In the early stages the concentration of butyric acid is negligible and the previous evaluation of $b$ is valid. By substituting the data for two points of a single kinetic curve and solving the resulting simultaneous equations, values of $r$ and $Ka$ are obtained.

The solid curves in Fig. 5 are the theoretically calculated curves representing the same data as Fig. 2. It is seen that the data are fairly well fitted, so that this alternate molecular mechanism is also acceptable. The butyric acid is here assumed to compete with crotonic acid for sites of type Y, thus progressively retarding the reaction.

Since the data can be expressed by either of the dual site rate laws, there seems to be no kinetic method of distinguishing between the two mechanisms. Since differential analysis of the data led to convenient evaluation of the constants of eq. 1, this rate law has been used throughout.

One can predict from either mechanism that the catalytic hydrogenation of crotonic acid follows zero-order kinetics initially and terminates by a first-order process. The duration of the zero-order portion of the curve is greater for high initial concentrations of acceptor. Butyric acid inhibits the reaction rate. The acceptable rate laws imply that the catalytic surface consists of two types of active centers, in agreement with the concepts of Balandin.[15]

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CHICAGO 16, ILLINOIS

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# The Fractionation of Insulin by Electrophoresis–Convection[1]

BY SERGE N. TIMASHEFF, RAYMOND A. BROWN AND JOHN G. KIRKWOOD

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An electrophoretic study, carried out in a whole spectrum of buffers, demonstrated that insulin is not a homogeneous protein, but is composed of a principal rapid moving component and a smaller amount of slower electrophoretically broadly spread out material. Fractionation by electrophoresis–convection at *p*H 7.5 resulted in a partial separation of the two components as demonstrated electrophoretically and by activity determinations.

## Introduction

Although in the last few years extensive work has been reported on the characterization of insulin, comparatively little literature is available on the problem of insulin homogeneity by physico-chemical criteria.[2-4]

When preliminary electrophoretic analyses, carried out in our laboratories, pointed to the electrophoretic non-homogeneity of insulin, it was decided to make a more complete investigation of this and to attempt the fractionation of this protein by the method of electrophoresis–convection. The results of these studies are reported in this paper.

## Experimental

The insulin used was Lilly Zinc-crystalline Insulin, Lot No. 515499 with an activity of 27 units/mg. and also a specially prepared sample of Lilly amorphous insulin No. 200-1B-11J, with an activity of 24 units/mg.

Electrophoresis experiments were performed at 2° in a Klett Tiselius electrophoresis apparatus and also at 0° in a Perkin–Elmer apparatus, Model 38. The electrophoresis–convection runs were carried out in a cold room at 3–4°. The details of construction and operation of the apparatus have been described previously.[5]

In each fractionation run, the insulin was dissolved in the appropriate volume of buffer and dialyzed for 24 hours against the same buffer in the cold room. In the case of zinc-crystalline insulin, in order to remove the zinc, the protein was dissolved first in a *p*H 3.0 HCl solution, the ionic strength of which had been adjusted to 0.1 with NaCl. This solution was then dialyzed for 72 hours against several changes of the HCl and finally brought to the conditions of the experiment prior to fractionation. At the end of each run the top and bottom fractions were removed out of the electrophoresis–convection cell and analyzed electrophoretically.

The samples for activity assay were prepared as follows. The solution was dialyzed against several changes of the *p*H 3.0 HCl solution, then several changes of distilled water and finally lyophilized. The insulin activity determinations were carried out for us at the Lilly Research Laboratories through the courtesy of Dr. E. D. Campbell and Dr. O. K. Behrens.

## Results

**Electrophoretic Analyses.**—The insolubility of insulin in its isoelectric region makes it impossible to carry out electrophoretic analyses in the *p*H zone between 4.4 and 7.0. A number of analyses were carried out, however, under various conditions in the solubility regions on both sides of the zone of insolubility.

In Fig. 1 are shown some typical patterns obtained at various *p*H's. The mobility data are summarized in Table I. From the pictures presented it can be seen that insulin does not migrate electrophoretically in a manner expected of a homogeneous protein, but considerable resolution into components may be observed.

In a *p*H 8.6 barbital buffer ($\Gamma/2 = 0.1$) (Fig. 1a) the pattern shows the presence of a principal component comprising 79% of the total protein with a mobility of $-6.74 \times 10^{-5}$, and a trailing shoulder with a mean mobility of $-5.55 \times 10^{-5}$. The component analyses of the rising and descending bound-

(1) This work was carried out partly with the help of funds provided by the Office of Naval Research, contract No. Nonr-659(00), and partly with a grant in aid from Eli Lilly and Co.

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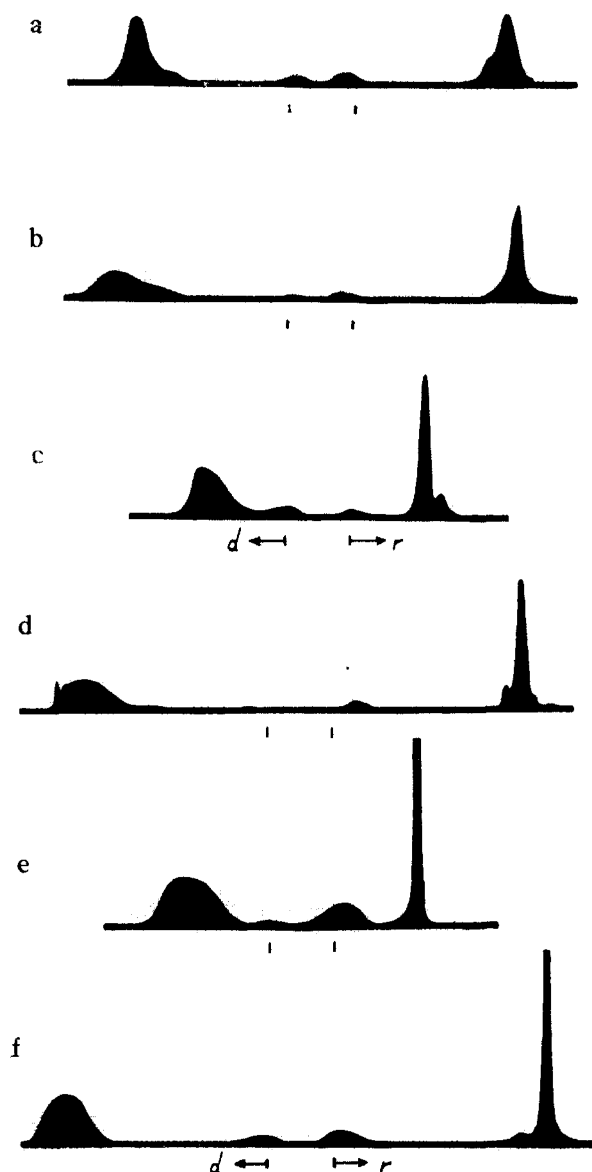


Fig. 1.—Electrophoretic pictures of insulin: (a) Zn-cryst. in $p$H 8.6 barbital ($\Gamma/2$ = 0.1), 120 min.; (b) Zn-cryst. in $p$H 7.5 phosphate ($\Gamma/2$ = 0.1), 150 min.; (c) Zn-cryst. in $p$H 7.0 cacodylate–chloride (Cac. $\Gamma/2$ = 0.02, NaCl $\Gamma/2$ = 0.08), 150 min.: (d) amorphous in $p$H 4.0 acetate ($\Gamma/2$ = 0.1), 238 min.; (e) amorphous in $p$H 4.0 acetate ($\Gamma/2$ = 0.023), 66 min.; (f) amorphous in $p$H 3.0 phosphate, ($\Gamma/2$ = 0.11), 185 min.

aries extrapolate to the same values at zero concentration. The mobility of insulin was found to be independent of concentration.

At $p$H 7.5, in a 0.1 ionic strength phosphate buffer (Fig. 1b) a similar type of heterogeneity is observed in the descending pattern. At this $p$H component analysis becomes difficult since the rising and descending patterns are no longer mirror images and the descending boundary spreads out considerably. It is possible, however, to estimate the composition of the descending pattern to be 73% of a principal component, the rest being a long spread out shoulder of slower moving material.

Considerable electrophoretic heterogeneity can also be observed at other conditions studied (Figs. 1c, 1d, 1e) down to $p$H 3.0 (Fig. 1f), at which point the pattern begins to take on a more homogeneous character.

That the demonstrated electrophoretic heterogeneity of the insulin is not due to residual zinc complexed with a fraction of the insulin molecules present, can be concluded from the observation that zinc-free amorphous insulin and zinc-crystalline insulin exhaustively dialyzed at $p$H 3.0 produced very similar electrophoretic patterns. It should also be pointed out that varying the time of dialysis of the zinc-crystalline insulin did not change the electrophoretic pictures.

Furthermore, it should be pointed out that no change in the electrophoretic pictures could be observed after the insulin had been allowed to stand for a period of three weeks dissolved at $p$H 7.5 in a 0.1 ionic strength phosphate buffer.

Even though the results reported have been obtained on a mixed species insulin, one can dismiss that factor as the cause of the observed heterogeneity from the fact that a sample of zinc-crystalline beef insulin (supplied to us by H. Gutfreund) displayed identical electrophoretic properties. It is also of interest to note that when this last sample was recrystallized in a zinc-free form in $p$H 2.65 phosphate[6] the electrophoretic pattern at $p$H 7.5 remained unchanged.

The electrophoretic heterogeneity of insulin was also demonstrated by a reversible spreading experiment. This was performed in phosphate buffer at $p$H 7.5 ($\Gamma/2$ = 0.1), since insulin is insoluble in its isoelectric region. The protein was allowed to migrate in the forward direction for 150 minutes, at which point the current was reversed and the boundaries run back to their starting positions. As the run was going forward, the pattern gradually resolved itself into a picture similar to that shown in Fig. 1b. Upon return to the starting positions, the boundaries assumed a pattern of homogeneity, the shoulders disappearing completely. This would point to the fact that the observed electrophoretic effects are not due to the concentration-dependent dissociation of insulin[7,8] at the protein–buffer boundary, but to the presence of real fractions.

**Fractionation.**—Since electrophoretically best resolution was obtained on the alkaline side of the isoelectric point, it was decided to carry out the fractionation of insulin in that $p$H region. Although successful fractionation was obtained at $p$H 8.6 in a borate buffer,[9] the main fractionation work was carried out in $p$H 7.5 phosphate ($\Gamma/2$ = 0.1), due to the reported loss of activity at higher $p$H's. The results are summarized in Table II.

Two sets of experiments were carried out. In the first run 1.6 g. of zinc-crystalline insulin was dissolved in 250 ml. of $p$H 3.0 HCl, dialyzed as described above and finally brought to $p$H 7.5. The volume was adjusted to 280 ml. and the solution fractionated in two electrophoresis–convection cells

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TABLE I

TISELIUS ELECTROPHORESIS DATA FOR INSULIN

| Sample | Buffer | Ionic strength | pH | Mobility,[a] cm./sec./v./cm. × 10[5] Rising | Descending Fast peak | Descending Slow peak | Mean | Rapid component, % (descending) |
|---|---|---|---|---|---|---|---|---|
| Zn-cryst. lot no. 515399 Lilly | Barbital | .1 | 8.6 | −6.60 | −6.74 | −5.55 | −6.49 | 79 |
| | Barbital | .02 | 8.45 | −6.51 | −6.67 | −5.43 | −6.45 | 83 |
| | NaSCN | .08 | | | | | | |
| | Borate | .1 | 8.5 | −6.23 | −6.37 | −4.98 | −6.25 | 84 |
| | Phosphate | .1 | 7.5 | −4.95 | −5.16 | −3.97 | −4.85 | 73 |
| | Cacodylate | .02 | 7.0 | −4.15 | | | −4.30 | |
| | NaCl | .08 | | | | | | |
| Amorphous no. 200-1B-11J Lilly | Barbital | .1 | 8.5 | −5.82 | −6.13 | −5.18 | −5.89 | 76 |
| | $PO_4$ | .1 | 7.5 | −4.98 | −5.19 | −3.78 | −4.85 | 76 |
| | Barbital | .01 | 7.5 | −4.72 | −4.82 | −3.61 | −4.56 | 78 |
| | NaCl | .09 | | | | | | |
| | $PO_4$ | .2 | 7.4 | −4.46 | −4.68 | −3.71 | −4.38 | 69 |
| | Acetate | .1 | 4.0 | 4.65 | | | 4.42 | |
| | Acetate | .023 | 4.0 | 6.74 | | | 6.87 | |
| | $PO_4$ | .22 | 3.0 | 3.99 | | | 3.90 | |
| | $PO_4$ | .11 | 3.0 | 7.96 | | | 7.78 | |
| Zn-cryst. beef T 2344 Lilly | $PO_4$ | .1 | 7.5 | −5.28 | −5.70 | −4.14 | −5.34 | 77 |
| | $PO_4$[b] | .1 | 7.5 | −5.35 | −5.72 | −4.12 | −5.36 | 78 |

[a] All runs were performed using the Klett Instrument. [b] Same sample recrystallized according to Gutfreund's method.

TABLE II

FRACTIONATION OF INSULIN IN pH 7.5 PHOSPHATE ($\Gamma/2$ = 0.1)

| Sample | Conditions | Fraction | Volume, ml. | Concn., mg./ml. | Activity, units/mg. | Mobility, cm./sec./v./cm. × 10[5] |
|---|---|---|---|---|---|---|
| Zn-cryst.[a] lot no. 515499 Lilly | 1.9 v./cm. 3, 12-hr. stages | Starting | 280 | 5.7 | 27 | −4.85 |
| | | Pooled tops | 432 | 0.4 | 16 | −4.58 |
| | | Final bottom | 156 | 7.1 | 25 | −4.86 |
| Recovery: protein, 82%; activity, 71%. Hyperglycemic factor present in all fractions | | | | | | |
| Amorphous[b] no. 200-1B-11J Lilly | 2.1 v./cm. 13 hr. | Starting | 125 | 6.5 | 24 | −5.06 |
| | | Top | 52 | 0.17 | 15 | −4.22 |
| | | Bottom | 78 | 11.0 | 23.5 | −5.10 |
| Recovery: protein, 106%; activity, 105%. Hyperglycemic factor present in all fractions. | | | | | | |

[a] Electrophoretic analyses carried out on the Klett instrument. [b] Electrophoretic analyses carried out on the Perkin-Elmer instrument.

set up in series in three 12-hour stages at a field strength of 1.9 v./cm. At the end of each stage the top fraction was removed, the bottom diluted to 280 ml. and rerun at the same conditions. The pooled tops and the final bottom were then analyzed electrophoretically and assayed for biological activity. These data, presented in columns 6 and 7 of Table II, indicate that fractionation has taken place, the top fraction being enriched with respect to the slower components and having a biological activity considerably lower than that of the original insulin.

In the second run, using the zinc-free insulin, 810 mg. of the protein was dissolved in 125 ml. of the pH 7.5 phosphate buffer and the fractionation was carried out for 13 hours at 2.1 v./cm. The electrophoretic and biological activity data again point to a high degree of fractionation.

It is of interest to note that in both runs hyperglycemic activity remained in both the top and bottom fractions.

Fractionation at pH 7.5 having led to the concentration of a less active fraction of insulin, attempts at fractionation were made on the acid side of the isoelectric point. Since at pH 3.0 insulin was found to present a nearly homogeneous picture electrophoretically, the fractionations were carried out at pH 4.0 in acetate buffer. Under these conditions, however, in a series of runs in which both the protein concentration and the ionic strength of the buffer were varied no fractionation occurred as the insulin was found to precipitate on the channel wall at the high concentration side. This gave rise to a strong osmotic influx into the cell which disrupted the convective flow in the channel.

## Discussion

Although previous studies on insulin have been carried out under the assumption of the homogeneity of this protein, electrophoretic analyses in a whole spectrum of buffers indicate that highly purified zinc-crystalline and zinc-free samples of insulin display a considerable degree of heterogeneity. It would seem, then, from the above-presented data, that insulin is composed of a principal electrophoretically rapid-moving component and a slow, highly heterogeneous minor component. Furthermore, from the fractionation data, one might conclude that the activity is concentrated mostly in the

rapid component. Using the theory of electrophoresis–convection,[10] it can be estimated that the top fraction in each case should have become enriched with respect to the slow component, until that material comprised *ca.* 40% of the total protein. This, along with the observed reduction in the biological activity of the top fraction would lead to the belief that the slower components are probably devoid of insulin activity. That the chemical differences between the two fractions must be very minute can be conjectured from the similarity of their behavior in electrophoresis, their lack of separation by the usual precipitation methods and their non-interference with the dissociation[7,8] and chemical composition[11] studies reported in the literature. It might also be of interest to add that, using the equation of Henry which relates electrophoretic mobility to charge, it has been possible to calculate that the difference of mobility between the two fractions can be accounted for by a difference of three electronic charges per insulin molecule, assuming a molecular weight of 36,000. Craig recently reported the fractionation of insulin into two fractions,[12] differing only to a very slight extent,[13] and both possessing equal insulin activity. The difference between these fractions consists in one amide group per 6,000 unit, the principal component having one less free carboxyl than the minor fraction. It would seem then that the fractions obtained by electrophoresis–convection are probably not identical with the ones separated by Craig due to the difference in activity and also since in the electrophoresis–convection fractions the major component is electrophoretically the more rapidly migrating one while one would expect just the opposite to be true in the case of Craig's insulin fractions.

Whether the hyperglycemic factor[14] found in insulin is concentrated mostly in one of the two fractions or is evenly distributed is impossible to judge from the data so far on hand. However, the recent report that this factor represents only a minute fraction of the total protein in crystalline insulin[15] and furthermore is insoluble in pure form at the *p*H used in this study could explain the lack of separation of the two activities in the electrophoresis–convection fractionations.

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[CONTRIBUTION NO. 1145 FROM THE STERLING CHEMISTRY LABORATORIES, YALE UNIVERSITY]

# Electrophoresis–Convection Applied to the Complexed Insulin–Protamine System[1]

BY SERGE N. TIMASHEFF AND JOHN G. KIRKWOOD

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The recently suggested method for the separation of electrophoretically similar proteins by electrophoresis–convection with the aid of complex formation has been applied to the fractionation of insulin. Using protamine as the complexing agent, it was possible to achieve a partial separation of the electrophoretic components of insulin in a *p*H 4.0 acetate buffer, demonstrating the applicability of the technique.

## Introduction

The method of electrophoresis–convection has been successfully applied to the fractionation of a number of systems of proteins. Thus, by its use it has been possible to carry out a number of very refined fractionations[2,3] and to make studies of biologically important systems.[4,5]

In some cases, however, fractionation by this method may become difficult due to either too close similarity of the isoelectric points of the proteins to be separated or to their insolubility near the isoelectric point and electrophoretic similarity in their solubility region.

For the fractionation of such systems by electrophoresis–convection, a modified technique has been suggested.[6] This technique is based upon the formation of soluble complexes between the protein to be fractionated and an interacting agent. Such complex formation would lead to a change in the constituent mobility of the protein,[7] with the result that its rate of transport in electrophoresis–convection would also change. Thus, if one had a mixture of proteins with similar mobilities, one of which could interact to a stronger degree than the others with a given complexing agent, electrophoretic resolution of the components, in their complexes, could be enhanced and their separation by electrophoresis–convection made easier. In a previous communication,[6] it has been shown that the formation of such soluble complexes does alter greatly the rate of transport of a protein out of the top reservoir of the electrophoresis–convection cell. It was also demonstrated that in such cases, the theory of electrophoresis–convection, as developed for two-

(1) This work was carried out partly with the help of funds provided by the Office of Naval Research, contract No. Nonr-659(00), and partly with a grant in aid from Eli Lilly and Co.

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