We have studied the metabolism of insulin to peptide intermediates which arise during the early stages of cellular hormone processing. [125I]iodoinsulin (prepared by direct radioiodination of the hormone) and [125I]iodotyrosylB]insulin (prepared by semisynthetic procedures and indirect radioiodination) were used as specific probes for the metabolic fates of the A and B chain domains of the hormone, respectively. Incubation of the A chain-labeled probe with isolated rat hepatocytes and subsequent analysis of the cell-associated products revealed low amounts of several insulin-degraded peptides. Mapping of these insulin fragments by chemical and enzymatic modification, electrophoresis, and autoradiography showed that they result from specific cleavages in both chains of the hormone. Two A chain-labeled fragments containing residues A14 to A20-21 in disulfide linkage to COOH-terminal portions of the insulin B chain were identified among the cell-associated metabolites. Other products contained intact A chains yet their elution positions during gel filtration were distinct from the position of [125I]iodoinsulin. Incubation of [125I]iodotyrosylB]insulin with hepatocytes resulted in cell-associated products containing a set of B chain-labeled fragments complementary to those described above. Structural analysis showed that these metabolites result from sequential cleavage of the insulin molecule at three sites between the interchain disulfide bonds of the B chain domain. Taken together, these A and B chain-labeled peptides form a series of cell-associated and structurally related metabolites. They likely represent the intermediate products which arise during the cellular processing of insulin.

The cellular metabolism of insulin proceeds through a receptor-mediated pathway involving endocytosis and cellular processing of the hormone. Biochemical studies using hepatocytes (1-4), adipocytes (5-7), and mononuclear blood cells (8) have shown that [125I]iodoinsulin is converted to low molecular weight, radiolabeled products (mostly [125I]iodotyrosine and 125I-) by a process which occurs subsequent to receptor binding and which likely involves lysosomal pro teasees. These findings have been extended through ultrastructural analysis which have directly examined the receptor-mediated endocytosis of insulin. In cultured fibroblasts, the hormone binds to insulin receptors which are diffusely distributed on the plasma membrane (9, 10). The insulin-receptor complexes rapidly aggregate to form cell surface patches; the patches, in turn, are internalized. As a consequence of internalization, the hormone becomes contained within endocytic vesicles and, eventually, within lysosomes. Similar conclusions have been obtained using electron microscopic autoradiography after incubation of 125I-insulin labeled with isolated hepatocytes (11, 12).

These ultrastructural and biochemical studies emphasize the participation of lysosomes in the ultimate conversion of insulin to low molecular weight products, but do not exclude the possibility that limited proteolysis of the hormone precedes its lysosomal degradation. For example, several investigators have shown that nonlysosomal enzymes isolated from target tissues can degrade insulin to well-defined peptides of relatively high molecular weight (13-16). Although prominent fragments of 125I-insulin have not been observed after incubation of the labeled probe with intact cells, the detection of these fragments is hindered by their potentially low steady state concentrations. Under the conditions of a typical cell incubation (10^-10 M insulin and 10^6 cells) only 10-20 fmol of the hormone would be cell-associated; even less would correspond to intermediates of hormone metabolism. In addition, the use of 125I-insulin as the radiolabeled probe has restricted detection of peptides to those containing the central or COOH-terminal portions of the A chain (see Ref. 17). Recent investigations describing the semisynthesis and receptor binding of [125I]iodotyrosylB]insulin (a B chain-labeled analog in which PheB is replaced by 125I-tyrosine) (4), however, now permit an assessment of the metabolic fate of the insulin B chain domain.

In order to study the role of limited proteolysis in the cellular metabolism of insulin, we have examined the processing of 125I-insulin and 125I-Tyr]insulin by isolated rat hepatocytes. Parallel use of these specifically labeled probes and analysis of cell-associated, radiolabeled products by enzymatic and chemical modification shows that insulin is processed through several cell-associated peptides which result from specific and limited proteolysis in both chains of the cell-bound hormone. The A chain domain of the insulin molecule is cleaved between amino acid residues LeuA11 and TyrA14, whereas the B chain domain is sequentially cleaved at three sites between its interchain disulfide bonds. These fragments form a series of related peptides which likely represent the intermediate products of cellular insulin metabolism.

**EXPERIMENTAL PROCEDURES**

Preparation of 125I-labeled Insulins—Porcine 125I-insulin (the A chain-labeled probe) was prepared by direct iodination using chloramine-T (18) and was purified by cellulose column chromatography (19) as described. However, iodination of the hormone was terminated by addition of N-acetyltirosine (1 μmol) rather than Na2S2O4. About

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The abbreviations used are: 125I-insulin, [125I]iodoinsulin; 125I-tyrosine, [125I]iodotyrosine; [125I]Tyr]insulin, (125I)iodotyrosylB]insulin.
RESULTS

Fig. 1, a and b, shows the gel filtration profiles of cell-associated radioactivity resulting from incubation of isolated hepatocytes with 125I-insulin (the A chain-labeled probe) at 30 and 37°C, respectively. The two profiles are similar with about 80% of the radioactivity eluting at the position taken by an 125I-insulin standard (fractions 65-90). Smaller amounts of radioactivity eluted at the void volume of the column (fractions 30-40, <1% of total cell-associated radioactivity) and as a partially resolved mixture of low molecular weight degradation products (fractions 122-180). Material appearing between fractions 140 and 150 (about 4% of the total) and between fractions 155 and 167 (about 6% of the total) eluted at the positions of 125I-tyrosine and Na125I standards, respectively; the identities of these components were confirmed by paper electrophoresis in 50% formic acid. Three regions of 125I-insulin-derived radioactivity eluted from 125I-tyrosine and 125I-tryptophan (corresponding to fractions 50-66, 90-105, and 122-140) were also apparent in the gel filtration profiles of Fig. 1, a and b. Components present in regions IA, IIA, and IIB (each comprising from 1 to 5% of the total, cell-associated radioactivity) were 90, 80, and 70% precipitable by 10% trichloroacetic acid, respectively. This result suggests the peptide nature of the 125I-labeled material and indicates the multiplicity of products generated during the cellular processing of 125I-insulin. Control experiments showed that incubation of 125I-insulin with the collagenase used for preparation of the cells did not generate any radiolabeled product, despite an enzyme concentration 1000 times greater than that which could have been present in the final cell preparation.

Further studies examined the structures of the cell-associated components identified above. Material corresponding to pools IA, IIA, and IIB was first subjected to Edman degradation to identify the positions of 125I-tyrosine within the insulin-derived fragments. As shown in Table I, a mock cycle of Edman degradation in the absence of phenylisothiocyanate released only negligible amounts of radioactivity from all three pools. The first cycle in the presence of phenylisothiocyanate released about 2, 14, and 32% of the radioactivity from pools IA, IIA, and IIB, respectively. No further release was seen during the second cycle. Since control studies (Table I) showed that one cycle of Edman degradation (a) removed 90% of the radioactivity from 125I-Tyr insulin (the probe with internally labeled tyrosines), and (b) removed no radioactivity from 125I-insulin (the probe with internally labeled tyrosines), the results of the Edman analysis indicate that portions of the radioactivity associated with peptide metabolites IIA and IIB result from NH2-terminal 125I-tyrosine.

As shown in the upper panel of Fig. 2, oxidative sulfotylsis of the peptide metabolite in pool IIA (a procedure which modifies peptides containing disulfide bonds by converting cystine residues to cysteic acid sulfonates; see Ref. 21) altered the electrophoretic mobility of the native material (lane 2) and generated a radiolabeled (therefore, A chain-derived) product with no net change at the low pH of the electrophoretic system (lane 1). This change in mobility demonstrates the presence of cysteine and, in conjunction with the results of the Edman analysis, indicates that the A chain-derived sequence of this radiolabeled metabolite is either Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-(Asn) or Tyr-Cys-(Asn). Residues 14 to 20-21 or 19 to 20-21 of the insulin A chain, respectively. In either case, the sulfotylized material would be electrophoretically neutral. To differentiate between these structures, the
were extracted with acetic acid, the four extracts were pooled, and incubated with hepatocytes for 30 min.

The chain that is radiolabeled in the peptide.

The percentage of total radioactivity extracted into butyl acetate during cycle 1 indicates the percentage of total \(^{125}\)I-tyrosine which occurs at the NH\(_2\)-terminal position.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sequence cycle</th>
<th>Butyl acetate-extractable radioactivity % total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool IA</td>
<td>0</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>Pool IIA</td>
<td>0</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>13.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>Pool IIA</td>
<td>0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>31.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.3</td>
</tr>
<tr>
<td>(^{125})I-[\text{Tyr}]insulin</td>
<td>0</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>87.2</td>
</tr>
<tr>
<td>(^{125})I-insulin</td>
<td>0</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2.2</td>
</tr>
</tbody>
</table>

peptide was treated with \(S. aureus\) protease, an enzyme which cleaves only at the COOH-terminal side of acidic residues (22). Since the electrophoretic mobility of the native material (lane 2) was sensitive to digestion with the enzyme (lane 3), a glutamic acid residue is present in the peptide. (Insulin lacks aspartic acid.) Moreover, the migration of the enzymatically treated peptide after oxidative sulfitolysis (lane 4) differed from that of the peptide treated by sulfitolysis alone (lane 1), indicating that glutamic acid occurs within the A chain-derived sequence. The A chain sequence is therefore \(\text{Tyr-Leu-Asn-Asp-Cys-(Asn)}\), residues A14 to A20-21. (Although small amounts of \(^{125}\)I are covalently bound to TyrB\(^{26}\) and TyrB\(^{27}\) in \(^{125}\)I-insulin, the results of Fig. 2 are consistent only with the radiolabeled peptide being derived from the insulin A chain. A radiolabeled B chain-derived fragment containing NH\(_2\)-terminal \(^{125}\)I-tyrosine, cysteine, and a site sensitive to \(S. aureus\) protease (a peptide having residues B16 to B22-30) would necessarily be cationic in 30% formic acid after oxidative sulfitolysis.)

Importantly, lane 4 of the upper panel of Fig. 2 also shows that the A chain-derived products obtained after sulfitolysis of the \(S. aureus\) protease-treated peptide IIA were resolved into two components, one of which migrated toward the cathode and one of which was electrophoretically neutral. Since these electrophoretic mobilities are in agreement with those expected for the reaction products A14 to A17 (\(\text{Tyr-Asp-Asn-Asp-Cys-(Asn)}\), and A18 to A20-21 [Asn-\(\text{Tyr-SulfoCys-(Asn)}\), respectively, and since control studies showed that the insulin carrier present in the reaction mixture was digested by the enzyme and converted to the S-sulfo derivative, the radiolabel in this insulin metabolite is likely distributed between the two tyrosine residues (A14 and A19) of its A chain-derived sequence. This distribution of \(^{125}\)I would be consistent with the results of the Edman degradation and with the data of others.

### TABLE I

Radiosequencing of A chain-labeled insulin metabolites

The amounts of NH\(_2\)-terminal \(^{125}\)I-tyrosine in \(^{125}\)I-[\(\text{Tyr}\])insulin, \(^{125}\)I-insulin, and the A chain-labeled insulin metabolites were determined by Edman degradation. Column fractions shown by the horizontal lines of Fig. 1a were combined to yield pools I, II, and III. Each pool, as well as standards of \(^{125}\)I-insulin and \(^{125}\)I-[\(\text{Tyr}\])insulin, were dried under vacuum in the presence of 50 nmol of native insulin carrier. The purification of pool IIIA (by extraction in dimethylformamide) and conditions for radiosequencing are described under “Experimental Procedures.” Each sample (containing 300-500 cpm for the metabolites and 1000-2000 cpm for the standards) was conditioned prior to analysis by performing a mock cycle of Edman degradation in the absence of phenylisothiocyanate. The percentage of total radioactivity extracted into n-butyl acetate without prior treatment with phenylisothiocyanate (cycle 0) or with treatment with phenylisothiocyanate (cycles 1 and 2) is shown below each for peptide. The percentage of total radioactivity extracted into butyl acetate during cycle 1 indicates the percentage of total \(^{125}\)I-tyrosine which occurs at the NH\(_2\)-terminal position.
enzymatic modifications as described under "Experimental Procedures;" the radiolabeled reaction products were then analyzed by paper electrophoresis in 30% formic acid and autoradiography. The upper panel shows, from left to right, the migrations of the sulfitolyzed peptide (lane 1), the native peptide (lane 2), the peptide treated with S. aureus protease (lane 3), and the peptide treated with S. aureus protease prior to sulfitolysis (lane 4), respectively. The horizontal arrows of the upper panel indicate, from top to bottom, the position taken by insulin, the position taken by the sulfitolyzed insulin B chain, the origin of the electrophoresis, and the position taken by the sulfitolyzed insulin A chain, respectively. The lower panel shows, from left to right, the migration of the native peptide (lane 1), the peptide treated with carboxypeptidase B (lane 2), and the peptide treated sequentially with trypsin and carboxypeptidase B (lane 3). The direction of electrophoresis in the lower panel was from anode to cathode.

As shown in Fig. 1, a and b, incubation of hepatocytes with $^{125}$I-insulin generates radiolabeled material which elutes as a broad descending shoulder on the peak of $^{125}$I-insulin (pool II). Fractions 90-105 (which contain products having a wide range of molecular weight) were chosen for study both to minimize contamination by material in the peak of $^{125}$I-insulin, and to include as many insulin-derived metabolites as possible. Fig. 3 shows an electrophoretogram of these A chain-labeled products. The native material (lane 2 of the upper panel of Fig. 3) migrated toward the cathode and consisted of a major and minor component. The electrophoretic mobilities of the two components were sensitive to both reduction and oxidative sulfitolysis (lanes 1 and 3 of the upper panel of Fig. 3, respectively); thus, these labeled insulin derivatives, like those in pool IIIA, represent peptides with at least one disulfide bond.

on the position of label in $^{125}$I-insulin (see Ref. 17). Nevertheless, these findings do not imply that both tyrosine residues are iodinated in any one insulin molecule.

Since the electrophoretic mobility of the insulin-derived peptide was sensitive to oxidative sulfitolysis (lane 1 of the upper panel of Fig. 2) as well as to reduction with dithiothreitol (data not shown), the radiolabeled A chain-derived portion of this metabolite (corresponding to residues A14 to A20-21 of the native hormone) is in disulfide linkage to an unlabeled B chain domain. The lower panel of Fig. 2 shows that the electrophoretic mobility of the peptide (lane 1) was also sensitive to sequential treatment with trypsin and carboxypeptidase B (lane 3). This result demonstrates that the B chain domain of the metabolite contains Arg$^{B22}$. As the electrophoretic mobility of the native material was not sensitive to treatment with the carboxypeptidase B alone (lane 2 of the lower panel of Fig. 2), the structure of the B chain domain must extend beyond Arg$^{B22}$ and include, at least, the sequence Cys-Gly-Glu-Arg-Gly, residues B19 to B23 of insulin.

Fig. 2. Structural analysis of the A chain-labeled insulin metabolite in pool IIIA. Fractions 122-140 of the profile shown in Fig. 1a were pooled and divided into aliquots for chemical and enzymatic modifications as described under "Experimental Procedures;" the radiolabeled reaction products were then analyzed by paper electrophoresis in 30% formic acid and autoradiography. The upper panel shows, from left to right, the migrations of the sulfitolyzed peptide (lane 1), the native peptide (lane 2), the peptide treated with S. aureus protease (lane 3), and the peptide treated with S. aureus protease prior to sulfitolysis (lane 4), respectively. The horizontal arrows of the upper panel indicate, from top to bottom, the position taken by insulin, the position taken by the sulfitolyzed insulin B chain, the origin of the electrophoresis, and the position taken by the sulfitolyzed insulin A chain, respectively. The lower panel shows, from left to right, the migration of the native peptide (lane 1), the peptide treated with carboxypeptidase B (lane 2), and the peptide treated sequentially with trypsin and carboxypeptidase B (lane 3). The direction of electrophoresis in the lower panel was from anode to cathode. 

Fig. 3. Structural analysis of the A chain-labeled insulin metabolites in pool II. Fractions 90-105 of the profile shown in Fig. 1a were combined and distributed into aliquots for chemical and enzymatic modifications as described under "Experimental Procedures;" the radiolabeled reaction products were analyzed by paper electrophoresis in 30% formic acid and autoradiography. The upper panel shows, from left to right, the electrophoretic mobilities of the peptides in pool II after treatment with dithiothreitol (lane 1), the untreated peptides of pool II (lane 2), the peptides in pool II after oxidative sulfitolysis (lane 3), the peptides in pool II digested with S. aureus protease prior to oxidative sulfitolysis (lane 4), and the peptide of pool IIIA digested with S. aureus protease prior to oxidative sulfitolysis (lane 5), respectively. The horizontal arrows of the upper panel indicate, from top to bottom, the position taken by insulin, the position taken by the sulfitolyzed B chain of insulin, the origin of the electrophoresis, and the position taken by the sulfitolyzed A chain of insulin, respectively. The minor band in lane 2 (which is discussed in the text) is identified by an arrowhead. The lower panel shows, from left to right, the electrophoretic mobilities of $^{125}$I-insulin (lanes 1 and 2), $^{125}$I-insulin after oxidative sulfitolysis (lanes 3 and 4), and $^{125}$I-insulin digested with S. aureus protease prior to oxidative sulfitolysis (lanes 5 and 6), respectively. The horizontal arrow of the lower panel shows the origin of the electrophoresis.
The major product obtained after oxidative sulfitolysis of the material in IIA migrated with the sulfitolyzed A chain of $^{125}$I-insulin during electrophoresis (Fig. 3, lane 3 of the upper panel), indicating that the majority of the insulin derivatives in IIA contained intact A chains. However, about 10% of the sulfitolyzed reaction products were electrophoretically neutral (Fig. 3, lane 3 of the upper panel). The existence of this neutral material was in contrast to the results obtained after oxidative sulfitolysis of $^{125}$I-insulin (Fig. 3, lanes 3 and 4 of the lower panel) and suggested that a portion of the metabolites in IIA contained A chains which had been proteolytically modified. Moreover, the electrophoretic neutrality of the modified A chain after sulfitolysis was consistent with the minor component containing the peptide Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-(Asn) (residues A14 to A20-21), as described above for the component in IIIA. (See lane 1 of the upper panel of Fig. 2.) Lane 4 of the upper panel of Fig. 3 shows that sulfitolysis of the products obtained from S. aureus protease digestion of the peptides in pool IIA generated, in addition to the characteristic S. aureus protease peptides of intact A chain (Fig. 3, lanes 5 and 6 of the lower panel), a cationic peptide having an electrophoretic mobility indistinguishable from that of the Tyr$^{14}$-Glu$^{17}$ fragment formed by similar treatment of the peptide present in pool IIIA (Fig. 3, lane 5 of the upper panel).

When Edman degradation was performed on an aliquot of the untreated material comprising IIA, about 14% of the radioactivity associated with the peptide was specifically removed in the first cycle (Table 1). This value corresponds well to the percentage of cleaved A chain as suggested by the autoradiogram of Fig. 3. Taken together, these results indicate that the insulin-derived peptides of IIA contain two forms of A chain: the major form is intact, but 10-15% of the material is proteolytically modified. Proteolytic events which produce the modified A chain yield the same A14 to A20-21 fragment that is present in the metabolite of IIIA. Since the molecular weights of the peptides in IIA are greater than the molecular weight of the peptide in IIIA (see Fig. 1a), the A chain-derived product in this descending shoulder of cell-associated $^{125}$I-insulin likely represents a metabolic intermediate in the cell-mediated conversion of insulin to the fragment of pool IIIA.

Cell-associated products arising from the interaction of $^{125}$I-[Tyr]insulin with hepatocytes (like those arising from $^{125}$I-insulin) appeared as a descending shoulder on the peak of radiolabeled hormone after gel filtration (Fig. 1c, pool IIIB). These peptides, which represent about 9% of the cell-associated radioactivity, are sensitive to oxidative sulfitolysis, but their B chain domains are insensitive to digestion with S. aureus protease (Fig. 4, upper, and Ref. 4). The peptide of lowest mobility after sulfitolysis has already been identified as extending from residue B1 (the position of the radiolabel) to a position between Cys$^{47}$ and Glu$^{111}$ (4). The electrophoretic mobility of the most rapidly migrating peptide, and its failure to be converted to the peptide B1-B13 by digestion with S. aureus protease, indicates that it too is not extended beyond residue B13. The co-migration of the peptide of intermediate mobility with the sulfitolyzed B1-B13 standard (Fig. 4, upper) precludes an easy determination of its length beyond residue B13. However, only two sulfitolyzed, B chain-derived peptides containing the requisite NH$_2$-terminal $^{125}$I-tyrosine residue would have the same charge as sulfitolyzed B1-B13 in 30% formic acid. These peptides (which might migrate with the B1-B13 standard) are sulfitolyzed B1 to B10-18 and sulfitolyzed B1 to B22-28. The latter peptide, B1-B22, was prepared by tryptic digestion and oxidative sulfitolysis of $^{125}$I-[Tyr]insulin, its electrophoretic mobility in 30% formic acid was less than that of intact, sulfitolyzed B chain (data not shown), whereas the sulfitolyzed metabolite of intermediate mobility migrated further than the sulfitolyzed B chain standard (see Fig. 4). Since the presence of amino acid residues B23 to B28 (Gly-Phe-Phe-Tyr-Thr-Pro) would not be expected to increase the mobility of the B1-B22 peptide in this electrophoretic system, the observed migration of the B chain derivative with intermediate mobility is consistent with its containing residues B1 to B10-B18 (4$^{125}$I-Tyr-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-(Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val]).

In order to examine the time-dependent formation of these B chain-labeled derivatives, hepatocytes were incubated with $^{125}$I-[Tyr]insulin for selected periods and the cell-associated products appearing in pool IIIB were analyzed as described above. The first metabolite to appear as a component in the cellular metabolism of $^{125}$I-[Tyr]insulin resulted in the sulfitolyzed peptide of intermediate mobility (Fig. 4, lane 1 of the lower panel; 5 min of incubation of $^{125}$I-[Tyr]insulin with hepatocytes). The second peptide to appear was the most rapidly migrating. It became predominant after 10 min of incubation of the B chain probe with the cells (Fig. 4, lane 2 of the lower panel). By 20 min of incubation (Fig. 4, lane 3 of

**Fig. 4. Structural analysis and kinetics of appearance of the B chain-labeled insulin metabolites in pool IIIB.** Fractions 90-105 of the profile shown in Fig. 1c were combined, distributed into aliquots, and dried in the presence of native insulin as carrier. These peptides, as well as standards derived from $^{125}$I-[Tyr]insulin, were subjected to chemical and enzymatic modifications, electrophoresis in 30% formic acid, and autoradiography as described under "Experimental Procedures." The upper panel shows, from left to right, the electrophoretic migrations of $^{125}$I-[Tyr]insulin after oxidative sulfitolysis (lane 1), the material in pool IIIB after oxidative sulfitolysis (lane 2), $^{125}$I-[Tyr]insulin digested with S. aureus protease prior to sulfitolysis (lane 3), and the material in pool IIIB digested with S. aureus protease prior to sulfitolysis (lane 4), respectively. The lower panel shows the time-dependent appearance of the three B chain-labeled metabolites of pool IIIB. $^{125}$I-[Tyr]insulin was incubated with isolated hepatocytes for selected periods of time. Acetic acid was added to collected cells, and the extracted, radiolabeled peptides were analyzed by gel filtration as described for Fig. 1. For each time point, the radiolabeled material corresponding to pool IIIB of Fig. 1c was pooled and dried in the presence of insulin carrier. Each sample was subjected to oxidative sulfitolysis and the radiolabeled products were analyzed by paper electrophoresis in 30% formic acid and autoradiography. (See "Experimental Procedures" for details.) Lanes 1 to 4 show, from left to right, the B chain-labeled peptides appearing in pool IIIB after 5, 10, 20, and 30 min of incubation of the $^{125}$I-[Tyr]insulin with isolated hepatocytes, respectively. The direction of electrophoresis in both panels was from anode to cathode.
the lower panel), the sulfotolysed B chain derivative that migrated most slowly appeared as a major form. By 30 min of incubation, this peptide had become the predominant B chain-labeled peptide present in pool IIB (Fig. 4, lane 4 of the lower panel). As expected from the kinetics of continuous pulse experiments, the early peptides remained detectable at all incubation periods, but their relative contribution to the total array of B chain-derived metabolites decreased during the more prolonged incubations. A similar analysis applied to the A chain-labeled peptide of pool IIIA showed that the modified and intact A chain forms were detectable at both early and late time points.

The time course of appearance of the three B chain-labeled metabolites described above provides information which supports the proposed structures of these products. In such pulse experiments, the first product to appear likely results from the initial proteolytic event (or from the event that immediately proceeds the rate-limiting step). As shown in the lower panel of Fig. 4, the metabolite resulting from this event yields the sulfotolysed B chain derivative of intermediate mobility (\( ^{125}\)I-Tyr-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-(Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val), B1 to B10-18; see above). Continued cellular processing of this product would result in the time-dependent formation of more extensively degraded forms. The greater electrophoretic mobility of the second major product to appear is, in fact, consistent with its arising from secondary proteolysis of the B1 to B10-18 derivative. (Removal of noncationic amino acid residues from the COOH-terminal region to the B1 to B10-18 peptide would result in an increased charge to mass ratio.) The mobility and time course of appearance of this peptide, as well as the constraints on its structure (determined by the data of Fig. 4, upper) suggest that its NH\(_2\)-terminal B chain domain extends from residue B1 to B10-12 \( ^{125}\)I-Tyr-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-(Leu-Val). Finally, continued proteolysis at the COOH-terminal region of the B1 to B10-12 fragment would remove His\(^{118}\) and generate a peptide product with decreased cationic charge. Such proteolysis is consistent with the electrophoretic migration of the last B chain derivative to appear during the time course. Note, however, that the Cys\(^{87}\) residue must be retained in this metabolite (as well as in the other two). Therefore, the NH\(_2\)-terminal portion of its B chain domain likely extends from residue B1 to B7-9 \( ^{125}\)I-Tyr-Val-Asn-Gln-His-Leu-Cys-(Gly-Ser). \( ^{125}\)I-Insulin.

The largest of the three insulin-derived metabolites identified in Fig. 1 (pool IA, Fig. 1a) eluted as an ascending shoulder on the peak of cell-associated \( ^{125}\)I-insulin. Examination of the structure of this material by paper electrophoresis in 30% formic acid (Fig. 5) showed that the native peptide (lane 2, upper) migrated slightly further than an \( ^{125}\)I-insulin standard (lane 1, upper). The migration of the peptide after chemical reduction (lane 4, upper), however, was indistinguishable from that of the reduced insulin A chain (lane 3, upper). Similarly, the S-sulfo derivatives of the peptide in pool IA and \( ^{125}\)I-insulin had identical electrophoretic mobilities (not shown). Further experiments showed that (a) \( S. aureus \) protease digestion of the metabolite prior to oxidative sulfotolysis yielded products (Fig. 5, lane 2 of the lower panel) which corresponded to the sulfotolysed, \( S. aureus \) protease peptides of intact \( ^{125}\)I-insulin (Fig. 5, lane 4 of the lower panel) rather than to the products obtained after similar modification of peptide IIIA (Fig. 5, lane 3 of the lower panel), (b) the metabolite did not contain NH\(_2\)-terminal \( ^{125}\)I-tyrosine (Table 1), and (c) the mobility of the metabolite during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (23) was the same as that of \( ^{125}\)I-insulin (not shown). Finally, gel filtration of the cell-associated products resulting from incubation of \( ^{125}\)I-[Tyr]insulin with hepatocytes failed to reveal a radiolabeled compound eluting as an ascending shoulder on the peak of intact hormone (Fig. 1c). Taken together, these findings indicate that pool IA contains an insulin metabolite having an intact A chain domain and a B chain domain which lacks a portion of its NH\(_2\)-terminal region.

**DISCUSSION**

Experiments described here have examined the metabolism of insulin by hepatocytes using hormone concentrations within the normal physiological range (2 to 5 \( \times \) \( 10^{-9} \) M). Since the amount of receptor-bound insulin under these conditions is only 10\(^{-21}\) to 10\(^{-20}\) mol/cell, analysis of the peptide products of cellular insulin metabolism cannot rely on conventional or micro approaches to structure determination. Our parallel use of \( ^{125}\)I-insulin and \( ^{125}\)I-[Tyr]insulin, combined with peptide mapping at the radiochemical level, however, has permitted the identification and characterization of A and B chain-
Peptide Products of Insulin Metabolism

derived peptides arising from cellular processing of the hormone.

Fig. 6 shows a model of cellular insulin metabolism which depicts a likely sequence in the time-dependent formation of the insulin-derived peptides described under "Results." As illustrated, the native hormone (compound 1) is cleaved at a peptide bond between the interchain disulfide bonds of the B chain to yield compound 2 (see Figs. 4 and 3). Processing near the newly formed COOH terminus of the split insulin derivative would result in the sequential appearance of metabolites with more extensively modified B chain domains, compounds 3 and 4 (see Fig. 4). Cleavage in the A chain domain of compound 4 (between residues LeuA13 and TyrA14) would then yield compounds 5 and 6 (see Fig. 2) which represent the NH2- and COOH-terminal domains of the insulin molecule as a whole, respectively. Alternatively, compound 2 (rather than compound 4) could be the substrate for the A chain-cleaving enzyme. Proteolysis of compound 2 between LeuA13 and TyrA14 would yield compounds 6 and 7 (see Figs. 2 and 4). The B chain domain of compound 7, which is identical with that of the NH2-terminal B chain domain of compound 2, could then be processed in a time-dependent manner to yield the two more extensively degraded insulin metabolites represented by compounds 8 and 5 (see Fig. 4). Thus, processing of insulin through either of the two branches of the pathway would ultimately yield the same peptide products.

The model of insulin metabolism presented in Fig. 6 is consistent with three important experimental observations. First, the time-dependent shortening of the NH2-terminal B chain domain in the overall metabolism of insulin could easily occur in either branch of the pathway. Second, the intact and split A chains forms would be present after both short (5-10 min) and long (20-30 min) periods of incubation due to hormone processing through the left and right branches of the model, respectively. Third, no product of insulin metabolism having an intact B chain would be detectable (see Figs. 4 and 5 and Ref. 4).

Two additional insulin metabolites (IA and the minor component of IIA) have not been included in the model of Fig. 6. The peptide in pool IA contains an intact A chain in disulfide linkage to a B chain which apparently lacks a portion of its NH2-terminal region. Its elution position during gel filtration (see Fig. 1) is consistent with its having undergone an additional proteolytic cleavage between the interchain disulfide bonds of its B chain thereby producing a molecule with decreased globular character and increased Stokes radius. The minor component in pool IIA resembles compound 6 of Fig. 6 in containing NH2-terminal 125I-tyrosine (residue A14 of insulin) and the COOH-terminal half of the insulin A chain. Its elution position during gel filtration (see Fig. 1) suggests that it, too, contains a portion of the B chain domain of insulin. An important question concerns the structure of the COOH-terminal region of the B chain domains in the insulin fragments identified above. Pools of metabolites IA, IIA, and IIB from gel filtration columns, unlike pools of metabolite IIA, contained material which inhibited the activities of both trypsin and carboxypeptidase B (as assessed by the failure of native insulin to be modified when it was mixed with the column pools and the above enzymes). Thus, methods useful for analysis of intermediate IIA could not be applied to determine the presence or absence of Argβ22 in these partially degraded forms of the hormone.

The site at which insulin is cleaved to generate the A chain-labeled fragment (LeuA13-TyrA14) is not easily assignable in terms of known enzyme specificities. It represents an acceptable site for a chymotryptic-like cleavage, but no more so than, for example, the bonds between TyrA14 and GlnA15, LeuA16 and GlnA17, or TyrA19 and CysA20. The specificity of this cleavage may well reside in elements of secondary or tertiary structure which limit enzymatic susceptibility when insulin is in association with its receptor or an intracellular organelle. An early cell-mediated cleavage in the B chain domain of the hormone between residues B10 and B18, however, is consistent with the ascribed specificity of insulin protease, a nonlysosomal enzyme which has been purified from kidney, muscle, and liver and which cleaves insulin between TyrB16 and LeuB17 in cell-free enzymes (16).

Sites for the proteolytic cleavage of insulin to the metabolites described here are identified on the crystallographically determined insulin structure of Fig. 7. Notably, these sites are segregated to a single domain of the insulin molecule. Whether considering loss of the NH2-terminal portion of the B chain (as in peptide IA), cleavage within the central domain of the A chain (as in the minor component of IIA and in the peptide of IIA), or the likely initial site for cleavage between the interchain disulfide bonds of the B chain (as in peptides IA and IIB), the sites for proteolytic modification are well separated from that portion of insulin which plays an important role in determining its receptor binding and biological activities. The proximity of these sites to each other and their separation from the receptor-binding region suggest that specific interactions of insulin with cellular components may well direct the specificity of insulin-degrading enzymes.

Experiments described here demonstrate that the cellular processing of insulin generates a series of peptide products which result from proteolysis at specific sites in both chains.

![Fig. 6](image-url)
Peptide Products of Insulin Metabolism

Our structural characterization of the peptide metabolites of insulin and placement of the metabolites in a sequential model for insulin processing permit further consideration of both the biochemical and cellular mechanisms which direct the metabolism of insulin and the uniqueness of this pathway for insulin degradation.

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REFERENCES


Fig. 7. Sites for limited proteolysis of insulin during its metabolism by hepatocytes. The structure of insulin shown represents molecule 2 of the two-zinc insulin hexamer viewed in a direction perpendicular to the 3-fold axis; it is derived from the crystallographic studies of Blundell et al. (17). The peptide backbones of the A and B chains are shown in thin and thick lines, respectively, and the sulfur atoms of the intra- and interchain disulfide bonds are shown by filled circles. Side chains of selected residues (LeuAs, TyrAl, PheBl, TyrB16, and LeuB15), are included on the right. Vertical arrows indicate peptide bonds likely cleaved during the early stages of insulin metabolism as described under "Results." The top arrow indicates the cleavage resulting in loss of the NH2-terminal portion of the B chain (in peptide IA); the middle arrow indicates the cleavage between LeuAl and TyrAl (resulting in the minor component of IIA and the peptide of IIIA); the bottom arrow indicates the cleavage between TyrBl and LeuB15 (the site of insulin protease action (16) and a likely site of cleavage resulting in peptide IA and the largest component of the peptides in IIB). The shaded area on the left shows that portion of the insulin molecule thought to be most important in conferring receptor binding specificity and affinity (26).