomasie brilliant blue R-250 containing 45.5 % methanol and 9.2 % acetic acid, followed by washing with a mixture of 25.5 % ethanol and 8.2 % acetic acid. The gel thus washed was stored in 5 % methanol containing 7.5 % acetic acid. The stained gel slab was enveloped by a sheet of well wet cellophane, then

sandwiched by two sheets of porous plastic plate in a slab gel dryer. The dring was carried out at 50 °C for 3 h under vacuum.

The molecular weight markers used were soybean trypsin inhibitor (Mr 20,500, Pharmacia) and the cyanogen bromide cleavage products of myoglobin (Mr 2,512 and 14,414, LKB).

Isoelectric focusing

For gel electrophoresis, samples were dissolved in a buffer containing 8 M urea, 3 % ampholytes (pH range 4-6), 2 % dithiothreitol (w/v), and 10 % glycerol (v/v). Analytical iso= electrophoresis was performed by the method of Lackner et al. (42), in 5 X 130 mm gels containing 8 M urea, 7.5 % acrylamide, and 7.5 % ampholytes (pH range 4-6). Electrophoresis was carried out for 18 h at 250 v at 4 °C. The gels were sliced into 5 mm segments and each segment was extracted with 1 ml of water for determination of the pH. Protein bands were stained with Coomassie brilliant blue R-250.

Gel filtration chromatography on Sephadex G-75 in 6 M guanidine hydrochloride

H-ISP and Cm-H-ISP were dissolved in 6 M guanidine hydrochloride, pH 5.0, and subjected to gel filtration through a Sephadex G-75 column (0.9 X 186 cm) equilibrated in 6 M guanidine hydrochloride at a constant flow rate of 10 ml / h, and fractions of 1 ml were collected. The void volume was determined with blue dextran (Pharmacia). Molecular weight markers were myoglobin (Mr 17,000, Sigma), oxidized bovine pancreatic ribonuclease A (Mr 13,000 Sigma), lima bean trypsin inhibitor (Mr 8,400 Cooper

Biomedical) and bovine pancreatic insulin (Mr 5,700, Sigma).

Fatty acid synthesis in adipose tissue explants

For fatty acid synthesis, ten explants (total wet weight, 8-10 mg) were prepared from epididymal fat pads of male Wister strain rats, weighing about 200 g, fed ad libitum. The explants were cultured on siliconized lens paper (43) in a sterile petri dish containing 3 ml of medium 199 supplemented with 0.05 μCi/ml of [U-^{14}C]glucose, 1.5 mg/ml of $NaHCO_3$ and antibiotics. After incubation for 20 h at 37 °C under 3 % CO_2 in air, unless otherwise specified, the explants were weighed and hydrolyzed in 1 ml of 2 N KOH in 50 % ethanol for 2 h at 100 °C. Fatty acids were extracted from the acidic hydrolyzate with 3 ml of petroleum ether (44). This assay method was used for the purification of H-ISP. The explants, though less sensitive to insulin than isolated adipocytes, were easily prepared and responded to insulin in a dose-dependent manner. In addition,this method had the advantage that 50 assays could be made at the same time, thereby avoiding variations between assays. Under the conditions for this method, conversion of labeled glucose to fatty acids proceeded linearly with time in the presence of 20 mU/ml of insulin for at least 22 h, while insulin at 0.2 mU/ml caused little, if any (Fig.15).

Preparation of isolated rat adipocytes

Epididymal fat pads were exised from male Wister rats weighing 120 to 150 g, which had been fed ad libitum. White fat cells were isolated from the fat pads by collagenase digestion as

described by Rodbell (45). Up to 1 g of adipose tissue was added to a polyethylene beaker containing 5 ml of Krebs-Ringer phosphate buffer supplemented with 10 mM Hepes, pH 7.4, 2.5 mg of collagenase, and 0.55 mM glucose. Incubation was carried out for 60 min at 37 C. The suspension of cells was centrifuged in polyethylene centrifuge tube for 1 min at 400 X g and the infranatant was removed. The fat cells were washed by suspending them in 10 ml of Krebs-Ringer phosphate Hepes buffer containing the desired concentration of glucose and centrifuged for 1 min at 400 X g. This procedure was repeated five times.

Glucose oxidation

Conversion of D-[1-^{14}C]glucose to $^{14}CO_2$ by adipocytes was measured essentially as described by Fain et al. (46). Reaction mixtures contained fat cells (5-9 X 10^5 cells/ml), D-[1-^{14}C]glucose (0.2 mM, 0.3 μCi/ml), and 10 mg/ml of bovine serum albumin in a total volume of 0.4 ml of Krebs-Ringer phosphate Hepes buffer, pH 7.4. $^{14}CO_2$ was collected and counted as described (47).

Lipid synthesis

The incorporation of D-[U-^{14}C]glucose (0.2 mM; specific activity, 2.5 μCi/μmol) into total lipids was measured after a similar incubation as for assay of glucose oxidation. The reaction was stopped by addition of 40 μl of 6 N H_2SO_4, and the medium containing adipocytes was then extracted by Dole's procedure (48). Aliquots were taken from the heptane layer for counting.

Glucose transport

Glucose transport activity was assayed by measuring the uptake of 2-deoxy-D-[1-^{3}H]glucose as described by Maloff and Lockwood (49). Adipocytes (5-7 X 10^{5} cells/ml) in 0.4 ml of Krebs-Ringer phosphate Hepes buffer, pH 7.4, containing 10 mg/ml of bovine serum albumin were preincubated at 37°C for 1 h with or without H-ISP in the presence of insulin. The reaction was started by the addition of 2-deoxy-D-[1-^{3}H]glucose (final concentration, 0.125 mM; specific activity, 15 mCi/mmol). The incubation was continued for 60 sec and terminated by rapid centrifugation through a layer of dinonyl phthalate (50).

Insulin binding

Binding of ^{125}I-labeled insulin to isolated adipocytes was assayed by the methos of Kikuchi et al. (51). Adipocytes (9 X 10^{5} cells/ml) in 0.4 ml of Krebs-Ringer phosphate Hepes buffer, pH 7.4, containing 10 mg/ml of bovine serum albumin and 0.55 mM glucose were preincubated at 37°C for 1 h with of without H-ISP. Then they were rapidly cooled to 25°C and incubated at this temperature for 30 min with ^{125}I-labeled insulin (final concentration, 70 pM; specific activity of insulin, 2.2 Ci/μmol) in the presence of various concentrations (0.03 to 100 nM) of native insulin. The reaction mixture was then subjected to rapid oil centrifugation (50). Specific binding was determined as the difference between total binding of labeled hormone and remaining binding in the presence of a large excess (58 μg/ml) of unlabeled hormone.

Insulin degradation

Degradation of ^{125}I-labeled insulin by isolated rat adipocytes was assayed by measuring the radioactivity precipitated with 10 % trichloroacetic acid, as described by Gammeltoft and Gliemann (52), and by radioimmunoassay (53) after removal of cells by oil centrifugation.

Pyruvate dehydrogenase activity in isolated adipocytes

Pyruvate dehydrogenase activity of intact adipocytes was measured essentially as described by Jarett et al. (54). Adipocytes (7-9 X 10^5 cells/ml) were incubated for 15 min at 37°C in a total volume of 400 µl of Krebs-Ringer phosphate buffer containing 10 mM $NaHCO_3$, 10 mM Hepes, 0.2 mM glucose, and 10 mg/ml of bovine serum albumin with H-ISP in the presence or absence of insulin. The cell suspension was centrifuged and the infranatant was discarded. The cell layer was washed once with 500 µl of Krebs-Ringer phosphate buffer containing 10 mM $NaHCO_3$, 10 mM Hepes and recentrifuged. The infranatant was discarded and the cell layer was extracted with 500 µl of ice-cold solution of 50 mM potassium phosphate buffer (pH 7.4), containing 0.2 % Triton X-100, and 2 mM dithiothreitol,and vortexed for 30 sec. The extract was recentrifuged, and chilled, and the lipid-free infranatant was used for assay of pyruvate dehydrogenase activity. Pyruvate dehydrogenase activity was assayed by measuring release of $^{14}CO_2$ from [1-^{14}C]pyruvic acid. The assay was begun by the addition of assay mixture to give final concentrations of 50 mM potassium phosphate buffer (pH 7.4), 50 µM

$CaCl_2$, 50 μM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM CoA, 0.1 mM thiamine pyrophosphate, 0.25 mM [1-^{14}C]pyruvate (1 μCi/μmol), and 0.5 mM β-NAD. After incubation for 3 min at 37°C, the reaction was stopped by adding 200 μl of 6 N H_2SO_4. $^{14}CO_2$ was collected and counted as described (47).

Subcellular fractionation of adipocytes

Plasma membranes and mitochondria were prepared from adipocytes (from 16-24 rats) as described by Mckeel and Jarett (55). The isolated fat cells were washed once with 10 volumes of homogenization medium (medium I: 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.25 M sucrose) to rid the cells of the Krebs-Ringer phosphate buffer. The cells were resuspended in at least 3.5 volumes of medium I at room temperature and transferred to a glass homogenizer fitted with a Teflon pestle. Potter-Elvehjem glass homogenizers fitted with Teflon pestles were used to resuspend all subsequent pellets. The cell suspension was homogenized with 10 up-and-down strokes by means of a variable-speed motor driven at 2600 rpm. The resulting homogenate was placed in ice, and all subsequent steps were carried out at 4°C.

The chilled homogenate was centrifuged at 16,000 X g for 15 min, yielding a pellet, a supernatant solution, and a congealed fat cake. The pellet was resuspended in 8 ml of medium I by six strokes at 1250 rpm and centrifuged at 1000 X g for 10 min. The supernatant was centrifuged at 17,000 X g for 20 min and the pellet was resuspended in 6 ml of medium I by 6 strokes at 1700 rpm. The resulting suspension was further diluted with medium I

and placed on a continuous sucrose gradient. The gradients formed were 1.05 density at the top and 1.25 density at the bottom, and contained 1 mM EDTA and 5 mM Tris-HCl, pH 7.4. Centrifugation at 59,000 X g for 90 min resulted in the separation of two finely particulate bands at densities of 1.14 and 1.18. The bands were removed separately and the 1.14 density band was diluted 4:1 (v:v) with medium I. After being centrifugation at 16,000 X g for 15 min, the pellet was resuspended in 1-2 ml of a solution (medium II) that contained 0.25 M sucrose and 10 mM Tris-HCl, pH 7.4. This was the plasma membrane fraction. Similarly, the 1.18 density band was removed, diluted 6:1 (v:v) with medium I and centrifuged at 16,000 X g for 15 min. The resulting pellet, resuspended in 1-2 ml of medium II, constituted the mitochondrial fraction. The purity of these two fractions showed less than 10 % cross-contamination.

Pyruvate dehydrogenase activity in subcellular systems

The plasma membranes (200 µg/ml) and mitochondria (500 µg/ml) were preincubated for 10 min at 37 °C in 200 µl of 50 mM potassium phosphate buffer (pH 7.4) containing 50 µM $CaCl_2$, 50 µM $MgCl_2$, and 250 µM ATP with insulin or H-ISP. The assay was begun by adding assay mixture as described above, and incubation was carried out for 3 min at 37 °C. Pyruvate dehydrogenase activity was expressed per mg of mitochondrial protein in the sample per min. Plasma membranes and mitochondrial fractions were generally used within 1-2 h after preparation.

Preparation of a solubilized insulin receptor fraction

Insulin receptors were solubilized and purified by wheat germ agglutinin affinity chromatography as described by Häring et al. (56). Adipocytes (50 ml of a suspension of approximately 3 X 10^6 cells/ml) were washed four times with phosphate-free Krebs-Ringer phosphate Hepes buffer containing 0.5 % bovine serum albumin, 12 mM $NaHCO_3$, and 0.55 mM glucose, and then immediately frozen. The cells were lysed by three cycles of freeze-thawing in liquid nitrogen and a water bath, respectively, and the resultant cell lysate was centrifuged for 90 min at 200,000 X g. The precipitate was dissolved in 25 mM Hepes, 1 % Triton X-100, 2 mM p-APMSF, and aprotinin (1 U/ml) and centrifuged for 60 min at the same speed as before. The supernatant (a solubilized membranous fraction) was then applied to a column of wheat germ agglutinin coupled to agarose and recycled five times through the column. The column was washed extensively with 25 mM Hepes buffer (pH 7.4) containing 0.1 % Triton X-100, and then bound material was eluted in a final volume of 2 ml with buffer supplemented with 0.3 M N-acetyl glucosamine and used as the solubilized insulin receptor fraction within two days after chromatography.

Phosphorylation and dephosphorylation of insulin receptor

Partially purified insulin receptors were incubated with or without insulin in the presence or absence of H-ISP at 25°C for 1 h. Then the phosphorylation reaction was induced by incubating the receptors with 5 μM [γ-^{32}P]-ATP in 50 mM Hepes buffer, pH

7.4, containing 10 m$\underline{M}$ $MgCl_2$ and 2 m$\underline{M}$ $MnCl_2$ at 4°C for 60 min (56). The reaction was terminated by adding NaF (100 m$\underline{M}$), sodium pyrophosphate (10 m$\underline{M}$), EDTA (5 m$\underline{M}$), and ATP (5 m$\underline{M}$). Tyrosine-O-phosphorylated proteins containing the β subunit of the insulin receptor were identified by immunoprecipitation with antibodies against phosphotyrosine (57). After 16 h at 4°C, 150 μl of Protein A (Pansorbin; 10 % w/v)was added and incubation was continued for 1 h at 4°C. The precipitate was then collected by centrifugation at 10,000 X g for 5 min at 4°C and washd twice with RIPA buffer (58) and once with 1 % Triton and 0.1 % SDS. Immunoprecipitates were boiled for 3 min in Laemmli's sample buffer (41) in the presence or absence of 2-mercaptoethanol. The solubilized samples were then analyzed by SDS/polyacrylamide gel electrophoresis with one of the following gel systems: 7.5 % acrylamide gel (41), a 4-18 % linear gradient of acrylamide (acrylamide:bis-acrylamide=20:1), and 20.9 % acrylamide containing a trace amount of NaCl (40). After electrophoresis, the slab gels were stained with Coomassie brilliant blue R250, destained, dried, and autoradiographed with Fuji RX film for 0.5 to 2 days at -70°C.

Dephosphorylation was studied by incubating the phosphorylated (for 15 min at 4°C), partially purified receptors in the presence of unlabeled ATP (1 m$\underline{M}$) with or without H-ISP at 4°C, as described by Tamura $\underline{et\ al}$. (59). At suitable times the reaction was terminated by adding a solution of NaF, EDTA, sodiumm pyrophosphate, and ATP at final concentrations of 100 m$\underline{M}$,

5 m$\underline{M}$, 10 m$\underline{M}$, and 25 m$\underline{M}$, respectively. Immunoprecipitation, SDS/polyacrilamide gel electrophoresis, and autoradiography were then carried out as described above.

RESULTS

A. Chemical characterization of an insulin-stimulating protein (H-ISP) from human plasma

A-I. Purification of H-ISP from human plasma

All purification steps are schematically shown in Fig. 1.

Fig. 1

Chilled ethanol (2 liters) was added to 1 liter of human plasma with stirring for 30 min in the cold. Insoluble material was removed by centrifugation at 10,000 X g for 30 min, and the resulting supernatant was concentrated to about 300 ml at 37°C under reduced pressure. The concentrated supernatant was shaken with an equal volume of chloroform, and the aqueous phase was concentrated to about 200 ml under reduced pressure, dialyzed overnight against two changes of distilled water (5 liters each) in a Spectrapor 3 (Spectrum Medical; 3500 Mr cutoff) and lyophilized.

The dried material (about 200 mg protein) was dissolved in 15 ml of 10 mM Tris-HCl buffer containing 0.1 M NaCl (pH 7.4) and passed through a Sephadex G-100 column (4.4 X 90 cm) in the same buffer at a flow rate of 20 ml/h. Fractions containing H-ISP activity were pooled, dialyzed, and lyophilized.

Fig. 2

The lyophilized material was dissolved in distilled water and fractinated on a TSK gel ODS-120A column (7.8 X 300 mm, Toyo Soda) with 0.1% TFA (solvent A) as the mobile phase, and 90% acetonitrile containing 0.1% TFA (solvent B) as a mobile-phase modifier. Elution was carried out with a linear gradient of solvent B as follows: 53% B for 20 min, 53-66% B in 30 min and 66-100% B in 10 min at a flow rate of 1 ml/min at 22-25°C. H-ISP activity was eluted from the column after 42 to 50 min under these conditions.

Fig. 3

Final purification was achieved on a Hi-Pore RP304 column (4.6 X 250 mm, Bio-Rad) with solvent A as the mobile phase, and solvent B as the mobile-phase modifier. Elution was effected with a linear gradient of solvent B as follows: 0-40% B in 5 min, 40-60% B in 40 min and 60-100% B in 5 min at a flow rate of 1 ml/min at 22-25°C (Fig. 4).

Fig. 4

Purified H-ISP was stable on heating at 60°C for 10 min and no loss of activity was observed on storage as an aqueous solution for at least 6 months at -20°C or for 24 h at 4°C at pH 2.0.

Homogeneity and molecular weight of the purified H-ISP

As shown in Fig. 5, the purified material in the non-reduced state gave only a single band with an apparent molecular weight of

16,000 on SDS-polyacrylamide gel electrophoresis (lane 1).

Fig. 5

After reduction with 2-mercaptoethanol, the material also gave only a single band, but with a lower molecular weight than that of the unreduced material (lane 2).

By gel filtration chromatography on Sephadex G-75 in 6 M guanidine hydrochloride, the molecular weight of H-ISP was estimated as 17,000 and that of the modified material (Cm-H-ISP) as 9,000 (Fig. 6).

Fig. 6

These findings suggest that H-ISP is composed of two polypeptide chains of equal or nearly equal molecular weight connected by one or more disulfide bonds.

Amino acid composition of H-ISP

The amino acid composition of H-ISP is shown in Table 1.

Table 1

A distinctive feature of the composition is the absence of histidine, arginine, and tryptophan. Assuming that the molecular weight is about 16,000, the results show that H-ISP contains two half-cystine residues, suggesting that it is composed of two polypeptide chains

of equal molecular weight connected by a single disulfide bond. The characteristic amino acid composition is similar to that of human plasma apolipoprotein A-II (apo A-II) (60), one of the two major apolipoprotein components of human plasma high density lipoproteins.

Apo A-II is polymorphic in human plasma, and four isoforms have been detected (42). Figure 7 shows the result of isoelectric gel focusing of H-ISP and apo A-II.

Fig. 7

The isoelectric point (pI) of H-ISP was estimated to be 4.91. Under the experimental conditions, apo A-II was polymorphic. The pI of the major apo A-II isoform was 4.91, while the pI values of the minor apo A-II isoforms were 4.67, 4.79, 5.00, and 5.17, respectively. Thus the pI of H-ISP was the same as that of the major apo A-II isoform.

Although the amino acid composition of the apo A-II used in this study was identical with that reported for apo A-II (60), the pI values of its minor apo A-II isoforms were different from those reported by Lackner et al. (42). It is not clear whether this difference was due to small amounts of impurities in our sample or due to the presence of some type of as yet unidentified form in human plasma. To further confirm the identity of H-ISP with apo A-II, we examined the amino acid sequence of H-ISP.

A-II. Amino acid sequence of H-ISP

For establishment of the amino acid sequence of H-ISP, Cm-H-ISP was first submitted to Edman-degradation. However, no N-treminal amino acid residue could be detected, suggesting that the N-terminus of H-ISP is pyrrolidone carboxylic acid or is blocked by an acyl group.

Then, Cm-H-ISP was digested with trypsin and a part of the digest was separated by reversed-phase HPLC, as shown in Fig. 8.

Fig. 8

The remainder of the tryptic digest was examined directly by FAB mass spectrometry (Fig. 9) as described by Takao et al. (39).

Fig. 9

From the observed amino acid ratios and mass values of the separated peptides, the integer values of the amino acid contents of the isolated peptides were determined to be as shown in Table 2.

Table 2

T-2 was a mixture of two peptides. The major component (T-2A) corresponded to the signal observed at m/z=635.4 and consisted of 5 amino acid residues, while the minor one (T-2B) corresponded to the signal at m/z=1199.6 and consisted of 11 amino acid residues.

T-2B contained one Val and one Lys residue more than T-1 and it had two Lys residues, presumably one of which was not cleaved by trypsin, because the carboxyl-proximal residue is proline, as reported below. T-4 had one Ser and one Lys residue more than T-5 and contained two Lys residues, one of which could not be cleaved for the same reason as that in T-2B. The recoveries of amino acids from T-4 and T-5 were less than those from T-1, T-2 and T-6, but their sum was almost the same as that of the other three peptides. Therefore, T-4 and T-5 were concluded to originate from the same sequence. T-8 and T-9 both corresponded to the signal at m/z=2385.3, because they had the same amino acid compositions except for Gln or Lys and their molecular weights were approximately the same. T-8, T-9, and T-10 contained one Gln, one Lys, and one Gln and one Lys residue, respectively, less than T-7. Since the recoveries of amino acids from T-7, T-8, T-9, and T-10 were all low and the amino acid composition of these peptides were similar, we deduced that they were derived from the same sequence. A signal observed at m/z=2968.5 in Fig. 9 did not correspond to any peptide isolated by HPLC in Fig. 8. However, this mass value coincided with the sum of the mass values of T-2A and T-6, indicating that T-2A and T-6 were consecutive peptides.

From amino acid analysis, Cm-H-ISP was deduced to contain a total of 77 amino acid residues, including 9 Lys residues. On the contrary, mass measurement of the tryptic peptides and amino acid analyses of the separated peptides indicated that it contained 74 amino acid residues with 8 Lys residues. Furthermore, mass measurement indicated that N-termini of the peptides isolated by HPLC were not

blocked. These results suggest that the N-terminal peptide of Cm-H-ISP was not detectable in the mass spectrum in Fig. 9 or by HPLC in Fig. 8, and that it consisted of about 3 amino acid residues.

Then Cm-H-ISP was cleaved by S. aureus protease V8, and the V8 peptides were separated by HPLC (Fig. 10) or measured directly by FAB mass spectrometry (Fig. 11).

Fig. 10

Fig. 11

The amino acid compositions of the separated peptides were determined, as described in Table 3, from the observed amino acid ratios and mass values as described for analyses of the tryptic peptides.

Table 3

Peptides V-4 seemed to have a common sequence with peptide V-3, because it had the same amino acid composition as V-3 except for one less Gln residue, and the recovery of V-4 was lower than those of other peptides, but the sum of the recoveries of V-3 and V-4 was nearly equal to those of other peptides. No signal with the mass value calculated from the amino acid composition of V-5 could be detected in the mass spectrum shown in Fig. 11. When isolated V-5 was submitted to FAB mass spectrometry (Fig. 12), it gave a signal at m/z=944.4, which was 18 atomic mass units (amu) amaller than the mass value calculated

from the observed amino acid composition.

Fig. 12

Furthermore, the signal in the mass spectrum in Fig. 11 was at m/z= 944.7. Since 18 amu corresponds to the mass value of H_2O, the results suggest that the N-terminus of this peptide is a pyrrolidone carboxy= lic acid (PCA). Moreover, when peptide V-5 was digested with pyrro= lidonecarboxylate peptidase (38) and the digest was measured by FAB mass spectrometry (Fig. 12), the signal of the peptide at m/z=944.4 disappeared and a new signal was observed at m/z=833.3. The difference of these mass values was 111, which correspoded to the residual weight of PCA. These results indicated that V-5 has PCA at the N-terminus and therefore, that the peptide is located in the N-terminal position of Cm-H-ISP.

Comparison of the total amino acid composition of the tryptic and V8 peptides with that of Cm-H-ISP indicated that a peptide composed of one residue each of Glu, Ala, and Lys should present among the tryptic peptides, although it could not be detected from the mass spectrum (Fig. 9) or by HPLC (Fig. 8) of the tryptic peptides. The N-terminal amino acid residue of these amino acid residues in the peptide was considered to be PCA, because no blocked peptide was found among the tryptic peptides in Table 2 and a peptide (V-5) with PCA as an N-terminal residue was found among the V8 peptides. On the other hand, a peptide consisting of one Glu, one Ser, and two Lys residues should be present among the V8 peptides, because mass

measurments and amino acid analyses of the V8 peptides indicated the presence of 73 amino acid residues. A peptide containing these four amino acid residues could not be isolated by HPLC (Fig. 10), but it was detected as a signal at m/z=491.3 in another mass spectrum (not shown).

These results were used as input data in a computer program developed and modified in our laboratory (61) and a candidate amino acid sequence of Cm-H-ISP was searched. The result is shown in Fig. 13.

Fig. 13

The sequence was compatible with the overlaps of fragments T-6/V-5, T-2A/V-8 and T-4 or T-5/V-7 with single residues of Cys, Met and Ile, respectively. The partial sequence and amino acid composition thus determined were compared with the known amino acid sequences of plasma proteins. The sequence determined was very similar to that of human plasma apo A-II (60). In fact, the amino acid compositions of the tryptic peptides were almost the same as those reported by Brewer et al. (60). To confirm the identity of our sequence with that of apo A-II, we examined the partial sequence of the tryptic peptides isolated in Fig. 8. As an example, the FAB mass spectrum of the CPase digest of T-2 is shown in Fig. 14.

Fig. 14

The result indicated that T-2B has Gln-Ala-Glu-Ala-Lys as its C-terminal sequence. This sequence was identical with that from 35 to 39 of apo A-II (60) except at position 37; our sequence had a Glu residue instead of Gln at this position. Moreover, the FAB mass spectrum of an aminopeptidase digest of T-2 indicated that T-2B had Ser and Pro in its N-terminal portion, which were compatible with the sequence described by Brewer et al. (60).

B. Biological activities of H-ISP

B-I. Effect of H-ISP on fatty acid synthesis in adipose tissue explants

As shown in Fig. 15, the purified H-ISP greatly stimulated the action of insulin added at a suboptimal concentration (0.2 mU/ml) on fatty acid synthesis, the value being close to that obtained with 20 mU/ml of insulin alone.

Fig. 15

Figure 16A shows the dose-response curve of H-ISP for fatty acid synthesis in the adipose tissue explant system. In the presence of 0.2 mU/ml of insulin, H-ISP was effective at over 2 μg/ml of H-ISP. H-ISP alone had no insulin-like activity in the concentration range tested.

Fig. 16A

Using the same system, the effect of H-ISP was tested as a function of the concentration of insulin. As shown in Fig. 16B, H-ISP shifted the concentration-resoense curve of insulin-stimulated fatty acid synthesis to the left.

Fig. 16B

B-II. Effect of H-ISP on glucose oxidation, lipid synthesis from glucose, and 2-deoxyglucose uptake by isolated rat adipocytes

Effect of H-ISP on glucose oxidation in the presence and absence of insulin

As shown in Fig. 17, H-ISP also stimulated $^{14}CO_2$ production from D-[1-^{14}C]glucose in the presence of insulin by isolated rat adipocytes. In the concentration range tested, H-ISP alone had no insulin-like activity measured as increase in $^{14}CO_2$ production from labeled glucose, but at 0.1 to 3 $\mu\underline{M}$ H-ISP stimulated $^{14}CO_2$ production from glucose in the presence of a submaximal concentration of insulin (67 $p\underline{M}$) (Fig. 17A).

Fig. 17A

Next, the effect of H-ISP was tested as a function of the concentration of insulin. As shown Fig. 17B, H-ISP at 1.2 $m\underline{M}$ shifted the insulin dose-response curve for $^{14}CO_2$ production to the left, and

increased the maximal effect of insulin on glucose oxidation significantly (40%).

Fig. 17B

As shown in Table 4, when H-ISP was reduced with 2-mercapto= ethanol and then carboxymethylated with iodoacetic acid (Cm-H-ISP) it showed no insulin-stimulating activity, but rather inhibited the action of insulin.

Table 4

Cm-H-ISP also exhibited no insulin-stimulating activity on fatty acid synthesis from labeled glucose by adipose tissue explants, but inhibited the action of insulin (data not shown). These facts suggest that the linkage of the subunits through a disulfied bond(s) is essential for manifestation of its action. Table 4 also shows that the apo A-II isolated from human plasma had insulin-stimulating activity under our assay conditions. Like H-ISP, apo A-II itself did not have insulin-like activity, but significantly enhanced the stimulatory effect of insulin on $^{14}CO_2$ liberation from labeled glucose. These characteristics of H-ISP correspond to those of human plasma apo A-II.

Effect of H-ISP on lipid synthesis from glucose by isolated rat adipocytes

As shown in Table 5, in the presence of both a maximal

(2.0×10^{-9} M) and a submaximal (6.7×10^{-11} M) concentration of insulin, H-ISP also enhanced total lipid synthesis from labeled glucose without influencing basal synthesis (synthesis measured in the absence of insulin).

Table 5

Effect of H-ISP on 2-deoxyglucose uptake by isolated rat adipocytes

Next, we examined the effect of H-ISP on 2-deoxyglucose uptake by isolated rat adipocytes. Table 6 shows that H-ISP did not change the basal rate of 2-deoxyglucose uptake, but significantly potentiated the maximal rate of uptake elicited by 1.7 nM insulin.

Table 6

B-III. Effect of H-ISP on the binding or degradation of ^{125}I-labeled insulin by isolated rat adipocytes

Effect of H-ISP on specific binding of ^{125}I-labeled insulin to its receptor

To determine the mechanism of action of H-ISP, we first examined its effect on the binding of labeled insulin to specific receptors of adipocytes, because enhaced binding of insulin was observed with insulin-potentiating fragments of insulin (4,5) and growth hormone (6). Results on displacement of labeled insulin

in the presence of unlabeled insulin with or without H-ISP are shown in Fig. 18A.

Fig. 18A

The displacement curve in a range of hormone concentrations of 0.03 to 100 nM revealed that H-ISP did not alter the specific binding of ^{125}I-labeled insulin to its receptor. Moreover, higher concentration of H-ISP (up to 5 μM) did not increase the specific binding of ^{125}I-labeled insulin to adipocyte receptors (Fig. 18B). It also did not affect nonspecific binding of ^{125}I-labeled insulin (Fig. 18B).

Fig. 18B

Effect of H-ISP on degradation of insulin by isolated rat adipocytes

Since prevention of degradation of insulin by adipocytes would result in apparent increase in insulin sensitivity, we next examined the effect of H-ISP on insulin degradation. For this, we measured the trichloroacetic acid precipitability of ^{125}I-labeled insulin in the medium after incubation under the conditions used for the assays described above. Figure 19A shows the time course of insulin degra= dation measured in this way.

Fig. 19A

H-ISP had no effect on insulin degradation. Similar results were

obtained by radioimmunoassay (Fig. 19B).

Fig. 19B

B-IV. Effect of H-ISP on pyruvate dehydrogenase activity of isolated rat adipocytes

Effect of H-ISP on pyruvate dehydrogenase activity of intact adipocytes

To examine the action of H-ISP on the representative insulin-sensitive intracellular enzymes, we next studies its action on mitochondrial pyruvate dehydrogenase. Under the conditions described here, insulin in the range 5 to 200 μU/ml induced a small but significant increase in pyruvate dehydrogenase activity, causing maximal stimulation at 200 μU/ml (data not shown). As shown in Fig. 20, insulin at 200 μU/ml caused 51.3% increase in the enzyme activity over the basal activity, an increase that is comparable to that reported by others (62,63).

Fig. 20

In the same system, H-ISP markedly stimulated the pyruvate dehydrogenase activity of intact cells in the absence of added insulin, and the magnitude of the activation was greater than that of insulin alone. H-ISP at 3 μM caused 193% stimulation of pyruvate dehydrogenase activity. Furthermore, as shown in Fig. 20, when cells were

incubated with insulin (200 μU/ml) plus H-ISP, the magnitude of the activation was greater than that obtained with insulin or H-ISP alone. The stimulatory effect of H-ISP was additive to maximal stimu= lation caused by insulin alone.

Figure 21 shows the concentration-response curve for the effect of H-ISP upon pyruvate dehydrogenase activity of intact cells.

Fig. 21

When adipocytes were incubated in the presence of increasing concent= rations of H-ISP, the pyruvate dehydrogenase activity increased linearly. H-ISP was effective at over 0.6 μM.

Effect of H-ISP on pyruvate dehydrogenase activity in subcellular systems

It has recently been demonstrated (14-16) that a simplified subcellular system consisting of plasma membranes and mitochondrial fractions from rat adipocytes could be used as a model system for studying the action of insulin or insulin mediator and that direct addition of insulin or insulin mediator to the mixture caused an increase in mitochondrial pyruvate dehydrogenase activity. We next examined whether H-ISP had pyruvate dehydrogenase-stimulating activity in this subcellular system. As shown in Table 7, the addition of H-ISP during preincubation of the plasma membrane-mitochondrial mixture in the presence of ATP resulted in stimulation of pyruvate dehydrogenase activity by 46.9%.

Table 7

In this system, insulin caused 33.3% ($p<0.005$) stimulation of pyruvate dehydrogenase, which was comparable to the stimulations reported by others (64,65). As shown in Table 7 (Experiment 2), H-ISP also stimulated pyruvate dehydrogenase activity when added to the isolated mitochondrial fraction in the absence of plasma memebrane. This is in sharp contrast with the observation that the effect of insulin on pyruvate dehydrogenase activity could not be produced by treatment of mitochondria alone (64).

Effect of sodium fluoride on the stimulation of pyruvate dehydrogenase by H-ISP

Next we examined the effect of sodium fluoride, a phosphatase inhibitor, on the stimulatory effect of H-ISP, since pyruvate dehydrogenase activity is known to be increased by activation of pyruvate dehydrogenase phosphatase and/or the inhibition of pyruvate dehydrogenase kinase (66,67). As shown in Table 8, addition of 75 mM sodium fluoride to the pyruvate dehydrogenase assay system had little effect on the basal activity, but almost completely inhibited the stimulatory effect of H-ISP on pyruvate dehydrogenase activity.

Table 8

These results suggest that H-ISP stimulate pyruvate dehydrogenase

activity through a sodium fluoride-sensitive mechanism, possibly through the pyruvate dehydrogenase phosphatase system.

B-V. Effect of H-ISP on the phosphorylation of rat adipocyte insulin receptor

Effect of H-ISP on phosphorylation of the solubilized insulin receptor fraction

To determine whether the potentiation of insulin-stimulated glucose metabolism by H-ISP was correlated with alteration of the tyrosine kinase activity of the insulin receptor, we studied the effect of H-ISP on the state of phosphorylation of the insulin receptor. For this we incubated solubilized rat adipose insulin receptors with an equal amount of wheat germ-purified proteins and a non-saturating concentration of [γ-^{32}P]ATP. Figure 22A shows autoradiograms of the phosphorylated glycoproteins. The major phosphorylated Mr 95,000 protein has been tentatively identified as the β subunit of the insulin receptor of rat adipocytes (56, 68).

Fig. 22A

On incubation of the solubilized fraction with insulin (10 nM), the incorporation of ^{32}P into this protein increased approximately 15-fold, as determined by scanning densitometry (Fig. 22A, lanes 1 and 3). On incubation with H-ISP (10 μM), the basal and insulin-

dependent phosphorylation were both clearly inhibited (Fig. 22A). To reduce the background of other proteins and clarify the tyrosine-specific phosphorylation, we used anti-phosphotyrosine antibodies in following experiments. These antibodies were highly specific for phosphotyrosine and did not cross-react with phosphoserine or phosphothreonine (57).

Preincubation of the same receptor fraction with insulin plus H-ISP (10 μM) caused 95% decrease in the extent of phosphorylation of the Mr 95,000 receptor subunit (Fig. 22B, lanes 7 and 8).

Fig. 22B

The decrease in autophosphorylation of the β subunit of the insulin receptor was not attributable to either decreased insulin binding or decreased immunoprecipitation, since H-ISP itself did not affect insulin binding to adipocyte receptors (Fig. 18) and the inhibition was also observed without antibodies (Fig. 22A).

Effects of the concentrations of H-ISP and insulin on phosphorylation of the insulin receptor

H-ISP inhibited 10 nM insulin-stimulated autophosphorylation of the β subunit dose-dependently (Fig. 23A), and was inhibitory at concentrations of over 0.5 μM.

Fig. 23A

Using the same system, we examined the influence of insulin

concentration on the effect of 10 μM H-ISP. Insulin caused marked and dose-dependent stimulation of ^{32}P incorporation into the Mr 95,000 protien, as observed by Häring et al. using anti-receptor antibodies (56). As shown in Fig. 22B, H-ISP (10 μM) strongly blocked the stimulation of phosphorylation by insulin at the concent= rations tested.

Fig. 23B

Phosphorylation of H-ISP by the solubilized insulin receptor

Next, we examined whether the inhibitory effect of H-ISP could be because it was a preferable substrate for phosphorylation, since it has eight possible sites per molecule that might be phosphorylated by a tyrosine kinase. However, we could not detected any phosphory= lated H-ISP in two SDS-polyacrylamide gel systems, NaCl-gel in the absence of 2-mercaptoethanol (Fig. 24B) and 4-18% gradient gel in the presence of the reductant (Fig. 24A), though both gel systems resolved H-ISP clearly (13).

Fig. 24A

Fig. 24B

These results exclude the possibility that H-ISP inhibits auto= phosphorylation of the solubilized receptor fraction by competiting as a substrate for the receptor tyrosine kinase.

Effect of H-ISP on dephosphorylation of insulin receptor

Another possibility was that H-ISP might activate phospho= tyrosine phosphatase, thus lowering the extent of phosphorylation of the insulin receptor. To examine this possibility, we stopped the incorporation of ^{32}P into the receptor protein by adding a high concentration of unlabeled ATP and then tested the effect of H-ISP on dephosphorylation. As shown in Fig. 25, H-ISP did not stimulate dephosphorylation of the phosphorylated β subunit of the insulin receptor.

Fig. 25

These results strongly suggest that H-ISP inhibited tyrosine-specific protein kinase associated with the insulin receptor.

DISCUSSION

This paper describes the presence of an insulin-stimulating protein, H-ISP, in human plasma for the first time.

H-ISP is composed of two identical subunits connected by a single disulfide bond and reduction of this linkage followed by carboxymethylation completely abolished its activity. This suggests that the disulfide linkage is essential for manifestation of the activity. The molecular weight, subunit composition, the amino acid composition, and the isoelectric point of this protein are comparable with those of human plasma apo A-II. In addition, apo A-II isolated from human plasma did not itself have insulin-like activity, but stimulated the action of insulin on CO_2 production from glucose in isolated rat adipocytes (Table 4). These results support the evidence that H-ISP is identical with human plasma apo A-II.

Although the deduced amino acid sequence of this protein was found to be identical with that of apo A-II, our sequence had a Glu residue instead of Gln at position 37. Recently, Sharpe, et al. (69) reported the nucleotide sequence of the gene encoding human plasma apo A-II and also found a difference at position 37 in the deduced amino acid sequence. Our sequence is consistent with the amino acid sequence deduced from the nucleotide sequence. However, our Cm-H-ISP showed heterogeneity at its C-terminus, about one-third of the preparation having no Gln residue, as shown in Fig. 8 and Tables 2 and 3. It is not clear whether this heterogeneity at the C-terminus is produced during

the purification procedure or whether natural apo A-II has this heterogeneity.

The action of insulin is reported to be modulated by alterations at three levels: before its interaction with its receptor, at the level of the receptor, and at steps after insulin-receptor interaction (1,2). Binding of insulin to its receptor is supposed to initiate increase in glucose utilization, the first and most rate-limiting step in this process being the rate of glucose transport. We found that H-ISP enhanced the action of insulin on glucose transport without modifying insulin-receptor binding. It also did not affect the rate of degradation of insulin by adipocytes. Thus H-ISP may modulate the action of insulin at a step after insulin-receptor interaction.

Change in insulin-receptor binding is thought to be associated with alteration of insulin sensitivity, whereas change at post-receptor levels is thought to result in alterations in insulin responsiveness (1,2). There are reports that insulin fragments (4,5) or fragments from human growth hormone (6) change the sensitivity of adipose tissue to insulin and potentiate the action of insulin in vitro. These fragments are thought to enhance the action of insulin by increasing the binding of insulin to the insulin receptor, since they enhance its specific binding to its receptor. On the other hand, insulin sensitivity may be altered without any change in insulin binding. For instance, ATP-deficiency results in decreased insulin sensitivity

due to a post-receptor defect in rat adipocytes (70), and conversely adenosine potentiates the action of insulin by increasing the sensitivity of adipocytes to insulin, without affecting insulin binding (71). As shown in the present study H-ISP had no affect on insulin-receptor binding (Fig. 18), but shifted the concentration -response curve of insulin-dependent fatty acid synthesis by adipose tissue preparations to the left (Fig. 16). In addition, it increased both the sensitivity and responsiveness to insulin, as assessed by CO_2 production from glucose (Fig. 17) and uptake of 2-deoxyglucose (Table 6) by rat adipocytes. These results suggest that the mechanism of action of H-ISP is closely related to that of adenosine. However, at higher concentrations, adenosine exhibits an insulin-like action in stimulating 2-deoxyglucose uptake (71) and also pyruvate dehydrogenase activity (72) in adipocytes, and its mechanism of action is suggested to be in some way similar or related to that of insulin (72).

In the present study, we demonstrated that H-ISP had an insulin mediator activity and thus that it caused dose-dependent stimulation of the activity of mitochondrial pyruvate dehydrogenase in intact adipocytes and also in the plasma membrane/mitochondria mixture in the absence of added insulin(Fig. 21 and Table 7). Furthermore, the effect of H-ISP on pyruvate dehydrogenase activity could be produced by treatment of the isolated mitochondrial fraction alone (Table 7, experiment 2).

Pyruvate dehydrogenase is known to be regulated by a phosphorylation-dephosphorylation mechanism: phosphorylation of the α subunit of the enzyme and concomitant inactivation is catalyzed by a cAMP-independent protein kinase, and dephosphorylation and concomitant reactivation by a phosphoprotein phosphatase, pyruvate dehydrogenase phosphatase (66,67). As shown in the present paper(Table 8), the action of H-ISP on pyruvate dehydrogenase was almost completely abolished by sodium fluoride, a phosphatase inhibitor, suggesting that H-ISP stimulates pyruvate dehydrogenase activity by activating pyruvate dehydrogenase phosphatase. In this connection, it is noteworthy that insulin mdiator was shown to activate mitochondrial pyruvate dehydrogenase in the absence or presence of ATP and its activation was also found to be inhibited by sodium fluoride, but not by the kinase inhibitor dichloroacetic acid (14-16,73). Newman et al. (74) showed that insulin mediator behaved as an activator of pyruvate dehydrogenase phosphatase, but had no effect on pyruvate dehydrogenase kinase activity. These findings are consistent with the view that H-ISP activates pyruvate dehydrogenase through a similar mechanism to that of insulin mediator. Further studies on the mechanism of pyruvate dehydrogenase activation by H-ISP are in progress.

Using rat adipocytes. Jarett et al. (62) showed that insulin induces production of a limited amount of the mediator per cell in a dose-dependent manner, that the amount determines the magnitude of stimulation of pyruvate dehydrogenase by insulin and

that this stimulation can be further enhanced by the presence of more mediator. As described in this paper, insulin caused a maximum stimulation of pyruvate dehydrogenase activity of about 50 % in adipocytes (Fig. 20), this value being comparable to those reported by others (62,63). On the other hand, H-ISP enhanced the enzyme activity approximately 3-fold (Fig. 21) and this enhancement was additive to the maximum stimulation obtained with insulin alone. These results also support the similarity of the mechanisms of pyruvate dehydrogenase activation by insulin mediator and by H-ISP.

As reported in this paper, H-ISP stimulate 2-deoxyglucose uptake, glucose oxidation and fatty acid synthesis, both in adipose explants and adipocytes in the presence of suboptimal concentrations of insulin. Since pyruvate dehydrogenase is one of the major rate-limiting enzymes in the pathway of fatty acid synthesis, it can be inferred that changes in its activity could mediate the short-term metabolic effects of insulin. However, Begum et al. (63) suggested from experiments using indomethacin that pyruvate dehydrogenase is not the rate-limiting step in glucose oxidation in fat cells. Therefore, the insulin-stimulating activity of H-ISP cannot be simply explained by activation of pyruvate dehydrogenase. Besides pyruvate dehydrogenase, some other insulin-sensitive enzymes related to glucose utilization are also known to be controlled by a phosphorylation-dephosphorylation mechanism and activated by dephosphorylation. These include glycogen synthase (18),

pyruvate kinase (75) and acetyl-CoA carboxylase (19), but little is known about the other key enzymes. If a phosphorylation-dephosphorylation control like that of pyruvate dehydrogenase is applicable to these key enzymes, an increase in glucose utilization by H-ISP would be expected in the presence of added insulin. Since H-ISP has little effect on glucose metabolism on adipocytes in the absence of insulin, it is likely that a glucose transport system is controlled by a different mechanism from that mentioned above.

In the present study, we showed that H-ISP inhibits tyrosine-specific protein kinase associated with the insulin receptor. The biological significance of the present results is not clear. It has been proposed that phosphorylation of the β subunit of the insulin receptor by the receptor tyrosine kinase may represent an intermediary step in the actions of insulin on metabolism and growth (1,76). In this connection, Kono and Barham (77) showed that trypsin causes similar stimulation to insulin of glucose oxidation in rat adipocytes. Larner et al. (78) reported that TLCK, an inhibitor of trypsin-like proteases, markedly inhibited glucose oxidation but not insulin binding in rat adipocytes. On the other hand, Tamura et al. (79) demonstrated the stimulation by trypsin of phosphorylation of the β subunit of the insulin receptor. As shown in Table 9, TLCK and leupeptin, a potent inhibitor of both serine proteases and thiol proteases with trypsin-like specificity, inhibited glucose oxidation in rat adipocytes 83 % and 51 %, respectively,

(Experiment 1) and they also blocked insulin-dependent phosphorylation to similar extents (Experiment 2).

Table 9

These data support the idea that activation of the receptor-associated tyrosine kinase is one of the integrated events in the action of insulin. Thus H-ISP may inhibit the phosphorylation through a feedback mechanism, since it potentiates the action of insulin and acts as an insulin mediator in the activation of pyruvate dehydrogenase both in intact adipocytes and in mitochondrial fractions in the absence of added insulin (Fig. 21 and Table 7). Further studies on the detailed mechanism of H-ISP action are now in progress.

Recently, Arner et al. (10) reported that glucose ingestion was accompanied by a marked increase in insulin sensitivity of human fat cells and they suggested that oral glucose stimulates the release of a circulatory factor(s) that potentiates the insulin effect. Livingston et al. (11) also observed an increase in insulin sensitivity of rat adipocytes after intravenous glucose infusion. However, little is known about the cellular processes that could enhance insulin sensitivity. Therefore, it might be of interest to examine the participation of H-ISP in these processes and also at the clinical level to

investigate the relationship between H-ISP and the etiology of non-insulin-dependent diabetes mellitus.

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Table 1. *Amino acid composition of H-ISP*
Values for H-ISP were calculated from average values for samples of 24-h, 48-h and 72-h hydrolyzates. The nearest integer values are shown in parentheses. Values for apo A-II are cited from (60). ½ Cys was determined as *S*-carboxymetylcysteine

Amino acid	Residues/molecule in	
	H-ISP	Apo A-II
Asp	6.17 (6)	6
Thr	12.09 (12)	12
Ser	11.97 (12)	12
Glu	32.19 (32)	32
Pro	8.18 (8)	8
Gly	6.42 (6)	6
Ala	10.10 (10)	10
Val	12.03 (12)	12
½ Cys	2.17 (2)	2
Met	1.57 (2)	2
Ile	1.84 (2)	2
Leu	16 (16)	16
Tyr	7.94 (8)	8
Phe	7.89 (8)	8
Trp	0.00 (0)	0
Lys	17.86 (18)	18
His	0.00 (0)	0
Arg	0.00 (0)	0
Total	154	154

Table 2. *Amino acid compositions and other data on tryptic peptides of Cm-H-ISP*
Tryptic peptides were separated by HPLC. T-*n* correspond to peak fractions in Fig. 8. Numbers of amino acid residues in parentheses indicate nearest integer values. Mass values without and with parentheses indicate observed mass values in Fig. 9 and theoretical mass values calculated from the amino acid compositions, respectively. Underlined values coincide with observed values

	T-1	T-2		T-3	T-4	T-5	T-6	T-7	T-8	T-9	T-10
		T-2A	T-2B								
CMC-Cys							1.02 (1)				
Asp		1 (1)					1.03 (1)	0.98 (1)	1.06 (1)	0.89 (1)	0.89 (1)
Thr					0.99 (1)	1.03 (1)	1.88 (2)	2.96 (3)	2.95 (3)	3.18 (3)	3.12 (3)
Ser	0.98 (1)	0.22 (1)		1.00 (1)	1.03 (1)		1.90 (2)	0.96 (1)	1.03 (1)	0.85 (1)	0.90 (1)
Glu	3.00 (3)	1.55 (1)	(3)	1.05 (1)	2.12 (2)	2.13 (2)	4.05 (4)	4.03 (4)	3.23 (3)	4 (4)	3 (3)
Gly							1.11 (1)	2.25 (2)	2.35 (2)	2.38 (2)	2.37 (2)
Ala	2 (2)	0.35 (2)						2 (2)	2 (2)	2.85 (2)	2.52 (2)
Val		0.13 (1)					2.95 (3)	1.77 (2)	1.81 (2)	1.58 (2)	1.59 (2)
Met		0.82 (1)									
Leu	0.99 (1)	1.15 (1)	(1)		2 (2)	2 (2)	1 (1)	2.72 (3)	2.61 (3)	2.52 (3)	2.46 (3)
Ile					0.82 (1)	0.86 (1)					
Tyr				1.00 (1)			2.06 (2)	1.00 (1)	1.03 (1)	0.90 (1)	0.94 (1)
Phe				1 (1)			1.01 (1)	1.75 (2)	1.81 (2)	1.60 (2)	1.62 (2)
Lys	1.01 (1)	1.29 (1)	(2)	1.01 (1)	1.87 (2)	0.88 (1)	1.04 (1)	1.00 (1)	0.97 (1)		
Pro	0.80 (1)	0.12 (1)			0.90 (1)	0.87 (1)	1.03 (1)	0.94 (1)	0.80 (1)	1.08 (1)	1.00 (1)
Recovery (nmol)	5.68	5.60		5.60	3.06	2.39	3.76	0.84	0.51	1.49	0.74
$[M+H]^+$	972.6	635.4	1199.6	673.2	1156.7	941.5	2351.2	2513.6	2385.3	2385.3	2257.1
	(970.5)	(633.3)	(1197.7)	(672.3)	(1155.7)	(940.6)	(2348.1)	(2511.3)	(2383.3)	(2383.3)	(2255.2)
	(971.5)	(634.3)	(1198.7)	(673.3)	(1156.7)	(941.6)	(2349.1)	(2512.3)	(2384.3)	(2384.3)	(2256.2)
	(972.5)	(635.3)	(1199.7)		(1157.7)	(942.6)	(2350.1)	(2513.3)	(2385.2)	(2385.2)	(2257.1)
	(973.5)		(1200.6)				(2351.1)	(2514.3)	(2386.2)	(2386.2)	(2258.1)
							(2352.1)	(2515.3)	(2387.2)	(2387.2)	(2259.1)
							(2353.0)	(2516.2)		(2388.1)	
Total amino acid residues	9	5	11	5	10	8	20	23	22	22	21

Table 3. *Amino acid compositions and other data on protease V8 peptides of Cm-H-ISP*
Protease V8 peptides were separated by HPLC. V-*n* denote peak fractions in Fig. 10. Numbers of amino acid residues in parentheses indicate nearest integer values. Mass values without and with parentheses indicate observed mass values in Fig. 11 and theoretical mass values calculated from the amino acid compositions, respectively. The theoretical values coinciding with observed values are underlinded

	V-1	V-2	V-3	V-4	V-5	V-6	V-7	V-8	V-9
CMC-Cys					1.02 (1)				
Asp								2.02 (2)	1.17 (1)
Thr			1.83 (2)	2.06 (2)			1.99 (2)	1.88 (2)	
Ser		1.22 (1)				0.94 (1)		1.86 (2)	1.16 (1)
Glu	1.95 (2)	1.34 (1)	1.89 (2)	1.12 (1)	2.96 (3)	1.07 (1)	2.03 (2)	3.09 (3)	1.16 (1)
Gly			0.99 (1)	1.15 (1)			1.01 (1)	1.09 (1)	
Ala	1 (1)		1 (1)	1 (1)	1 (1)	1 (1)	1 (1)		
Val		1 (1)			0.93 (1)			2.03 (2)	1.99 (2)
Met								0.91 (1)	
Leu	0.95 (1)		0.95 (1)	1.03 (1)			1.94 (2)	2 (2)	2 (2)
Ile							0.80 (1)		
Tyr						1.02 (1)		2.04 (2)	1.10 (1)
Phe						0.95 (1)		1.01 (1)	2.01 (2)
Lys		2.72 (2)			0.99 (1)	1.05 (1)	1.83 (2)	1.00 (1)	
Pro		1.16 (1)	0.83 (1)	0.92 (1)	0.77 (1)		0.79 (1)		
Recovery (nmol)	5.76	4.05	3.73	1.86	5.56	5.42	4.11	3.81	1.15
$[M+H]^+$	460.3	687.6	815.6	687.6	944.7	744.6	1298.9	2224.4	1230.9
	(459.3)	(686.4)	(815.4)	(687.4)	(959.5)	(743.4)	(1297.8)	(2221.1)	(1229.7)
	(460.2)	(687.4)	(816.4)	(688.4)	(960.4)	(744.4)	(1298.8)	(2222.1)	(1230.6)
	(461.2)		(817.4)		(961.4)		(1299.8)	(2223.1)	(1231.6)
					(962.4)			(2224.0)	
								(2225.0)	
								(2226.0)	
Total amino acid residues	4	6	8	7	8	6	12	19	10

Table 4. *Effect of Cm-H-ISP and apo A-II on CO_2 liberation from glucose by isolated rat adipocytes*
Experimental conditions were as described in the text. Total values are given as means ± SEM ($n = 4$), except those of experiments with Cm-H-ISP which are means for duplicate determinations in two separate experiments. For insulin-stimulated values, P (vs. respective control activity) was calculated by the paired t test

Addition	Insulin (30 μU/ml)	$^{14}CO_2$ liberated from D-[1-^{14}C]glucose	
		total	insulin-stimulated
		nmol $h^{-1}/10^6$ cells	
None	−	9.59 ± 0.37	
	+	49.97 ± 1.03	40.38 ($P < 0.005$)
H-ISP (1.2 μM)	−	9.88 ± 0.54	
	+	64.61 ± 1.40	54.73 ($P < 0.01$)
Apo-A-II (1.2 μM)	−	9.76 ± 0.18	
	+	60.13 ± 1.50	50.37 ($P < 0.001$)
Cm-H-ISP (2.4 μM)	−	8.63	
	+	8.94	

Table 5. Effect of H-ISP on lipid synthesis from glucose by isolated adipocytes. Experimental conditions were described in the text. Values are means ± SEM for three separate experiments.

Insulin (M)	H-ISP (1.2 μM)	Total lipid synthesized from D-[U-^{14}C]glucose (nmol/10^6 cells/h)	
		Total	Insulin-stimulated
0	-	15.0 ± 1.8	
	+	14.8 ± 1.6	
6.7×10^{-11}	-	21.2 ± 1.6	7.2 ± 0.3
	+	25.3 ± 1.5	10.5 ± 0.3[a]
2.0×10^{-9}	-	42.4 ± 2.7	27.4 ± 1.7
	+	49.0 ± 2.7	34.2 ± 1.4[b]

[a] $p < 0.005$, [b] $p < 0.02$ (vs. respective value without H-ISP) by the paired t test.

Table 6. *Effect of H-ISP on 2-deoxyglucose uptake by isolated rat adipocytes*
H-ISP, 1.2 μM. Values are means ± SEM ($n = 5$)

Insulin	H-ISP	2-Deoxyglucose uptake	
		total	insulin-stimulated
		nmol $min^{-1}/10^6$ cell	
0	−	0.81 ± 0.05	
	+	0.76 ± 0.07	
1.7 nM	−	4.87 ± 0.41	4.06 ± 0.36
	+	5.87 ± 0.47	5.11 ± 0.44[a]

[a] $P < 0.05$ (vs value without H-ISP) by paired-data analysis.

Table 7. *Stimulation of pyruvate dehydrogenase activity by insulin or H-ISP in subcellular systems*

In experiment 1, the mixture of plasma membrane (200 μg/ml) and mitochondria (500 μg/ml) was incubated for 10 min with ATP (250 μM); in experiment 2, the mitochondria (500 μg/ml) were substituted for the plasma membrane/mitochondria mixture. Then pyruvate dehydrogenase activity was assayed for 3 min as described in the text. Values are means ± SEM ($n = 4$). P values were calculated (vs. respective control activity) by the paired t test

Expt	Addition	Pyruvate dehydrogenase activity	Stimulation
		nmol (mg mito protein)$^{-1}$ min^{-1}	%
1.	Control	4.48 ± 0.08	
	Insulin (200 μU/ml)	5.97 ± 0.18	33.3 ($P < 0.005$)
	H-ISP (2.0 μM)	6.58 ± 0.20	46.9 ($P < 0.001$)
2.	Control	3.65 ± 0.09	
	H-ISP (2.0 μM)	5.15 ± 0.21	41.1 ($P < 0.002$)

Table 8. *Effect of sodium fluoride on the stimulation of pyruvate dehydrogenase by H-ISP*

The mixture of plasma membrane (200 μg/ml) and mitochondria (500 μg/ml) was preincubated for 10 min at 37°C with ATP (250 μM) and with or without sodium fluoride (75 mM). Then pyruvate dehydrogenase activity was assayed for 3 min as described in the text. Values are means ± SEM ($n = 4$)

Expt	Pyruvate dehydrogenase activity	
	no NaF	75 mM NaF
	nmol (mg mito protein)$^{-1}$ min^{-1}	
Control	4.47 ± 0.06	4.24 ± 0.15
H-ISP (2.0 μM)	6.64 ± 0.44	4.58 ± 0.38

Table 9. Effects of TLCK or leupeptin on glucose oxidation and insulin receptor phosphorylation

Inhibitor	Experiment 1			Experiment 2		
	Insulin (0.2 nM)	Insulin-stimulated [a] $^{14}CO_2$ liberation from D-[1-^{14}C]glucose (nmol/10^6 cells/h)	Inhibition (%)	Insulin (10 nM)	Phosphorylation [b] (arbitrary units)	Inhibition (%)
None	+	34.66		+	36.5	
TLCK (250 μM)	+	5.84 ($p<0.001$)	83.2	+	7.8	78.6
Leupeptin (50 μg/ml)	+	16.94 ($p<0.001$)	51.1	+	17.3	52.6

[a] Values are means of those obtained with 4 rats.

[b] Values are means of two separate experiments.

In Experiment 1, adipocytes were incubated at 37°C for 2 h with or without insulin (0.2 nM) in the presence or absence of TLCK or leupeptin. The values without insulin were 6.70 ± 0.13 (none), 6.30 ± 0.31 (TLCK) and 6.86 ± 0.27 (leupeptin) nmol $^{14}CO_2/10^6$ cells/h, respectively. For insulin-stimulated values, p(vs. value with insulin alone) was calculated by the paired t test. In Experiment 2, phosphorylation was assayed as described in the text. The autoradiograms were scanned with a Joyce-Loebl microdensitometer and the peak areas corresponding to the Mr 95,000 band were calculated and expressed in arbitrary units.

Fig. 1. Purification of H-ISP from human plasma.

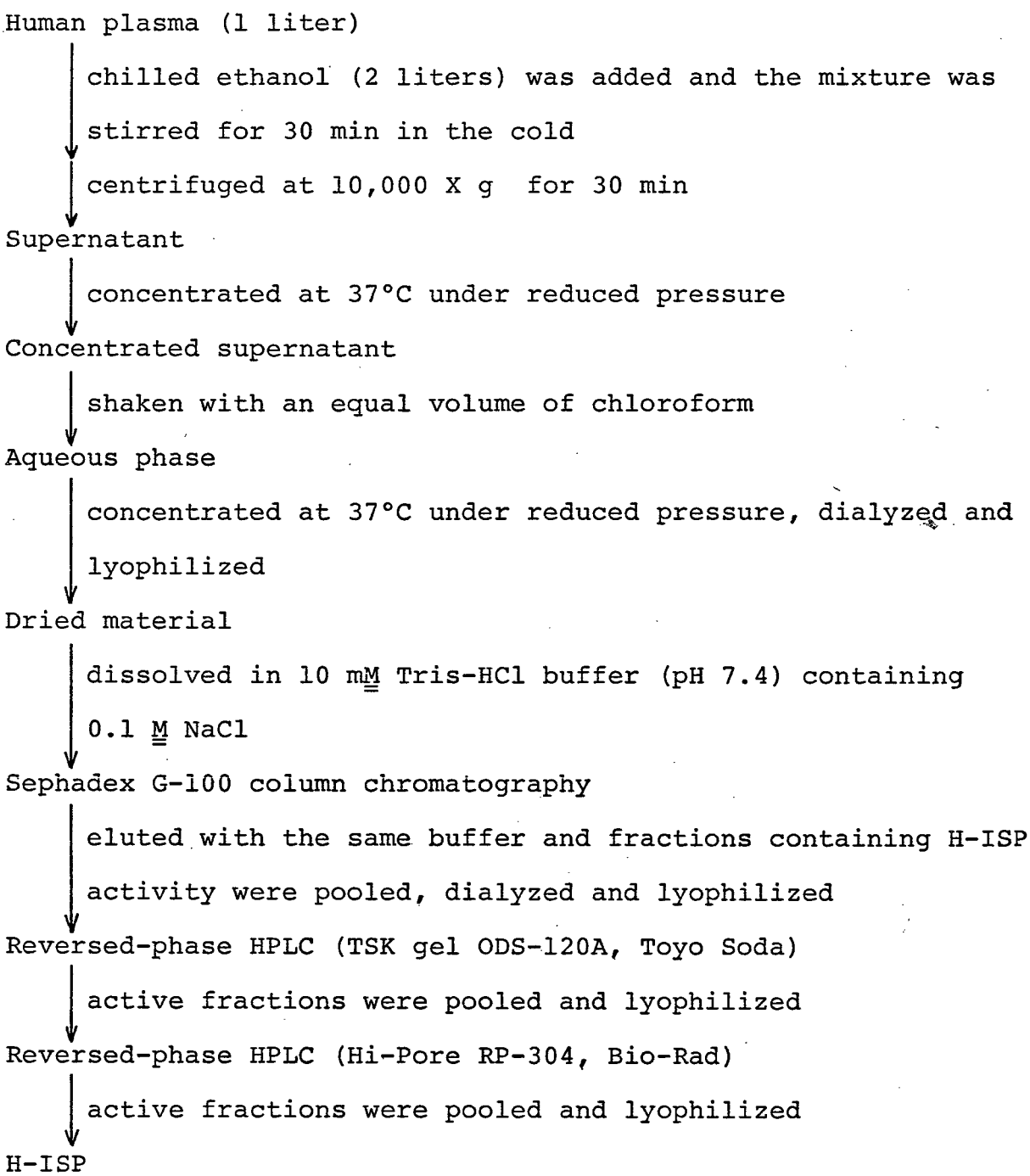

Fig. 2. Sephadex G-100 column chromatography. The fractions pooled for subsequent purification are indicated by a horizontal bar.

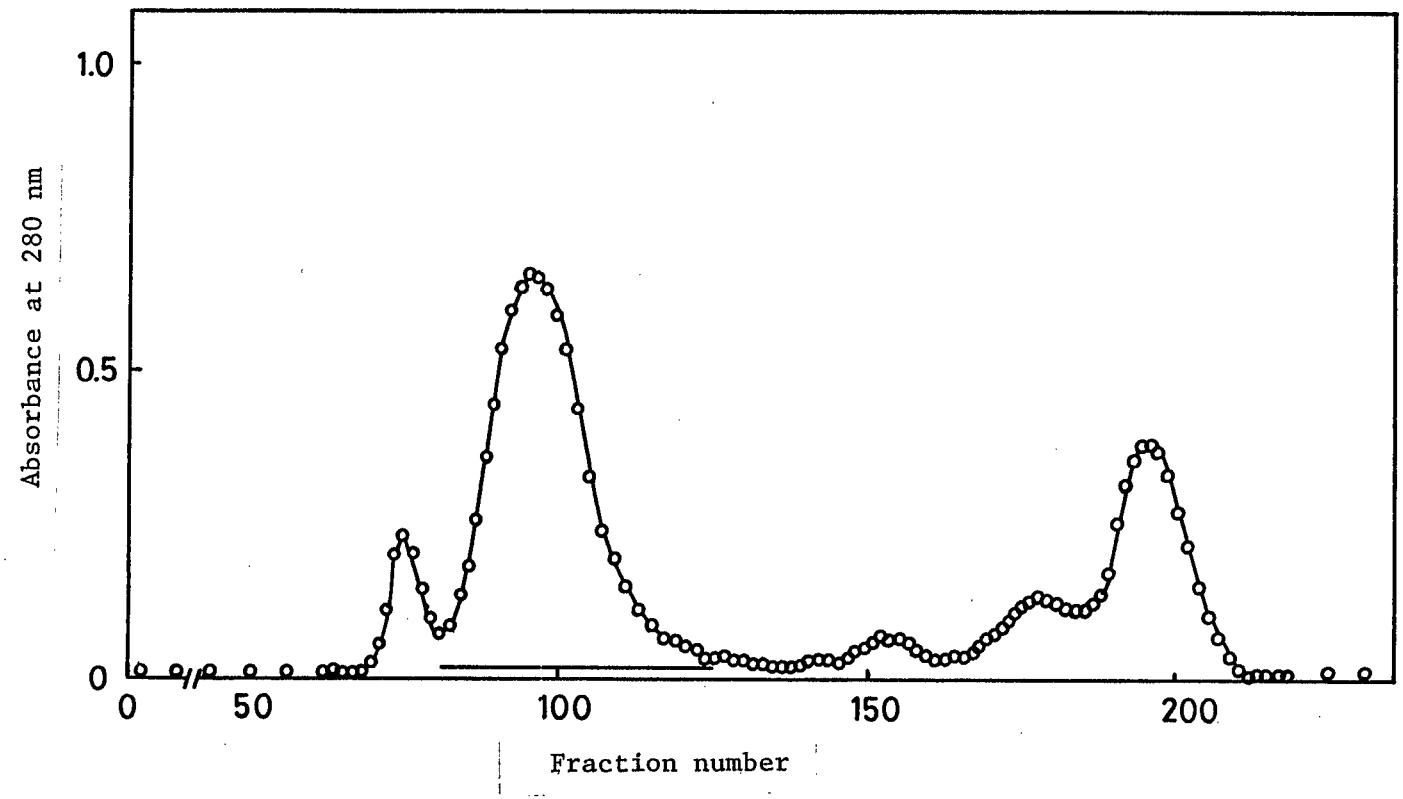

Fig. 3

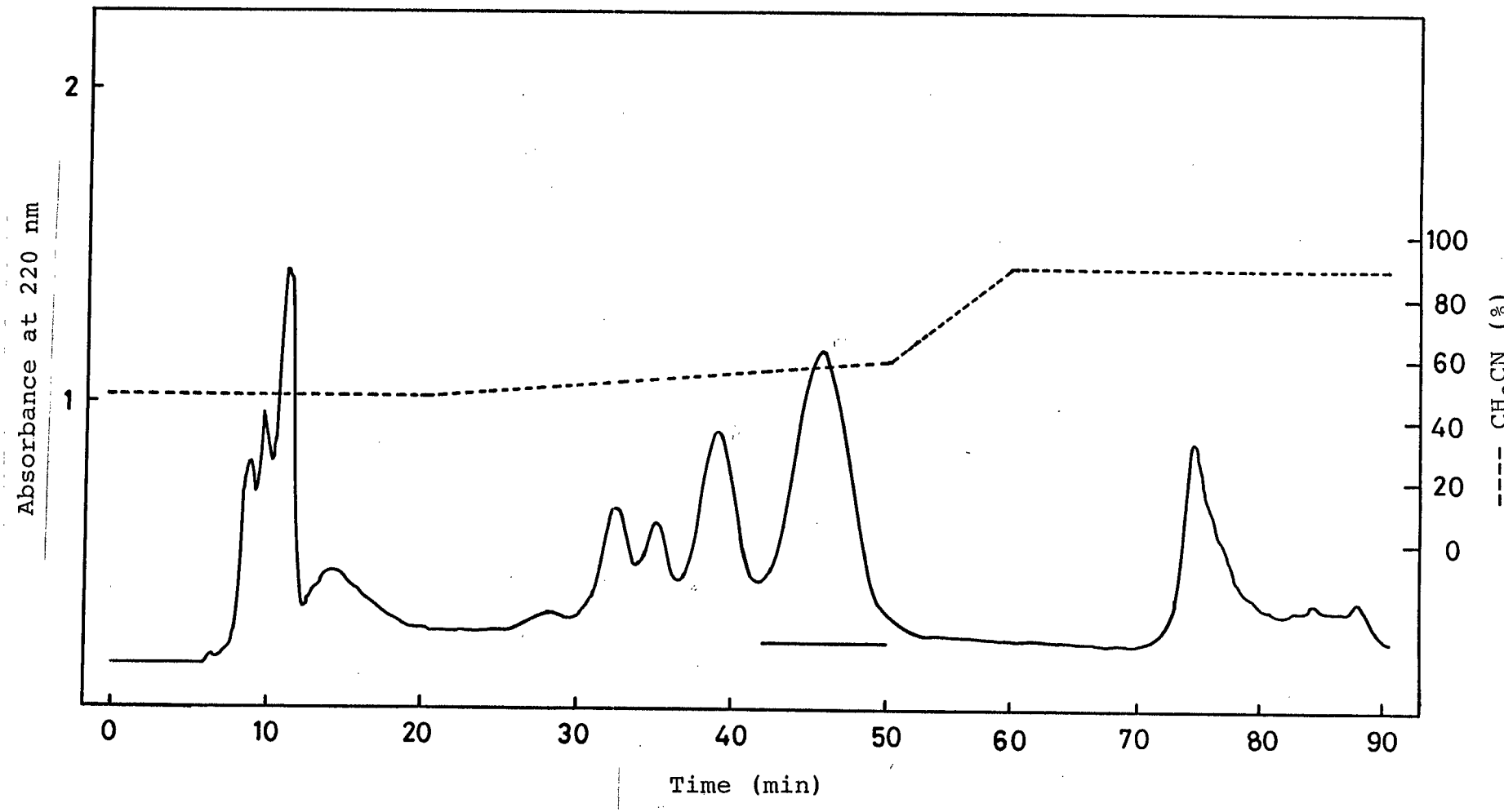
Absorbance at 220 nm
2
1
0
10
20
30
40
50
60
70
80
90
Time (min)
100
80
60
40
20
0
---- CH_3CN (%)

Fig. 3. Reversed-phase HPLC on a TSK gel ODS-120A column. The horizontal bar indicates the fractions of H-ISP pooled.

Fig. 4. HPLC of H-ISP on a Hi-Pore RP-304 column. The horizontal bar indicates the fractions of H-ISP pooled and used for further experiments.

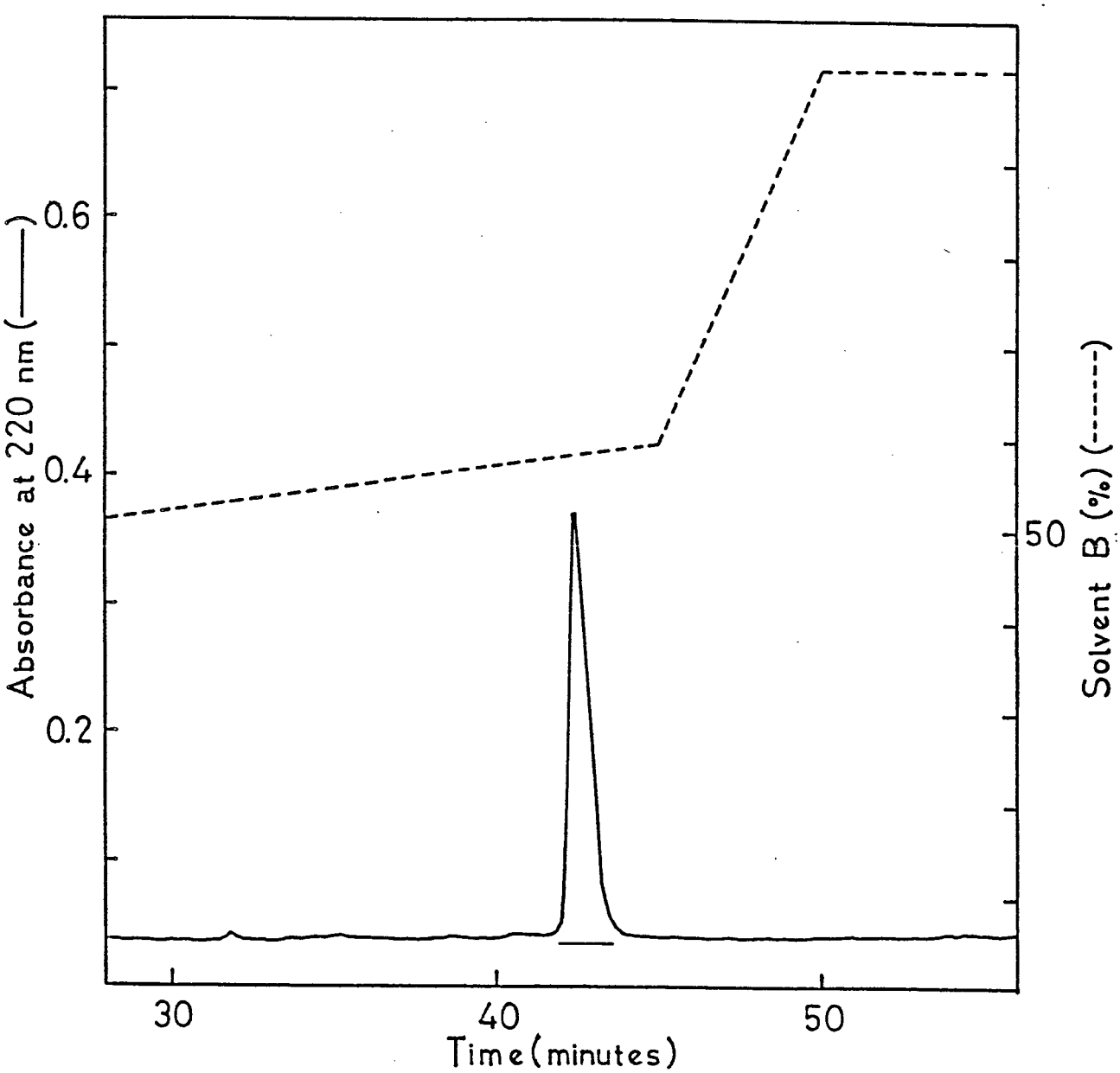

Fig. 5. SDS-polyacrylamide gel electrophoresis of purified H-ISP. Purified H-ISP (10 μg) was boiled for 3 min in Laemmli's sample buffer (41) in the absence (lane 1) or presence (lane 2) of 5% 2-mercaptoethanol. For other conditions, see text.

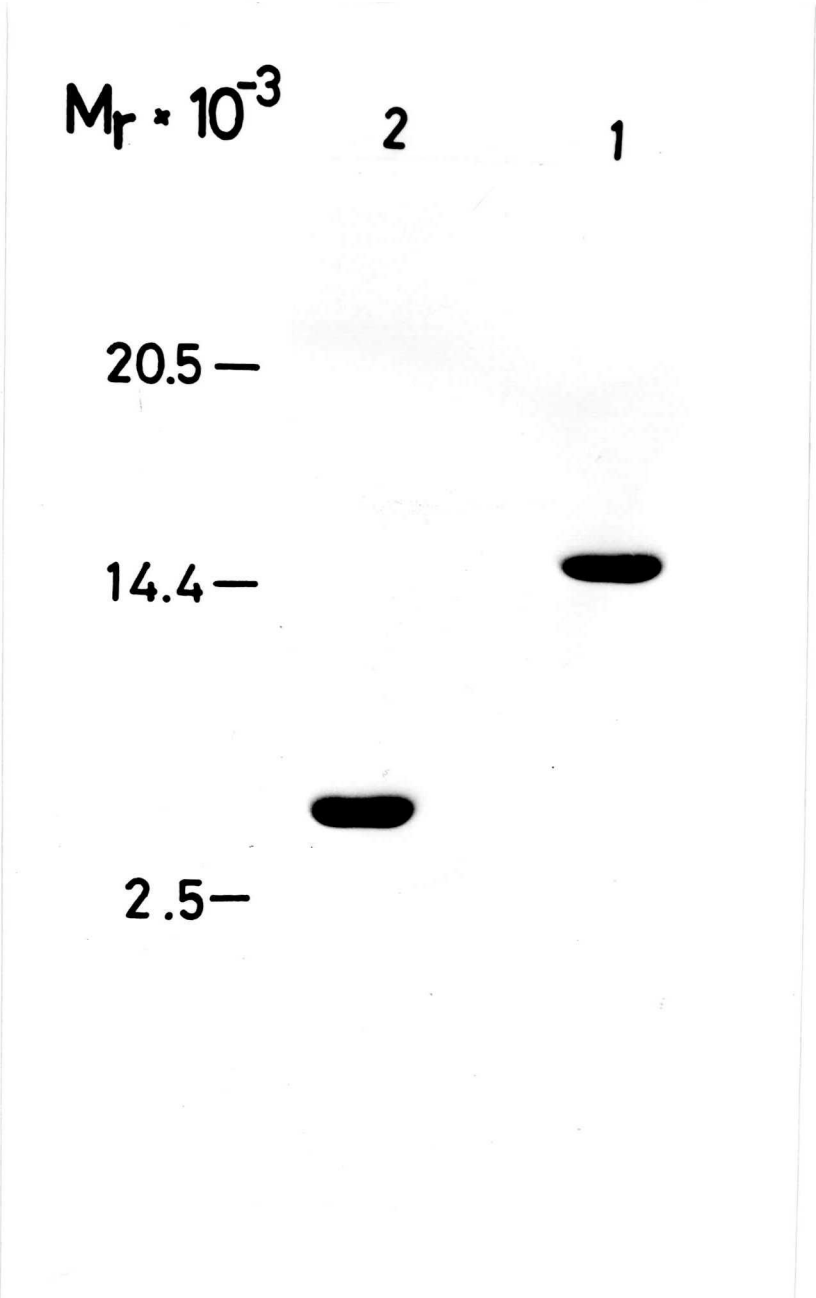

Fig. 6. Gel filtration chromatography of H-ISP and Cm-H-ISP on Sephadex G-75 in 6 M guanidine hydrochloride. Approximately 1 mg of each protein was dissolved in 1 ml of 6 M guanidine hydrochloride and loaded on a Sephadex G-75 column (1.0 X 180 cm). Materials were eluted with 6 M guanidine hydrochloride at a flow rate of 10 ml/h and fractions of 1 ml were collected. Elution patterns of H-ISP (—●—) and Cm-H-ISP (---○---) are indicated. The molecular weight standards used were: myoglobin (Myo., 17,000), oxidized bovine pancreatic ribonuclease A (RNaseA, 13,500), lima bean trypsin inhibitor (T.Inh., 8,400), and bovine pancreatic insulin (Ins., 5,700). The positions of the standard proteins eluted are indicated.

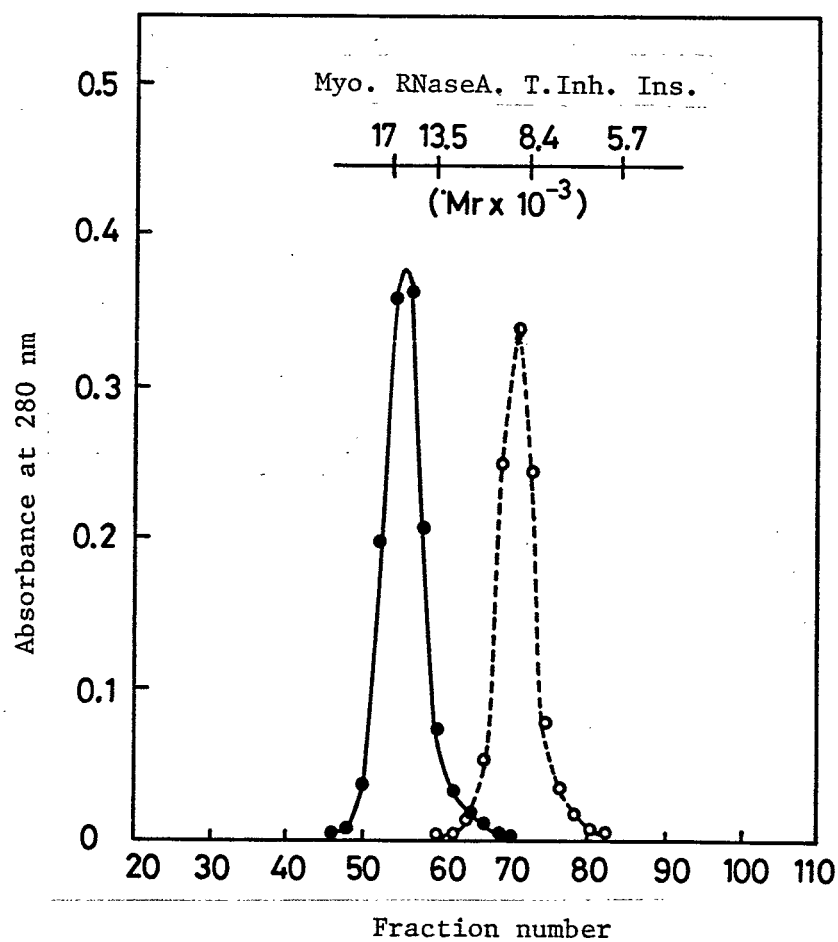

Fig. 7. Isoelectric gel focusing of H-ISP and apo A-II. Samples (50 μg) were subjected to isoelectric gel focusing as described in the text. For estimation of pI values, the gel was sliced into 5 mm segments and each segment was extracted with 1 ml of water. Gels were stained with Coomassie brilliant blue R-250.

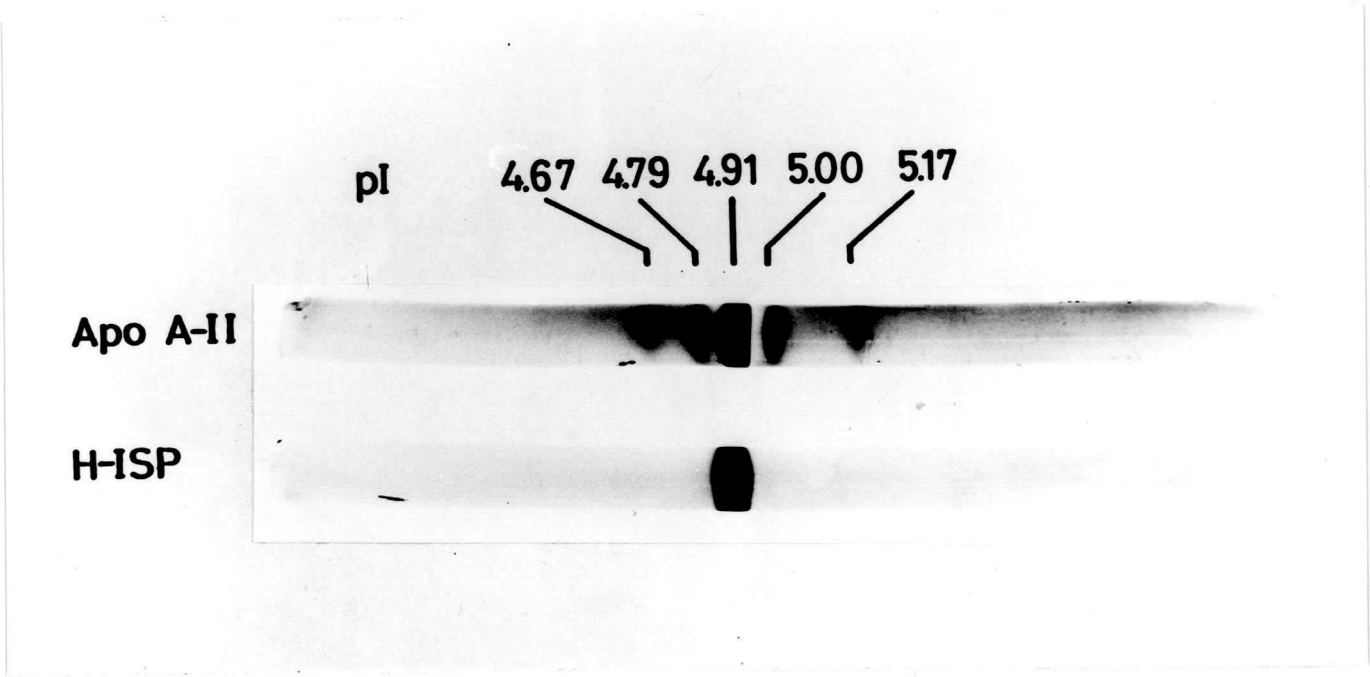

Fig. 8. HPLC profile of the tryptic digest of Cm-H-ISP. Chromatographic conditions were as described in the text.

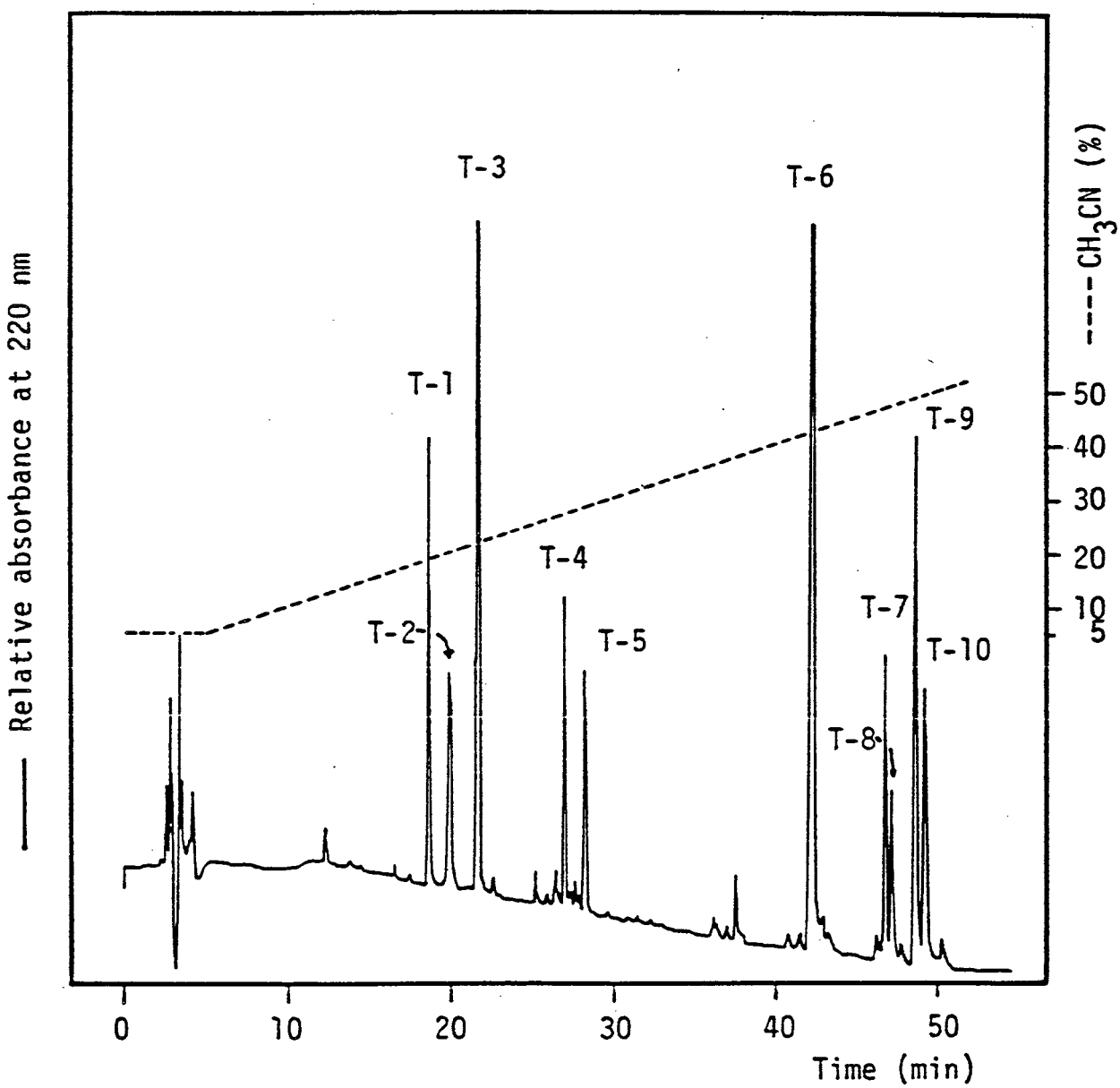

Fig. 9

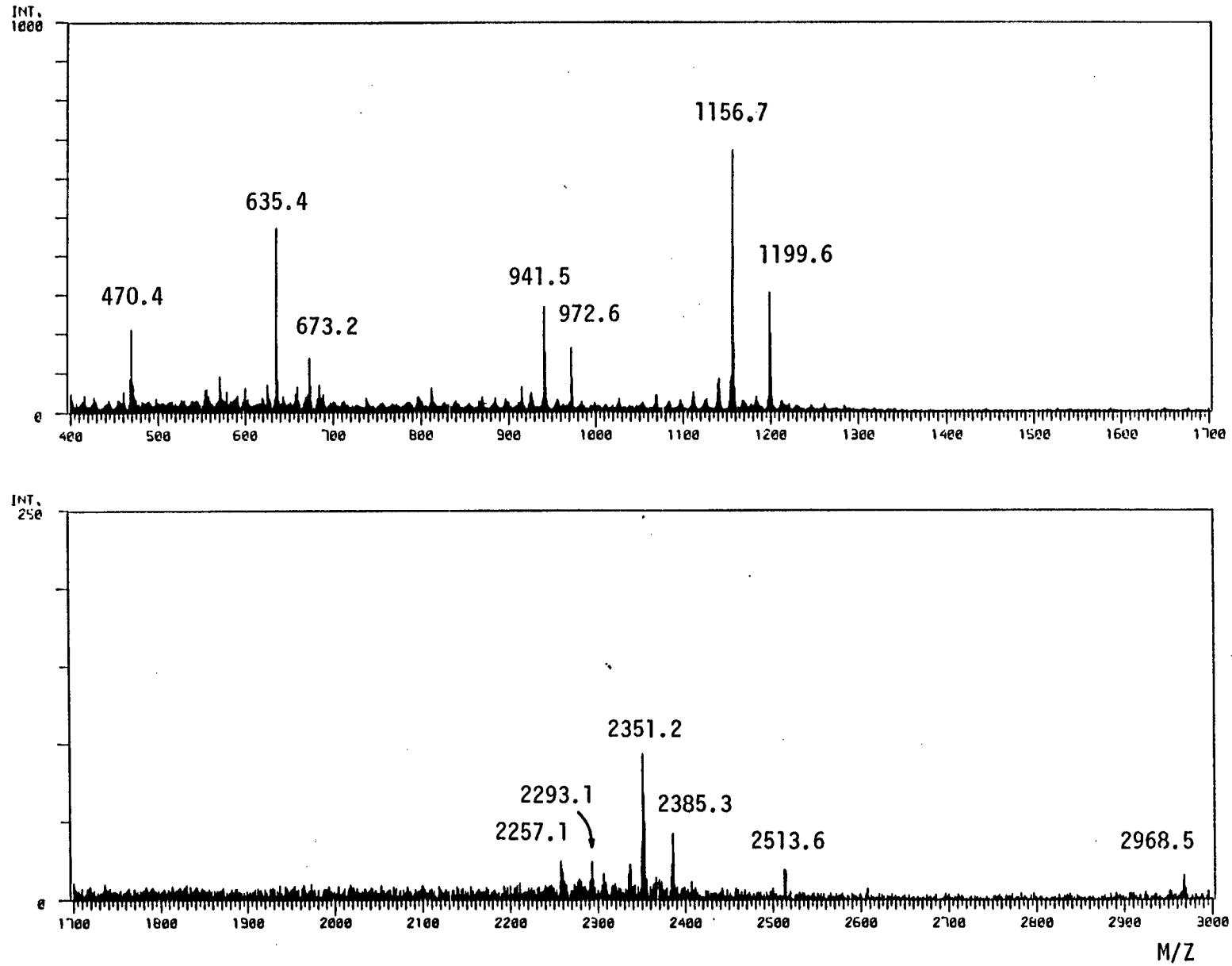
INT.
1000
0
470.4
635.4
673.2
941.5
972.6
1156.7
1199.6
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
INT.
250
0
2293.1
2257.1
2351.2
2385.3
2513.6
2968.5
1700
1800
1900
2000
2100
2200
2300
2400
2500
2600
2700
2800
2900
3000
M/Z

Fig. 9. FAB mass spectrum of the tryptic digest of Cm-H-ISP.

Fig. 10. HPLC profile of the protease V8 digest of Cm-H-ISP.

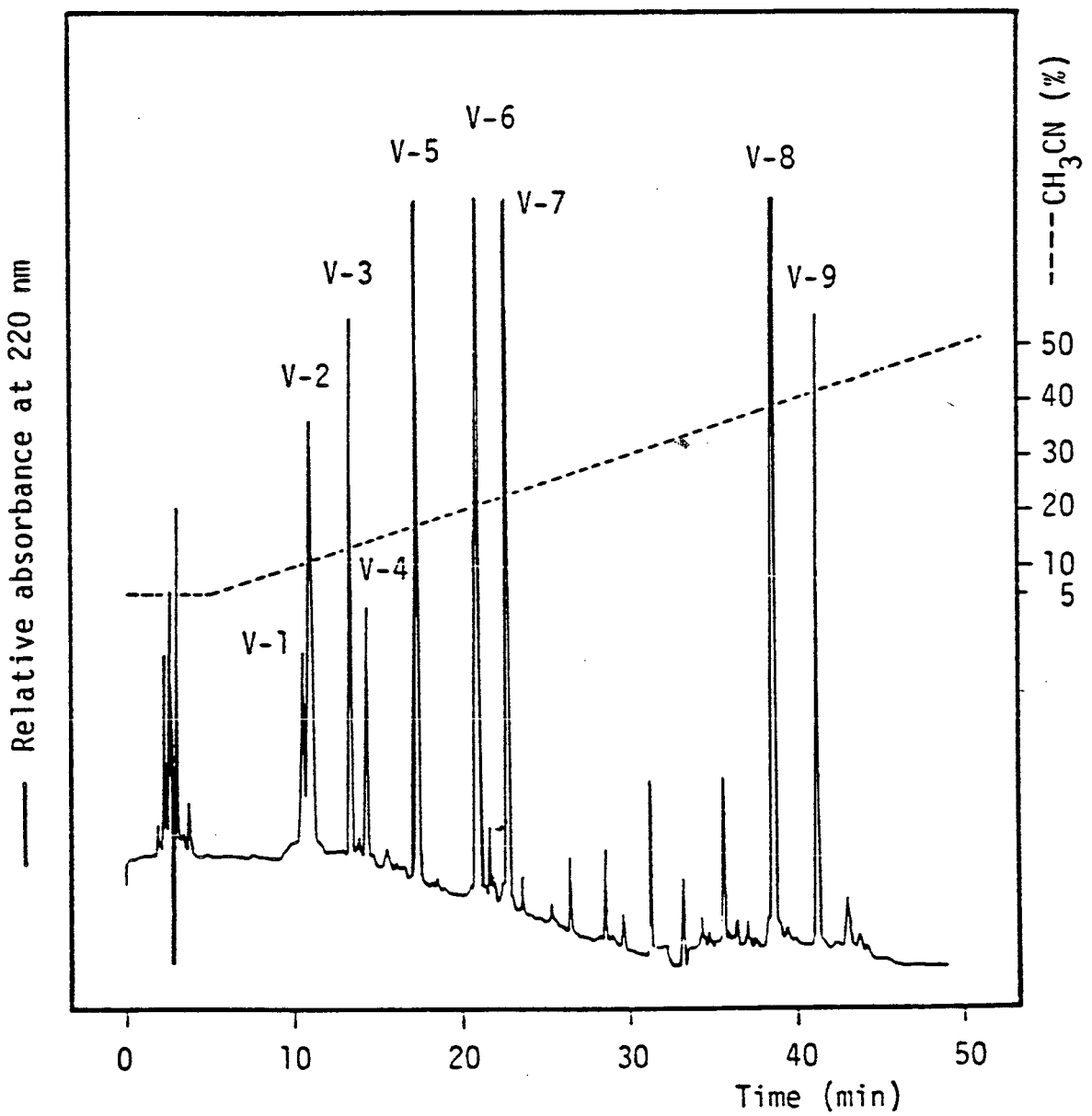

Fig. 11

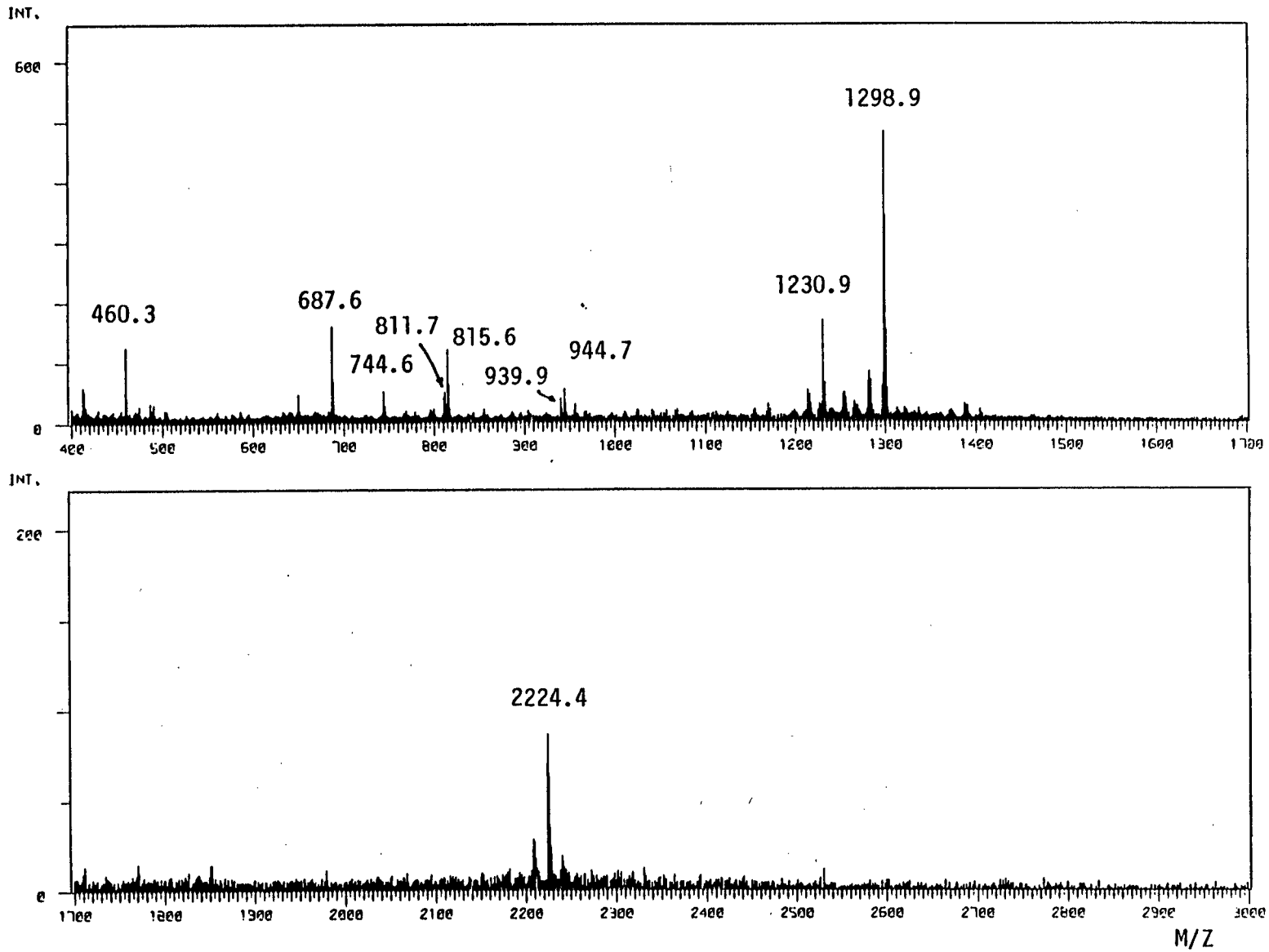
INT.
600
0
460.3
687.6
744.6
811.7
815.6
939.9
944.7
1230.9
1298.9
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
INT.
200
0
2224.4
1700
1800
1900
2000
2100
2200
2300
2400
2500
2600
2700
2800
2900
3000
M/Z

Fig. 11. FAB mass spectrum of the protease V8 digest of Cm-H-ISP.

Fig. 12. FAB mass spectrum of: (A) V-5 and (B) the digest of V-5 with pyrrolidonecarboxylate peptidase.

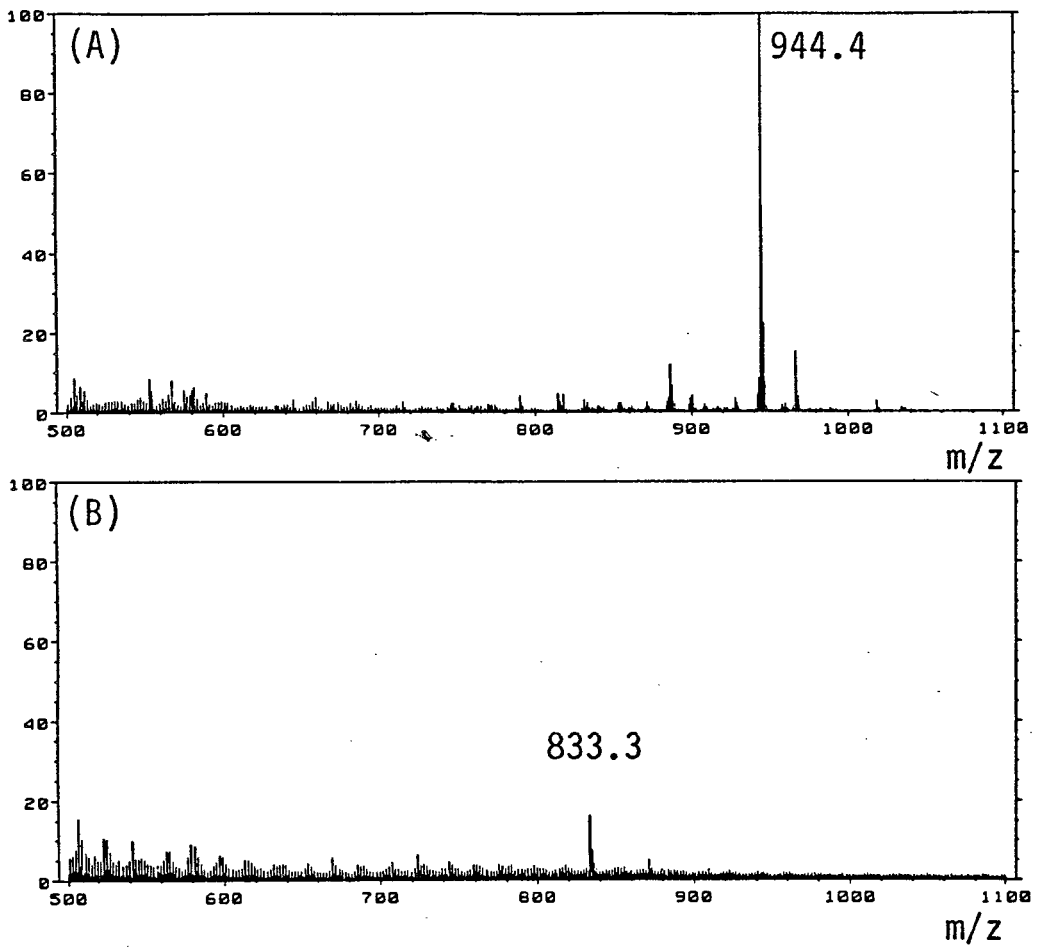

Fig. 13

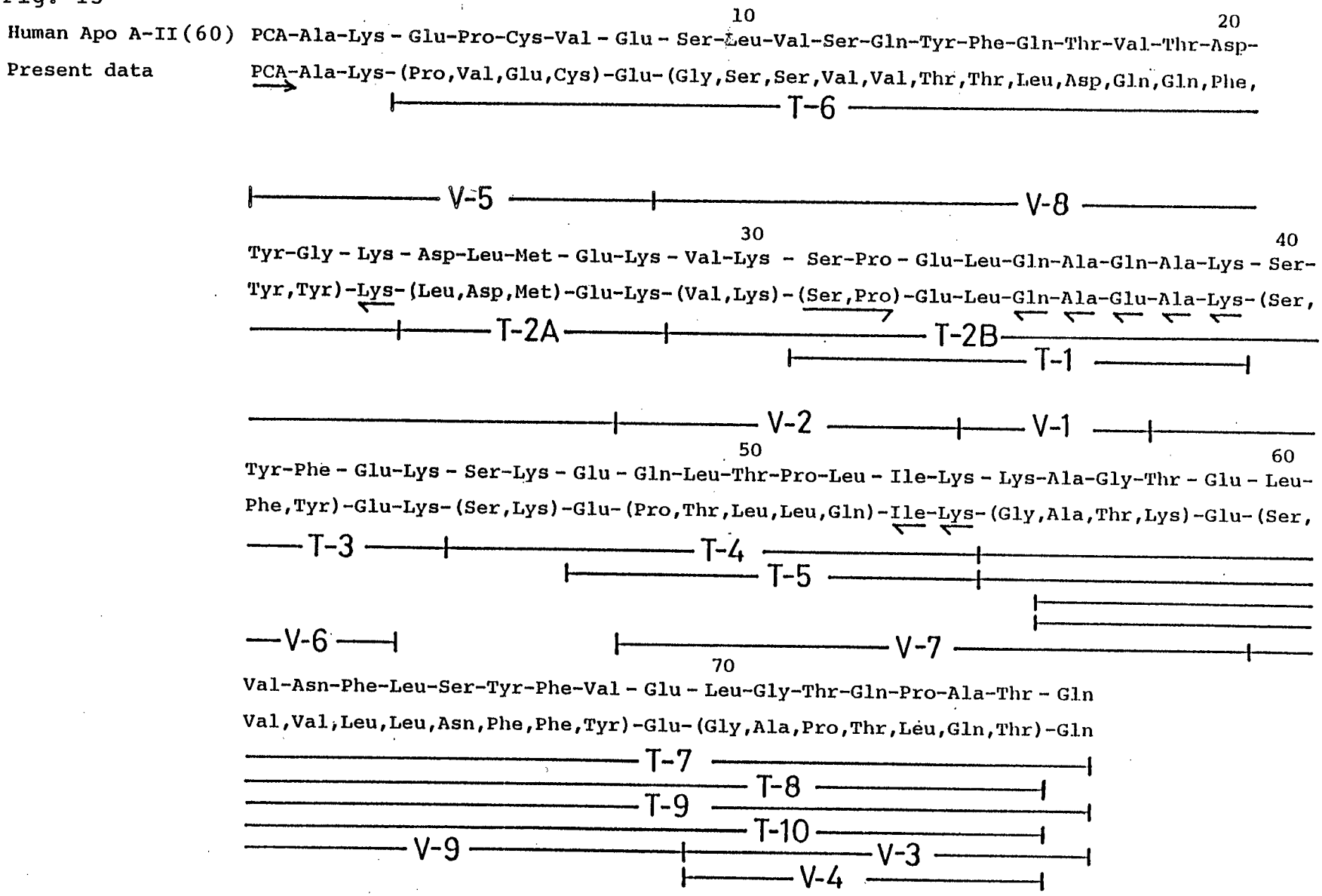
10
20
Human Apo A-II(60) PCA-Ala-Lys - Glu-Pro-Cys-Val - Glu - Ser-Leu-Val-Ser-Gln-Tyr-Phe-Gln-Thr-Val-Thr-Asp-
Present data PCA-Ala-Lys-(Pro,Val,Glu,Cys)-Glu-(Gly,Ser,Ser,Val,Val,Thr,Thr,Leu,Asp,Gln,Gln,Phe,
T-6
V-5
V-8
30
40
Tyr-Gly - Lys - Asp-Leu-Met - Glu-Lys - Val-Lys - Ser-Pro - Glu-Leu-Gln-Ala-Gln-Ala-Lys - Ser-
Tyr,Tyr)-Lys-(Leu,Asp,Met)-Glu-Lys-(Val,Lys)-(Ser,Pro)-Glu-Leu-Gln-Ala-Glu-Ala-Lys-(Ser,
T-2A
T-2B
T-1
V-2
V-1
50
60
Tyr-Phe - Glu-Lys - Ser-Lys - Glu - Gln-Leu-Thr-Pro-Leu - Ile-Lys - Lys-Ala-Gly-Thr - Glu - Leu-
Phe,Tyr)-Glu-Lys-(Ser,Lys)-Glu-(Pro,Thr,Leu,Leu,Gln)-Ile-Lys-(Gly,Ala,Thr,Lys)-Glu-(Ser,
T-3
T-4
T-5
V-6
V-7
70
Val-Asn-Phe-Leu-Ser-Tyr-Phe-Val - Glu - Leu-Gly-Thr-Gln-Pro-Ala-Thr - Gln
Val,Val,Leu,Leu,Asn,Phe,Phe,Tyr)-Glu-(Gly,Ala,Pro,Thr,Leu,Gln,Thr)-Gln
T-7
T-8
T-9
T-10
V-9
V-3
V-4

Fig. 13. Partial amino acid sequence of Cm-H-ISP searched by the computer program and its comparison with that of human apo A-II (60). Arrows indicate residues determined by a combination of CPase Y (←) or aminopeptidase M (→) or pyrrolidonecarboxylate peptidase (→) digestion and FAB mass spectrometry.

Fig. 14. FAB mass spectrum of the CPase Y digest of T-2 in Fig. 8.

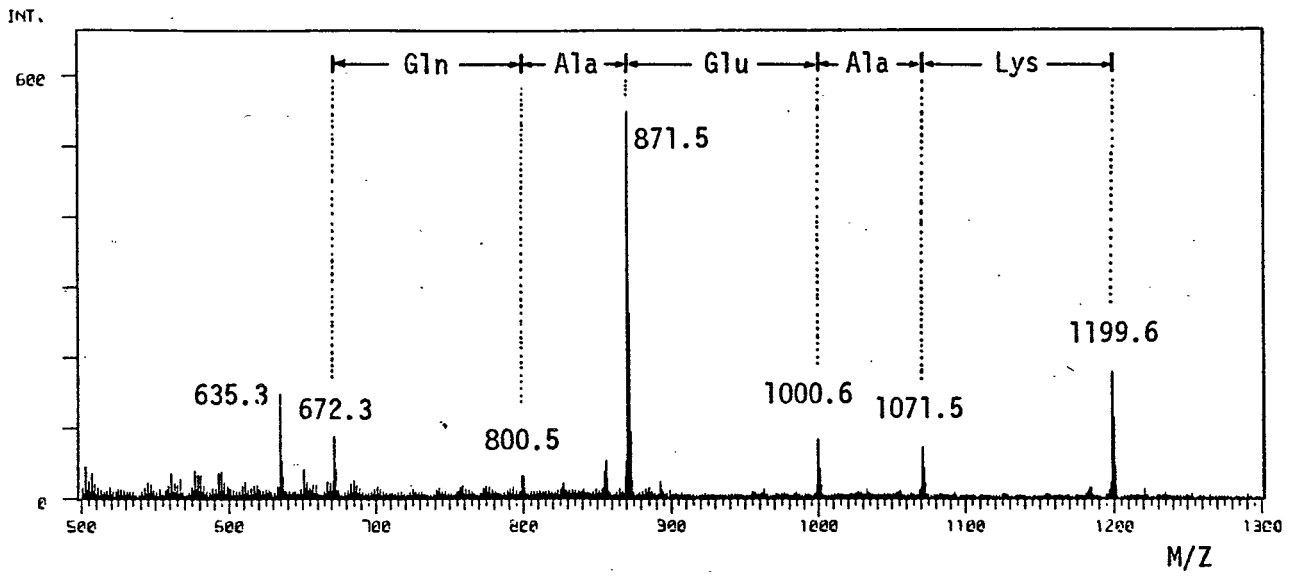

Fig. 15. Effects of insulin and H-ISP on the time course of fatty acid synthesis by explants of adipose tissue. Incubations were carried out as described in the text. Values are means for tri= plicate determinations. ○, without insulin; ●, insulin (20 mU/ml); △, insulin (0.2 mU/ml); X, insulin (0.2 mU/ml) plus H-ISP (20 µg/ml); □, H-ISP alone (20 µg/ml).

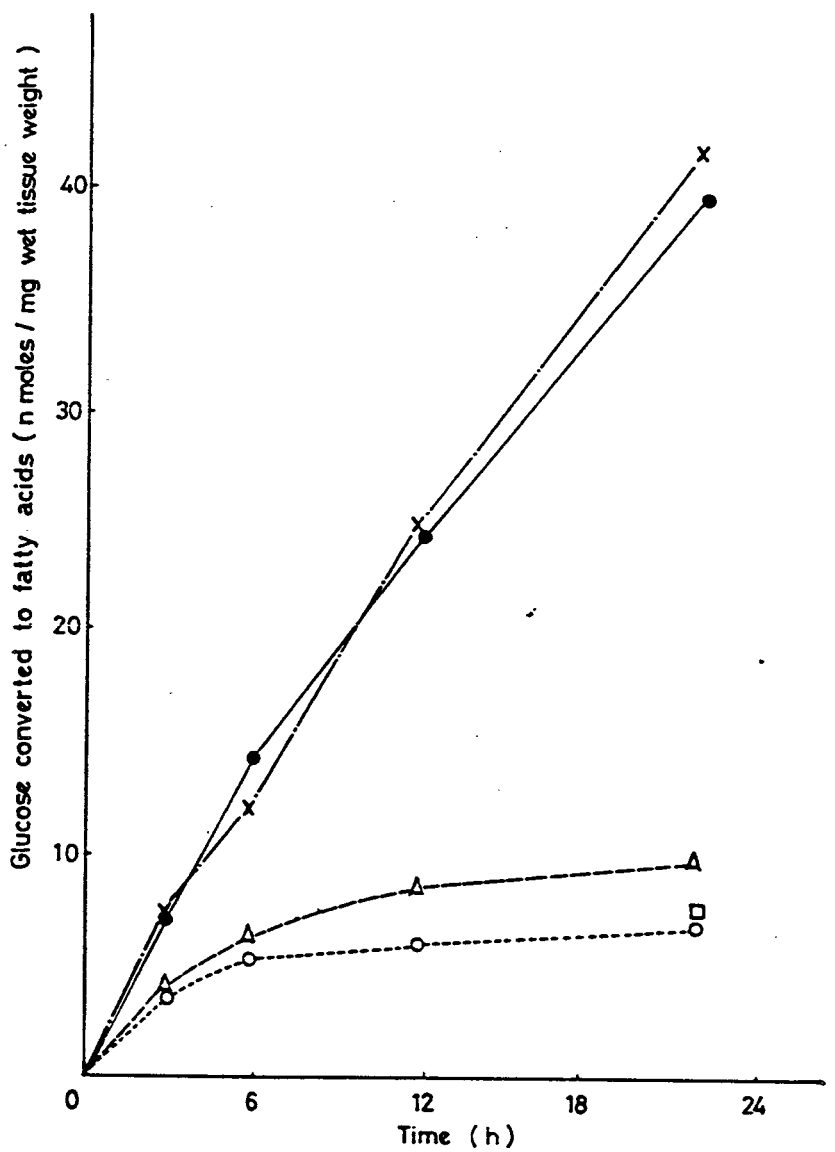

Fig. 16

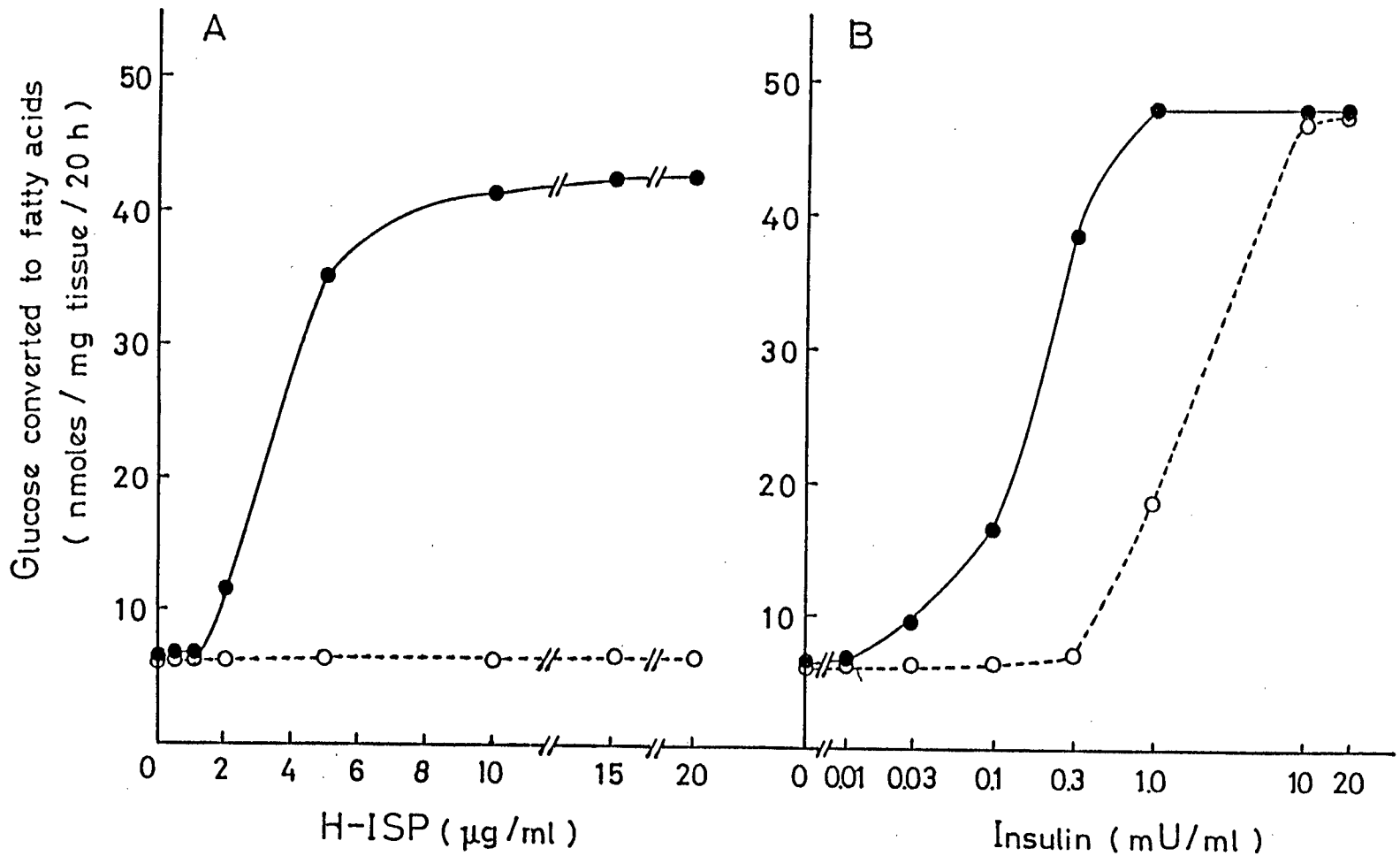
A
B
Glucose converted to fatty acids
(nmoles / mg tissue / 20 h)
50
40
30
20
10
0
2
4
6
8
10
15
20
H-ISP (μg /ml)
0
0.01
0.03
0.1
0.3
1.0
10
20
Insulin (mU/ml)

Fig. 16. (A) Effect of the concentration of H-ISP on fatty acid synthesis from glucose in the presence of insulin. Adipose tissue explants were incubated at 37°C for 20 h with the indicated concent= rations of H-ISP in the absence (○) or presence (●) of insulin (0.2 mU/ml). Values are means for duplicate determinations in two separate experiments. (B) Dose-response curves for the effects of insulin with and without H-ISP. Adipose tissue explants were incu= bated with the indicated concentrations of insulin alone (○), or with H-ISP (15 μg/ml) (●). Values are means for duplicate determi= nations in two separate experiments.

Fig. 17

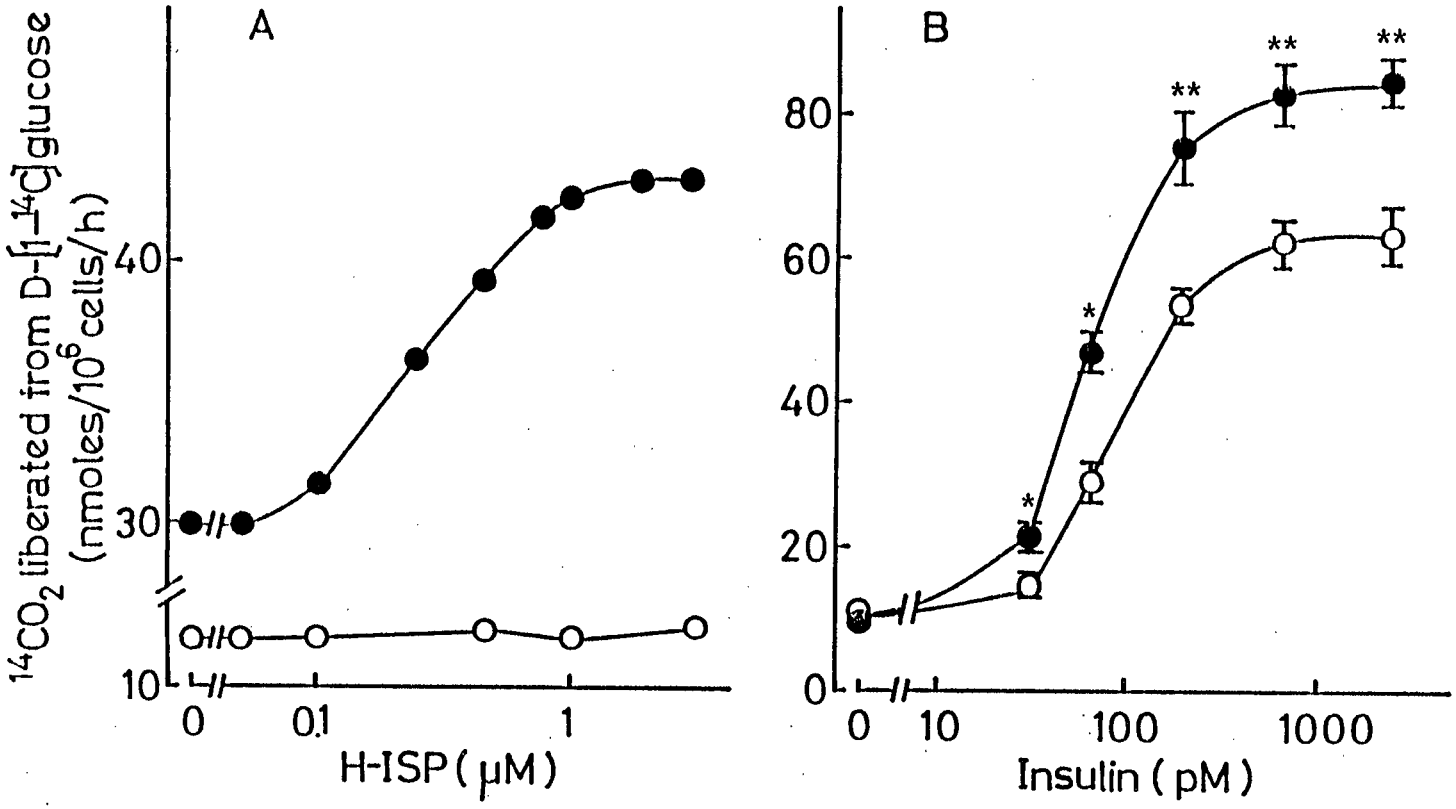
A
B
$^{14}CO_2$ liberated from D-[1-^{14}C]glucose (nmoles/10^6cells/h)
40
30
10
0
0.1
1
H-ISP (μM)
80
60
40
20
0
10
100
1000
Insulin (pM)
*
**

Fig. 17. A, Effect of H-ISP on glucose oxidation in the absence and presence of insulin. Cells were incubated at 37°C for 2 h in medium containing 0.2 m$\underline{M}$ D-[1-^{14}C]glucose (0.3 μCi) with the indicated concentrations of H-ISP without (○) or with (●) 67 p$\underline{M}$ insulin. Values are means for duplicate determinations in two separate experiments. B, Concentration-response curve for the effect of insulin on glucose oxidation with and without H-ISP. Cells were incubated with the indicated concentrations of insulin alone (○), or with H-ISP (1.2 μ$\underline{M}$) (●). Data are means ± SEM for duplicate determinations in four separate experiments. *, $\underline{p} < 0.01$; **, $\underline{p} < 0.002$ (for difference from value with insulin alone).

Fig. 18

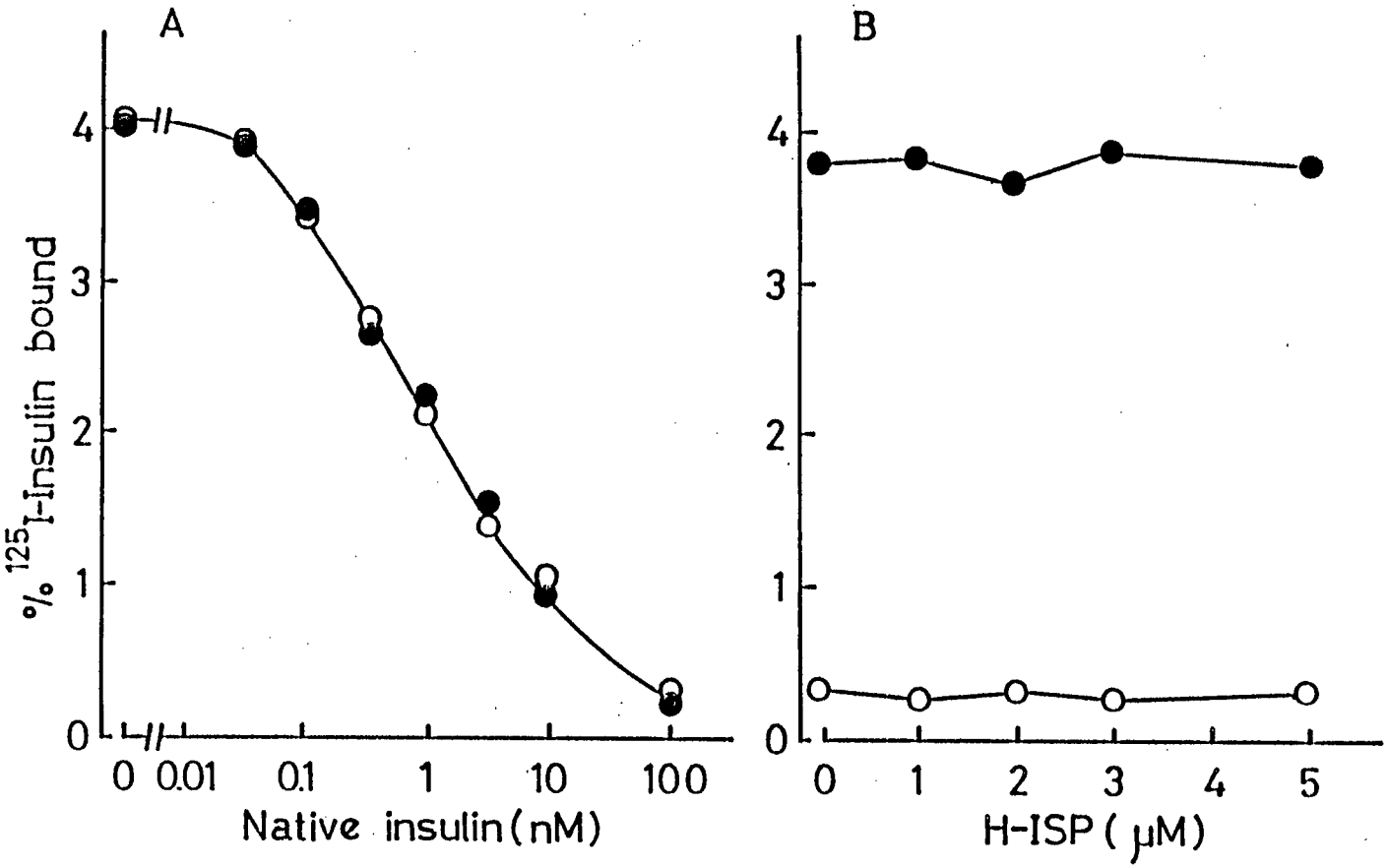
A
B
% ^{125}I-Insulin bound
4
3
2
1
0
0 0.01 0.1 1 10 100
Native insulin (nM)
0 1 2 3 4 5
H-ISP (μM)

Fig. 18. A, Displacement of ^{125}I-labeled insulin by unlabeled insulin. Cells were preincubated at 37°C for 1 h without (○) or with (●) 1.2 μM H-ISP, and ^{125}I-labeled insulin binding to adipocytes was determined as described in the text. Values are corrected for nonspecific binding. B, Effect of H-ISP on ^{125}I-labeled insulin binding to adipocytes. Cells were preincubated with the indicated concentrations of H-ISP at 37°C for 1 h and then incubated at 37°C for 30 min with ^{125}I-labeled insulin (70 pM) in the absence (●) and presence (○) of cold native insulin (58 μg/ml). Values are means for triplicate determinations.

Fig. 19

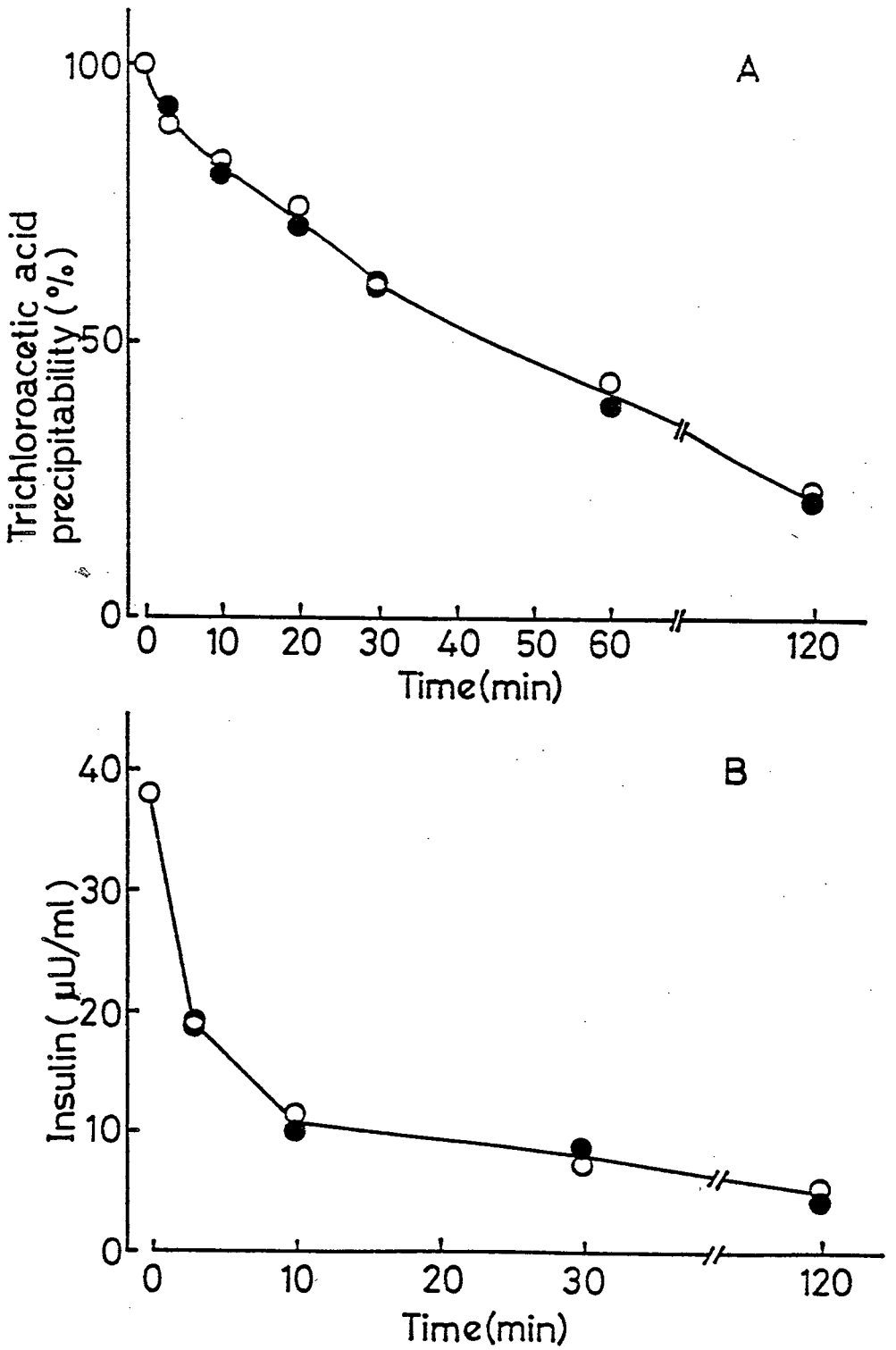
A
100
50
0
Trichloroacetic acid precipitability (%)
0
10
20
30
40
50
60
120
Time(min)
B
40
30
20
10
0
Insulin (μU/ml)
0
10
20
30
120
Time(min)

Fig. 19. Effect of H-ISP on degradation of insulin by adipocytes. A, Cells (7 X 10^5 cells/ml) were incubated at 37°C for the indicated times without (○) or with (●) 1.2 μM H-ISP in the presence of ^{125}I-labeled insulin (70 pM) plus native insulin (130 pM). Then tri=chloroacetic acid-presipitable ^{125}I activity in the medium was measured. Results are plotted as percentages of values for ^{125}I-labeled insulin in the medium before the addition of cells against time. B, Cells (7 X 10^5 cells/ml) were incubated at 37°C for the indicated times without (○) or with (●) 1.2 μM H-ISP in the presence of cold insulin (37 μU/ml). The content of insulin in the medium was determined by radioimmunoassay as described in the text.

Fig. 20. Effects of insulin and H-ISP on pyruvate dehydrogenase activity of intact adipocytes. Adipocytes (7-9 X 10^5cells/ml) were incubated for 15 min at 37°C in Krebs-Ringer phosphate buffer containing 10 mM $NaHCO_3$, 10 mM Hepes, 0.2 mM glucose and 10 mg/ml of bovine serum albumin with H-ISP (3 μM) in the presence or absence of insulin (200 μU/ml). The cell layer was washed and extracted and pyruvate dehydrogenase activity was assayed for 3 min as described in the text. Values are means ± SEM (n=4). *, $p < 0.005$, **, $p < 0.001$ (vs. respective control activity) by paired t test.

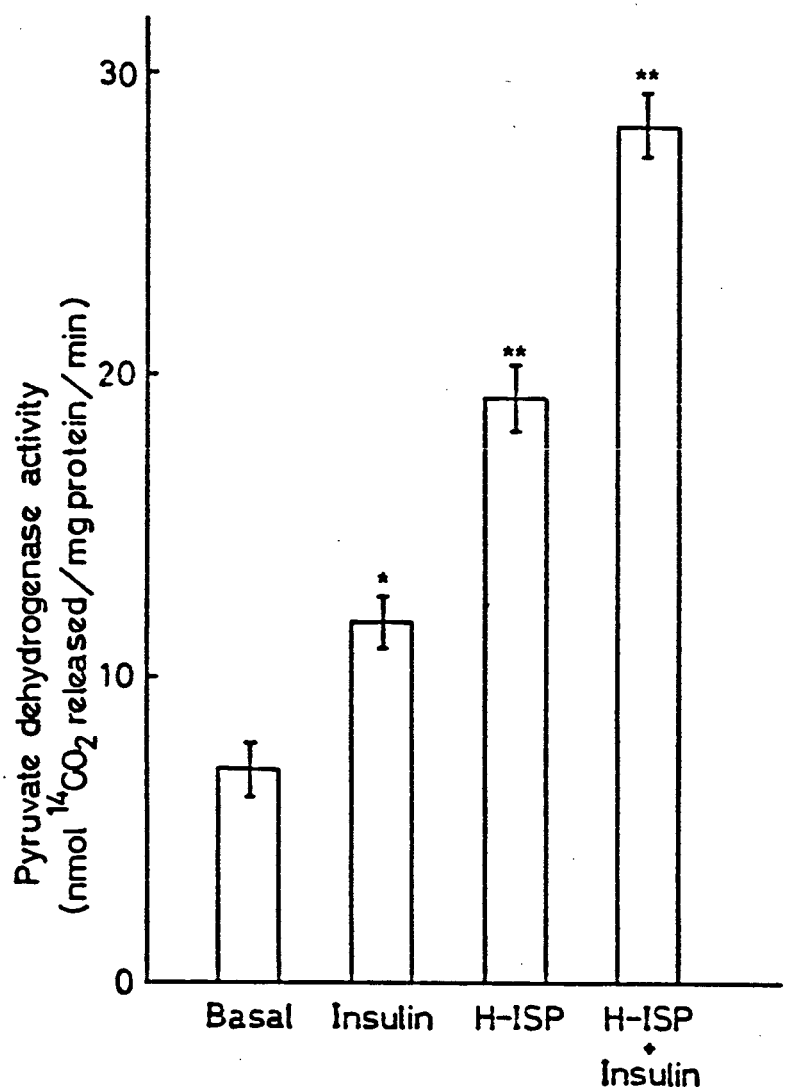

Fig. 21. Effects of various concentrations H-ISP on pyruvate dehydrogenase activity of intact adipocytes. Adipocytes (7-9 X 10^5 cells/ml) were incubated for 15 min at 37°C with the indicated concentrations of H-ISP as described in Fig. 20. Then pyruvate dehydrogenase activity was assayed for 3 min as described in the text. Values are means ± SEM (n=4).

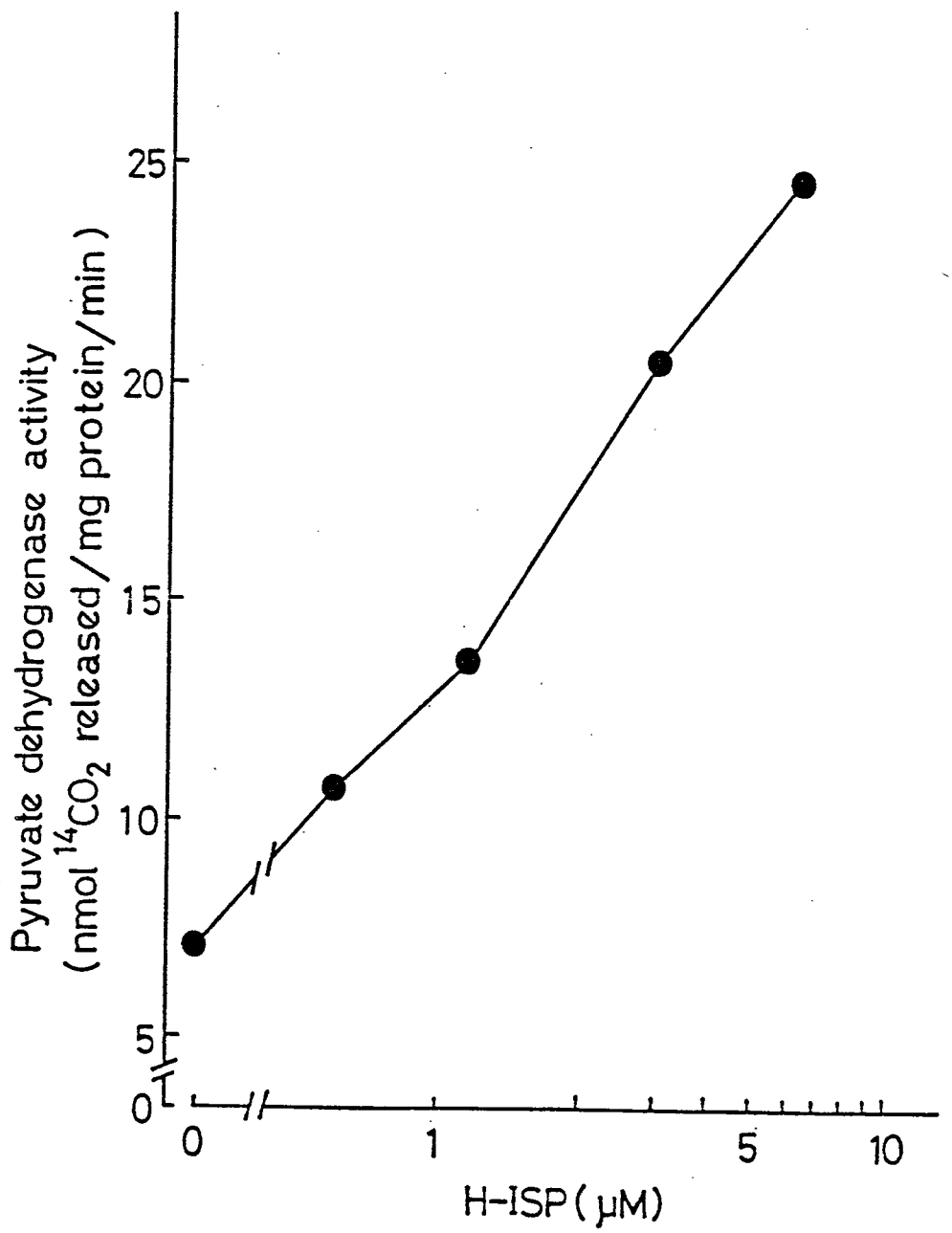

Fig. 22. Effect of H-ISP on phosphorylation of the solubilized insulin receptor fraction. Insulin receptor purified on wheat germ agglutinin as described in the text was preincubated with or without 10 n$\underline{\underline{M}}$ insulin in the presence or absence of 10 $\mu\underline{\underline{M}}$ H-ISP at 25°C for 1 h. Phosphorylation was assayed as described in the text using 8.5 μg protein and 0.1 mCi [γ-^{32}P]ATP per lane. For (B), samples were treated with anti-phosphotyrosine antibodies. Lanes 1 and 5, no addition; lanes 2 and 6, H-ISP (10 $\mu\underline{\underline{M}}$); lanes 3 and 7, insulin (10 n$\underline{\underline{M}}$); lanes 4 and 8, insulin (10 n$\underline{\underline{M}}$) and H-ISP (10 $\mu\underline{\underline{M}}$).

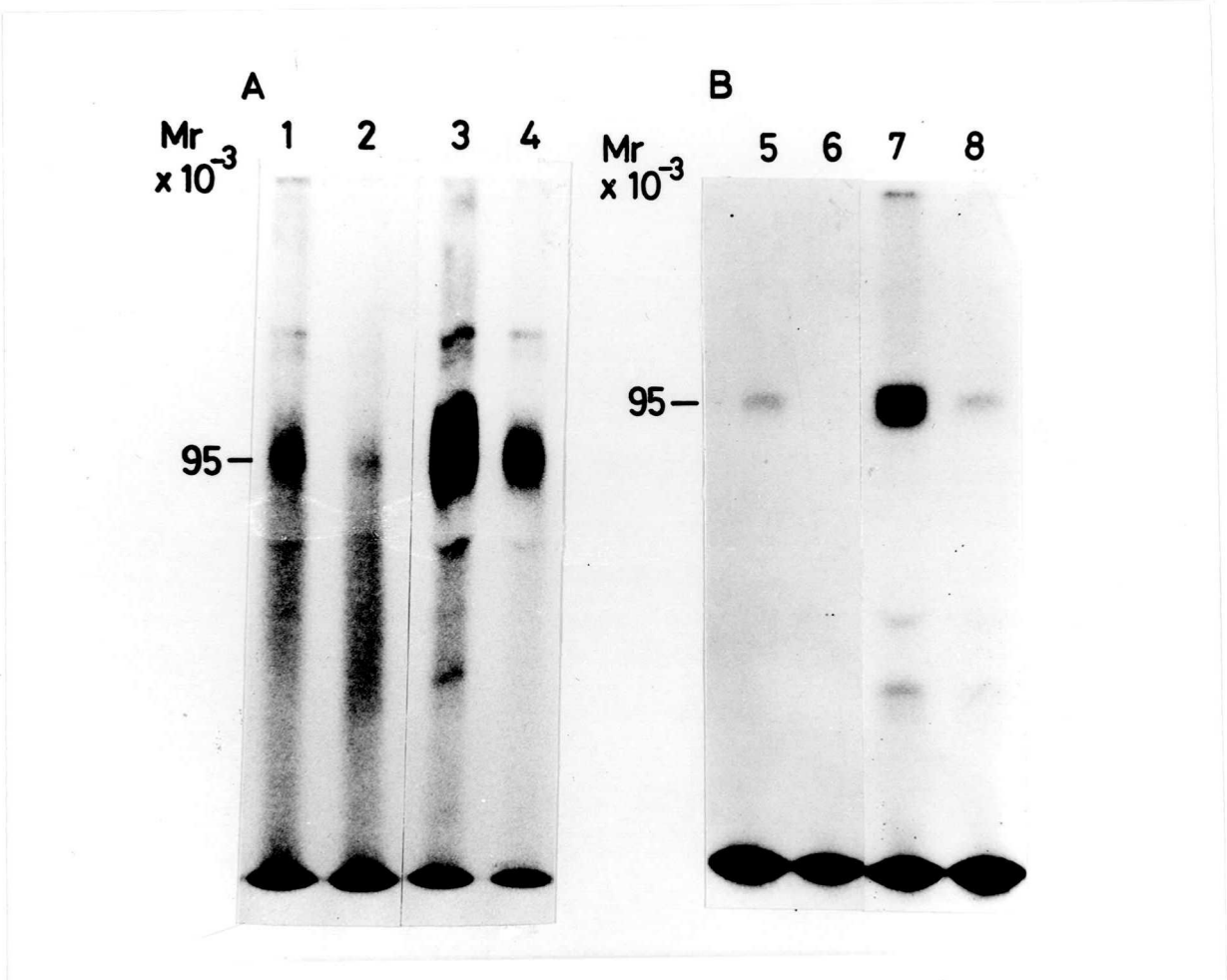

Fig. 23. Effects of the concentrations of H-ISP and insulin on phosphorylation of the insulin receptor. (A) Effect of H-ISP concentration. Samples of receptor fraction (8.5 μg) were pre= incubated with (lanes 2-5) or without (lane 1) 10 n$\underline{\underline{M}}$ insulin in the presence of increasing concentrations of H-ISP at 25°C for 1 h. Phosphorylation, immunoprecipitation, SDS-polyacrylamide (7.5%) gel electrophoresis, and autoradiography were performed as described in the text. (B) Effect of insulin concentration. After pre= incubation for 1 h with the indicated concentrations of insulin in the absence (lanes 1,3,5 and 7) or presence (lanes 2,4,6 and 8) of H-ISP (10 μ$\underline{\underline{M}}$), the solubilized insulin receptor (8.5 μg) was incubated for 1 h with 5 μ$\underline{\underline{M}}$ [γ-^{32}P]ATP, 2 m$\underline{\underline{M}}$ $MnCl_2$, and 10 m$\underline{\underline{M}}$ $MgCl_2$. Other experimental conditions were as described in the text.

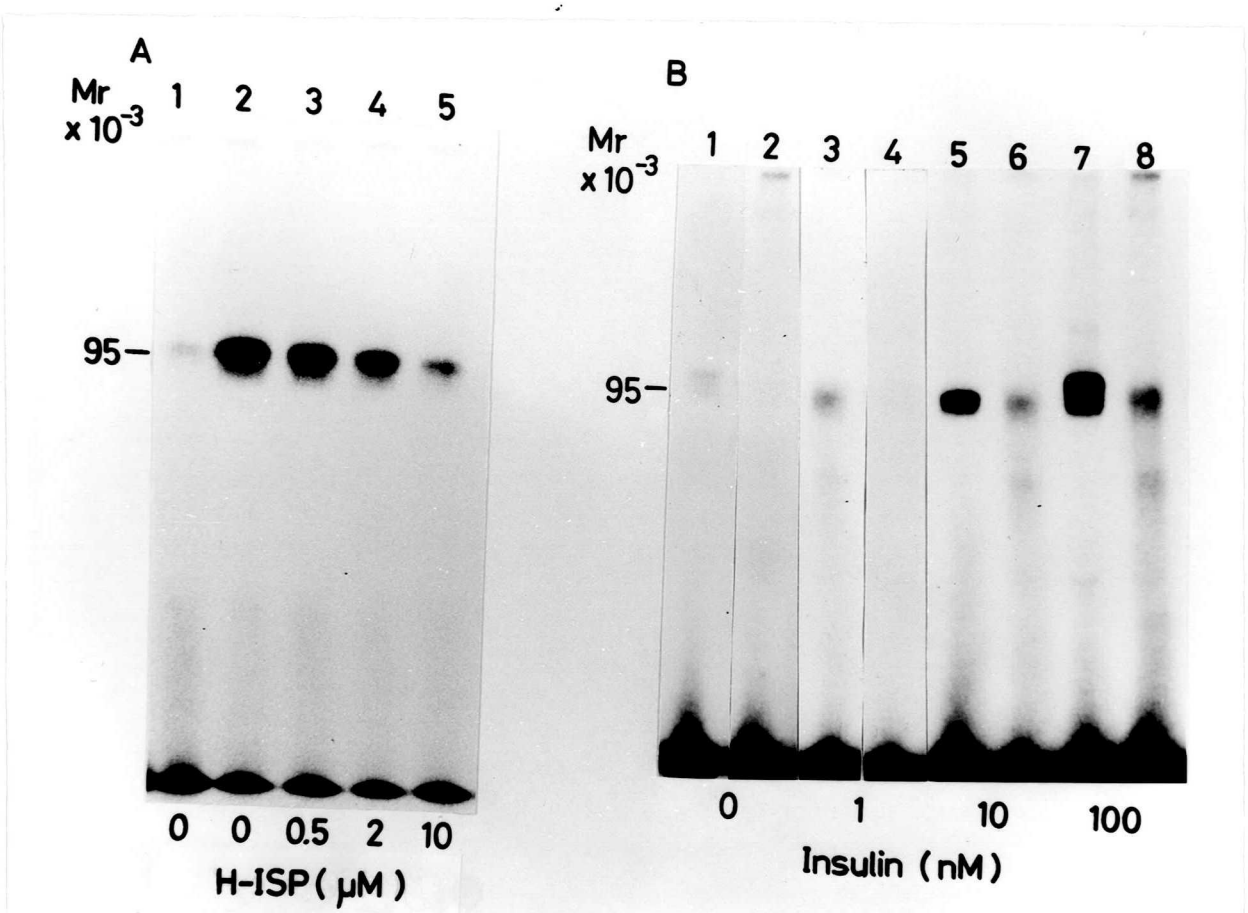

Fig. 24. Phosphorylation of H-ISP by the solubilized insulin receptor. Samples of receptor fraction (8.5 μg) were preincubated with 10 n$\underline{M}$ insulin in the presence (1) or absence (2) of 10 μ$\underline{M}$ H-ISP. Then, the phosphorylated and immunoprecipitated proteins were analyzed in one of the two following systems: A), a linear gradient of 4-18% acrylamide (acrylamide : bis-acrylamide=20 : 1); B), 20.9% acrylamide containing a trace amount of NaCl. Arrows indicate bands of H-ISP.

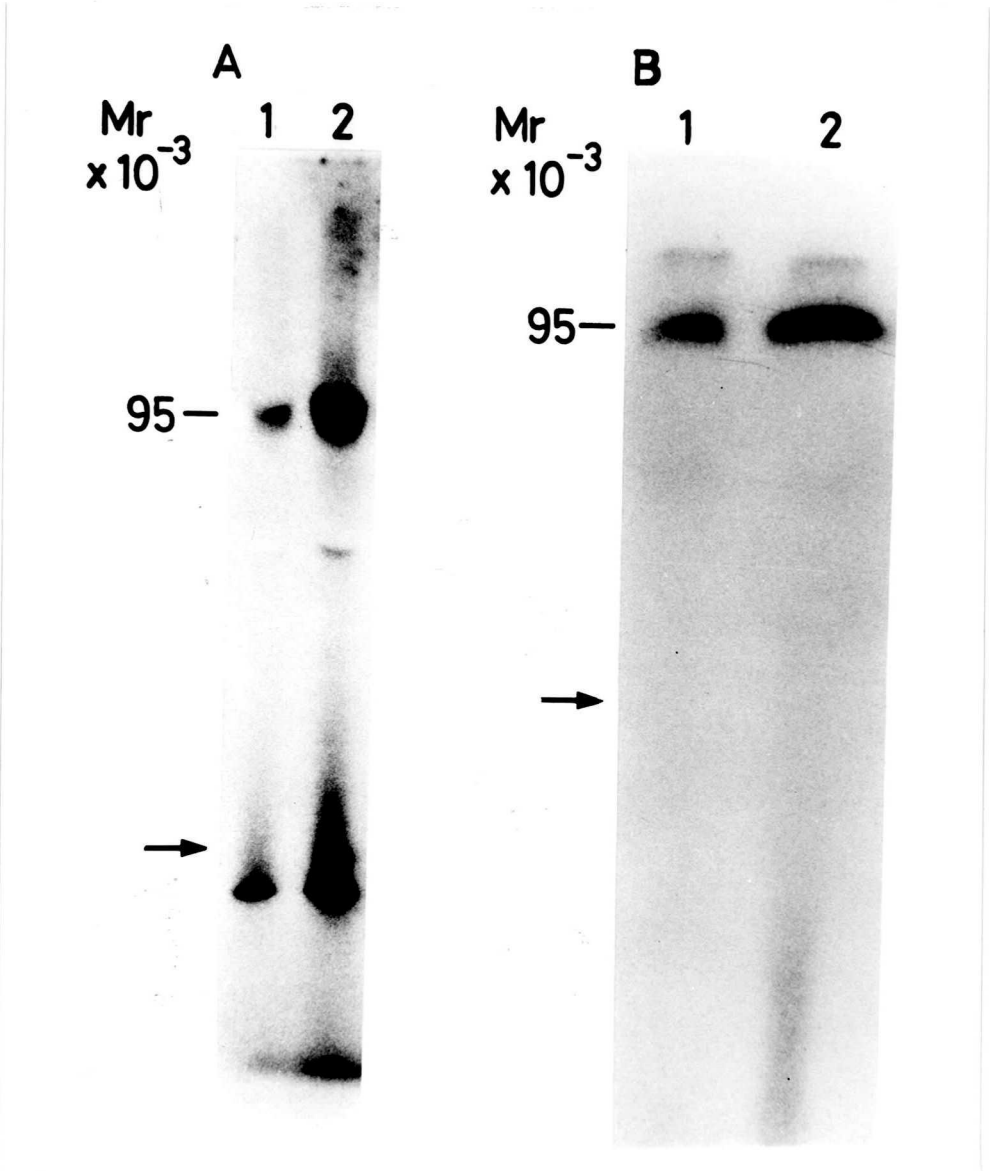

Fig. 25. Effect of H-ISP on dephosphorylation of insulin receptor. The receptor fractions (9 μg) were incubated without (lane 1) or with (lanes 2 to 6) insulin (100 n$\underline{\underline{M}}$) at 25°C for 1 h, and then incubated with [γ-^{32}P]ATP (5 μ$\underline{\underline{M}}$, 160 μCi/nmol), $MnCl_2$ (2 m$\underline{\underline{M}}$), and $MgCl_2$ (10 m$\underline{\underline{M}}$) for 15 min at 4°C. ATP was then added to a final concentration of 1 m$\underline{\underline{M}}$ with (lanes 4 and 6) or without (lanes 1,2,3 and 5) H-ISP (10 μ$\underline{\underline{M}}$) and the samples were incubated for 0 min (lanes 1 and 2), 15 min (lanes 3 and 4), or 60 min (lanes 5 and 6) at 4°C. The reaction was stopped as described in the text.