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Synthesis of Insulin

Availability of A and B chains readily leads to the synthesis of this protein.

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Great strides in degradative techniques spearheaded by Sanger's pioneering work on insulin, and impressive advances in methods of isolating amino acids, peptides, and proteins during the last 20 years have exerted a dramatic influence on our knowledge of the structure of proteins. On the other hand, spectacular accomplishments in synthetic methodology and in the development of methods enabling the separation of closely related peptides (and of highly sensitive analytic tools for assessing their chemical and stereochemical homogeneity) have set the stage for the synthesis of large polypeptide chains (*1*). On this basis we undertook the synthesis (*1–3*) of sheep insulin according to the structure postulated by Sanger (*4*) (Fig. 1).

In this structure two polypeptide chains are present: the A chain containing 21 amino acid residues and the B chain containing 30. In the insulin molecule the two chains are linked by two disulfide bridges; there is also an intrachain disulfide bridge in the A chain that results in formation of a 20-membered cyclic system. We made the assumption that if chemically synthesized A and B insulin chains, with their sulfhydryl groups free, were available, it might be possible to obtain insulin, among the probable isomers that might be formed, by air-oxidation of a mixture of the sulfhydryl forms of these chains.

While the work on synthesis toward that goal was in progress, independent studies with natural insulin chains, originally by Dixon and Wardlaw (*5*) and later by Du *et al.* (*6*) confirmed this assumption. These investigators, following procedures described by Bailey and Cole (*7*) and Swan (*8*), cleaved insulin to its two chains by treatment with sodium sulfite in the presence of a mild oxidizing agent such as sodium tetrathionate. As a result of that treatment, known as oxidative sulfitolysis, the A and B chains of insulin are obtained as the *S*-sulfonated derivatives in which each cysteinyl residue has been converted into an *S*-sulfocysteinyl residue ($Cys{\cdot}H \rightarrow Cys{\cdot}SO_3^-$).

The *S*-sulfonated chains are stable compounds and can be purified by various methods: ion-exchange chromatography (*5, 6*), gel filtration (*9–11*), and continuous-flow electrophoresis (*11, 12*) have been used. On treatment with a thiol such as mercaptoethanol or thioglycollic acid, the *S*-sulfocysteinyl residues of these derivatives are transformed to cysteinyl residues ($Cys{\cdot}SO_3^- \rightarrow Cys{\cdot}H$), and thus the *S*-sulfonated chains are converted to their sulfhydryl forms. Air-oxidation of a mixture of the sulfhydryl forms of the two chains generated insulin, which subsequently was crystallized and shown to be identical with the natural hormone. This overall process of cleavage and regeneration of insulin is shown in Fig. 2.

Recombination Yields of Natural Chains

The insulin yielded by recombination of its separated chains depends greatly on the conditions employed for the preparation of the *S*-sulfonated chains, for the conversion to their sulfhydryl forms, and in the air-oxidation step. Consequently, the recombination yields reported by various laboratories vary considerably. With chains prepared by oxidative sulfitolysis of insulin, Dixon and Wardlaw obtained insulin in a yield of 1 to 2 percent of the theoretical prediction (as are all unspecified percentages mentioned in this article), whereas Du *et al.* reported a yield of 5 to 10 percent.

Recently Du *et al.* (*13*) indicated a yield of 50 percent in production of insulin upon recombination of natural chains of bovine insulin; but it appears that in this instance the yield was calculated on the basis of not the amounts of the starting chains but the specific activity of the final oxidation product. Since the final product had undergone a certain degree of purification—precipitation at acidic *p*H's—its specific activity cannot be considered a true measure of the recombination yield. On the basis of the amount of the B chain used, the yield of insulin, according to the method of Du *et al.*, was approximately 12 to 16 percent (*11, 14*).

In all three recombination methods that I have mentioned, the overall principle in obtaining insulin is air-oxidation of a mixture of the sulfhydryl forms of the A and B chains. These methods, however, differ from each other in the conditions employed to prepare the sulfhydryl forms of the chains from the *S*-sulfonated derivatives and for the air-oxidation step. Another recombination method reported recently by Zahn (*15*) entails oxidation of the sulfhydryl form of the A chain for 60 to 100 minutes before its reaction with the sulfhydryl form of the B chain; average yields of 20 to 30 percent, with a maximum up to 44 percent, are re-

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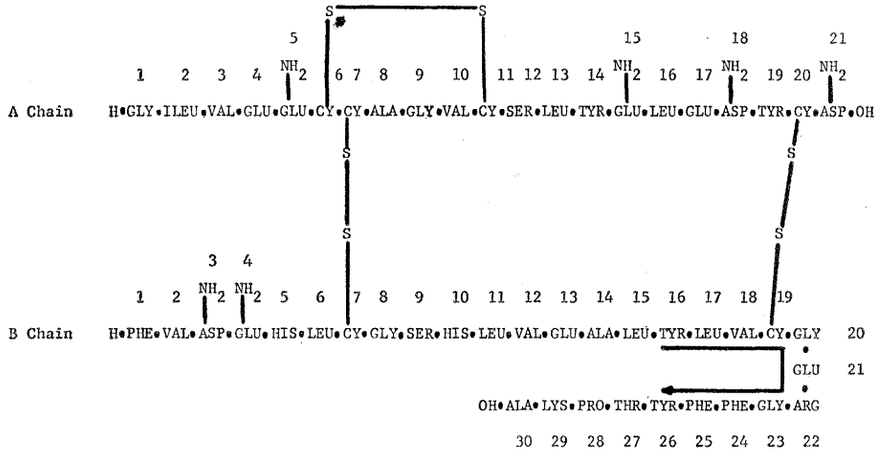


Fig. 1. Structure of sheep insulin.

ported. Again, however, the yield is calculated on the basis of not the amount of the starting chains but the specific activity of the final oxidation product.

An entirely different principle underlies the method (Fig. 3) we have recently developed for recombining A and B chains to produce insulin (*14*): an excess of the sulfhydryl form of the A chain reacts directly with the *S*-sulfonated derivative of the B chain. On the basis of the amount of the B chain used, the yield of insulin is 60 to 80 percent. It is therefore apparent from the high recombination yields that the necessary information for the folding and orientation of the A and B chains in a way that enables their spontaneous combination to form predominantly insulin, among the several possible isomers, is embodied within the primary structure of the chains.

The yields of insulin from recombination of natural chains are significantly lower than those just mentioned when the chains are prepared by cleavage of insulin with sodium in liquid ammonia (*16*), followed by oxidative sulfitolysis, according to the scheme shown in Fig. 4. The results of studies in two laboratories (*9, 14, 17*) indicate that the efficiency of insulin formation from natural chains, prepared by the sodium–liquid ammonia treatment of insulin, is approximately 10 to 20 percent of that obtained by recombination of chains prepared by oxidative sulfitolysis of insulin.

The nature of this phenomenon is still unclear. It is possible that, during the sodium–liquid ammonia treatment, by-products are formed that are closely related to the A and B chains and that cannot be separated from these chains by the methods employed. However, this observation is of considerable importance when one considers the fact that sodium–liquid ammonia treatment is an obligatory step in the chemical synthesis of the insulin chains; later I shall discuss this point. Thus it is quite obvious that synthesis of the *S*-sulfonated derivatives of the A and B chains readily leads to synthesis of insulin by the route shown in Fig. 2 or 3.

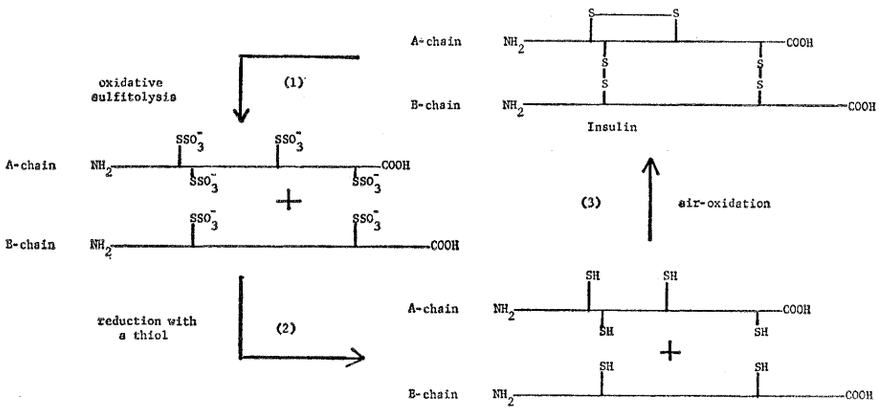


Fig. 2. Cleavage and resynthesis of insulin: sulfitolysis of insulin produces the *S*-sulfonated A and B chains (1); the *S*-sulfonated chains with mercaptoethanol are converted to their sulfhydryl form (2); air-oxidation of a mixture of the sulfhydryl chains produces insulin (3).

Synthesis of the A and B Chains of Insulin

Synthesis of the *S*-sulfonated derivatives of the A and B chains of sheep insulin was first undertaken and completed in our laboratory in 1963 (*18–20*). For this synthesis the "fragment condensation approach" is utilized (*1*), in which peptide subunits, containing from 4 to 10 amino acid residues, are synthesized stepwise by addition of one amino acid residue at a time to the amino terminus of the chain. Experience, gained predominantly from the studies of synthesis by du Vigneaud *et al.* (*21*) in connection with the posterior pituitary hormones, indicates that, in the stepwise synthesis of polypeptides, racemization, if any, is kept to a minimum. The peptide subunits synthesized stepwise are then condensed to form larger peptide subunits. This process is repeated until the desired polypeptide chain is constructed.

We have implemented the fragment-condensation approach by first undertaking the synthesis of the *S*-sulfonated derivative of the A chain of sheep insulin. The original synthetic route (*18, 20*) followed for the construction of that chain is illustrated in Fig. 5. We have prepared two peptide subunits (I and II), with the secondary functional groups of the constituent amino acids protected with blocking agents that can be readily removed once the peptide chain is synthesized. Thus the sulfhydryl functions of the cysteine residues are protected with benzyl groups (*22*), which are cleaved from a peptide chain upon treatment with sodium in liquid ammonia (*16*). Amino and carboxyl functions are protected with groups that can be removed from the peptide chain either by sodium in liquid ammonia or on exposure to trifluoroacetic acid. Subunit I contains the nine amino acid residues found at the amino terminus of the A chain (positions 1 to 9), and subunit II contains the 12 amino acid residues found at the carboxyl terminus of that chain (positions 10 to 21). Coupling of subunits I and II affords the protected heneicosapeptide III.

This polypeptide derivative contains the entire amino acid sequence of the A chain of sheep insulin. Successive

treatments of the protected heneicosapeptide III with trifluoroacetic acid and with sodium in liquid ammonia results in the removal of all the protecting groups. Oxidative sulfitolysis of the resultant reduced material affords the desired product: the *S*-sulfonated derivative of the A chain of sheep insulin (IV). Two consecutive chromatographic steps on G-25 Sephadex, with 1*M* pyridine and then 1*M* acetic acid used as the eluting solvents, suffice to purify the crude product (*9, 11, 20*).

Paper chromatography in two solvent systems, paper electrophoresis at two *p*H values, and amino acid analysis of an acid hydrolyzate established the chemical purity of the synthetic chain, whereas complete digestibility by the proteolytic enzyme leucine aminopeptidase established its stereochemical homogeneity (*20*). Recently devised alternative routes produce the same synthetic chain in higher yields (*20*).

The fragment-condensation approach was also employed in synthesis of the *S*-sulfonated derivative of the B chain of sheep insulin. The original route (*19*) followed for the construction of this chain is illustrated in Fig. 6. Two peptide fragments (I and II) were prepared having the functional groups of the constituent amino acid residues blocked with groups that can be removed in one step by treatment with sodium in liquid ammonia once the peptide chain is synthesized. Fragment I contains the nine amino acid residues found at the amino terminus of the B chain (positions 1 to 9), and fragment II contains the 21 amino acid residues found at the carboxyl-terminal end of that chain (positions 10 to 30).

Condensation of these two fragments yields the triacontapeptide derivative III, which embodies within its structure the entire amino acid sequence of the B chain of sheep insulin. Sodium–liquid ammonia treatment of the triacontapeptide III caused the cleavage of all the protecting groups from this compound and produced the sulfhydryl form of the B chain, which in turn, by sulfitolysis, was converted to the desired *S*-sulfonated derivative (IV). Originally, ion-exchange chromatography on Dowex-50 [later, continuous-flow electrophoresis (*11*)] was used to purify the synthetic chain. This product, on paper electrophoresis and paper chromatography, behaved as a single component and, after acid hydrolysis, gave the constituent amino acids in the correct ratios (*19*).

Soon after completion of the syn-

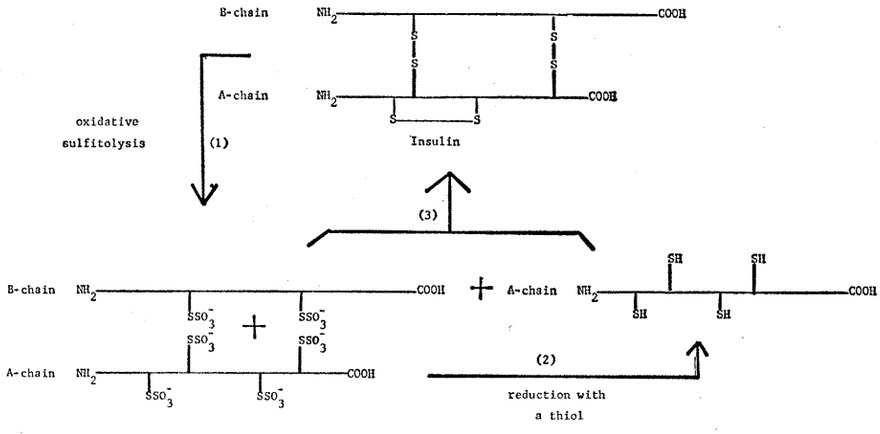


Fig. 3. Cleavage and resynthesis of insulin by the new procedure: sulfitolysis of insulin produces the *S*-sulfonated A and B chains (1); the *S*-sulfonated A chain with mercaptoethanol is converted to its sulfhydryl form (2); the sulfhydryl A chain reacts with the *S*-sulfonated B chain to produce insulin (3).

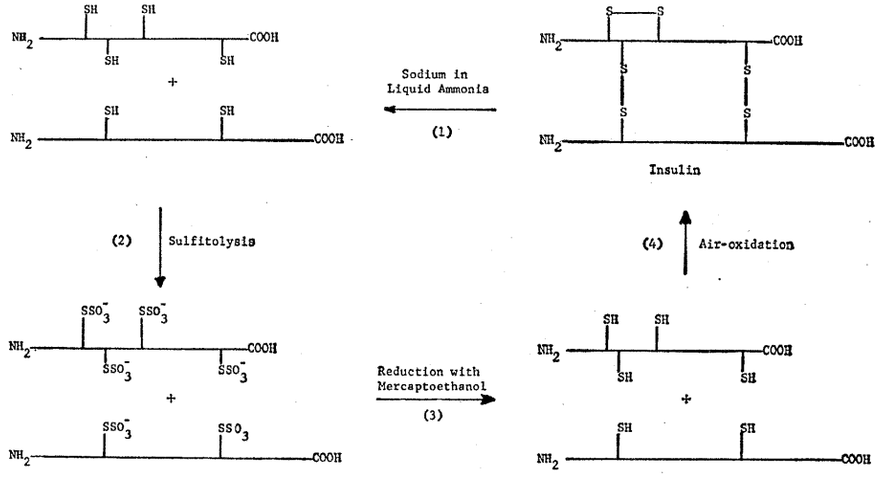


Fig. 4. Cleavage and resynthesis of insulin by the sodium–liquid ammonia route: on exposure to sodium in liquid ammonia insulin is cleaved and forms the sulfhydryl forms of the A and B chains (1); sulfitolysis converts the sulfhydryl chains to the *S*-sulfonated derivatives (2); on treatment with mercaptoethanol, the *S*-sulfonated chains are converted to their sulfhydryl forms (3); air-oxidation of the sulfhydryl chains regenerates insulin (4).

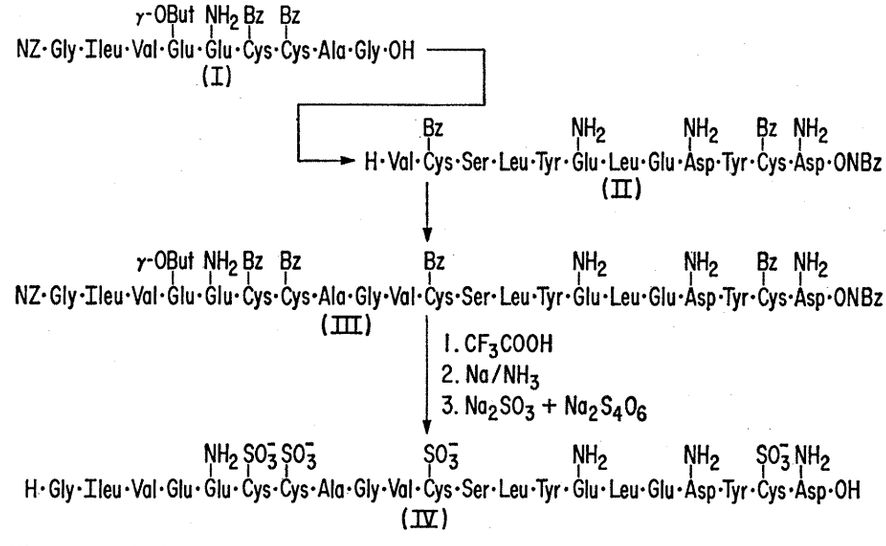


Fig. 5. Synthetic route to the *S*-sulfonated A chain of sheep insulin. Abbreviations: *NZ*, *p*-nitrocarbobenzoxy group (amino protector); *But*, tertiary butyl group (carboxyl protector); *Bz*, benzyl group (sulfhydryl protector); *NBz*, *p*-nitrobenzyl group (carboxyl protector).

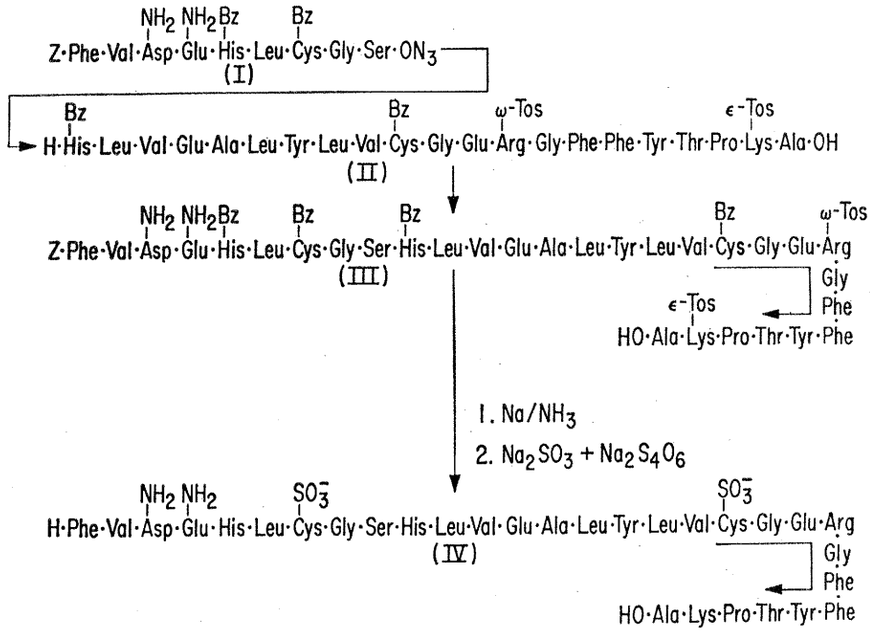


Fig. 6. Synthetic route to the *S*-sulfonated B chain of sheep insulin. Abbreviations: *Z*, carbobenzoxy group (amino protector); *Bz*, benzyl group (imidazole and sulfhydryl protector); *Tos*, *p*-toluenesulfonyl group (guanido and ϵ-amino protector).

thesis of the *S*-sulfonated derivatives of the A and B chains of sheep insulin in our laboratory, Zahn and co-workers (*23–25*) also reported the preparation in the fully protected form of the A and B chains of the same protein. In their case, however, the chains were not converted to the *S*-sulfonated derivatives for purification and identification purposes, but were transformed to their sulfhydryl form by sodium in liquid ammonia and used directly for combination experiments (*26*).

Within 2 years of the completion of synthesis of the A and B chains of sheep insulin, Niu *et al.* (*27*) and Wang *et al.* (*28*) also reported synthesis of the *S*-sulfonated derivatives of the A and B chains of bovine insulin; they used essentially the synthetic plan employed in the preparation of the chains of sheep insulin. The B chains of sheep and bovine insulins are identical, whereas the A chains of these proteins differ in one amino acid residue: instead of a glycine residue at position 9, the bovine A chain contains a serine residue.

More substantial are the differences in amino acid sequence between sheep and human insulins (*29*). These differences are confined to positions 8, 9, and 10 of the A chain and position 30 of the B chain (Fig. 1): instead of the sequence alanine-glycine-valine in positions 8 to 10, the human A chain contains threonine-serine-isoleucine; instead of alanine at position 30, the human B chain contains threonine.

Following the general overall plan that we employed in construction of the sheep insulin chains (Figs. 5 and 6), we have recently completed the synthesis and isolation, in the *S*-sulfonated form, of the A and B chains of human insulin (*10, 30*). For construction of the human A chain, we have thus prepared two subunits: one containing the nine amino acid residues found at the amino terminus of the chain; the other containing the 12 amino acid residues found at the carboxyl-terminal end of that chain. Condensation of these two fragments yielded the fully protected form of the human-insulin A chain, which, after exposure to sodium in liquid ammonia and sulfitolysis, is converted to its *S*-sulfonated derivative (*10*). Similarly, for construction of the human B chain we have prepared one peptide subunit containing the nine amino acid residues found at the amino terminus and another peptide subunit containing the 21 amino acid residues found at the carboxyl terminus. Coupling of these two subunits and unblocking of the resultant product with sodium in liquid ammonia afford the sulfhydryl form of the human B chain, which, by sulfitolysis, is converted to the *S*-sulfonated derivatives (*30*). Purification of the *S*-sulfonated derivatives of the human-insulin chains is accomplished by the same procedures employed in the purification of the sheep-insulin chains. Conversion of the *S*-sulfonated chains to their sulfhydryl form and air-oxidation of a mixture of the latter compounds led to formation of human insulin (*10*).

Combination Yields of Synthetic Chains

Once the synthetic *S*-sulfonated derivatives of A and B chains of sheep insulin became available (*18, 19*), they were submitted to combination experiments according to the method of Dixon *et al.* (*5*)—initially by these workers. At first, the synthetic sheep-insulin A chain was combined with the natural bovine-insulin B chain. The yield of hybrid insulin (one chain synthetic, the other natural) produced from this combination, as measured by the mouse hemidiaphragm method of assay and by immunological assays, was approximately 40 percent of the yield obtained when sulfitolytically prepared natural chains are recombined (*31*). When the synthetic sheep-insulin B chain was combined with natural bovine A chain by the same method, the yield of hybrid insulin ranged from 44 to 100 percent of that obtained from natural insulin chains (*31*). Finally, combination of the two synthetic sheep-insulin chains produced all-synthetic insulin in a yield that was 11 percent of that obtained by combination of the sulfitolytically prepared natural chains (*31*). Thus the efficiency of the combination of the all-synthetic chains to form insulin is within the range of the efficiency of natural chains prepared by the sodium–liquid ammonia route (Fig. 4). Under the experimental conditions used by Dixon (*31*), the recombination yield of sulfitolytically prepared natural chains was 0.65 percent.

Improvement of the conditions for recombination of natural chains (*13*) enabled us thereafter to increase further the yield of insulin from synthetic chains. Thus all-synthetic human insulin was formed in 2-percent yield (based on the starting amounts of synthetic chains used), and hybrid insulins, consisting of one synthetic human-insulin chain (A or B) and one natural bovine-insulin chain, were produced in yields ranging from 4 to 8 percent (*10, 30*). The yield of synthetic insulin is most dramatically increased, however, when our new method (*14*) for combining

chains is employed: the yields now range from 25 to 33 percent (*14*)—most recently up to 38 percent (*20*)—based on the amount of the B chain used when synthetic sheep- or human-insulin A chain is combined with natural bovine-insulin B chain; and yields range from 10 to 13 percent (*14*)—recently, up to 32 percent (*32*)—when synthetic B and natural A chains are combined. Finally, all-synthetic insulin in yields up to 6 percent (*20*)—recently up to 16 percent (*33*)—is now obtained upon combination of synthetic A and B chains.

Comparison of the yields of insulin obtained in our laboratory, by combination of synthetic chains, with the yields obtained in other laboratories emphasizes the importance of both the methods employed for synthesis and purification of the synthetic chains and the conditions used in the combination steps.

Zahn *et al.* (*24, 25*) prepared the A and B chains of sheep insulin in the protected form, as was done in our synthesis. However, in contrast with our synthetic route, after removing the blocking groups by exposure to sodium in liquid ammonia, they did not convert the resultant sulfhydryl forms of those chains to the *S*-sulfonated derivatives for further purification but used them directly for combination experiments (*26*). Thus the yields of insulin from the combination of synthetic A and natural B chain, of natural A and synthetic B, and of all-synthetic A and B ranged from 0.2 to 1 percent.

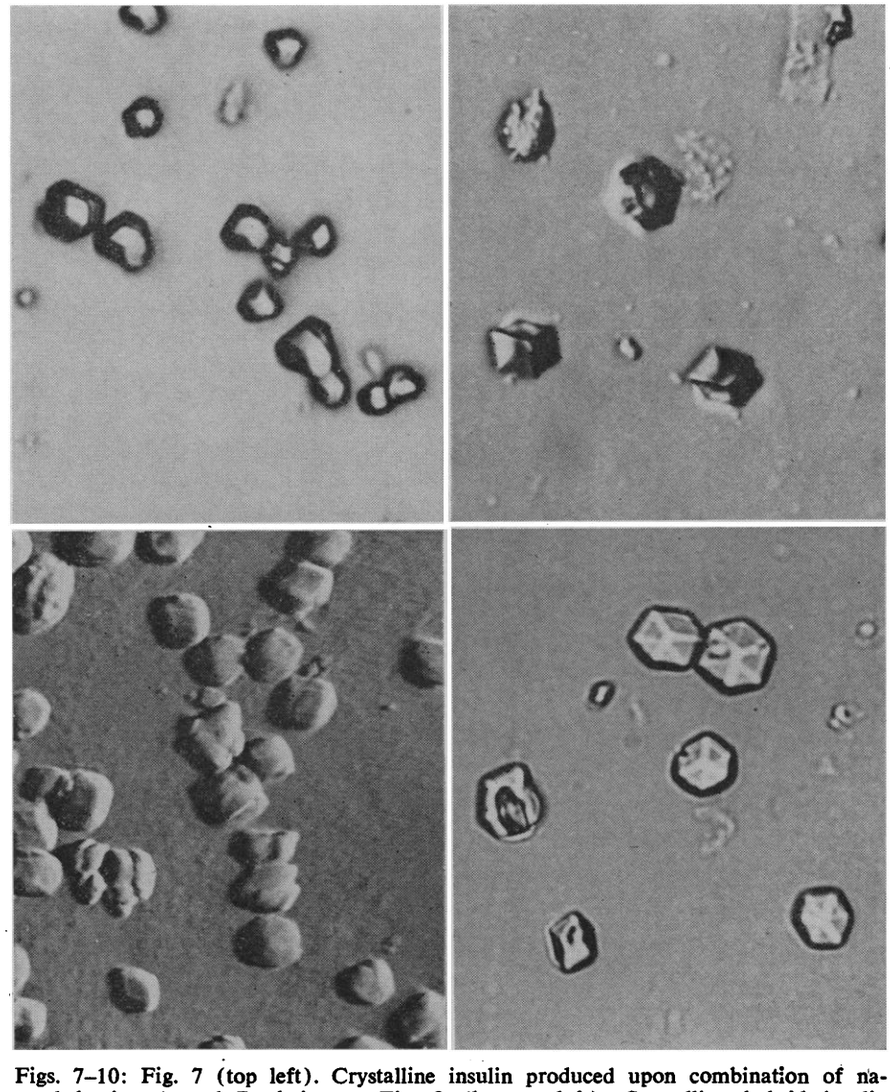
Niu *et al.* (*27*) and Wang *et al.* (*28*) prepared the A and B chains of bovine insulin also in the protected form, although they employed certain protective groups different from those used by us. Removal of the blocking groups with sodium in liquid ammonia and sulfitolysis of the resultant reduced chains provided the respective *S*-sulfonated derivatives, which were then submitted to various purification procedures. The *S*-sulfonated chains were subsequently converted to the sulfhydryl form and used for combination experiments. The yield of insulin, calculated not on the basis of the amounts of initial chains but on the specific activity of the final oxidation product, was approximately 7 percent when synthetic A and natural B chains were combined (*28*), 5 percent when natural A and synthetic B chains were combined (*27*), and from 1 to 2 percent when all-synthetic chains were combined (*34*).

It is obvious, therefore, that freedom in selection of methods of synthesis and of purification procedures for the synthesis of large polypeptide chains is not unlimited. Indiscriminate use of routes for synthesis and isolation of polypeptide chains does not necessarily lead always to the same product. Subtle chemical transformations and slight changes in optical purity and conformation of the chains may arise that may not be detected by the routine procedures of peptide chemistry. Such differences, however, could have pronounced effects in the case of insulin, where specific folding and orientation of the individual chains are critically important for appropriate alignment and ultimate combination to form the protein.

Isolation of Insulin from Combination Mixtures of A and B Chains

The need for the isolation of insulin from different species, in pursuit of elucidation of structure, demanded the availability of techniques for preparing this protein in small quantities. To this end several methods have been devised; those of Randall (*35*) and Smith (*36*) seem to serve.

In Randall's procedure insulin is extracted from crude mixtures with acidified (H_3PO_4) ethanol, precipitated with picric acid, converted to insulin hydrochloride, and finally subjected to isoelectric precipitation and crystallization. In Smith's method insulin is extracted from the crude mixture with acidified (HCl) ethanol and precipitated at an



Figs. 7–10: Fig. 7 (top left). Crystalline insulin produced upon combination of natural bovine A and B chains. Fig. 8 (bottom left). Crystalline hybrid insulin produced upon combination of synthetic sheep A chain and natural bovine B chain. Fig. 9 (top right). Crystalline hybrid insulin produced upon combination of natural bovine A chain and synthetic sheep B chain. Fig. 10 (bottom right). All-synthetic crystalline sheep insulin.

acidic *p*H with NaCl; the crude precipitate is then chromatographed on a carboxymethyl-cellulose column, and insulin is isolated from the effluent by precipitation with picric acid. The insulin picrate subsequently is converted to the hydrochloride and crystallized in the presence of zinc.

By applying a modification of Smith's method for isolating insulin from the combination mixtures of A and B chains, we have isolated in milligram quantities crystalline insulin (*11, 33*) from combination mixtures of natural bovine A and B chains (Fig. 7), of synthetic sheep A and natural bovine B chains (Fig. 8), and of natural bovine A and synthetic sheep B chains (Fig. 9). In all these experiments, the recoveries of crystalline insulin were approximately 40 percent of the insulin present in the combination mixture.

A third method for isolating insulin from combination mixtures of natural A and B chains was reported by Du *et al.* (*6*): insulin was extracted from the crude mixture with acidified secondary butanol, reextracted into dilute acetic acid, and eventually crystallized from citrate buffer containing zinc. This procedure has been used by Wang *et al.* (*27, 28*) and Kung *et al.* (*34*) for isolating microgram quantities of crystalline insulin from combination mixtures of synthetic and natural chains and of all-synthetic chains of bovine insulin. However, by this method also the recoveries of crystalline insulin were 37 percent of the insulin present in the combination mixture of natural chains (*6*) and approximately 5 percent of that present in the combination mixture of synthetic chains (*34*). Since in the latter instance the combination yield is 2 percent and of that amount of insulin only 5 percent is obtained in crystalline form. The overall yield of synthetic crystalline insulin is 0.1 percent of the theoretical prediction.

Of course the isolation of insulin from combination mixtures of its individual chains presents a situation different from that presented by isolation from natural sources, inasmuch that in the former case a protein is to be separated from closely related polypeptide chains. However, this consideration alone would hardly account for the low recoveries of insulin. A more likely explanation of this phenomenon may be provided by data recently obtained in our laboratory.

We have now developed (*33*) a new procedure for isolating insulin from combination mixtures of its individual chains, by which we can recover up to 70 percent of the insulin present in the combination mixture, as well as the unreacted A and B chains. We have further found that only part of the recovered insulin is readily crystallizable, whereas the remainder—often as much as 50 percent of the total—requires certain manipulations for crystallization. Whether this latter portion is formed during the combination reaction or is a product of subtle transformation of the crystallizable portion during isolation and handling remains to be shown. Recent experiments indicate that this insulin fraction gives the correct amino acid analysis for insulin and has a specific activity comparable to that of the natural hormone. The new procedure has been used with very favorable results in the isolation both of crystalline insulin from combination mixtures of synthetic and natural chains and of all-synthetic crystalline insulin (Fig. 10) (*33*).

In summary, preparation of the individual chains of insulin led us to its synthesis. Once synthesis was accomplished, the following questions remained: (i) Can the A and B chains be combined in high yield to produce insulin as the predominant final product? (ii) Can an effective procedure be developed for isolating the insulin so produced from the mixture of the individual chains? Our recent work that I have just described seems to answer both questions affirmatively.

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