long-chain bromide with an alkynyllithium or (b) reaction of a ketone with a Grignard complex or an alkyllithium. Dehydration and hydrogenation steps were applied to yield the saturated alkanes.

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Inhibition of Insulin Activity in Mitochondrial Systems and in Normal Rats by Reduced Insulin B Chain–Albumin Complex*

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ABSTRACT: A complex consisting of reduced bovine insulin B chain with crystalline bovine albumin inhibits the swelling effect of insulin on rat liver mitochondria. The inhibition is concentration dependent and is not seen with S-sulfo B chain or with reduced insulin A chain. The mitochondrial swelling effect of bovine growth hormone is not inhibited by reduced B chain–albumin, and pretreatment of mitochondria with reduced B chain–albumin does not result in subsequent inhibition of insulin activity. These two findings suggest that the B chain may be acting directly on insulin rather than on its site of action. Intraperitoneal injection of the reduced B chain–albumin complex into fasted normal rats significantly increases blood glucose levels, presumably by inhibiting endogenous insulin. Blood glucose is not increased by S-sulfo B chain or when reduced B chain is not complexed with albumin prior to injection. These results obtained by different experimental procedures further confirm the demonstration by J. W. Ensinck, R. J. Mahler, and J. Vallance-Owen (1965, *Biochem. J. 94*, 150) of a reduced insulin B chain insulin-inhibition mechanism.

The observation of inhibition of insulin activity associated with the albumin fraction of diabetic plasma (Vallance-Owen *et al.*, 1958a,b) and the identification of this factor as a complex of insulin B chain with albumin (Ensinck *et al.*, 1965) have presented an attractive new approach to the problem of diabetes.

In the work reported here, we complexed the reduced B chain of purified bovine insulin with crystalline bovine albumin and determined the effect of the complex under various conditions on the swelling of rat liver mitochondria. In addition, we determined the extent of the *in vivo* inhibition of endogenous insulin activity by the reduced B chain–albumin complex in fasted normal rats by measurement of blood sugar levels.

Materials

Crystalline Bovine Insulin (approximately 24 IU/mg). Insulin concentrations were calculated on the basis of a molecular weight of 36,000; thus a 5×10^{-6} M concentration of insulin contained 0.9 mg of insulin in a 5-ml test system. *Insulin, crystalline* L-*cysteine*, and *Trizma*, pH 7.3 [tris(hydroxymethyl)aminomethane hydrochloride, reagent grade] were obtained from the Sigma Chemical Co.

S-Sulfo A and B chains of insulin were prepared from

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crystalline insulin according to a described procedure (Dixon and Wardlaw, 1960). After the Dowex 50W-X2 column treatment and separation of the A and B chains had been accomplished, removal of urea was effected by dialysis of the solution with agitation for 3 hr against four changes (13 l. each) of cool (18°) distilled water. The solutions were then shell frozen and lyophilized. A small amount of urea (0.1–1.0 mg/mg) was retained with the B chain preparations. Urea determinations were made on weighed aliquots of the A and B preparations by an automated procedure (Technicon AutoAnalyzer Method N-10, based on the method of Skeggs, 1957) so that the amount of A or B chain present with urea in each weighed aliquot of each preparation could be determined by difference. The small quantity of urea remaining with the B chain preparation appeared to aid in its solubilization.

Paper electrophoretic examination on Whatman No. 3 paper strips of purified S-sulfo A and S-sulfo B chains of insulin in pH 8.6, Veronal buffer, $\mu = 0.05$, for 2.25 hr in an E-C apparatus at 8 ma, 430 v, at 4°, indicated a single band for each component. The components were stained by spraying with 1% diazotized sulfanilic acid in 5% sodium carbonate. S-Sulfo A chain appeared as a single orange band that migrated 4.8 cm toward the anode from the point of application while S-sulfo B chain appeared as a single orange band which migrated 2.2 cm toward the anode under these conditions..

Reduction of S-sulfo B chain was accomplised by a modification of the Dixon and Wardlaw (1960) procedure. The separated and purified S-sulfo B chain was solubilized in 0.0062 M cysteine–0.02 M Tris solution, pH 8.6–8.9, at a concentration of 1 mg/ml. Tubes containing 1-mg/ml aliquots of B chain were placed in a near-boiling hot water bath for 2 min and then in a beaker of water to cool to room temperature. The maximum temperature attained was 70°. As soon as the solutions reached room temperature, bovine crystalline albumin was added to make a concentration of 1 mg of albumin/mg of reduced B chain. This ratio provided sufficient albumin to stabilize the B chain without interfering with mitochondrial swelling. B chain reduction was also accomplished by allowing S-sulfo B chain at a concentration of 1 mg/ml in Tris–cysteine reducing solution, pH 8.6–8.9, to stand at room temperature (25°) for 90 min. In some of the experiments this solution was mechanically dialyzed for 1.75 hr against a 40:1 gradient of pH 8.6, 0.125 M KCl–0.02 M Tris solution to remove the excess cysteine.

Because the full extent of the B chain reduction could not be determined, reduced B chain concentrations could not be calculated on a molar basis, and therefore a weight basis was used. Since 5×10^{-6} M insulin represents 0.9 mg of insulin in the 5-ml mitochondrial test system, the reduced and other forms of B chain were employed at this concentration.

Growth hormone was prepared from bovine anterior pituitary glands (Wilhelmi *et al.*, 1948). A molecular weight of 44,000 was used for calculating the growth hormone concentration. Crystalline bovine albumin (lot no. 11) was obtained from the Pentex Corporation.

Methods

Mitochondria were prepared from the livers of male Sprague–Dawley rats (150–250 g) that had been maintained on a standard laboratory pellet chow diet and fasted for 18 hr. The animals were decapitated, the livers were immediately removed and chilled in an ice bath, and 5-g portions were homogenized in pH 7.3, 0.25 M sucrose–0.001 M ethylenediaminetetraacetic acid solution (Judah, 1961). This same solution maintained at 0–2° was used throughout the isolation of the mitochondria using a standardized differential centrifugation procedure (Schneider, 1948). The mitochondria were washed three times, then suspended in the same medium so that 1.0 ml contained the mitochondria from 0.6 g of liver, and they were used immediately after preparation (Neubert *et al.*, 1962).

In the experiment in which mitochondria were preincubated with either reduced B chain–albumin or albumin alone, the preincubation was accomplished during the isolation of the mitochondria. After the first wash, the mitochondria were divided into two equal portions and resuspended in 10 ml of pH 7.8, 0.125 M KCl–0.02 M Tris solution that contained either a 1 mg/ml concentration of albumin or a 1 mg/ml concentration of both dialyzed reduced B chain and albumin. The suspensions were incubated (with gentle mixing) for 5 min at room temperature (25°), centrifuged, washed by resuspension in sucrose–Versene solution, and recentrifuged.

The effects of insulin and its A and B chains on mitochondrial swelling were determined in a Beckman Model B spectrophotometer at 520 mμ (Lehninger, 1959). Insulin, the purified insulin chains, and albumin were suspended in pH 7.3, 0.125 M KCl–0.02 M Tris–0.1% partially hydrolyzed gelatin buffer so that the specified weight, or molar concentration of each of these agents, was contained in 5 ml of buffer. When reduced B chain–albumin or reduced A chain–albumin was used in the test system, they comprised 0.9 ml of the 5 ml total and were added to the system after solubilization in Tris–cysteine reducing solution or after dialysis in 0.125 M KCl–0.02 M Tris solution. These solutions were placed in matched 15 × 100 mm test tubes. If the addition of 0.02–0.05 ml of the stock mitochondrial suspension to 5 ml of KCl–Tris buffer did not give an initial optical density of 0.49–0.52, or if it gave an abnormal spontaneous swelling curve, it was discarded. The same concentration of albumin was added to the control tubes for all experiments in which albumin was present in the experimental tubes, and all experimental and control determinations were run in triplicate.

The *in vivo* effects of insulin B chain on blood glucose levels were determined in 180–210-g male Sprague–Dawley rats that had been fasted 18 hr prior to the experiment. Reduced B chain, alone or with albumin, was injected intraperitoneally in 1 ml of reducing solution. A control blood sample (0.1 ml) and samples at 30-min intervals after injection were obtained by external heart puncture under light ether anesthesia. Blood glucose was measured by Technicon Auto-

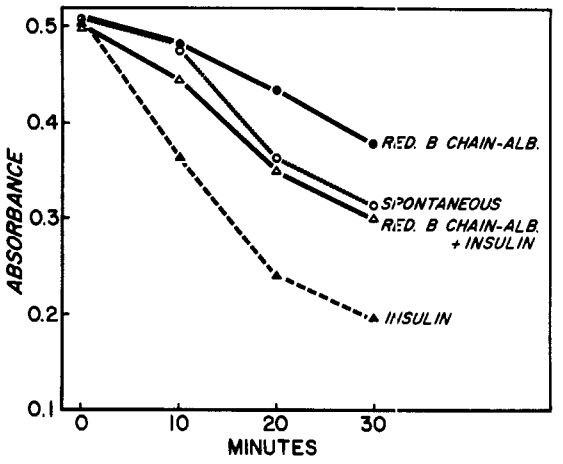

FIGURE 1: Effect of the dialyzed reduced insulin B chain–albumin complex on the mitochondrial swelling activity of insulin. Concentrations: 0.9 mg of reduced B chain, 0.9 mg of albumin, 5×10^{-6} M insulin.

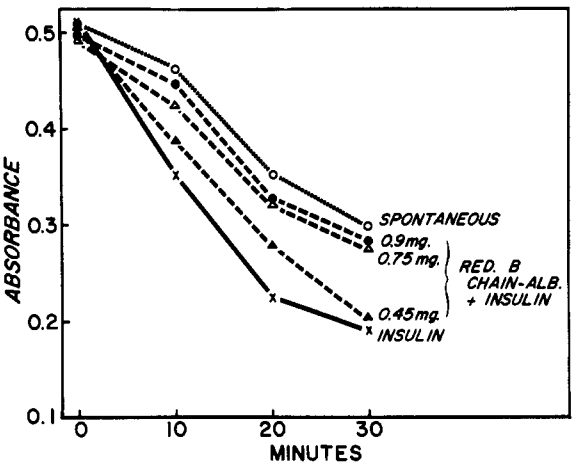

FIGURE 2: Concentration effect of dialyzed reduced insulin B chain–albumin complex on the mitochondrial swelling activity of insulin. Albumin concentration same as that of the reduced B chain; insulin concentration 5×10^{-6} M.

Analyzer Method N-9, based on the procedure of Hoffman (1937).

Results

In the mitochondrial system the reduced B chain–albumin markedly inhibited the swelling activity of insulin. Although cysteine, present in excess in the initial experiments, tended by its swelling effect (Lehninger and Schneider, 1959) to mask the inhibitory effect of B chain, the B chain inhibition of insulin swelling was apparent. In later experiments, after nearly complete removal of cysteine by dialysis (Figure 1), the reduced B chain–albumin inhibition of insulin activity is clearly demonstrated. The concentration dependence of the inhibition is shown in Figure 2.

Neither the S-sulfo B chain nor the mixture of S-sulfo A and B chains altered the mitochondrial swelling activity of insulin (Table I). S-Sulfo B chain and the mixture of S-sulfo A and B chains elicited moderate and pronounced mitochondrial swelling, respectively.

Reduced A chain did not inhibit the mitochondrial swelling activity of insulin, and reduced B chain, not complexed with albumin, had only limited insulin-inhibitory activity (Table II). Depression of mitochondrial swelling below the spontaneous level by the reduced B chain–albumin complex appears to be a nonspecific effect, since reduced A chain–albumin which does not inhibit the swelling activity of insulin depressed mitochondrial swelling to a similar degree.

Bovine growth hormone is known to markedly stimulate mitochondrial swelling in a manner similar to insulin (Melhuish and Greenbaum, 1961), and therefore it was of interest from the mechanism standpoint to see whether the B chain–albumin complex would inhibit this growth hormone activity. No inhibition of growth hormone activity was found, however, under conditions which produce marked inhibition of insulin activity

TABLE I: Mitochondrial Swelling Obtained with S-Sulfo B Chain and with a Mixture of S-Sulfo A and B Chains Alone and in Combination with Insulin, Compared with the Swelling Obtained with Insulin.

Fraction[a]	Concn of Fraction in Test System (mg/5 ml)	$-\Delta OD \times 10^3$ at 520 mμ 10 min	20 min	30 min
S-Sulfo B chain	0.9	0	48	90
S-Sulfo A + B chains	0.9	63	154	170
S-Sulfo B chain + 5×10^{-6} M insulin	0.9	74	124	143
S-Sulfo A + B chains + 5×10^{-6} M insulin	0.9	44	124	142
5×10^{-6} M insulin	—	68	140	143

[a] Bovine albumin present together with fraction at equivalent weight.

(Table III). In an attempt to determine whether the effect of B chain was at the insulin receptor sites or directly on insulin, the mitochondria were preincubated for 5 min at room temperature with dialyzed reduced B chain–albumin and then washed. No inhibition of insulin was observed under these conditions (Figure 3).

In the *in vivo* experiments, significant increases in blood sugar were obtained 30 and 60 min after intraperitoneal injection of 2.5 and 5 mg/kg of reduced B

TABLE II: Mitochondrial Swelling Obtained with Reduced, Albumin Complexed, and Dialyzed A Chain, B Chain, and A and B Chains Alone, and in Combination with Insulin, Compared with the Swelling Obtained with Insulin.[a]

Fraction[b]	Concn of Fraction in Test System (mg/5 ml)	$-\Delta OD \times 10^3$ at 520 mμ		
		10 min	20 min	30 min
Reduced A chain–albumin	0.9	18	−64[c]	−59
Reduced A chain–albumin + 5×10^{-6} M insulin	0.9	117	139	137
5×10^{-6} M insulin + albumin	—	115	153	140
Reduced B chain–albumin	0.9	10	−27	−50
Reduced B chain–albumin + 5×10^{-6} M insulin	0.9	52	56	43
5×10^{-6} insulin + albumin	—	120	128	90
Reduced B chain	0.9	42	26	42
Reduced B chain + 5×10^{-6} M insulin	0.9	90	110	111
5×10^{-6} M insulin	—	140	155	145
Reduced A and B chains–albumin	0.9	4	−29	−49
Reduced A and B chains–albumin + 5×10^{-6} M insulin	0.9	50	48	59
5×10^{-6} M insulin + albumin	—	134	175	136

[a] Each experiment is referred to its own insulin control. [b] Bovine albumin, when present, was added with fraction at equivalent weight. [c] Negative sign (−) indicates less mitochondrial swelling than spontaneous.

TABLE III: Effect of the Dialyzed Reduced B Chain–Albumin Complex on the Mitochondrial Swelling Activity of Bovine Growth Hormone.

Fraction[a]	Concn of Fraction in Test System (mg/5 ml)	$-\Delta OD \times 10^3$ at 520 mμ		
		10 min	20 min	30 min
Dialyzed reduced B chain–albumin + 5×10^{-6} M growth hormone	1.0	88	133	139
Dialyzed reduced B chain–albumin + 5×10^{-6} M growth hormone	0.5	104	145	157
5×10^{-6} M growth hormone + albumin	—	115	159	165
Dialyzed reduced B chain–albumin + 5×10^{-6} M insulin	1.0	52	108	136
5×10^{-6} M insulin + albumin	—	129	192	203

[a] Bovine albumin present together with fraction at equivalent weight.

chain–albumin into fasted rats (Table IV). Nonreduced S-sulfo B chain–albumin prior to injection had little effect on the blood sugar levels. Dialyzed reduced B chain–albumin (5 mg/kg) produced only a slight increase in blood sugar (Table V).

Discussion

These experiments show that it is possible to prepare a potent inhibitor of insulin activity by the addition of purified reduced insulin B chain to an equal weight of bovine crystalline albumin. The complex interfered

TABLE IV: The Effect of Intraperitoneal Administration of Different Doses and Forms of Insulin B Chains on the Glucose Level (mg %) of Fasted Rats.

No. of Animals	Injection[a]	Mean ± Std Error					
		Control	30 min	60 min	90 min	120 min	180 min
10	5 mg of albumin/kg	73 ± 1.9	72 ± 2.8	74 ± 2.2	70 ± 1.3	73 ± 2.1	75 ± 2.2
5	5 mg of S-SO_3 B chain + 5 mg of albumin/kg	83 ± 3.9	91 ± 3.9	90 ± 4.7	83 ± 3.9	88 ± 3.0	—
3	5 mg of reduced B chain/kg	71 ± 2.8	76 ± 1.9	60 ± 5.0	73 ± 2.1	72 ± 1.8	73 ± 3.8
9	5 mg of reduced B chain + 5 mg of albumin/kg	74 ± 1.4	91 ± 4.0	95 ± 2.1	85 ± 3.0	89 ± 3.2	—
3	2.5 mg of reduced B chain + 2.5 mg of albumin/kg	80 ± 1.6	97 ± 2.6	93 ± 6.8	—	80 ± 3.4	87 ± 0.6

[a] All injections were 1 ml except for the 2.5-mg dose which was in 0.5 ml. All of the solutions administered contained 0.0062 M cysteine–0.02 M Tris solution.

TABLE V: Effect of Intraperitoneal Administration of Dialyzed Reduced B Chain–Albumin on the Glucose Level (mg %) of Fasted Rats.[a]

No. of Animals	Injection[a]	Mean ± Std Error					
		Control	30 min	60 min	90 min	120 min	180 min
4	5 mg of albumin/kg	65 ± 1.1	74 ± 1.6	74 ± 1.6	80 ± 2.0	78 ± 1.0	76 ± 3.1
6	5 mg of dialyzed reduced B chain + 5 mg of albumin/kg	72 ± 4.1	87 ± 7.5	76 ± 5.3	89 ± 6.7	90 ± 5.1	83 ± 2.8

[a] All injections were 1 ml. The solutions contained 0.0062 M cysteine–0.02 M Tris solution prior to dialysis against pH 8.6, 0.125 M KCl–0.02 M Tris solution.

with the ability of insulin to promote mitochondrial swelling and, in normal rats, interfered with control of blood glucose levels by insulin.

The mitochondrial swelling activity of insulin and other disulfide hormones has been investigated extensively by Lehninger and Neubert (1961). They have expressed the view that the disulfide groups of insulin are the active groups in stimulating water uptake by mitochondria, and that the specific amino acid sequence and peptide chain conformation on both sides of the disulfide bridges are important factors in the mitochondrial swelling activity of the hormone. Neither S-sulfo B chain nor a mixture of S-sulfo A and B chains interfered with the mitochondrial swelling activity of insulin. Insulin chain reduction alone was not sufficient to inhibit the mitochondrial swelling activity of insulin, since reduced A chain–albumin did not affect this mitochondrial swelling activity of insulin. Reduced B chain did inhibit insulin activity, but in combination with albumin had considerably greater inhibitory activity. The most likely mechanism for the B chain inhibition would seem to be either blocking of the mitochondrial binding sites of insulin or alteration of the configuration of insulin so that it can no longer complex with its binding sites. While this still remains an unanswered question, the failure of preincubation of reduced B chain–albumin solution with mitochondria to prevent the swelling action of insulin would suggest that the second mechanism is operating, since it seems unlikely that the washing step would remove B chain if it were specifically bound to the receptor sites. Further evidence for this viewpoint is the absence of inhibition of growth hormone by reduced B chain–albumin, assuming that the same sites are involved.

The absence in the mitochondrial studies of any interference by A chain in the B chain inhibition of insulin activity fits in with the work of Ensinck and co-workers (1964) who reported that after incubation of

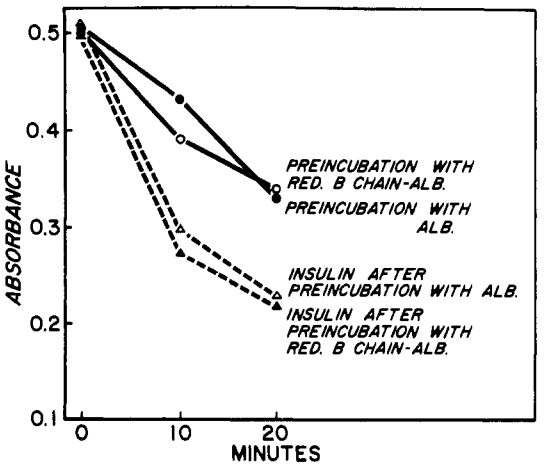

FIGURE 3: Effect of preincubating mitochondria with the dialyzed reduced B chain–albumin complex. Insulin concentration is 5×10^{-6} M.

labeled insulin in serum with glutathione–insulin transhydrogenase, electrophoresis showed B chain to be complexed with albumin while A chain was not.

Although the relative insolubility of S-sulfo B chain and reduced B chain in physiological buffers has presented a problem in previous studies (Ensinck *et al.*, 1965), in the work reported here the problem was minimized by cysteine reduction of a low concentration of B chain (1 mg/ml), the presence of urea with B chain, and the combination of reduced B chain with albumin.

The fact that reduced B chain alone showed no activity *in vivo* suggests the possibility that on intraperitoneal injection, the B chain is oxidized or otherwise inactivated before it can complex with the circulating albumin. The observation that even in the presence of albumin removal of the excess reducing agent by dialysis tends to nullify the insulin-inhibitory activity of reduced B chain–albumin might be explained on the basis that cysteine sustains the reduced state of the complex at the intraperitoneal site.

This work therefore provides further *in vitro* and *in vivo* evidence for the mechanism proposed by Ensinck *et al.* (1965) in which insulin is markedly inhibited by reduced insulin B chain complexed with albumin.

Acknowledgment

We thank Mr. Robert Whitley for the urea and blood glucose analyses and Mrs. Joanne Schmitz for valuable technical assistance.

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