chemical reactions involved in bile acid formation in mammalian liver.

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The Biologic Activity of Insulin A and B Chains as Determined by the Rat Diaphragm and Epididymal Fat Pad*

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ABSTRACT: This study demonstrates that both purified insulin A and B chains inhibit the uptake of glucose by the rat hemidiaphragm, but neither chain prevents the expected stimulation of glucose uptake in the presence of unmodified insulin. A chain but not B chain is capable of stimulating the conversion of labeled glucose to carbon dioxide in the rat epididymal fat pad, and this simulates the action of the intact insulin molecule. This effect of A chain on adipose tissue is abolished by the addition of insulin antiserum. The integrity of the A and B chains was established by the demonstration of significant insulinlike activity in both muscle and adipose tissue when the chains were recombined and by the neutralization of this activity by insulin antiserum.

These results suggest that insulin A chain alone is capable of producing an insulinlike action upon rat adipose tissue and that, in the absence of insulin, both A and B chains are capable of inhibiting the uptake of glucose in muscle, but this action does not appear to be dependent upon the actual antagonism of unreduced insulin.

The insulin molecule is composed of two polypeptide units, A chain having 21 amino acids with glycine at its NH_2 terminus, and B chain having 31 amino acids with a phenylalanine residue at its NH_2 terminus. These two chains are linked together by two disulfide bonds, and it had been generally accepted that when these disulfide bonds are broken hormone activity is abolished (du Vigneaud *et al.*, 1931). Dixon and Wardlaw (1960) have reported that separated A or B chains did not stimulate glycogen formation in the mouse diaphragm, nor were they active in the mouse convulsion test. Recently, however, conflicting reports have suggested that synthetic A chain produces slight insulinlike activity upon rat diaphragm muscle (Volfin *et al.*, 1964) and that B chain is capable of stimulating adipose tissue (Langdon, 1960). In addition, it has been suggested that B chain, when in combination with serum albumin, behaves as an insulin antagonist and is capable of inhibiting glucose uptake in muscle but not in adipose tissue (Ensinck and Vallance-Owen, 1963).

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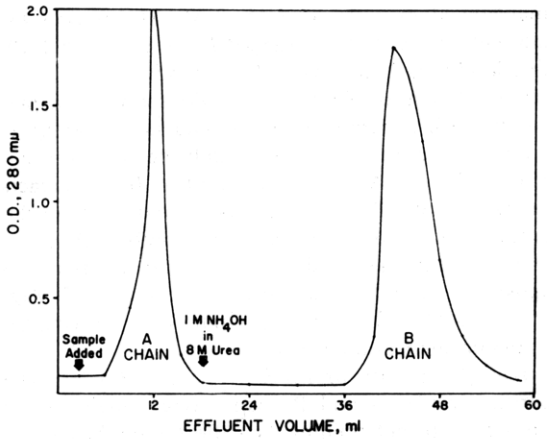

FIGURE 1: Separation of 100 mg of reduced, sulfonated insulin mixture by Dowex 50W-X2, 200–400 mesh resin, equilibrated with 2.6 M formic acid in 8 M urea; sample size, 2 ml. Eluting buffer changed to 1 M NH_4OH in 8 M urea immediately following collection of initial unabsorbed peak.

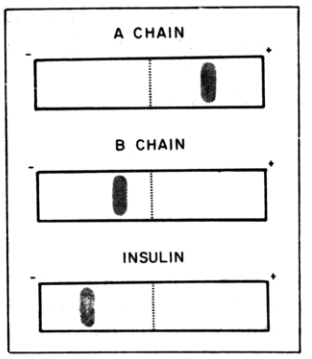

FIGURE 2: Electrophoresis of S-sulfonate A and B chain and crystalline insulin performed on cellulose acetate strips in 1% pyridine–10% acetic acid–8 M urea buffer. The dotted line represents the point of sample application. Electrophoresis continued for 2.5 hr at 4 v/cm and 27°. (For clarity the electrophoretic strips are represented by drawings.)

Because this question of biologic activity of the insulin polypeptide chains has not been resolved, it was considered worthwhile to examine this problem by testing the purified chains in two *in vitro* insulin assay systems: the determination of glucose uptake by the rat hemidiaphragm and the production of CO_2 from glucose by rat adipose tissue.

Experimental Procedures and Results

Preparation of Insulin A and B Chains. Crystalline bovine insulin (approximately 24 IU/mg) was obtained from Sigma Chemical Co. and was subjected to sulfitolysis by the method of Swan (1957). Immediately prior to sulfitolysis an 8 M urea solution was deionized by passage through Amberlite MB-1 resin to remove cyanate (Cole, 1960). The sulfitolysis mixture contained 1.0 g of insulin in 20 ml of an 8 M urea solution containing 0.2 M sodium sulfite and 0.08 M cupric sulfate. The pH of this reaction mixture was adjusted to 10.2 with 27% NH_4OH. The reaction mixture was allowed to stand at room temperature for 2.5 hr following which it was acidified with 90% formic acid to pH 3.2. The chain mixture was precipitated by adding 10 volumes of 95% ethanol. The precipitate was collected by centrifugation and dried *in vacuo* under N_2.

The S-sulfonate derivatives of the insulin A and B chains were separated by passage through a 1.2 × 30 cm column of Dowex 50W-X2, 200–400 mesh, resin which had been equilibrated with 2.6 M formic acid in 8 M urea (Dixon and Wardlaw, 1960).

As indicated in Figure 1, two separate protein peaks were obtained. The first peak represents A chain which is not retained by the column. The second peak, containing B chain, was obtained following elution of the resin with 1 M NH_4OH in 8 M urea. The appearance of B chain corresponded to the change in pH of the eluate from 4 to 9. A chain was separated from urea by precipitation in an acetone–ethanol mixture. B chain was freed of urea by passage through a Sephadex G-50 column (2.0 × 34 cm) which had been equilibrated with 1 M NH_4OH. B chain was then precipitated by neutralization with 90% formic acid.

Electrophoretic and N-Terminal Analysis of the Insulin A and B Chains. The S-sulfonate chain derivatives were studied for purity by means of electrophoresis and N-terminal amino acid analysis. Crystalline insulin and the chain derivatives were dissolved in 0.02 M formate buffer, pH 3.8, and 8 M urea, and 1.0 mg was applied to cellulose acetate strips. The electrophoresis buffer was 1% pyridine and 10% acetic acid in 8 M urea. Electrophoresis was performed at 4 v/cm for 2.5 hr at room temperature. The strips were dried and stained with a diazotized sulfanilic acid–sodium carbonate solution. Figure 2 indicates the presence of a single anodal migrating band representing A chain, a single slow-moving cathodal spot by B chain, and a faster moving negative component produced by insulin.

The N-terminal amino acid of each chain was determined by the fluorodinitrobenzene method (Sanger, 1945). The dinitrophenyl (DNP) derivatives of each chain were subjected to a separate acid hydrolysis of 6 and 18 hr in order to detect both glycine and phenylalanine in each chain. It was found that the isolated A chain preparation contained only DNP-glycine and the B chain product contained solely DNP-phenylalanine at its NH_2 terminus.

Immunologic Specificity of Insulin A and B Chains. Insulin and the S-sulfonated A and B chains were iodinated with ^{131}I by the method of Greenwood *et al.* (1963) which labeled the insulin molecule and the chain derivatives with 90–95% efficiency and resulted in specific activities ranging from 70 to 95 mc/mg (Meek *et al.*, 1966). The labeled antigens were purified

by gel filtration through Sephadex G-25 and G-75. Groups of four guinea pigs were immunized with insulin and the S-sulfonated A and B chains by the method of Yagi *et al.* (1965).

The reaction of the antibodies with the labeled antigens was determined by the double antibody technique of Schalch and Parker (1964). The concentration of each labeled antigen was approximately 0.1 mμg/600 μl of reaction mixture at pH 8.6, and the antibody concentrations ranged from a 1:10 to 1:10^5 dilution for each antiserum. Table I indicates the maximum percentages of the radioactivity found in the precipitates which represents the bound labeled antigen–antibody complex. The results indicate that A chain binds only with A chain antibody; that B chain reacts primarily with B chain antibody; and that insulin antibody does not cross-react significantly with either of the S-sulfonate insulin chains. These results are in agreement with the published observations of Yagi *et al.* (1965) and establish the purity of the isolated chains on an immunologic basis.

TABLE I: Reaction of ^{131}I-Labeled Insulin and S-Sulfonated Chains with Specific and Control Guinea Pig Antiserum as Determined by the Two Antibody Immunoprecipitation Method.

Labeled Antigen	Antibody			
	A	B	I	C[a]
A	++++	−	−	−
	(41)[b]	(0.8)	(2.3)	(0.4)
B	−	+++	±	−
	(3.1)	(25)	(6.3)	(1.9)
I	±	+++	++++	−
	(7.5)	(21)	(60)	(1.0)

[a] Control (nonimmunized) guinea pig serum. [b] Percentage of labeled antigen bound to antiserum.

Biologic Activity of Insulin A and B Chains. The purified insulin A and B chains were initially tested for activity by determining their effect upon the glucose uptake of the isolated rat hemidiaphragm by the method of Willebrands and Groen (1954). Immediately prior to the incubation of the diaphragm with the test material, the insulin chains were treated with excess mercaptoethanol (6.2 mM) to remove the sulfonate and regenerate the free sulfhydryl groups (Dixon and Wardlaw, 1960). In order to obtain statistic validity a minimum of five animals was used in each assay. Glucose concentrations of the media were determined by the oxidase method (Keilin and Hartree, 1948) both prior to and following a 90-min incubation

TABLE II: Activity of Insulin A and B Polypeptide Chains as Determined by the Uptake of Glucose in the Rat Hemidiaphragm.

Expt	Hemidiaphragm		Concn of Chain (μg ml)	No. Diaphragms	Change in Glucose (mg%)	σD[a]	P	Net Effect[b]
	Control	Test						
1	Buffer	Insulin[c]	—	20	+18	±1.3	<0.001	+
2	Buffer	Insulin + antiserum[d]	—	15	+0.8	±1.8	>0.5	0
3	Buffer	A chain	40	35	−7.3	±1.6	<0.01	−
4	Buffer	B chain	40	15	−10	±2.6	<0.005	−
5	Buffer	A (S-sulfo)	40	5	+2.2	±2.7	>0.05	0
6	Buffer	B (S-sulfo)	40	5	+2.5	±2.2	>0.05	0
7	Buffer	Thiol	—	5	+0.8	±2.3	>0.05	0
8	Buffer	A + B	80	10	+7.6	±2.3	<0.01	+
9	Buffer	A + B + antiserum	80	5	−13	±1.6	<0.005	−
10	Insulin	Insulin + A	40	20	−1.7	±1.7	>0.05	0
11	Insulin	Insulin + B	40	5	−3.4	±4.2	>0.05	0
12	A chain	A + Insulin	40	5	+17	±2.7	<0.005	+
13	B chain	B + Insulin	40	5	+15	±3.3	<0.005	+
14	Antiserum	Antiserum + A	40	20	−10	±1.3	<0.001	−
15	Antiserum	Antiserum + B	40	10	−11	±2.9	<0.005	−

[a] $\sigma D = \sqrt{(\Sigma X)^2/N(N-1)}$. [b] Net effect, significant difference ($P < 0.01$) in glucose uptake of test diaphragm indicated as stimulated (+) or inhibited (−). [c] Insulin concentration, 40 mμg ml. [d] Guinea pig antiinsulin serum, 0.06 ml/2.0 ml of media.

TABLE III: Activity of Insulin A and B Polypeptide Chains Determined by [^{14}C]CO_2 Production in the Rat Epididymal Fat Pad.

Expt	Substance	Amt (μg/ml)	Act. (log micro units of insulin/ml)	SD[a]	N[b]	Net Effect[c]
1	Insulin	0.0002	0.70	0.27	24	0
2	Insulin	0.02	2.70	0.27	24	+
3	Insulin + antiserum	0.02	0.00	0.22	12	0
4	A chain	40.0	2.27	0.32	21	+
5	A chain + antiserum	40.0	0.13	0.23	12	0
6	A chain	80.0	2.36	0.25	6	+
7	A chain + antiserum	80.0	0.00	0.35	3	0
8	A chain + insulin	40.0 + 0.02	2.37	0.40	6	+
9	B chain	40.0	0.10	0.39	9	0
10	B chain + insulin	40.0 + 0.02	2.54	0.38	3	+
11	B chain + antiserum	40.0	0.00	0.38	3	0
12	A + B chains	80.0	2.90	0.34	6	+
13	A + B chains + antiserum	80.0	0.60	0.23	3	0
14	A chain (S-sulfo)	40.0	0.00	0.06	3	0
15	Thiol[d]		0.15	0.29	6	0
16	Thiol + antiserum		0.95	0.20	3	0
17	Thiol + insulin	0.02	2.70	0.36	3	+

[a] $SD = \sqrt{(\Sigma x^2 - (\Sigma x)^2/N)/(N-1)}$. [b] N, numbers of assays. [c] Net effect, response indicating significant insulinlike activity ($p < 0.01$), designated as +. [d] Thiol, 6.2 mM β-mercaptoethanol.

of the paired hemidiaphragms under aerobic conditions. One incubation flask contained the test substance; the other contained the control buffer. The results were expressed as changes in glucose concentration in milligrams per cent, which reflects the difference in glucose uptake by the test hemidiaphragm compared to that of the control tissue.

The results of the effect of the insulin chains upon glucose uptake by the rat diaphragm are listed in Table II. Both A and B chains when tested in the concentration of 40 μg/ml significantly inhibited the uptake of glucose by the muscle tissue. This effect was noted only with the free sulfhydryl forms of the chains since no appreciable change in glucose concentration was obtained following incubation with the S-sulfonate derivatives of the insulin chains. Likewise, the presence of thiol alone (6.2 mM mercaptoethanol) did not inhibit glucose uptake. The inhibitory effect of the individual insulin chains was abolished by the preincubation of an equal mixture of S-sulfonate A and B chain with thiol. The demonstration of a significant insulin effect of such a mixture indicated that the chains were capable of recombination to form the intact and biologically active insulin. This finding was further supported by the abolishment of the insulin activity from the recombination of the chains by the addition of guinea pig antiinsulin serum.

The relationship between whole insulin and its component chains was studied in a separate series of experiments. When either A or B chain was added to the system in which both control and test hemidiaphragms were stimulated by intact insulin (40 mμg/ml), no significant difference in glucose uptake by the rat diaphragm was obtained. Conversely, when whole insulin was added to the test hemidiaphragm in a system in which equal concentrations of either A or B chain were present in both the control and experimental flasks, the expected insulin stimulation of glucose uptake was observed.

Although insulin antiserum in the concentration of 0.06 ml/2.0 ml of incubation media completely neutralized the stimulation of glucose uptake by whole insulin or following resynthesis from A and B chains, identical concentrations of antiserum had no effect upon the inhibition of glucose uptake by A or B chain alone. These experiments indicate that whereas both insulin chains inhibit glucose uptake by the resting diaphragm, neither chain at a concentration of 40 μg/ml alters the expected insulin stimulation when incubated with 0.04 μg/ml of insulin.

Effect of Insulin A and B Chains upon [^{14}C]CO_2 *Production by the Rat Epididymal Fat Pad.* The purified sulfhydryl derivatives of the insulin chains were tested for their effect upon adipose tissue by the method

of Renold (Renold *et al.*, 1960) which is based upon the production of $[C^{14}]CO_2$ from C-1-labeled glucose by the rat epididymal fat pad. The results are listed in Table III. In contrast to the effect upon the rat diaphragm, A chain in a similar concentration of 40 μg/ml produced marked insulinlike activity upon the fat pad. This insulin effect, although not clearly dose related, was completely abolished by insulin antiserum at both 40- and 80-μg/ml concentration of A chain.

B chain did not display significant insulinlike activity nor did it antagonize the expected stimulation of adipose tissue by 20 mμg of insulin. As was noted with the diaphragm studies, recombination of insulin A and B chains prior to incubation produced a marked insulin effect which was significantly, although not completely, abolished by addition of insulin antiserum. The S-sulfonate derivative of A chain had no insulinlike effect and mercaptoethanol alone (in a concentration equal to that used to regenerate the free sulfhydryl forms of the chains) was without effect upon the assay, either alone or in combination with insulin.

Discussion

The biologic activity of the two polypeptide chains of the insulin molecule has been subject to conflicting reports in the past. Following Sanger's isolation and identification of the insulin chains (Sanger, 1949), it was generally assumed that hormone activity was abolished when insulin was split into its A and B chains by breaking the disulfide bonds (du Vigneaud *et al.*, 1931). In recent years, however, reports have appeared which suggest that insulin chains may possess some of the activity associated with intact insulin (Volfin *et al.*, 1964; Langdon, 1960; Nichol, 1959). Part of the confusion as to biologic activity of the chains stems from the method of preparation. Langdon's work (1960), which suggested that B chain in the sulfhydryl form has insulin activity in adipose tissue, has been criticized (Dixon and Wardlaw, 1960) on the grounds that his B chain preparation was significantly contaminated with A chain which allowed recombination to form intact insulin at the time of biologic testing. Recently Volfin *et al.* (1964) have indicated that when A chain is prepared in a form which keeps the 6–11 disulfide intact, the A chain unit then is capable of stimulating glucose uptake by the rat diaphragm. The degree of stimulation obtained, however, is small (less than 0.6% based on native insulin). In addition, it is not clear that the A chain peptide by Volfin *et al.* (1964) differs significantly from that of Dixon and Wardlaw (1960) since the latter group treated the S-sulfonate derivatives of the A and B chains with excess thiol prior to assay to reestablish the free sulfhydryl groups. The same technique was employed in the present study, and the results indicate that the sulfhydryl groups must be free of sulfonate in order for A chain to exert biologic activity in adipose tissue.

The same criticisms concerning purity of the insulin chain derivatives can be directed at the present work. It was for this reason that three independent criteria of purity were applied to the isolated chain peptides. Both N-terminal amino acid analysis and cellulose acetate electrophoresis indicated that the chains were isolated in a form free of significant cross-contamination. To further check the degree of purity of the isolated chains, antibodies were produced in guinea pigs against the purified A and B chains of insulin as well as intact insulin by the method of Yagi *et al.* (1965). The antigen–antibody reactions were detected by the use of the ^{131}I-labeled antigen–double antibody precipitation method (Meek *et al.*, 1966). This procedure is capable of detecting and differentiating millimicrogram amounts of either insulin chain or the intact molecule. The results indicated that neither B chain nor insulin could be detected in the A chain preparation. There was significant cross reaction of B chain antibody with labeled insulin, but B chain labeled antigen did not bind significantly with insulin antibody. Similar results were noted by Yagi *et al.* (1965) which indicate certain common features of B chain and insulin antibody; however, the results indicate immunologic specificity of the labeled chain preparations and establish their purity on this basis.

The finding of a difference in biologic activity of A chain when tested in adipose tissue *vs.* muscle is interesting in view of the recent developments suggesting possible biologic modifications of insulin in pathologic states (Antoniades, 1961; Samaan and Fraser, 1963; Alp and Recant, 1964). On the basis of the present study it is difficult to assign a role to one or both insulin chains as possible insulin antagonists. Certainly B chain appears to be biologically inactive since it does not stimulate glucose uptake by diaphragm muscle nor adipose tissue. It does share in common with A chain the ability to depress glucose uptake by the noninsulin-stimulated rat diaphragm. The activity of A chain as demonstrated in this *in vitro* work would more properly serve as an insulin antagonist since it displays the dual ability to stimulate glucose uptake by adipose tissue, yet inhibit glucose uptake in muscle when additional insulin is absent.

It should be pointed out that much larger concentrations of A chain compared to insulin were used to demonstrate biologic activity of the former upon adipose tissue. Although the minimum concentration of A chain capable of adipose tissue stimulation was not determined, it is possible that significantly larger concentrations of the insulin chains in relationship to the complete insulin molecule are present in biologic fluids. Preliminary results of the application of a sensitive radioimmunoassay to the detection of insulin A and B chains in human serum suggest that such a relationship exists (Meek *et al.*, 1966). There are at least two factors which make this a possibility. Mirsky *et al.* (1955) have described an insulin-degrading enzyme in liver which has now been purified (Tomizawa, 1962) and acts to cleave the insulin molecule into its A and B chains. This could serve as one source of the insulin chains. In addition, evidence has recently been presented (Humbel, 1965) that in the biosynthesis of insulin the two chains are first synthesized as separate polypeptide

chains and then combined to form the intact insulin molecule. This finding of the separate synthesis of the two insulin chains suggests that situations might exist in which there is a relative overproduction of either chain. There is no evidence at the present time that an abnormal production or degradation of the insulin chains actually exists; however, it seems appropriate to suggest that the *in vitro* biologic activities demonstrated in this work be correlated with *in vivo* concentrations of the insulin chains in normal and pathologic states.

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