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.

(10) J. G. Kirkwood, J. R. Cann and R. A. Brown, *Biochim. Biophys. Acta*, **5**, 301 (1950); **6**, 606 (1951).

(11) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951); F. Sanger and E. O. P. Thompson, *Proc. Biochem. Soc.*, Sept., iii (1952).

(12) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3083 (1952).

(13) L. C. Craig, Sixth Lilly Insulin Symposium, May, 1952.

(14) E. W. Sutherland and C. F. Cori, *J. Biol. Chem.*, **172**, 737 (1948).

(15) A. Staub, Seventh Lilly Insulin Symposium, May, 1953.

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

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

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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.

(2) J. R. Cann, R. A. Brown and J. G. Kirkwood, THIS JOURNAL, **71**, 2687 (1949).

(3) M. Bier, J. A. Duke, R. J. Gibbs and F. F. Nord, *Arch. Biochem. Biophys.*, **37**, 491 (1952).

(4) J. R. Cann, D. H. Campbell, R. A. Brown and J. G. Kirkwood, THIS JOURNAL, **73**, 4611 (1951).

(5) J. R. Cann, R. A. Brown, J. G. Kirkwood, P. Sturgeon and D. W. Clarke, *J. Immunology*, **68**, 243 (1952).

(6) S. J. Singer, S. N. Timasheff and J. G. Kirkwood, THIS JOURNAL, **74**, 5985 (1952).

(7) L. G. Longsworth and D. A. MacInnes, *J. Gen. Physiol.*, **25** 507 (1942).

component systems,[8,9] can be applied if now the constituent mobilities of the components are used.

In order to establish the applicability of this method in actual fractionation, it was decided to use an electrophoretically inhomogeneous protein, insoluble in its isoelectric region, but the components of which are electrophoretically similar at *p*H's removed from the isoelectric point, and which therefore is difficult to fractionate by usual electrophoresis–convection. Insulin is a protein which has been shown to satisfy these requirements.[10] The complexing agent selected was protamine since that protein is known to precipitate insulin out of solution in the *p*H region above the latter's isoelectric point.[11,12] The results of the fractionation of insulin by electrophoresis–convection in the presence of protamine are reported in this paper.

Experimental

The insulin was a specially prepared sample of Lilly amorphous insulin No. 200-1B-11J, possessing an activity of 24 units/mg. The protamine was Lilly Salmine sulfate, Lot No. 552227.

Electrophoretic 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 experiments were carried out in a cold room at 3–4°. The details of construction and operation of the apparatus have been reported previously.[13]

Prior to fractionation or electrophoretic analysis, the insulin and protamine, dissolved in the appropriate buffer, were dialyzed for 24 hours against the same buffer in the cold room. At the end of each fractionation run the top and bottom fractions were removed out of the electrophoresis–convection cell and analyzed electrophoretically in *p*H 3.1 phosphate ($\Gamma/2 = 0.1$) to establish the concentration of the two proteins in each fraction. The insulin was then separated from the protamine by the following procedure. The insulin–protamine solution was dialyzed against *p*H 3.1 phosphate to which thiocyanate had been added (phosphate, $\Gamma/2 = 0.02$; thiocyanate, $\Gamma/2 = 0.08$). This resulted in the precipitation of the insulin, which is known to bind SCN^- ions,[14,15] while the protamine remained in solution. The insulin was then centrifuged down, redissolved in the *p*H 3.1 phosphate and the process repeated twice more.

The fractions were analyzed electrophoretically in a *p*H 7.6 phosphate buffer ($\Gamma/2 = 0.1$). The insulin solutions were then adjusted to *p*H 3.1, dialyzed against several changes of distilled water and lyophilized. The activity determinations were carried out for us at the Lilly Research Laboratories through the courtesy of Dr. O. K. Behrens.

Results

Electrophoresis.—In Fig. 1 is presented a typical electrophoretic pattern obtained with the insulin–protamine system in *p*H 4.0, $\Gamma/2 = 0.023$ acetate buffer. The mobility data on a number of runs under various conditions are presented in Table I.

From careful analysis of the electrophoretic data, it can be seen that the system insulin–protamine forms soluble complexes at *p*H 4.0 and above at low ionic strengths, while at higher ionic strength no interaction takes place. It should be noted that at *p*H 4.0 in a 0.023 ionic strength acetate buffer the mobility of the slow boundary on the descending side has lower values in the mixture than that of pure insulin, while in the case of rapid equilibrium the mobilities should be identical. Furthermore, this mobility changes as the insulin:protamine ratio is changed, increasing as the insulin concentration increases and approaching that of pure insulin at a high insulin:protamine ratio. This can be attributed to variation in the salt concentration and *p*H in the cell behind the fast moving boundary at which the protamine disappears. That the slow boundary on the descending side contains only insulin, was shown by direct sampling of the contents of the region between the slow and fast peaks and subsequent electrophoretic analysis of that solution. It should also be noted that in all cases on the descending side the area under the slow boundary was found to be considerably less than that calculated for the insulin present in the system, while that under the fast boundary was greater than that calculated for the protamine present. This would point to the disappearance of part of the insulin at the fast boundary in the form of complexes. It is true that the Dole theory of moving boundaries[16] predicts a similar distribution of the areas even in the absence of complexes between the components. However, the magnitude of the area changes predicted by Dole is of a much smaller order than that observed in the present case. Further evidence for the existence of complexes can be derived from the low mobilities of the fast descending boundaries

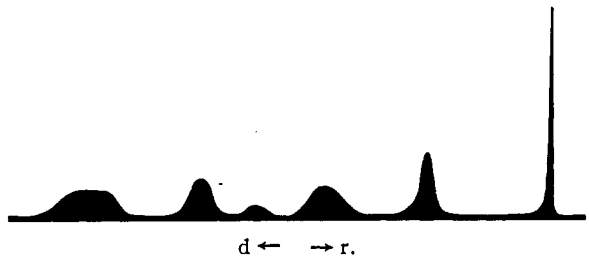

Fig. 1.—Electrophoretic picture of the insulin–protamine system in *p*H 4.0 acetate buffer ($\Gamma/2 = 0.023$); 0.26% insulin, 0.33% protamine.

TABLE I

TISELIUS ELECTROPHORETIC DATA FOR THE INSULIN–PROTAMINE SYSTEM

Protein insulin, mg./ml.	Concn. protamine, mg./ml.	Acetate *p*H	Buffer $\Gamma/2$	Electrophoretic mobilities,[a] cm./sec./v./cm. × 10^5 Descending Fast	Descending Slow	Rising Fast	Rising Slow
5.18		4.0	0.023		6.87		6.74
	3.78	4.0	.023	15.24		17.67	
0.58	4.32	4.0	.023	11.22	3.81	17.70	8.38
1.73	4.46	4.0	.023	12.74	4.18	19.30	8.87
2.59	3.34	4.0	.023	13.50	4.84	17.98	8.22
3.46	2.22	4.0	.023	14.23	5.17	18.98	8.58
4.32	1.11	4.0	.023	14.32	5.83	18.12	7.68
4	4	4.0	.05	12.92	4.49	16.31	7.41
10	3	4.0	.1	12.78	5.83	12.83	5.23
4	4	4.3	.04	11.97	3.73	17.32	8.09
4	4	4.4	.04	13.34	3.46	14.33	7.37

[a] All runs were performed using the Klett Instrument.

(8) J. G. Kirkwood, J. R. Cann and R. A. Brown, *Biochim. Biophys. Acta*, **5**, 301 (1950); **6**, 606 (1951).

(9) R. A. Brown, J. B. Shumaker, S. N. Timasheff and J. G. Kirkwood, THIS JOURNAL, **74**, 460 (1952).

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

(11) H. C. Hagedorn, B. Norman Jensen, N. B. Krarup and I. Wodstrup, *J. Am. Med. Assoc.*, **106**, 177 (1936).

(12) D. H. Scott and A. M. Fisher, *J. Pharamacol.*, **58**, 78 (1936).

(13) J. R. Cann, J. G. Kirkwood, R. A. Brown and O. J. Plescia, THIS JOURNAL, **71**, 1603 (1949).

(14) E. Volkin, *J. Biol. Chem.*, **175**, 675 (1948).

(15) E. Fredericq and H. Neurath, THIS JOURNAL, **72**, 2684 (1950).

(16) V. P. Dole, *ibid.*, **67**, 1119 (1945).

when both insulin and protamine are present in the solution.

Fractionation.—In a preliminary run, carried out in *p*H 4.25, $\Gamma/2 = 0.04$ acetate, it was found that the transport occurred exactly as expected for a complexed system in electrophoresis–convection. In this case it was found that when 75% of the total protein had been transported out of the top reservoir, the experimental value for the separation factor,[17] f_2, was 2.4. The corresponding theoretical value, assuming interaction, was calculated to be 2.1, while if no interaction had been present, f_2 would have had a value of 17.

Due to the limited solubility of insulin above *p*H 4.0, the final fractionations were carried out at *p*H 4.0, in $\Gamma/2 = 0.023$ acetate buffer. The results of two such runs are summarized in Table II.

TABLE II

FRACTIONATION OF INSULIN IN THE PRESENCE OF PROTAMINE IN ACETATE BUFFER; *p*H 4.0, $\Gamma/2 = 0.023$

Run	Conditions	Fraction	Insulin activity, units/mg.	Mobility,[a] cm./sec./v./cm. × 10^5
3	0.6 v./cm.	Starting	24	−5.71
	24 hours	Top	9	−5.21
		Bottom	21.5	−5.92
4	0.5 v./cm.	Starting	24	−5.71
	13.5 hours	Top	15	−5.16
		Bottom	20	−5.89

[a] Tiselius electrophoresis analyses carried out on the Perkin–Elmer instrument, in phosphate buffer, *p*H 7.6, $\Gamma/2 = 0.1$.

In the first run, 560 mg. of insulin and 980 mg. of protamine were dissolved in 120 ml. of buffer and fractionated for 24 hours at a field strength of 0.6 v./cm. The top and bottom fractions were then removed out of the cell and the insulin separated from the protamine as described above. The electrophoretic and insulin activity data, presented in columns 4 and 5 of Table II, show that the top fraction has become strongly enriched with respect to the slow component.

In the second experiment, 540 mg. of insulin and 740 mg. of protamine, dissolved in 130 ml. of the buffer, were fractionated for 13.5 hours at 0.5 v./cm. Once again the electrophoretic and activity data point to a considerable degree of fractionation.

Although in both runs only 80% of the protein has been recovered with a corresponding activity loss of 25%, it is noteworthy that the electrophoretic mobility and activity changes in the top fractions are in the same direction as those observed for the fractionation in *p*H 7.5 phosphate.[10] It would appear that the less active fraction of insulin is bound to a lesser degree by protamine than the principal active material and therefore transported less rapidly out of the top reservoir.

Discussion

The complexing of insulin with protamine has resulted in the rather easy fractionation of the former by electrophoresis–convection. It is quite apparent that not only does the protamine binding increase the transport of insulin in electrophoresis–convection, but that it also aids to keep the insulin in solution, in view of the fact that all previous attempts at fractionation at *p*H 4.0 had met with failure due to the precipitation of insulin on the walls of the channel.[10]

The demonstrated fractionation of insulin by electrophoresis–convection *via* its interaction with protamine, illustrates a new approach to the problem of protein separation. This technique should prove of great use in the fractionation of some biologically important systems. For example, by its application it should be possible to separate a mixture of complete and incomplete antibodies which, although similar electrophoretically, would bind differentially a suitable protein antigen. Another possible application might be in the isolation and purification of enzymes, where the enzyme could be caused to form soluble complexes with an inhibitor or, in the case where a double substrate is required for enzyme action, it might be possible to fractionate the enzyme *via* its interaction with one of the two substrates.

The complexing agents used in this method do not necessarily have to be macromolecular in nature, but small ions which interact with the protein in question also could be used. In such case the interacting ion would have to be present also in the buffer solution circulating on the outside of the electrophoresis–convection cell since it would diffuse through the semipermeable membrane walls of the channel. This might require in some cases a slight modification in the instrument such as the replacement of the platinum electrodes by another type or the introduction of salt bridges between the electrodes and the cell.

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(17) The separation factor is defined as

$$f_2 = (C_2/C^0_2)/(C_1/C^0_1)$$

where C_1 and C_2 are the concentrations of the two components in the top reservoir at a given time during the run, and C^0_1 and C^0_2 are the initial concentrations. The theoretical value of f_2 may be calculated using the equation

$$(1 + X^\circ_2(f_2 - 1))\beta/f = \gamma\beta$$

where $\gamma = (c_1 + c_2)/(c^0_1 + c^0_2)$, $X^0_2 = c^0_2/(c^0_1 + c^0_2)$ and $\beta = 1 - \mu_2/\mu_1$, where μ_1 and μ_2 are the electrophoretic mobilities of the two components.