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STUDIES ON ISOLATED ISLETS OF LANGERHANS (BROCKMANN BODIES) OF TELEOST FISHES

II. EVIDENCE FOR INSULIN BIOSYNTHESIS *IN VITRO*

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SUMMARY

Isolated islet tissue of toadfish (*Opsanus tau*) has been incubated *in vitro* and the incorporation of radioactive substrates into islet tissue protein has been studied. Using ^{14}C-labeled substrates, high specific activities were found in insulin which was purified from crude extracts by a combination of paper chromatography, isoelectric precipitations and cellulose adsorption. The highest specific activity of insulin labeled with ^{14}C achieved in these studies was $5.7 \cdot 10^5$ counts/min/mg. The biologic activity of this preparation was 7.7 units/mg. Using [^{14}C]isoleucine as radioactive substrate, preferential labeling of the A chain of insulin could be demonstrated, as would be expected from the absence of isoleucine from the B chain.

INTRODUCTION

In recent years, a large body of information has been gathered on the mechanism of protein biosynthesis. Major attention has been devoted, and is still being devoted to the pathways which lead from amino acids to completed protein, and to the mechanisms by which the genetic information stored in DNA is transcribed into the specific amino acid sequence of the finished protein. Less effort has been devoted to the question of how peptide chains are linked with each other through, for instance, disulfide bridges, and essentially nothing is known of the intimate mechanisms by which rates of synthesis are regulated in accord with biologic needs.

The time sequence and the intricate processes by which the amino acid chains are put together in the proper three-dimensional array are best studied in systems capable of synthesizing few or, preferably only one protein of precisely known structure. The hemoglobin-synthesizing reticulocytes are as yet the only system described fulfilling these requirements. Since the structure of insulin is precisely known, being made up of two peptide chains linked by two inter-chain disulfide bridges[1,2] and since insulin requires a precise regulation of its rate of synthesis and its rate of

Abbreviations: POPOP, 5-phenyloxazole; PPO, 2,5-diphenyloxazole.

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secretion, insulin biosynthesis might similarly provide a suitable system for study. However, the study of insulin biosynthesis is complicated by the fact that islet tissue represents only 1/60000 of the entire body mass and only approx. 0.01 of the mass of the exocrine pancreas, in which the islets of Langerhans are imbedded. The study of insulin biosynthesis *in vivo* is thus limited by the requirement for large amounts of labeled amino acids of very high specific activity[3], while protein synthesis *in vitro* in homogenates or cell-free systems of mammalian pancreas have not, as yet, been observed*, perhaps because of the inhibitory action of RNAase of exocrine tissue. Although pancreas slices have been used by VAUGHAN AND ANFINSEN[5], as well as by LIGHT AND SIMPSON[6] the specific activity of the isolated insulin remained low.

The occurrence of islet tissue as a separate organ in certain fishes (see ref. 7 for review) suggests a further approach to the problem. In a preliminary communication, BAUER AND LAZAROW[8] have reported on the incorporation of [^{14}C]amino acid into an ethanol-soluble protein fraction of goosefish islets *in vitro*. In previous communications[7,9,10] we have demonstrated the incorporation of ^{14}C-labeled substrate into an acid ethanol-soluble protein fraction of islets from toadfish (*Opsanus tau*) incubated *in vitro*. The protein was shown to behave like insulin on paper chromatography and to exhibit biological activity. The purpose of the work to be reported here was to further purify the insulin-containing fraction, to demonstrate the possibility of obtaining insulin of high specific activity, and to achieve selective labeling of one of the two peptide chains.

MATERIAL AND METHODS

The experimental animals used as well as the incubation procedure were as described in the preceding communication[8], *i.e.*, freshly excised islets from toadfish (*Opsanus tau*) were incubated unsliced in Eagle's medium for 36 h at 15°. The fish were obtained from a Narragansett, Rhode Island, fisherman, and were stored in continuously circulating sea water at the American Lobster Company, in Boston.

After incubation, the islets were washed twice in 0.9% NaCl, homogenized in 10% trichloroacetic acid by means of an all glass Potter–Elvehjem homogenizer, and washed five times in 5% trichloroacetic acid at room temperature. The precipitate was extracted three times with 0.1 ml acid ethanol/mg tissue (1.56 N HCl in 65% ethanol[11]) at room temperature for 30, 20 and 10 min, respectively. The insoluble residue was further extracted with 5% trichloroacetic acid at 90° for 15 min and washed with ethanol, ethanol–ether and ether. The final precipitate was designated residual trichloroacetic acid-precipitable protein. Ethanol from the acid ethanol extract was evaporated *in vacuo* and the aqueous residue brought to pH 4 with 1.0 M sodium citrate. The residue was extracted three times with equal volumes of methylene chloride to remove lipid. The aqueous phase was dialyzed in Visking tubing 23/32 (see ref. 12) for 36 h at 4° against six changes of distilled water, lyophilized, dissolved in 0.05 N HCl, streaked on Whatman No. 1 paper and subjected to ascending chromatography with *sec.*-butanol–1% acetic acid (1:1) at 4° for 16 h[13,14]. A small guiding strip was stained with bromocresolgreen and the material with R_F similar to that of beef and tuna-bonito insulin[10] was eluted with 0.05 N HCl.

The eluate was concentrated *in vacuo* to a small volume, 0.2 volume acetone was

* Amino acid incorporation into microsome protein was obtained by using cell-free preparations of pancreas from young pigeons by WEISS *et al.*[4].

added, pH was brought to 5.35 with 1.0 N NaOH in the cold, and the mixture was left at 4° overnight[15]. The resultant precipitate was redissolved in 0.05 N HCl and, after a second isoelectric* precipitation, it was dissolved in a small volume of 0.01 N NaOH and added to 3 volumes of 0.04 M Tris buffer (pH 7.6). The solution was added to a slurry of DEAE-cellulose (Schleicher and Schuell, Keene, New Hampshire) equilibrated with Tris buffer. Unadsorbed material was decanted off three times after addition of washes of Tris buffer. The adsorbed material was then eluted with 0.3 M NaCl in Tris buffer (pH 7.6), dialyzed, lyophilized and subjected to a third isoelectric precipitation. The precipitate was dissolved in a small volume of 0.05 N HCl and added to three volumes of 0.04 M citrate buffer (pH 3.3). The solution was added to a slurry of CM-cellulose (Schleicher and Schuell), equilibrated with citrate buffer. Adsorbed material was eluted with 0.25 M NaCl in citrate buffer (pH 3.3), dialyzed and lyophilized. The lyophilisate was dissolved in 0.05 N HCl and precipitated for a fourth and fifth time at pH 5.35, washed with acetone and dried.

For the separation of the two chains of insulin, the third pH-5.35 precipitate was oxidized in performic acid (9 volumes 98 % formic acid and 1 volume 30 % H_2O_2, used 1 h after preparation at room temperature) for 15 min at room temperature[16]. An equal volume of water was then added and the peptides were precipitated with 20 volumes acetone at 4°, washed three times with acetone and dried. The material was dissolved in 0.1 M NH_4OH and chromatographed on Dowex-50 X2 (200–400 mesh) according to BAILEY AND COLE[17], *i.e.*, the solution was brought to pH 2.2 with 1 N HCl, and enough solid urea (previously deionized by passing a 8 M solution through a mixed bed, Amberlite MB-3, resin) was added to redissolve the partially precipitated B chain. The fraction which remained unadsorbed at pH 2.2 (containing the A chain) and the fraction eluted at pH 7.6 (containing the B chain) were both dialyzed for 2 h at room temperature against several changes of distilled water and lyophilized. The two fractions were applied on Whatman No. 1 paper, and subjected to electrophoresis in 20 % formic acid[18] at 5 V/cm for 16 h at room temperature in a horizontal tank by the hanging strip method. The paper was stained with Pauly's reagent using the method described by BROWN *et al.*[19]. Equal paper areas containing either A or B chains were cut out and eluted with 0.1 M NH_4OH. Aliquots were dried in counting vials and dissolved in 1.0 M hyamine.

After each purification step, aliquots were taken for protein determination according to LOWRY *et al.*[20], using bonito-tuna insulin as protein standard. Aliquots for assay of radioactivity were dissolved in 0.5 ml M hyamine 10-X (Packard Instrument Company, La Grange, Illinois), and added to 20 ml toluene containing PPO (Packard Instrument Company) and POPOP (Pilot Chemicals, Waltham, Massachusetts) and counted in a Nuclear-Chicago 3-channel liquid scintillation counter. For the double label experiment, ^{14}C and ^{3}H being present in the same samples, duplicate samples were counted before and after addition of either [^{14}C]toluene or [^{3}H]toluene standards. Measurements and calculations were carried out using the discriminator-ratio method described by OKITA *et al.*[21]. All results were corrected for background and for quenching by the use of internal standards and are expressed as counts/min at 100 % efficiency.

Determination of $^{14}CO_2$ released by ninhydrin before and after hydrolysis of

* The term "isoelectric" is used here, although the isoelectric point of this particular insulin has never been determined.

protein samples was performed by the VAN SLYKE procedure[22], adding carrier alanine to the labeled protein. The liberated CO_2 was trapped with NaOH and counted as $BaCO_3$ in a proportional flow counter. Protein hydrolysis was carried out in 6 N glass-distilled HCl at 110° for 12 h. Radioactivity in amino acids having a free amino group was determined by using SANGER's dinitrophenyl method[23].

Assays for biological activity of insulin followed the procedure described by RENOLD *et al.*[24]. Statistical evaluation of the assays was done as described by SHEPS *et al.*[25].

[$^{14}C_6$]Glucose and DL-[1-^{14}C]leucine were obtained from New England Nuclear Corporation, Boston, Massachusetts. Randomly labeled L-[$^{14}C_6$]leucine, L-[$^{14}C_6$]isoleucine and DL-[$^{3}H_{12}$]tryptophan were obtained from Nuclear-Chicago Company, Chicago, Illinois. Crystalline beef insulin, batch number 719106, assaying 25.2 units/mg, was kindly supplied by Dr. W. R. KIRTLEY of the Lilly Research Laboratories, Ind. (U.S.A.). A mixture of purified bonito and tuna insulin, 16.3 units/mg, was the gift of Shimizu Seiyaku Company, Ltd., Shimizu (Japan).

RESULTS

Labeling of insulin with ^{14}C

To rule out exclusive binding of labeled amino acids to free amino or carboxyl groups of the protein, or binding by non-covalent bonds, protein samples of islets incubated in DL-[1-^{14}C]leucine were treated with ninhydrin before and after acid hydrolysis. As is shown in Table I, only 1.3 % of the total ninhydrin-labile carboxyl-carbon could be released from the protein before hydrolysis. Similarly, only 0.5 % of the total protein counts were found in dinitrophenylamino acids, after coupling the free amino groups of the protein to dinitrofluorobenzene. These results were accepted as evidence for incorporation of a major part of trichloroacetic acid-precipitable leucine into islet protein in true peptide linkage.

TABLE I

NINHYDRIN-LABILE CARBON AND DINITROFLUOROBENZENE-REACTIVE AMINO ACID OF A SAMPLE OF ^{14}C-LABELED PROTEIN OF TOADFISH ISLETS INCUBATED WITH DL-[^{14}C]LEUCINE

Ninhydrin-labile $^{14}CO_2$	*Counts/min*	*%*
Before acid hydrolysis	56	1.3
After acid hydrolysis	4340	100
Dinitrophenyl-^{14}C-protein		
DNP-amino acids (ether extract)	43	0.5
Water phase of ether-extracted hydrolyzate	8500	99.5

The experiment to be described was so designed as to yield insulin of relatively high specific activity and purity. As an index of insulin purification, an ^{3}H-labeled amino acid not present in insulin, namely tryptophan, was incubated together with the ^{14}C-labeled substrates glucose and L-leucine.

A total of 200 toadfish islets from as many fish was used for this experiment. The incubation medium contained [$^{14}C_6$]glucose (5 mM, 5 μC/μmole), L-[$^{14}C_6$]leucine (1.77 mM, 7.07 μC/μmole), and DL-[$^{3}H_{12}$]tryptophan (1.9 mM, 659 μC/μmole). After incubation, protein was precipitated with trichloroacetic acid and further treated

as outlined under METHODS. Upon purification the specific activity with regard to ^{14}C of the insulin-containing fractions gradually increased until it reached a plateau after step number 6 (Table II). The fractions presumably devoid of insulin (fractions 1 and 3a) exhibited lower specific activity. The fact that the specific activity of the insulin fraction after paper chromatography (3b) does not increase despite removal of a considerable portion of non-insulin protein (3a) of lower specific activity is probably due to the concomitant removal of labeled lipids which are not accounted for in the protein determination. The drop in specific activity in fraction 7 is due to the addition of carrier insulin (bonito-tuna) in the ratio of 9:1 on a protein weight basis.

TABLE II

SPECIFIC ACTIVITIES OF PROTEIN FRACTIONS OBTAINED ALONG A PURIFICATION PROCEDURE FOR INSULIN FROM TOADFISH ISLETS AFTER INCUBATION WITH [^{14}C]GLUCOSE, [^{14}C]LEUCINE AND [^{3}H]-TRYPTOPHAN

Fraction		*^{14}C-activity (counts/min/mg)*	*^{3}H-activity (counts/min/mg)*	*$^{14}C/^{3}H$**	*Protein (mg)*
No.	*Description*				
1	Residual trichloroacetic acid precipitate	248 000	863 000	0.29	18.1
2	Acid-ethanol extract	395 000	1 432 000	0.28	5.5
3	Paper chromatography				
a	Origin	119 000	274 000	0.44	2.21
b	"Insulin"	375 000	390 000	0.96	3.25
c	Front	**	**	0.08	**
4	1st pH-5.35 precipitate	457 500	497 500	0.92	1.76
5	2nd pH-5.35 precipitate	474 000	431 500	1.10	1.76
6	DEAE-cellulose adsorbed	568 000	221 000	2.58	0.88
7	3rd pH-5.35 precipitate	57 000 (× 10)***	21 100	2.73	7.09***
8	CM-cellulose adsorbed	57 500 (× 10)	18 000	3.16	2.84
9	4th pH-5.35 precipitate	57 800 (× 10)	17 400	3.33	2.30
10	5th pH-5.35 precipitate	57 300 (× 10)	16 800	3.42	2.16

* Expressed as ratios of total counts/min.
** This fraction did not contain measurable amounts of protein.
*** Carrier insulin added to fraction No. 6 in the ratio of 9:1.

Since tryptophan is not present either in mammalian[3,4] or in fish insulin[26–29], a decrease of ^{3}H-activity (derived from [^{3}H]tryptophan) was expected during the purification procedure. This was indeed the case, although removal of ^{3}H-activity was not as complete as had been hoped. The residual trichloroacetic acid precipitate, containing no appreciable quantities of insulin (fraction 1) and the insulin-containing acid-ethanol extract (fraction 2) exhibited approximately the same $^{14}C/^{3}H$ ratio, again suggesting contamination of the acid-ethanol extract with lipid despite the previous lipid extraction. Indeed, the lipid fraction (fraction 3c) had a very low $^{14}C/^{3}H$ ratio, suggesting that comparatively small amounts of lipid would decrease the $^{14}C/^{3}H$ ratio quite significantly. In considering the $^{14}C/^{3}H$ ratio, the 100-fold difference in the specific activities of the ^{14}C and the ^{3}H substrates used must be kept in mind.

Upon assaying an aliquot of fraction 6 (after DEAE-cellulose adsorption) for insulin activity, a value of 7.7 units/mg protein was found (95 % confidence limits: 4.8–12.6).

Labeling of the A chain of insulin with ^{14}C

Attempts to subject the "insulin-fraction" as isolated by paper chromatography

(corresponding to fraction 3b of Table II) to electrophoresis were unsuccessful, in as much as the material stuck to the origin. Only after 3 isoelectric precipitations and DEAE-cellulose chromatography was electrophoresis of the oxidized material satisfactory. As can be seen in Fig. 1, the electrophoretic mobility of beef (I) differed from that of tuna-bonito (II) insulin, tuna-bonito insulin moving somewhat faster toward the cathode than beef insulin. Both were electrophoretically homogeneous in this system. After splitting the disulfide bonds by oxidation, beef insulin gave rise to two fractions, the sulfonic acid derivatives of the A and the B chain respectively (III), whereas both tuna-bonito (IV) and toadfish insulin (V) gave rise to 4 fractions. Separation of oxidized tuna-bonito insulin into A chains (VI) and B chains (VII) by ion-exchange chromatography yielded fractions which were slightly contaminated with each other (VI and VII).

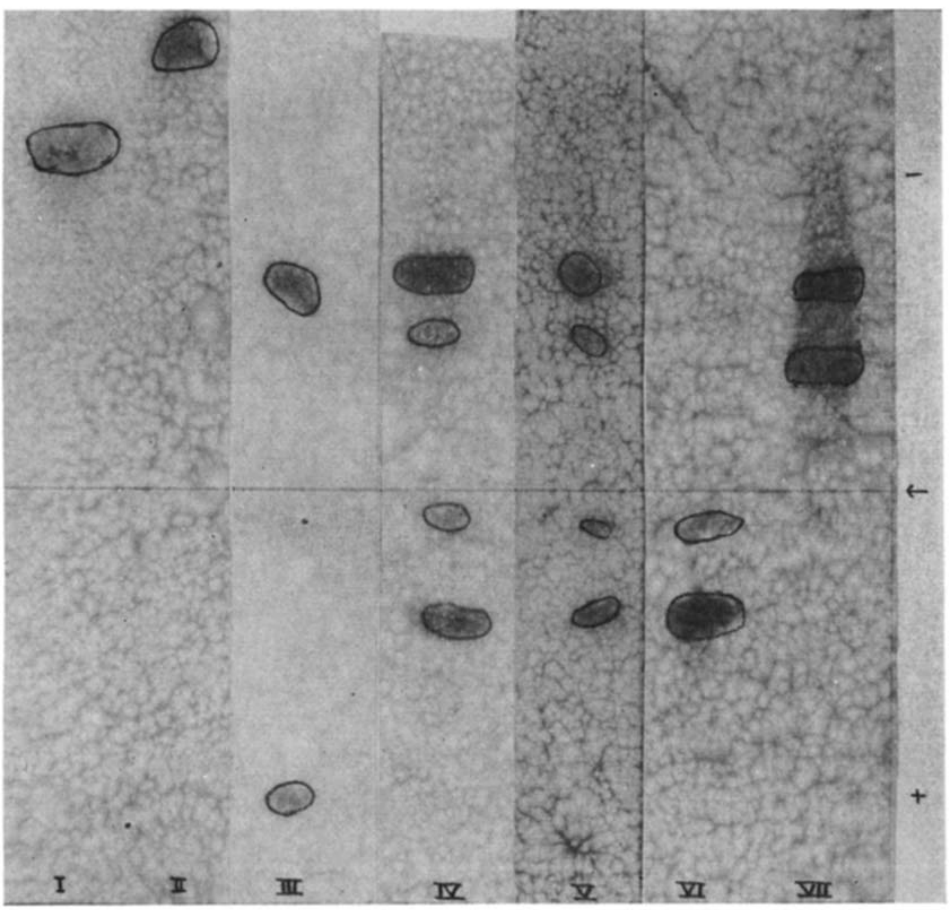

Fig. 1. Electrophoretic mobility of; I, beef insulin; II, tuna-bonito insulin; III, oxidized beef insulin; IV, oxidized tuna-bonito insulin; V, oxidized toadfish insulin; VI, A-chain fraction of oxidized tuna-bonito insulin; VII, B-chain fraction of oxidized tuna-bonito insulin. Experimental conditions of oxidation, separation and electrophoresis described under MATERIAL AND METHODS in the text.

After these preliminary experiments, 45 islets were incubated in 3 batches in the presence of L-[$^{14}C_6$]isoleucine (2.7 mM, 6.16 μC/μmole), an amino acid known to be present only in the A chain of all species so far studied[1,2,26-30]. After incubation, protein was isolated and purified as described in the METHODS section. The third

isoelectric precipitate (corresponding to Fraction 7 in Table II) was oxidized and subjected to electrophoresis. Of a total of 11950 counts/min/mg insulin, an aliquot of 0.4 mg (4790 counts/min) was applied on paper. After separation of the chains, 4200 counts/min (88 %) were recovered in the A chains and 390 counts/min (8 %) in the B chains. Paper areas showing only background color from staining did not exhibit any measurable radioactivity.

DISCUSSION

Serious objections can be raised against indiscriminate equation of incorporation of amino acids into proteins with *de novo* protein synthesis. The most serious one is the possibility of a mere replacement of old with new amino acids in a completed protein. Results obtained with simple amino acid-insulin systems suggest that this type of exchange process may indeed contribute to the total amino acid incorporation[31,32]. A further pitfall is the possibility of adsorption of amino acids rather than incorporation into the peptide chain by peptide bond formation. To evaluate the extent of binding by non-covalent bonds, the ninhydrin reaction has been used as a measurement of amino acid residues with a free carboxyl and amino group[22,33]. In addition to this, the amount of radioactivity present in dinitrophenylamino acids after dinitrophenylation of the protein[23], was determined, a measurement which provided an estimate of amino acid residues with a free amino group. The results of both measurements are evidence for only minimal binding of leucine to islet protein either by C- or N-terminal incorporation, or by non-covalent bond formation. That leucine is incorporated into islet protein mostly as leucine and not as transamination or degradation product has been shown previously[7].

Satisfactory purification of a labeled protein during isolation can be demonstrated either by reaching constant specific activity or, in the case of enzymes or hormones, by determining the biological activity. In the case of insulin, it was tempting to add another test, namely the gradual removal of tryptophan from the crude protein during the purification due to the complete absence of this amino acid from insulin. This test failed to demonstrate the purity of the final product conclusively. Although the $^{14}C/^{3}H$ ratio was increased during the purification by a factor of 12, complete absence of tryptophan (or ^{3}H derived from tryptophan) was never achieved.

It should be pointed out, however, that the insulin content of whole homogenates of toadfish islets is about 75 mg/g protein. Thus a 12-fold purification comes close to the maximum purification theoretically possible. It is known, moreover, that glucagon which contains tryptophan is a contaminant of many insulin preparations. A relatively small amount of glucagon of the order of 0.3 % present in the final insulin preparation could account, therefore, for the residual ^{3}H-activity found, bearing in mind the 100-fold greater specific activity of the ^{3}H substrate over the ^{14}C substrates. On the other hand, the determination of biological activity of a purified fraction yielded 7.7 units/mg, a rather low value when compared with crystalline beef insulin (best preparations approx. 26 units/mg) and to bonito insulin (16–24 units/mg) (see ref. 28). Rather than assuming low biological activity of toadfish insulin or crudeness of the preparation tested, it is felt that the most likely explanation is partial loss of the biological activity during purification. Similar losses of activity for beef insulin during paper chromatography have been previously observed in this laboratory[34], and by TAYLOR[35].

The purification of a synthesized labeled protein to constant specific activity is a generally accepted procedure and has been previously used in the case of insulin[3,5,6]. In the data presented, constant specific activity was reached after DEAE-cellulose adsorption (step 6). It is felt, therefore, that the subsequent protein fractions represent insulin of considerable purity, despite the relatively low biological activity and the continuous small decrease of ^{3}H-activity during further purification steps. Further support for this view is provided by the electrophoretic behavior of the oxidized insulin, which gave rise to 4 fractions of the same electrophoretic mobility as that of fractions of purified bonito-tuna insulin. The ^{14}C-labeled insulin thus obtained *in vitro* has a specific activity two orders of magnitude greater than the ^{35}S-insulin obtained by VOELKER *et al.*[3] *in vivo* and three to four orders of magnitude greater than the ^{14}C-insulin obtained by VAUGHAN AND ANFINSEN[5] and LIGHT AND SIMPSON[6] with slices of mammalian pancreas *in vitro*. The specific activity could probably be further raised by substituting all amino acids present in the incubation medium by labeled amino acids.

The fact that certain amino acid residues are present only in either the A chain or the B chain of insulin offers the possibility of preferential labeling of one chain, provided that the amino acid chosen is not easily transformed into other amino acids. In the case of toadfish insulin, there are two isoleucine residues present in the A chain, whereas no isoleucine is present in the B chain*. Our results show that incubation with labeled isoleucine resulted in preferential labeling of the A chains after their separation by paper electrophoresis. Radioactivity in the B chains was only 9% of that found in the A chains, an observation which is probably accounted for by contamination with A chains. Also, unspecific adsorption of isoleucine on the B chain, or incorporation of a partly degraded carbon-skeleton of isoleucine in the form of another amino acid must be considered**. Certainly, the possibility of selective labeling of one chain of insulin is of considerable theoretical interest. It should prove of special merit in studies designed to unravel the biosynthetic process of assembling the two chains of insulin, a process which might be similar to that occurring in other proteins with disulfide bridges, and therefore of more general importance.

It is realized that more elaborate studies are needed to achieve the goals initially commented upon. Particularly, the use of a cell-free system would seem necessary Studies in this direction are now in progress.

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* Only one A chain and one B chain have been completely analyzed so far[26]. From the results at hand, it seems reasonable to expect that the other A and B chains do not differ to any great extent from the ones analyzed. In rat insulin, where two different B chains are present, the only difference is the substitution of a lysine residue for a methionine residue in position 29 (see ref. 31).

** We are presently planning hydrolysis of the isolated chains and isolation of the labeled amino acid(s).

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