that these two peptide hormones are also structurally identical, at least with respect to amino acid sequence. In addition, it can be said that the carboxyl group of the C-terminal amino acid residue, valine, is blocked, probably by an amide structure, since very mild acid hydrolysis leaves the valine susceptible to removal by carboxypeptidase digestion. Both α_p-MSH[15,16] and α_b-MSH[11,12] have been shown to be blocked at the N-terminus by an acetyl residue. The failure to react with ninhydrin on the part of the N-terminal amino group of α_e-MSH indicates similar blocking at this terminus of the equine hormone as well, possibly also by an acetyl group.

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[CONTRIBUTION FROM THE BIOCHEMISTRY SECTION, ARMOUR AND COMPANY, CENTRAL RESEARCH LABORATORIES, CHICAGO 9, ILLINOIS]

The Fractionation of Insulin on Diethylaminoethylcellulose

BY MARGUERITE VOLINI AND MILTON A. MITZ

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Insulin was separated into two components on the anion exchanger diethylaminoethylcellulose, using stepwise elution with carbon dioxide–water solution and 0.1 *M* ammonium phosphate. The fractions were compared as to biological properties, crystallizability, solubility, amino-acid content, optical rotation and electrophoretic mobility. They were identical in qualitative amino-acid content but displayed significant differences in physical configuration as well as in net charge. Their apparent interrelationship is that of native and partially denatured protein forms. It is postulated that the two insulins are composed of polymeric forms each containing a different monomeric species.

Introduction

Heterogeneity in purified insulin preparations has been demonstrated by means of several criteria.[1–7] In his sedimentation studies, Fredericq[1] has obtained evidence that the component differences lie in the elementary sub-units of the insulin polymer. The classical work of Sanger[8] shows that these differences are not concerned with the nature or sequence of the constituent amino acids; the fractionation studies of Fredericq,[1] Harfenist and Craig[2], and Timasheff, Brown and Kirkwood[3] collectively suggest that the differences can be attributed to variations in a few chemical groups, resulting in slight differences in net molecular charge. Indeed, Harfenist and Craig[2] have actually showed that their A and B components, isolated by countercurrent distribution, differ by only a single amide group per 6000 molecular-weight unit.

The purpose of the present paper is to report the separation of purified insulin into two components apparently unlike those obtained in prior investigations. Fractionation was accomplished on the anion exchanger diethylaminoethylcellulose (DEAE-cellulose)[9] by using stepwise elution with carbon dioxide–water solution[10] and 0.1 *M* ammonium phosphate. The relation of the two fractions to each other as well as to the end products of other fractionation procedures is discussed.

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(2) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3083 (1952); *ibid.*, **75**, 3528 (1953).

(3) S. N. Timasheff, R. A. Brown and J. G. Kirkwood, *ibid.*, **75**, 3121, 3124 (1953).

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(7) D. F. Waugh, D. F. Wilhelmson, S. L. Commerford and M. L. Sackler, THIS JOURNAL, **75**, 2592 (1953).

(8) F. Sanger, *Advances in Protein Chem.*, **7**, 1 (1952).

(9) E. A. Peterson and H. A. Sober, THIS JOURNAL, **78**, 751 (1956).

(10) M. A. Mitz and S. S. Yanari, *ibid.*, **78**, 2649 (1956).

Materials

Adsorbent.—The DEAE-cellulose preparations were prepared by the method of Peterson and Sober,[9] using Solka-Floc SW-40B. The preparations ranged in capacity from 0.4 to 0.6 meq. per g. After use, they were regenerated with 0.1 *M* sodium hydroxide.

Insulin.—The insulin preparations studied in detail were: (1) Armour crystalline zinc insulin, Lot 1, 24.2 units per mg.; (2) Armour crystalline zinc insulin, Lot 2, 24.5 units per mg.; (3) Armour crystalline zinc insulin, Lot 3, 22 units per mg.; (4) U.S.P. Reference Standard, crystalline zinc insulin, 24.2 units per mg.; and (5) IUPAC crystalline zinc insulin, Batch 2189, 23 units per mg. The preparations were of beef origin with the exception of the U.S.P. Reference Standard which was a mixture of beef and pork insulins.

Methods

Chromatographic Procedures.—The adsorbent column was prepared from an aqueous slurry of DEAE-cellulose in the free base form. Approximately 3 g. of exchanger were used for a column 33 × 0.9 cm. To insure even distribution of the exchanger, gentle air pressure was used during packing. The column was washed with one to two hold-up volumes of distilled water and a freshly prepared solution containing 90–100 mg. of insulin (adjusted to *p*H 3.0 with hydrochloric acid) was added. Distilled-water wash was continued until the effluent, which showed a small *p*H shift, returned to *p*H 7. The water reservoir was then saturated with carbon dioxide gas at atmospheric pressure. At the breakthrough volume of the carbon dioxide solution, conveniently identified by a sharp change in effluent *p*H (minimum, *p*H 4.1 at 25°), the initial protein peak (Insulin I) appeared in the effluent. After elution of Insulin I was complete, as determined by optical density measurement, the eluent was changed to 0.1 *M* ammonium phosphate, *p*H 4.3.[11] After the carbon dioxide had been displaced, the 0.1 *M* ammonium phosphate solution was adjusted to *p*H 3.3 with phosphoric acid and the second protein fraction (Insulin II) was then eluted.

(11) This solution satisfied two important requirements as an intermediate eluent. (a) It removed carbon dioxide as the bicarbonate ion and thus prevented the column collapse which results when carbon dioxide gas is liberated. (b) It prevented elution of the remaining protein at undesirable alkaline *p*H since it did not permit the effluent *p*H to rise above 7.1.

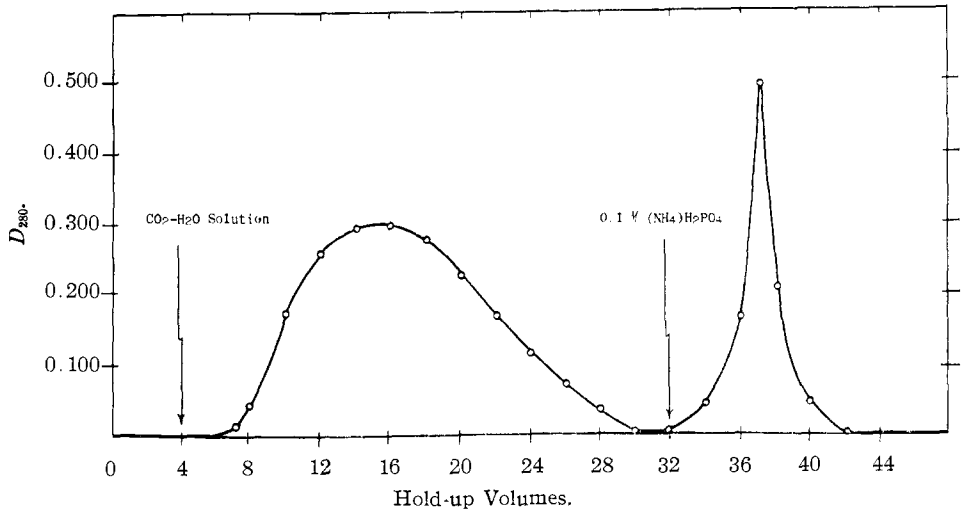

Fig. 1.—Effluent diagram of Armour crystalline zinc insulin, lot 2. 90 mg. in 3.5 ml., *p*H 3.0, applied to 33 × 0.9 cm. column containing 3 g. DEAE-cellulose, 0.4 meq. per g.; hold-up volume 15 ml. and flow rate 1 ml. per min.

Effluent Analysis.—The protein concentrations of the effluent fractions were determined by measuring their ultraviolet absorption at 280 mμ on a Beckman Model DU spectrophotometer. Protein recoveries were calculated as the per cent of total reference absorption added in the sample solution.

Isolation of Insulins I and II.—The Insulin I solution was lyophilized directly from the effluent solution after escape of the carbon dioxide. The Insulin II solution was dialyzed in the cold against several changes of distilled water before lyophilization.

Results

Chromatography.—Separation of crystalline zinc insulin into two principal fractions was obtained with all preparations examined. In Fig. 1 is shown an elution diagram from a typical experiment. Insulin I, the fraction removed with carbon dioxide solution, appears as a very broad asymmetrical peak comprising 78% of the total protein added. Insulin II, the fraction eluted with 0.1 *M* ammonium phosphate, displays itself as a single, comparatively sharp peak corresponding to 23% of the total protein added. Similar elution patterns were also obtained when the same insulin preparation was sorbed on DEAE-cellulose either from alkaline solution (*p*H 10) or in the zinc-free form after dialysis against 0.001 *M* hydrochloric acid.

Table I summarizes the recoveries of Insulins I and II obtained from several crystalline preparations. The last two samples, which yielded only 40 and 55% of Insulin I, respectively, were substandard (<20 units per mg.) The remaining ones had initial biological activity close to that of the U.S.P. reference insulin.

The behavior of Insulins I and II, when rechromatographed, agreed closely with that observed for the starting mixture. These experiments were conducted in the same manner as those carried out on the starting material, *i.e.*, with Insulin I, carbon dioxide–water elution was followed by 0.1 *M* ammonium phosphate elution; with Insulin II, 0.1 *M* ammonium phosphate elution was preceded by carbon dioxide–water elution. Typical rechromatograms of Insulins I and II are presented in Figs. 2a and 2b, respectively. The recovery of Insulin I

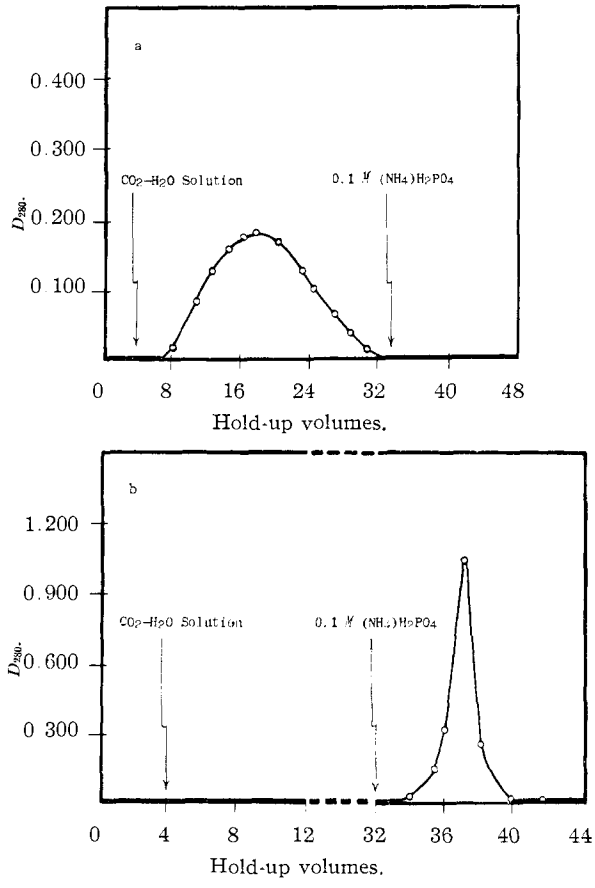

Fig. 2.—Effluent diagrams of Insulin I and Insulin II derived from Armour crystalline zinc insulin, lot 2: (a) 45 mg. of Insulin I in 3.8 ml., pH 3.0, applied to column 33 × 0.9 cm. containing 3 g. DEAE-cellulose, 0.4 meq. per g.; hold-up volume 15 ml. and flow rate 1 ml. per min.; (b) 38 mg. of Insulin II in 9.0 ml., pH 3.0 applied to column 33 × 0.9 cm.; other conditions as in Fig. 2a.

was nearly quantitative, more than 96% of the protein added. The rechromatogram of Insulin II

TABLE I

THE CONTENT OF INSULIN I AND INSULIN II IN VARIOUS CRYSTALLINE PREPARATIONS

Insulin preparation	Insulin I (%)[a,b]	Insulin II (%)[a]
U.S.P. Reference Standard	76	26
IUPAC, Batch 2189	79	19
Armour, Lot 1	81	19
Armour, Lot 2	78	23
Armour, Lot 3	83	18
Armour, Lot 4	40	..[c]
Armour, Lot 5	55	..[c]

[a] Based on optical density readings at 280 mμ. [b] The ready precipitation of Insulin I upon escape of carbon dioxide from solution required that extraordinary care be employed for the quantitation of recoveries by ultraviolet spectrophotometry. As a confirmatory measure in these experiments, optical density readings of the effluent fractions were also taken after hydrochloric acid had been added to make the *p*H constant. [c] Not quantitated.

did not show any Insulin I contamination. However, the recovery was somewhat low, totaling 90% of the protein added.

Insulin II, when rechromatographed, gave low recovery because it was leached from the column with carbon dioxide–water solution. It yielded an insulin solution whose concentration was below that detectable by optical density measurement. This fact was established in studies in which DEAE-cellulose columns containing adsorbed Insulin II were submitted to washing with excessive amounts of carbon dioxide–water solution. A summary of one such study is presented in Table II. Prolonged carbon dioxide–water wash reduced the recovery of Insulin II more than 50% (Step A). The recovery was reduced still further by continued washing (Step B). Optical density readings with the carbon dioxide–water wash did not rise above the minimum detectable level, 0.010 per ml. However, when the washings were combined and added to a second DEAE-cellulose column, after escape of the carbon dioxide, 80% of the "lost" Insulin II could be recovered by direct elution with dilute acid.

The weight of Insulin II leached from the column was directly proportional to the ml. of carbon dioxide–water wash used. The total amount of Insulin II contamination in the Insulin I fraction, therefore was small.

TABLE II

INSULIN II RECOVERIES AFTER PROLONGED CARBON DIOXIDE–WATER WASH

Step	CO_2–H_2O wash (ml.)	Recovered Insulin II[b] (%)	Lost Insulin II mg./ml. CO_2–H_2O wash
A[a]	11,200	47	0.004
B[c]	17,100	23	0.004

[a] 92 mg. of Insulin II derived from Armour, Lot 3, applied to column 12 × 2 cm. containing DEAE-cellulose, 0.5 meq. per g.; hold-up volume, 27 ml. [b] After carbon dioxide–water wash, the exchanger was extruded from the column and eluted batchwise with 0.1 *N* hydrochloric acid. 0.1 *N* hydrochloric acid was used to insure complete desorption of all retained materials. [c] Acid eluate from Step A was added to the regenerated DEAE-cellulose column and washing with carbon dioxide solution was continued.

Characterization.—Insulins I and II were identical in qualitative amino-acid content, as demonstrated by comparative analyses. Their biological activity, crystallizability, solubility, optical rotation and electrophoretic behavior differed significantly.

The biological activity of the individual fractions obtained from three different insulin preparations is shown in Table III. Insulin I, in every case, exhibited biological activity equal to that of the starting material. The three Insulin II fractions, however, displayed from 27 to 73% lower activity than their respective starting materials.

TABLE III

BIOLOGICAL ACTIVITY OF INSULIN I AND INSULIN II DERIVED FROM THREE CRYSTALLINE PREPARATIONS

Preparation	Activity-units per mg.[a] Starting material	Insulin I	Insulin II
IUPAC, Batch 2189	23	23.3	14.8
Armour, Lot 1	24.2	24.6	8.9
Armour, Lot 2	24.5	25.8	18.0

[a] Determined by the sloped screen-mouse convulsion method.[12] Experimental error within 10% for all assays.

Conventional crystallization procedures, employing citrate or acetate buffer and soluble zinc salts such as zinc chloride or zinc acetate, yielded well-defined rhombohedral crystals from all Insulin I preparations. Insulin II solutions, subjected to the same conditions, showed no evidence of crystallinity throughout a *p*H range of 5 to 7.

Gross differences in solubility were noted between the two fractions at *p*H 4.3 in 0.1 *M* ammonium phosphate solution. The average solubility of three Insulin I fractions was 1.2 mg per ml., whereas that of the corresponding Insulin II fractions was 0.2 mg. per ml.

The electrophoretic patterns of Insulins I and II preparations derived from a combined lot of insulin are shown in Fig. 3.[13] As one would expect from the column separation, Insulin II migrated more rapidly in the electrophoretic field than Insulin I. The mobility of the Insulin II peak was -8.00×10^{-5} in contrast to -6.95×10^{-5} for the Insulin I peak. Contrary to expectation, the 50–50 mixture of the two fractions resulted in a single peak with a mobility of -7.00×10^{-5}, suggesting interaction between the two components.

Optical rotation measurements for Insulins I and II fractions are presented in Table IV. The specific rotation of Insulin I was somewhat lower than that of the starting material, whereas that of Insulin II exhibited a variable increase. The difference in the specific rotation of Insulin II as compared to Insulin I ranged from 26 to 99%.

Discussion

Fractionation on DEAE-cellulose has resulted in the separation of insulin into two components differing in biological and physicochemical properties. The elution of Insulins I and II in the order of their increasing electrophoretic mobilities confirms that ion exchange was the dominant factor in their separation. It can, thus, be concluded that Insulin I possesses a higher net positive charge than Insulin II.

(12) R. E. Thompson, *Endocrinol.*, **39**, 62 (1946).

(13) We are indebted to Miss Joan R. Anesey for the electrophoretic analyses.

TABLE IV

OPTICAL ROTATION OF INSULIN I AND INSULIN II DERIVED FROM THREE CRYSTALLINE PREPARATIONS

Preparation	Starting material	$-[\alpha]^{24}D$ Insulin I	Insulin II
IUPAC, Batch 2189[a]	25.3	22.3	28.2
Armour, Lot 2[b]	23.7	19.4	38.6
Armour, Lot 3[a]	24.9	22.1	43.8

[a] Determined at 1% concentration at *p*H 3.0. [b] Determined at 0.67% concentration. The Insulin II fraction derived from this preparation was not soluble in 1% concentration at *p*H 3.0.

Other experimental criteria indicate that the two fractions display differences in physical configuration. Their interrelationship as suggested by solubility, biological activity, crystallization and optical rotation studies is that of native and partially denatured forms. The variation noted in biological activity and optical rotatory power among Insulin II fractions suggests further that the degree of denaturation varies with different starting preparations.

It is of especial interest that Insulins I and II appear to be unlike the fractionation products obtained by other investigators. The two fractions obtained by Timasheff, Brown and Kirkwood using electrophoretic convection, like Insulins I and II, displayed differences in their biological activities. However, their minor component was the less rapidly migrating one, indicating a charge difference not only from Insulin II but also from the B component of Harfenist and Craig. The A and B components of Harfenist and Craig, in contrast with Insulins I and II, crystallized isomorphously and exhibited similar biological activities. The three fractions, separated by Fredericq through the use of differences in their solubility properties, also crystallized isomorphously.

These several fractions indicate that a degree of heterogeneity exists which is unexpected for a protein as well characterized as insulin. If, however, one assumes free interaction between like and unlike monomeric species, as few as two forms, bearing slight differences in charge and physical configuration, can account for this heterogeneity. For example, let us consider the insulin monomers, A_1 and A_2. Association at the dimer level would give rise to three polymeric forms: A_1A_1, A_1A_2 and A_2A_2. At higher levels of association, a variety of polymeric forms would be obtained, the total number being dependent on *p*H and other factors which affect the stability of the association equilibria. By fractionation under selective conditions, it would be possible to separate, as a minor component,

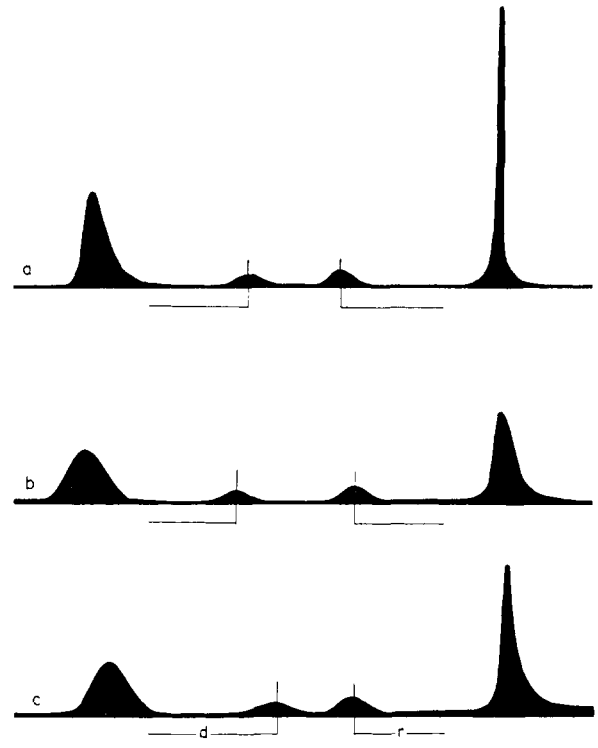

Fig. 3. Electrophoretic patterns of Insulins I and II derived from a combined lot of three crystalline zinc insulin preparations (IUPAC, 0.67 g.; Armour lot 1, 2.0 g.; Armour lot 2, 1.0 g.). All analyses were carried out at *p*H 8.5 in veronal buffer (r/2 = 0.1) at 3.2° in the standard Klett apparatus. (a) Insulin I, 180 min. (b) Insulin II, 150 min. (c) 50-50 mixture of Insulins I and II, 180 min.

polymeric forms composed predominantly of one monomeric species or the other. The end products would display increased uniformity. On the other hand, only fractionation at the monomer level would result in the separation of completely homogeneous materials.

It appears likely that DEAE-cellulose by virtue of its high density of accessible binding groups reacts with the insulin monomer by displacing it from its internal complexes. Moreover, it appears that the monomer-exchanger complexes thus formed (to cite the example, A_1-DEAE-cellulose and A_2-DEAE-cellulose) display sufficient difference in their binding affinities to permit selective elution by carbon dioxide–water solution. Thus, it is postulated that Insulins I and II differ from the end products of other fractionation procedures in that they are composed of polymeric forms each containing a single monomeric species.