protein synthesis in the absence of added mRNA (Fig. 4). The curve for absorbancy shows the separation of polysomes into at least seven classes with respect to size. The amount of amino acid that had been incorporated was determined for each class. The single ribosomes exhibited one-tenth to one-twentieth the specific activity of the larger polysomes, and this suggests that some of the ribosomes still contain mRNA or fragments of mRNA. These fragments support amino acid incorporation in this system (*1*). As expected, synthetic mRNA (polyuridylic acid) primarily stimulates only the single ribosomes in which C^{14}-phenylalanine incorporation rose about 15-fold.

The amount of labeled amino acid incorporated can be used as a measure of the accumulation of peptide product on the ribosomes. Plotting the specific activity of each peak fraction against polysome size (Fig. 5) shows regularly increasing amounts of peptide per ribosome unit as the polysome size increases. This is the predicted result if the larger polysomes contain longer strands of mRNA and produce higher molecular weight protein. From the theory of modulation of polycistronic messages by release of product protein by ribosomes at the end of a cistron (*7*) one can predict that the specific activity would increase at a diminishing rate as the polysome size increased. In the *Escherichia coli* system which was first depleted of basal mRNA and then supplemented with polycistronic messenger RNA from turnip yellow mosaic virus, the amino acid incorporation per ribosome particle rose initially, remained generally constant up to the octomer, and then decreased (*8*). In our experiments the largest polysome clusters were of such size that many clusters would contain more than one cistron per mRNA. In these large polysomes the rate of increase of specific activity with polysome size was undiminished. Our results could lose some of their significance if the mechanism of regulation responsible for polycistronic controls is not preserved by the system in vitro.

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Antibodies against the Component Polypeptide Chains of Bovine Insulin

Abstract. *Antibodies were produced in guinea pigs against A and B chains of bovine insulin; the chains were prepared by cleavage of all the disulfide bonds with sulfite. Antibodies against A chain reacted with A chain, but not with B chain or native insulin. Antibodies against B chain reacted strongly with both B chain and insulin, but not with A chain. Antibodies against insulin reacted strongly with insulin, weakly with B chain, and not at all with A chain. The results indicate that the insulin-reactive antibodies in antiserums to B chain and to insulin differ and are directed to differing individual antigenic regions of the insulin molecule.*

The primary structure of bovine insulin is well known; insulin is composed of two polypeptide chains connected by two interchain disulfide bonds (*1*). The A chain consists of 21 amino acid residues with *N*-terminal glycine and *C*-terminal asparagine and has one intrachain disulfide bond between the two cysteine residues at 6 and 11. The B chain consists of 30 amino acid residues with *N*-terminal phenylalanine and *C*-terminal alanine. Insulins of other species of mammals as well as of fishes have chains closely related in structure (*2*). Antibodies against these component chains of insulin, which are of such a small molecular size and of known amino acid sequence, could serve as models for studying the interaction of more complex protein antigens and antiprotein antibodies. In addition, these antibodies should be useful in biological studies of insulin and perhaps of the insulin antagonist in serum (*3*).

We now report on the antibodies produced in guinea pigs against the A and B chains of bovine insulin. The chains were obtained by cleavage of all the disulfide bonds with sulfite. Antigens labeled with I^{125} were used to detect antibodies. Antigens bound to antibodies were separated from free antigens by precipitation with rabbit antiserum against components of guinea pig serum.

Crystalline bovine zinc insulin (*4*) was freed of most zinc by isoelectric precipitation in the presence of ethylenediamine tetraacetate. The product was submitted to chromatography on diethylaminoethyl cellulose in *tris*-phosphate buffer containing 6*M* urea (*5*) at 5°C to remove desamidoinsulin and two minor contaminants (*6*). The purified insulin showed a sharp single band on electrophoresis on paper in veronal buffer containing 6*M* urea (*p*H 8.6, $\Gamma/2 = 0.075$) and on disc electrophoresis at *p*H 8.9 (*7*).

The *S*-sulfonated A and B chains (SS-A and SS-B chains) were prepared from the chromatographically purified bovine insulin with sulfite and tetrathionate at *p*H 7.4 in 6*M* urea (*5*) in the manner of Bailey and Cole (*8*), with minor modifications. On paper electrophoresis in urea-veronal buffer, the SS-A chain gave a single band of fast mobility; the SS-B chain showed essentially a single band at a position slightly slower than that of insulin. Occasionally, another component was observed migrating more slowly in small quantity. The results of amino acid analysis were in good agreement with the values expected for the sulfonated chains. Threonine, proline,

Table 1. Reactions of I^{125}-labeled insulin and *S*-sulfonated chains with guinea pig antiserums. Signs (in parentheses) indicate the densities of arcs on the radioautographs obtained by radioimmunodiffusion. Values indicate the percentages of added radioactivity found in the precipitates by coprecipitation. The labeled antigen in *p*H 8.0 borate buffer containing 2 percent bovine serum albumin (20 μl) was incubated with 200 μl of 20-fold diluted guinea pig antiserum for 3 hours at 37°C. Antigen concentrations in the mixtures were 4.8, 4.3, and 4.5 $\times$ 10^{-8} mole/liter for I^{125}-labeled insulin, SS–A chain, and SS–B chain, respectively. RAGP (500 μl) was then added to precipitate guinea pig globulin. The amount of labeled antigen carried down with the guinea pig globulin was calculated from the radioactivity remaining in the supernatant.

Labeled antigen bound to the serum globulin		
Insulin (%)	SS–A chain (%)	SS–B chain (%)
Normal serum		
0.4 (–)	2.6 (–)	0 (–)
Antiserum to insulin		
83 (++++)	0.9 (–)	11 (+)
Antiserum to SS–A chain		
2.3 (–)	42 (++++)	0 (–)
Antiserum to SS–B chain		
78 (++++)	2.3 (–)	79 (++++)

phenylalanine, lysine, histidine, and arginine were entirely absent from the SS-A chain preparation, and isoleucine was absent from the SS-B chain preparation, indicating that these preparations were essentially pure.

The *S*-carboxymethylated A and B chains (SC-A and SC-B chains) were prepared from the corresponding sulfonated chains by reduction in 0.1*M* β-mercaptoethanol and 8*M* urea (*5*) at *p*H 8.6 for 2 hours at room temperature, followed by alkylation with 0.2*M* iodoacetate for 30 minutes. The *S*-carboxymethylated chains were isolated by passage through Sephadex G-25 in $(NH_4)_2CO_3$ at *p*H 8.5.

Insulin was iodinated with I^{125} by the use of iodine monochloride, ICl (*9*); the SS-A and SS-B chains, by the method of Greenwood *et al.* (*10*). The iodinated preparations contained less than 1 atom of iodine per molecule—10 to 30 mc of I^{125} per milligram of protein; they were kept frozen in the presence of 2 percent bovine serum albumin until used.

Groups of four to eight guinea pigs were immunized with the chromatographically purified insulin, SS-A chain, or SS-B chain by two or three intramuscular injections at 2-week intervals. Prior to injection, the purified insulin was converted to insoluble protamine-zinc-insulin, and the suspension was emulsified with complete Freund's adjuvant (Difco); the chain preparations were also incorporated in the adjuvant. Each dose (0.4 ml) contained 0.33 mg of insulin and 1 mg of either SS-A or SS-B chain. The animals were bled 7 to 10 days after the last injection. Insulin and SS-B chain produced antiserums of moderate to high titers in most animals. SS-A chain produced antiserums of moderate titers in only about 25 percent of the animals. Rabbit antiserums against guinea pig globulin (RAGP) were obtained by repeated injections of crude normal globulin (precipitate at 50 percent saturation of ammonium sulfate at *p*H 7) emulsified with Freund's complete adjuvant.

The reaction of antibodies with the antigens was determined at *p*H 8.0 by two methods: radioimmunodiffusion and coprecipitation. Insulin and its component chains do not directly precipitate with the antibodies. For radioimmunodiffusion, the guinea pig antiserum (2 to 6 μl) was diffused in agar against a mixture of RAGP and I^{125}-labeled antigen (1 μg/ml of rabbit serum). Among the precipitate arcs formed, the arcs containing guinea pig antibody components bound the radio-iodinated antigen; these radioactive arcs were revealed by x-ray films (*11*).

The coprecipitation technique of Skom and Talmage (*12*) yielded more quantitative data. The guinea pig antiserum was incubated for 3 hours at 37°C with I^{125}-labeled antigens which had been prepared by diluting the original radio-iodinated antigens with the corresponding unlabeled materials. It was then mixed with RAGP to precipitate guinea pig globulins. The mixture stood overnight in the cold before the amount of antigen bound to the precipitate was determined from the radioactivity remaining in the supernatant (*13*).

The reactions of insulin and SS-A and SS-B chains (all labeled with I^{125}) with the guinea pig antiserums (Table 1) indicate that the antiserum against A chain is specific and reacts only with the homologous A chain. The antiserum against insulin reacts strongly with insulin, only weakly with B chain, and not at all with A chain. The antiserum against B chain reacts equally well with B chain or insulin, but not at all with A chain. Antiserums from other animals gave essentially the same results.

Reactions of antibodies with unlabeled antigens and related compounds were determined by inhibition of the radioimmunodiffusion and coprecipitation. In the radioimmunodiffusion, increasing amounts of the test material were added to the guinea pig antiserum (30 to 3000 μg/ml of antiserum) and the mixtures were reacted in agar with RAGP containing I^{125}-labeled antigen. With the coprecipitation, a binding curve was established by addition of increasing amounts of the homologous unlabeled antigen. Inhibition of the binding was then determined by replacing the unlabeled antigen with a test compound; inhibitory activity was expressed as the ratio of the molar amount of the homologous antigen to that of the test material producing the same degree of inhibition.

The homologous unlabeled antigen strongly inhibited fixation of the labeled antigen by the corresponding antibody

Table 2. Inhibitory activity of unlabeled antigens and related compounds. Signs (in parentheses) indicate the degrees of inhibition observed in radioimmunodiffusion with unlabeled antigens or related compounds up to 3000 μg per milliliter of antiserum. Values represent the activity, relative to the homologous antigen, of related compounds in inhibiting the binding of the labeled antigen to the corresponding antibody. Experiments with the homologous unlabeled antigen are underlined. Concentration of the labeled antigens in the reaction mixtures (270 μl) was approximately 3.5 to 3.9 $\times$ 10^{-8} mole/liter; 3.3 μl of antiserum to insulin, 10 μl of antiserum to A chain, and 5.0 μl of antiserum to B chain were used. The homologous unlabeled antigen produced significant inhibition when added to concentrations of 5 to 10 $\times$ 10^{-8} mole/liter; compounds which produced no significant inhibition at concentrations more than 20 times this concentration are marked 0.

Unlabeled compound	Labeled antigen-antibody system				
	Insulin versus anti-insulin	SS–A versus anti-A	SS–B versus anti–B	Insulin versus anti–B	SS–B* versus anti-insulin
Insulin	100 (++)	0 (–)	~100 (++)	(++)	(++)
SS–A chain	0 (–)	100 (++)	0 (–)	(–)	(–)
SC–A chain	0 (–)	6 to 25 (+)	0 (–)	(–)	(–)
SS–B chain	0 (–)	0 (–)	100 (++)	(++)	(++)
SC–B chain	0 (–)	(–)	~100 (++)	(++)	(++)
Cys–*S*–sulfonate	(–)	(–)	(–)	(–)	(–)
Cys–sulfonate	(–)	(–)	(–)	(–)	(–)

* Reaction of I^{125}-SS-B chain and the antiserum to insulin was much weaker than that in other systems (see Table 1). Anti-insulin, antiserum to insulin; anti-A, antiserum to A chain; anti-B, antiserum to B chain; Cys, cysteine.

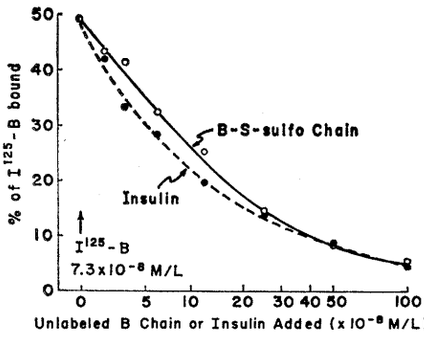

Fig. 1. Inhibition by unlabeled SS-B chain and insulin of the binding of I^{125}-labeled SS-B chain with antiserum to B chain; the concentration of I^{125}–B chain in the reaction mixtures was 7.3×10^{-8} mole/liter. Abscissa represents the percentage of the added radioactivity found in the precipitate by coprecipitation. M/L, mole per liter.

(Table 2). No inhibition was observed with L-cysteine *S*-sulfonate, L-cysteine sulfonate (L-cysteate) (*4*), or *S*-sulfonated chains other than the homologous antigen, indicating that the cysteine *S*-sulfonate residue itself is not a determinant. However, the *S*-carboxymethylated A (SC-A) chain showed a significantly lower activity than the homologous SS-A chain in inhibiting the reaction of I^{125}–SS-A chain with the corresponding antiserum. This difference suggests that the nature of the altered cysteine residue does affect the determinant region of the SS-A chain, either by direct participation or by indirect effect on the conformation (*14*). On the other hand, the SC-B chain showed an inhibitory activity as strong as that of the homologous SS-B chain, indicating that *S*-sulfonate groups are not involved in the determinant region of the SS-B chain.

The reaction of I^{125}-labeled B chain with the antiserum to the B chain was inhibited almost completely by both unlabeled insulin and B chain; very similar inhibition curves (Fig. 1) indicate that most of the antibodies formed against B chain react equally well with B chain or the native insulin. On the other hand, the reaction of I^{125}-insulin with the antiserum to insulin was inhibited strongly by unlabeled insulin, but not at all by B chain (Table 2); radioimmunodiffusion showed no sign of inhibition even when 3000 μg of B chain was used per milliliter of the antiserum, whereas inhibition was obvious with as little as 30 μg of unlabeled insulin. This is strong evidence that the preparation of SS-B chain used here is not contaminated with insulin. Both insulin and B chain showed inhibition of the cross-reacting systems: I^{125}-insulin with the antiserum to B chain, and I^{125}–B chain with the antiserum to insulin. The A chain did not inhibit any reaction with either antiserum.

These results clearly indicate the difference in specificity between the insulin-reactive antibodies in the antiserum to insulin and in the antiserum to B chain. The insulin-reactive antibody formed against B chain must be directed against certain structures common to both SS-B chain and native insulin. On the other hand, the insulin-reactive antibody in the antiserum to insulin must be directed largely against regions present only in the native insulin molecule, which were altered either chemically or by a conformational change accompanying the sulfitolysis. Whether this region involves the A chain alone (as present in intact insulin) or the B chain alone, or both, is not known. Berson and Yalow (*15*) and Wilson *et al.* (*15*) indicate that the A-chain part of the insulin molecule seems to contain the determinant region reacting with the antiserum to insulin.

The absence of cross-reaction between A chain–insulin and the antiserums must be due to great change in the structure of the A chain caused by the sulfitolysis.

A slight reaction of the antiserum to insulin with I^{125}–B chain when tested directly (Table 1) may indicate that a small portion of antibodies in the antiserum to insulin is similar to those in the antiserum to B chain and is directed to the region common to B chain and insulin. The content of such antibodies in the antiserum to insulin may be too small to be detected by inhibition of the I^{125}-insulin–anti-insulin system with unlabeled B chain (Table 2). Alternatively, all the insulin-binding antibody in the antiserum to insulin may combine with B chain, but with an association constant much lower than that with the native insulin. Here, B chain would be much less effective than insulin in inhibiting the system. However, no inhibition was observed by either method, even at high concentrations of B chain.

Thus, only B chain produces this cross-reacting antibody; native insulin does not. In the course of immunization, the B chain may be modified in vivo to leave the antigenic regions common to both the antigens, before antibody formation, whereas the native insulin may be modified to leave only the antigenic regions characteristic of the native insulin molecule. Antibodies to insulin apparently directed to differing antigenic regions of the molecule were reported recently by Arquilla *et al.* in inbred strains of guinea pigs and in rabbits (*16*).

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5. Fresh urea solution was deionized by passing through Dowex-MB1 to remove cyanate; appropriate salts or reagents were added, and the solution was used immediately [G. R. Stark, W. H. Stein, S. Moore, *J. Biol. Chem.* **235**, 3177 (1960); D. Cole, *ibid.* **236**, 2670 (1961)].
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14. Because both *S*-sulfonate and *S*-carboxymethyl groups are fully charged at *p*H 8.0, the difference between SS-A and SC-A chains must be due to differences other than charge.
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