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Analysis of solvent-mediated conformational changes of insulin by radioimmunoassay (RIA) techniques

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Abstract: Stationary phase radioimmunoassay (RIA) (i.e. antibodies bound to polystyrene test tubes) techniques are used as an analytical probe of secondary and tertiary structural changes of radiolabelled ^{125}I porcine insulin. The effects of temperature, buffer composition, pH and ionic strength and solvents on insulin binding are studied. Optimum insulin–antibody binding occurred at 22°C, pH 6 and a buffer strength of 0.1 M or less. Results of experiments with three pH 6 buffers (0.005 M phosphate, 0.1 M acetate and 0.1 M Tris) showed no statistical difference in binding properties. For all solvents tested, increasing the solvent concentration decreased the amount of insulin binding. Comparison of the various solvents tested indicated that ethylene glycol and methanol are the least denaturant whilst 1-propanol and acetonitrile are among the most denaturant.

Keywords: *Radioimmunoassay (RIA); insulin conformational analysis; solvent-mediated denaturation.*

Introduction

The production and analysis of proteinaceous drugs (e.g. human insulin and growth hormone) is becoming increasingly important to the pharmaceutical community because of advances in recombinant DNA (rDNA) technology. Proteinaceous drugs produced by rDNA techniques should be analysed for impurities, such as nucleic acids and microbial proteins, and controlled for biological activity. Additionally Smith and Lee [1] have proposed that a hybrid of high-performance liquid chromatography (HPLC) and analytical affinity chromatography might be useful for the simultaneous determination of homogeneity and of biological activity of proteinaceous drugs. The analytical affinity chromatography part of the system is only possible, however, if the initial measurements do not alter the native conformation of the proteins. Any changes in the conformation of the proteinaceous drugs prior to the analytical affinity chromatographic step could lead to errors in the assay.

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In order to develop non-denaturing HPLC analyses, the effect of buffers and solvents on protein conformation need to be determined. Alterations in protein conformation have been examined by optical rotatory dispersion (ORD) [2, 3], ultra-violet spectroscopy (UV) [2, 4], circular dichroism (CD) [2, 5, 6] and by high resolution nuclear magnetic resonance spectroscopy (NMR) [2, 6]. Additionally, Lazdunski [8] has proposed the use of antibodies as probes in the analysis of protein conformational changes.

The ability of a protein antigen to combine with its corresponding antibody is a structurally and conformationally specific interaction. Therefore it would be expected that if either the protein or the antibody were conformationally altered (i.e. denatured), a less than optimal protein–antibody interaction would occur. MacPherson and Heidelberger [9] found that heat, acid or alkali denaturation of ovalbumin greatly reduced its interaction with antibodies for native ovalbumin. Similar findings were reported with urea treatments of diptheria antitoxin [10–12] and staphylococcus antitoxin [13], thereby causing inhibition of binding to their respective toxins.

In this study antibodies and stationary phase radioimmunoassay (RIA) techniques were used to monitor the effects of temperature, buffers (composition, molarity and pH) and solvents (composition and concentrations) on the conformation of a model protein, porcine insulin. It was proposed that the results of this research effort would be useful in developing non-conformation altering chromatographic procedures.

Experimental

Equipment

Gamma counts were determined by means of a LKB 1282 Compugamma Counter (LKB-Produkter AB, Bromma, Sweden) equipped with a Texas Instruments 700 Data terminal printer.

Materials

Insulin RIA kits were purchased from Micromedic Systems, Inc. (Horsham, PA, USA). The buffer systems used were a 0.1 M acetate system (pH 6) made of sodium acetate (MCB, Cincinnati, OH, USA) and acetic acid (Fisher Scientific, Dallas, TX, USA) and a 0.1 M Tris system (pH 6) made from Tris base and Tris–HCl (Sigma Chemical Co., St Louis, MO, USA). The 0.05 M monobasic potassium phosphate–sodium hydroxide (pH 6) buffer system and all other buffer systems (pH 4–10) were purchased from Fisher Scientific. Methanol, 1-propanol, 2-propanol, acetonitrile (Fisher Scientific) and methoxyethanol and ethoxyethanol (Burdick–Jackson, Muskegon, MI, USA) were glass-distilled HPLC grade. Ethylene glycol, tert-butanol, trifluoroacetic acid (TFA), and trichloroacetic acid were Fisher Scientific certified reagent grade. Absolute ethanol was from Midwest Solvents Co. (Perkin, IL, USA). The 2-mercaptoethanol was obtained from Bio-Rad Labs (Richmond, CA, USA).

Methods

When the radiolabelled (^{125}I) porcine insulin solution was prepared according to directions (Micromedics Systems, Inc.) it gave gamma-counts in the range 1000–2500 cpm for a 2 h incubation. A more concentrated solution, with 50% less buffer, was prepared; this concentrated tracer solution gave counts of 2500–5000 cpm after a 2 h incubation. The insulin tracer solution and the test tubes containing antibody were allowed to equilibrate to room temperature before starting all assays. A typical assay

involved adding 0.25 ml of radiolabelled porcine insulin solution and 1.00 ml of various solvent/buffer mixtures to a test tube containing antibody. The tubes were then vortexed and incubated for 2 h (except test 1 in which the effect of incubation time was studied). Following incubation each tube was rinsed (3 × 5 ml) with water and gamma radiation counted for 60 s.

Effect of incubation time

Insulin tracer (0.25 ml) and 1.00 ml mixtures of 0.05 M phosphate buffer, pH 6, and varying methanol concentrations (0–67%, v/v) were added to antibody-containing test tubes. The tubes were vortexed, incubated for 1, 2, 3, 5 or 18 h at 22 ± 3°C, rinsed and counted.

Effect of pH

Insulin tracer (0.25 ml) and 1.00 ml of buffer were added to antibody-containing test tubes. The buffers used had pH values in the range of 4–10. The tubes were vortexed, incubated for 2 h at 22 ± 3°C, rinsed, and counted. The effect of pH on only the antibodies was tested by pretreating antibody-containing test tubes with 2.00 ml of buffer, pH 4–10. The tubes were incubated for 2 h at 22 ± 3°C and rinsed. Insulin tracer was then added to the test tubes and reincubated for 2 h at 22 ± 3°C, then rinsed and counted.

Effect of temperature

The insulin tracer (0.25 ml) and 1.00 ml mixtures composed of buffer and 15% methanol, ethanol, 1-propanol, 2-propanol, or tert-butanol were added to antibody-containing test tubes. The tubes were vortexed and incubated for 2 h at 5, 20, 30 or 40°C, rinsed and counted. Temperature effects on the antibodies only were studied by incubating the antibody-containing tubes at 5, 20, 30 and 40°C for 2 h. Insulin tracer was then added and the tubes were reincubated as above.

Effect of buffer molarity

Insulin tracer (0.25 ml) and 1.00 ml mixtures containing 15% methanol, ethanol, 1-propanol, 2-propanol or tert-butanol and varying strengths of acetate pH 6 buffer (0.01 M, 0.05 M, 0.1 M, 0.2 M, 0.5 M, 1.0 M) were added to antibody-containing test tubes. The tubes were then vortexed and incubated for 2 h at 22 ± 3°C, rinsed and counted. The effect of buffer molarity on the antibodies alone was also tested. Antibody-containing tubes were incubated with 2.00 ml of acetate pH 6 buffer of the above molarities at 22 ± 3°C for 2 h and then rinsed. Insulin tracer was added to the tubes and reincubated, rinsed and counted as stated previously.

Effect of solvents

Insulin tracer (0.25 ml) and 1.00 ml mixtures of buffer and varying concentrations (0–80%) of solvents were added to antibody-containing test tubes. The solvents tested included methanol, ethanol, 1-propanol, 2-propanol, tert-butanol, acetonitrile, ethoxyethanol, methoxyethanol, ethylene glycol, and a 0.10 M aqueous solution of trifluoroacetic acid. The tubes then were vortexed, incubated for 2 h at 22 ± 3°C, rinsed and counted. The effect of the various solvents on the antibodies was also studied. Antibody-containing tubes were pretreated with 2.00 ml of solvent for 2 h and rinsed. Insulin tracer was added next and the tubes reincubated as described above, rinsed and counted.

Reversibility of denaturation

Radioactive I^{125} insulin solution (0.20 ml) and solvent (0.20 ml) were added to plain test tubes and incubated for 0.5 h. Aliquots of these mixtures were diluted 1 to 50 with 0.1 M acetate buffer pH 6. Samples (1.00 ml) of the diluted solution were added to antibody-containing tubes, incubated for 2 h at 22 ± 3°C, rinsed and counted. The solvents used in this experiment included those stated above plus 5% trichloroacetic acid and 12.5% mercaptoethanol. Also studied was thermal denaturation, in which insulin tracer was heated (80°C) in a water bath for 0.5 h and then cooled to 22°C and diluted (1:50).

Results and Discussion

At the outset, it is important to clarify that radioiodinated porcine insulin was used in all the experiments. Iodinated insulin could have a slightly different native conformation than non-iodinated insulin. However, the conformational difference is possibly negligible because the I^{125} porcine insulin binds significantly to antibodies produced from treatment of animals with non-iodinated insulin. It is also important to note that in experiments described, both the insulin and antibodies were exposed to the various treatments. In order to elucidate whether the treatments were denaturing to the insulin or the antibodies, experiments were conducted in which only the antibodies were treated. It was not possible to treat the insulin alone because solvent-mediated denaturation was reversible in most instances upon removal of the solvent. This reversible denaturation phenomenon is discussed later.

The effect of incubation time on insulin binding in the RIA procedure was initially studied because of the long (18 h) incubation time recommended by the manufacturer. Since the present determinations involved insulin in aqueous solution instead of in plasma it was thought that incubation times could be shortened from overnight to a few hours because the aqueous solutions do not contain plasma proteins and other substances which may interfere with the insulin–antibody equilibrium process. The results of these experiments are shown in Table 1. The counts of the 2 h standard (0% solvent) tubes were roughly half of the counts of the overnight (18 h) standard tubes. In an attempt to decrease the probability of counting errors and to compensate for the lower gamma-counts (i.e. a range of 1000–2000 cpm) when using a 2 h incubation time, a more concentrated tracer solution was made by adding 50% less buffer. The concentrated tracer solution gave counts in the range 2500–5000 cpm when incubated for 2 h. Under these conditions a 1-h incubation time yielded binding ratios that were significantly different to the corresponding binding ratios for a 2-h incubation time, but the binding ratios obtained after a 3-, 5- or 18-h incubation time were statistically the same as the comparable 2-h values. Thus, for all subsequent assays a 2-h incubation time was chosen since incubation times of greater than 2 h had no significant effect on binding ratios.

The conformation of proteins in solution can be affected by pH because of ionization changes in amino acid residues. The effects of different pHs on insulin conformations were determined by studying antibody–insulin binding as a function of pH. The results of these experiments (Fig. 1) indicated that a pH of 6.00 was the optimum for insulin binding. Therefore, a pH 6 buffer system was employed in all remaining experiments. As previously stated, to help determine whether the pH variations were affecting the insulin or the antibodies, experiments treating only the antibodies were conducted. As indicated in Fig. 1, pH had less of an effect on the antibodies than on the insulin. A possible

Table 1
Effect of incubation time on radioactive insulin binding ratios* (± relative standard deviations); $n = 5$ (duplicate determinations)

Methanol (%)	1 h	2 h	3 h	5 h	18 h
Standard (cpm)	754 ±1.67%	1056 ±2.01%	1263 ±2.11%	1644 ±1.79%	2684 ±1.87%
10	0.906† ±3.55%	0.923 ±2.55%	0.883† ±0.41%	0.984† ±9.69%	0.925† ±6.38%
15	0.765 ±0.57%	0.811 ±4.15%	0.849† ±2.61%	0.820† ±2.69%	0.843 ±2.84%
20	0.548 ±4.10%	0.636 ±2.37%	0.663† ±6.63%	0.717† ±11.0%	0.727 ±5.94%
25	0.531 ±3.05%	0.580 ±1.57%	0.564† ±5.92%	0.566† ±2.35%	0.608† ±2.45%
30	0.244 ±1.60%	0.279 ±1.15%	0.245 ±3.69%	0.258† ±5.55%	0.330 ±0.51%
35	0.097† ±11.0%	0.109 ±4.66%	0.079 ±8.60%	0.087† ±7.55%	0.108† ±4.89%
40	0.037 ±11.9%	0.044 ±7.34%	0.027 ±11.1%	0.027 ±7.55%	0.024 ±2.59%
45	0.029 ±6.43%	0.021 ±21.4%	0.022 ±6.14%	0.014 ±12.8%	0.011 ±6.66%
50	0.021 ±30.6%	0.016 ±10.4%	0.022 ±17.2%	0.012 ±31.1%	0.009 ±17.3%
67	0.034 ±11.2%	0.022 ±7.28%	0.017 ±22.1%	0.018 ±25.0%	0.013 ±12.9%
Thermally denatured‡	0.042 ±12.5%	0.036 ±7.61%	0.029 ±22.7%	0.020 ±15.5%	0.013 ±2.80%

*Binding ratios were determined as follows:

$$\frac{\text{Gamma-counts of the solvent-treated tubes}}{\text{Gamma-counts of the standard non-treated tubes}}.$$

†Indicates that the values are statistically equivalent to the comparable 2-h values. Binding ratios below 0.100 could not be interpreted without error because of their low gamma-counts.
‡Thermal denaturation was accomplished by heating insulin in a water bath at 80°C for 0.5 h.

explanation for this difference may be stabilization of the antibodies caused by the binding to polystyrene tubes.

Moderate temperatures (i.e. >60°C) denature some proteins. Therefore, the effect of temperature on insulin–antibody binding was studied (Table 2). The counts of the standard tubes increased with increasing temperature up to 40°C which may be due to an accelerated equilibrium effect. Interestingly, the solvent-treated insulin tubes showed the opposite effect (i.e. decreased insulin binding). The temperature-mediated decrease in binding ratios may be due to the effects of alcohols in lowering the thermal transition

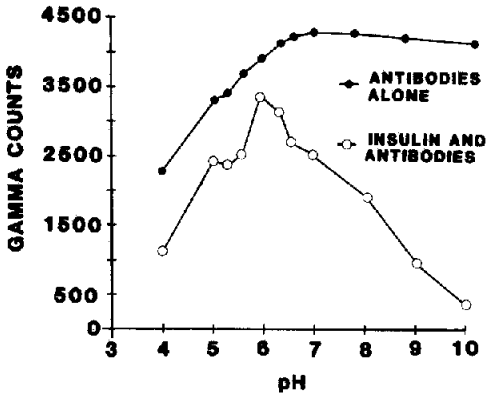

Figure 1
Effect of pH on insulin–antibody binding; $n = 5$ (duplicate determinations).

Table 2
The effect of incubation temperature on radioactive insulin binding ratios (± relative standard deviation); $n = 5$ (duplicate determinations)

Treatment	5°C	20°C	30°C	40°C
Standard (cpm)*	1494 ±2.52%	2161 ±2.10%	2720 ±3.01%	2841 ±1.98%
15% Methanol	0.826 ±3.46%	0.743 ±3.76%	0.686 ±4.47%	0.648 ±3.02%
15% Ethanol	0.700 ±4.84%	0.711 ±3.14%	0.624 ±3.50%	0.566 ±3.83%
15% 1-Propanol	0.390 ±4.57%	0.360 ±4.33%	0.265 ±3.78%	0.100 ±3.16%
15% 2-Propanol	0.614 ±2.75%	0.622 ±4.24%	0.516 ±4.56%	0.445 ±3.16%
15% tert-Butanol	0.407 ±6.11%	0.457 ±3.88%	0.372 ±2.88%	0.321 ±6.01%
Effect on only the antibodies (cpm)	3875 ±2.26%	3550 ±1.87%	3894 ±3.17%	4023 ±3.61%

*cpm = counts per minute.

temperature of insulin. Similar results have been reported for lysozyme [14] and ribonuclease [15–17]. In the presence of solvents, however, the observed decrease in insulin binding is not totally a thermal effect because the standard tube counts increase as temperature increases. Therefore, it is proposed that the decrease in binding ratios is due to an increased temperature destabilization of tertiary structure (slight thermal denaturation). This destabilization would expose the interior hydrophobic groups to the solvent which could lead to more solvent-mediated denaturation and the lower binding ratios. When the antibodies alone were incubated at the various temperatures, a statistically insignificant change in insulin binding occurred (Table 2). Therefore any effect of temperature on lowering binding ratios is believed to be due to the denaturation of the insulin.

High ionic concentrations can alter the secondary and tertiary structure of proteins by interrupting ionic and hydrogen bonds. Consequently the effects of buffer strength on insulin binding were tested (Table 3). The results obtained with pH 6 acetate buffers of 0.01 and 0.05 M were statistically equivalent with the results obtained with the 0.10 M acetate buffer. As buffer strengths are increased above 0.10 M, insulin binding and binding ratios decrease. The decrease in insulin binding as a function of buffer strength could be due to denaturation of insulin, the antibody, or interference of insulin–antibody binding by the increasing ionic concentration. As indicated in Table 3, the higher ionic strength buffers had no effect on the antibodies, therefore, the decrease in insulin binding can be attributed to either denaturation of insulin or interference of insulin–antibody binding.

Table 3
The effect of buffer molarity (sodium acetate–acetic acid pH 6) on insulin binding ratios (± relative standard deviation): $n = 5$ (duplicate determinations)

Treatment	0.01 M	0.05 M	0.10 M	0.20 M	0.50 M	1.00 M
Standard (cpm)	2204* ±1.86%	2260* ±2.97%	2111 ±2.10%	1830 ±2.19%	1471 ±2.63%	1253 ±2.25%
15% Methanol	0.790* ±3.77%	0.812* ±3.01%	0.776 ±3.81%	0.737* ±5.50%	0.699 ±4.66%	0.619 ±4.43%
15% Ethanol	0.754* ±1.79%	0.759* ±4.89%	0.732 ±2.63%	0.714* ±3.71%	0.615 ±4.45%	0.417 ±4.69%
15% 1-Propanol	0.435 ±4.68%	0.424 ±2.05%	0.367 ±3.48%	0.303 ±4.61%	0.210 ±6.84%	0.110 ±2.70%
15% 2-Propanol	0.700* ±3.33%	0.670* ±2.05%	0.682 ±2.52%	0.584 ±2.84%	0.448 ±2.25%	0.272 ±5.29%
15% tert-Butanol	0.545* ±2.83%	0.490* ±3.54%	0.509 ±2.61%	0.397 ±4.88%	0.283 ±4.27%	0.173 ±11.5%
Effect on only the antibodies (cpm)	3122* ±2.87%	3431* ±3.16%	3468 ±2.04%	3579* ±2.71%	3706* ±2.21%	3595* ±2.56%

*Indicates that the values are statistically equivalent to the comparable 0.10 M values.

The results of solvent effects on insulin binding (binding ratio) are shown in Table 4. The solvents were compared by BR 50% values. The BR 50% value is the solvent concentration required to yield a 50% decrease in binding relative to incubations with no solvents. The solvent experiments were conducted with three different pH 6 buffer systems (0.05 M phosphate, 0.1 M acetate and 0.1 M Tris). The BR 50% of each solvent with each buffer were statistically equivalent and therefore buffer composition (i.e. phosphate vs acetate vs Tris) appears to have no effect on insulin–antibody binding (Table 4).

The alcohol series of solvents exhibited the following denaturing order: methanol < ethanol < 2-propanol < tert-butanol ≅ 1-propanol. Plots of the effects of increasing alcohol concentration on insulin binding are contained in Fig. 2.

Organic solvents such as alcohols are believed to induce protein conformational changes to produce apparently more ordered helical forms [2, 18, 19]. It is known that

Table 4
Binding ratio 50%* values of solvents in three different pH 6 buffer systems. The values reported were determined from the graphs of solvent concentration vs insulin binding ratio (i.e. Figs 2–4)

Solvent	0.10 M Acetate Molarity	% (v/v)†	0.05 M Phosphate Molarity	% (v/v)	0.10 M Tris Molarity	% (v/v)
Methanol	6.14	24.8	5.68	23.0	6.36	25.7
Ethanol	3.74	21.8	3.67	21.4	3.24	18.9
1-Propanol	1.60	12.0	1.46	11.0	1.41	10.6
2-Propanol	2.45	18.7	2.30	17.5	2.50	19.1
tert-Butanol	1.69	15.8	1.45	13.6	1.45	13.6
Acetonitrile	2.18	11.4	2.26	11.8	2.36	12.3
Methoxyethanol	2.19	17.2	2.51	19.8	2.34	18.2
Ethoxyethanol	1.54	14.9	1.79	17.3	1.59	15.4
Ethylene glycol	5.10	28.4	5.20	29.0	—	—
Trifluoroacetic acid	0.04	0.31	0.05	0.37	—	—

*Binding ratio 50% (BR 50%) is the amount of solvent required to yield a binding ratio of 0.500.
†The v/v % are not adjusted for the small change in volume, due to hydrogen bonding, that occurs when solvents and water are mixed.

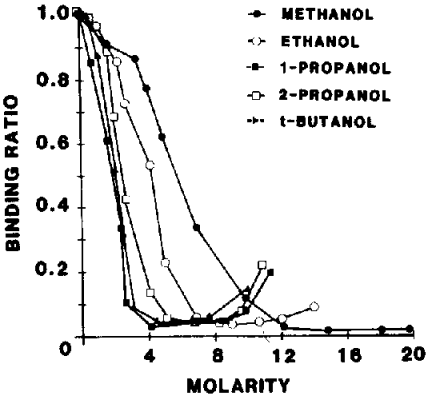

Figure 2
The effect of alcohols on insulin–antibody binding; $n = 4$ (quadruple determinations).

increasing chain length of an alcohol and thus increasing its hydrophobocity increases the denaturing ability of the alcohols [2, 18, 19]. Additionally, increasing the amount of branching (i.e. decreasing ability for hydrophobic interactions) of alcohols with equal carbon content decreases the ability to denature. The effects of branching and chain length (i.e. same rank order) on the ability of alcohols to denature proteins, as monitored by UV and CD spectroscopy, have been shown with proteins such as myoglobin, cytochrome *c* and chymotrypsinogen [19]. Similar effects of chain length and branching of alcohols have been reported for their effects on the thermal stability of lysozyme [14] and ribonuclease [15–17]. This chain length/hydrogen bonding phenomenon correlates with the proposal that solvent-mediated secondary and tertiary structural changes are due to the organic solvents interfering with hydrophobic interactions between non-polar amino acid side chains [2, 19, 20].

Other solvents tested in this study included acetonitrile, methoxyethanol and ethoxyethanol (Table 4 and Fig. 3). The two-carbon alcohol series had the following order of denaturation: ethylene glycol < ethanol < methoxyethanol < ethoxyethanol; a

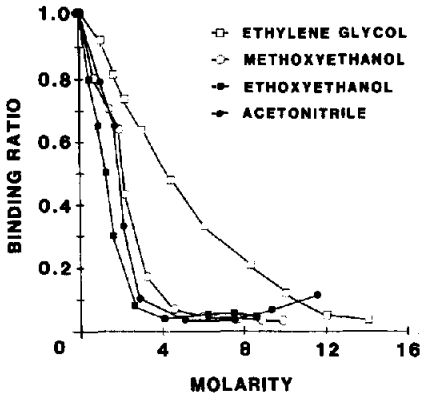

Figure 3
The effect of various solvents on insulin–antibody binding; $n = 4$ (quadruple determinations).

rank order that is related to the hydrogen bonding characteristics of the solvents. That is, the greater the extent of hydrogen bonding, the greater the solvent interaction with polar surface amino acids and a decreased chance of solvent interaction with the internal non-polar amino acid side chains. These results suggest that the greater the hydrogen bonding tendency of solvents, the less the effects on denaturation of insulin. Consequently, the finding that ethylene glycol was the least denaturant was not surprising. Similar results have also been reported for other proteins such as cytochrome *c* and myoglobin [2, 19].

The effect of TFA on the apparent denaturation of insulin was also tested because of the common use of TFA as a protein solubilizer and counter-ion in mobile phases used in protein separations (see Table 4 and Fig. 4). The TFA experiments indicated that up to 0.030 M TFA can be used without large changes in insulin binding ratios. Furthermore, the range 0–0.030 M TFA tested is in accord with the amounts of TFA typically employed in mobile phases [21]. In summary, of the solvents tested in this study, acetonitrile and 1-propanol were found to be among the most denaturing and are the most widely used in protein HPLC.

The effects of solvents on insulin antibodies alone also were tested to determine whether the solvents were affecting insulin or the antibodies. The results (see Figs 5 and 6) were similar to what was observed when both the insulin and antibodies were incubated with the solvents. The major difference was the higher insulin binding (i.e. higher binding ratios) observed when only the insulin antibodies were treated as

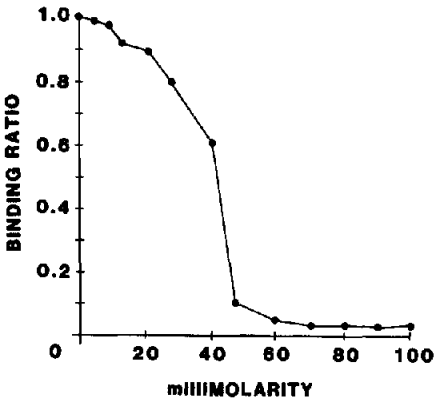

Figure 4
The effect of trifluoroacetic acid on insulin–antibody binding; $n = 4$ (quadruple determinations).

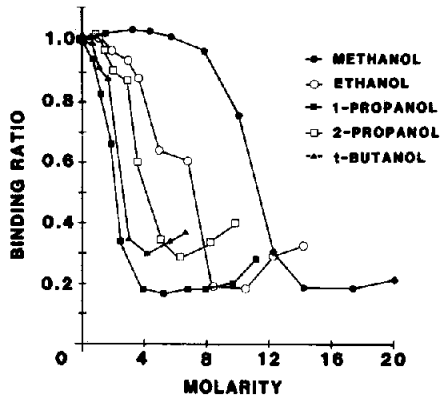

Figure 5
Alcohol treatment of the antibodies alone and its effect on insulin–antibody binding; $n = 4$ (quadruple determinations).

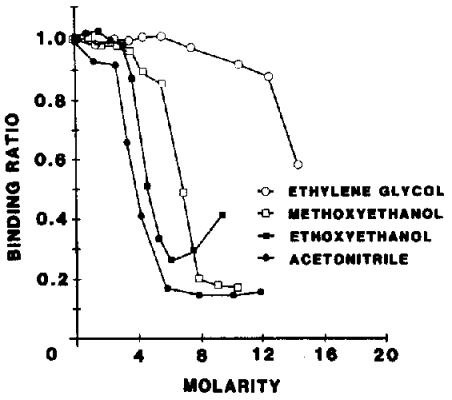

Figure 6
Solvent treatment of the antibodies alone and its effect on insulin–antibody binding; $n = 4$ (quadruple determinations).

compared to the insulin binding recorded when both insulin and antibodies were treated. Again, this increase in insulin binding in the experiments where only the antibodies were treated was probably due to stabilization of the antibodies against denaturation due to linkage to a stationary phase. The effects of the solvents on insulin alone were not tested because removal of the solvents after the incubation period resulted in reversal of insulin denaturation, as noted below.

Examining the results of solvent treatment of the insulin antibodies alone and the insulin/antibodies together helped to elucidate whether the antibodies or insulin or both were being denatured. It appears that at lower solvent concentrations (i.e. <20%) a major portion of the decrease in binding ratios is due to denaturation of the insulin. This proposal is reinforced by the finding that treatment of the antibodies alone resulted in higher insulin binding compared to simultaneous treatment of insulin and antibodies. This also would be in agreement with the proposal of the antibodies being stabilized by linkage to a support. At high solvent concentrations (i.e. >20%) both the antibodies and insulin were denatured, resulting in very low insulin binding. In Figs 2 and 3 some of the curves exhibit an upswing in insulin binding at the very high solvent concentration (e.g. 2-propanol > 9 M). The upswing in insulin binding was apparently due to non-specific binding of precipitated insulin to the polystyrene tubes. This was demonstrated by adding radioactive insulin and solvent to non-antibody-containing polystyrene test tubes.

The average radioactivity measured (cpm) in the 80% 2-propanol-treated tubes was 1925 ± 19.8%, whereas the average value of the standard (0% solvent) tubes was 127 ± 8.61% cpm. This insulin precipitation and subsequent non-specific binding was also seen with 80% 1-propanol (1657 ± 13.3%), 70% acetonitrile (3506 ± 21.8%), and 80% tert-butanol (1131 ± 16.3%).

Denaturation of insulin by the solvents studied was reversible upon dilution, as indicated by data found in Table 5. As previously stated, this reversal of denaturation prevented the direct evaluation of solvent effects on the binding properties of insulin.

Table 5
The reversibility of solvent-mediated denaturation by 1 to 50 dilution of solvent

Treatment*	Binding ratio No dilution of solvent	Binding ratio 1 to 50 dilution of solvent
80°C	0.043 ±6.88%	0.032† ±7.14%
12.5% Mercaptoethanol	0.113 ±4.59%	0.182 ±5.26%
5% Trichloroacetic acid	0.091 ±11.2%	0.941 ±6.04%
50% Methanol	0.031 ±6.65%	0.870 ±1.10%
50% Ethanol	0.031 ±18.8%	0.856 ±2.27%
50% 1-Propanol	0.038 ±39.5%	0.961 ±1.34%
50% 2-Propanol	0.037 ±12.2%	0.986 ±2.00%
50% tert-Butanol	0.046 ±7.79%	1.021 ±2.25%
50% Acetonitrile	0.073 ±11.8%	0.953 ±2.76%
50% Ethylene glycol	0.208 ±4.35%	1.010 ±1.49%
50% Methoxyethanol	0.034 ±5.61%	0.937 ±1.51%
50% Ethoxyethanol	0.038 ±3.87%	0.936 ±1.51%
50% Trifluoroacetic acid (0.1 M)	0.080 ±1.89%	1.008 ±1.62%

* All treatments were conducted for 30 min.
† Cooled to 22°C and then diluted 1 to 50.
The insulin binding ratios (± relative standard deviation) are the average values of two assays each with eight tubes.

Reversibility of protein denaturation was first reported by Anfinsen [22] in the early 1960s. Singer [18] noted that denaturation of trypsin by methanol, ethanol and other organic solvents could be reversed. More recently Sadler *et al.* [23] reported that 1-propanol-induced conformational changes of several proteins were reversible upon dilution or dialysis of the 1-propanol.

Fisher and Porter [24] note that insulin can undergo physical and chemical changes on storage that are not detected by immunological reaction. However, Fisher (personal communication) suggests that solvent-mediated denaturation could be expected to cause a reduction in binding even if it were less than that caused by other factors.

The effects of 2-mercaptoethanol were not reversible by dilution as indicated in Table 5. This apparently irreversible denaturation was possibly due to the cleavage of the disulphide bonds of insulin by 2-mercaptoethanol.

Although only one protein, insulin, was used in this research it has been demonstrated that antibodies have the potential to be used as probes in the analysis of protein conformational changes. Additionally we hope the information discovered in this study will be of help to researchers who need to develop non-denaturing mobile phases for use in protein HPLC.

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