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Isoelectric Fractionation, Analysis, and Characterization of Ampholytes in Natural pH Gradients

IV. Further Studies on the Resolving Power in Connection with Separation of Myoglobins

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The resolving power of the method of stationary electrolysis for isoelectric focusing of proteins is studied on a system of myoglobins, which is found to contain two components with a difference in isoelectric point (pI) of only 0.06 pH units. With the aid of a new system of carrier ampholytes, these components are found to be completely resolvable, and their zone breadths satisfy a theoretically derived equation. Further, by introduction of an exact definition of a resolving power, a mathematical equation for the latter is derived. It shows that the resolving power improves with the shallowness of the pH course and with the field strength, for both variables with a square root dependence. There is no theoretical limit to the resolving power. With the present apparatus and technique, a resolving power of 0.02 pH units is calculated for proteins physico-chemically similar to myoglobin. With some technical improvement, a resolving power of 0.01 pH unit should be possible. Thus, if two proteins have a pI difference big enough to be picked up by a pH meter of standard accuracy, the proteins can be resolved by stationary electrolysis.

In part III of this series, Svensson [1] showed, by the use of various hemoglobins, that the resolving power of this separation method is better than 0.2 pH units, since hemoglobins with such a pI difference could be fully resolved. This conclusion is, however, restricted to pH regions in which good carrier ampholytes (Svensson [2]) are available. A good carrier ampholyte can most simply be described as an ampholyte with an appreciable conductance in its isoelectric state. As has been shown, a good conductance is always accompanied by a good buffering capacity. The great difficulties in finding a sufficient number of such ampholytes among commercially available chemicals have for a long time restricted applications of the method and severely hampered its further development. So far, most of the necessary carrier ampholytes have to be made in the laboratory.

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For theoretical reasons, it could be expected that peptides containing acidic and/or basic amino acids, and especially histidine, would have the desired properties. Only few such peptides are commercially available, however, and those which can be bought are far too expensive to be generally applied for this purpose. Since undefined peptide mixtures serve the purpose almost as well as pure peptides, which would have to be mixed anyway, a program was contemplated for production of partial protein hydrolyzates on a laboratory scale. Hemoglobin or whole blood was used as a starting material due to the high histidine content of hemoglobin. Various hydrolytic agents were used, such as sulphuric acid, baryta, and proteolytic enzymes. After hydrolysis, the sulphuric acid was neutralized with baryta, and *vice versa*, and the inorganic precipitate was removed. The partial hydrolyzate was then dialyzed through cellophane in order to remove unattacked high-molecular material, and the dialyzate was concentrated by evaporation. It was finally desalted and fractionated by electrolysis in an apparatus essentially according to Williams and Waterman,[3] but with 20 compartments separated by filter paper membranes, and with efficient water-cooling in each compartment.

Peptide mixtures prepared in this way proved to be useful since they gave shallow pH gradients on electrolysis, and continued research on the method could thus be performed. This was done on various proteins, and it turned out that the resolving power could be improved to about 0.1 pH unit. The peptides also made it possible to fractionate and analyze proteins isoelectrically over the entire pH scale.

Nevertheless, several severe practical difficulties still persisted. Thus it proved practically impossible to obtain quite colourless peptide preparations, even if most of the dyestuffs in the black, crude hydrolyzate stuck to and were removed by the copious precipitate of barium sulphate. This implied that electrolysis of carrier ampholytes alone gave rise to a number of coloured zones. This proved to be a great nuisance especially in work with myoglobins since the tinge of several peptide zones could not be distinguished from that of myoglobins. This necessitated blind experiments without added protein in all cases where myoglobins were involved. Moreover, oligo-peptides display most of the typical protein reactions, the light absorption at 280 nm included, and hence proteins could not be quantitatively analyzed until the peptides had been dialyzed away. Although only dialyzable peptides were used, the bigger members dialyzed out very slowly, and the protein analyses thereby became a time-consuming business. Finally, the peptides isoelectric between pH 5.0 and 6.5, although obtained in great quantities, proved to possess poor carrier ampholyte properties. As a consequence thereof, the conductance in this pH region of the column became exceedingly low, and the electric field strength correspondingly high. This part of the column thereby became susceptible to disruption by thermal convection even at a very low electric current, while other parts got only a small fraction of the available voltage. A resonable field strength must, however, be available for focusing protein zones in every part of the column. This emphasizes the importance of having an essentially constant conductance throughout the column, and hence the desire of having access to good carrier ampholytes with pI values distributed over the entire pH scale. The peptides left a void between pH 5.0 and 6.5.

During this period of work with oligo-peptides, it thus became increasingly evident that a still more useful system of carrier ampholytes was highly desirable. The ideal system should not in any way interfere with the most common and useful analytical reactions of proteins. Consequently, it should not display any light absorption in the vicinity of 280 nm, where proteins have a light absorption maximum, and it should not include any members with peptide linkages. If these conditions are satisfied, protein analyses can be done without preceding dialysis. Nevertheless, dialyzable carrier ampholytes are preferable for the easy preparation of non-contaminated proteins.

A synthetic procedure for a system of ampholytes satisfying these demands has recently been invented by one of the authors (Vesterberg, to be published shortly). The product obtained by this procedure is a mixture of low-molecular aliphatic poly-amino-poly-carboxylic acids. Like partial protein hydrolyzates, the new system of carrier ampholytes contains a very great number of chemical individuals in unknown proportions. As is evident from previous articles, a great multitude of individual ampholytes is very essential for the success of protein fractionation and analysis by stationary electrolysis: as the number of available carrier ampholytes increases, the pH course obtainable by stationary electrolysis improves. For this reason, the synthetic procedure adopted for production of carrier ampholytes is quite unconventional from the standpoint of a research worker in organic chemistry. Whereas the latter always tries to synthesize pure compounds and has in general no interest in synthetic procedures liable to give complicated mixtures of isomers and homologues, our goal is quite the opposite: the more isomers and homologues we get, the happier we are, because such mixtures display a whole spectrum of pK and, consequently, pI values. They can be expected to give more favourable pH gradients, more shallow pH courses, on stationary electrolysis than mixtures of a few individual ampholytes.

The synthetic procedure adopted rules out the formation of highmolecular compounds; hence the ampholytes can easily be removed from protein fractions by dialysis. Further, they are colourless and transparent to radiation well below the absorption maximum of proteins, 280 nm, since they are altogether saturated, aliphatic compounds. Thus, as a matter of principle, isoelectric spectra of protein mixtures may be obtained simply by analyzing the column in light of the proper wavelength. Finally, the conductance and buffering capacity in the isoelectric state of these new ampholytes are much superior to those of peptides obtainable by partial hydrolysis of proteins. After introduction of the new ampholytes, the conditions for protein separations could be much improved. Extremely shallow pH courses could be obtained, and the field strength distribution between the electrodes became much more favourable.*

In this situation it was felt worth while to reinvestigate the resolving power of the method. A system of myoglobins, which had already been analyzed with peptides as carrier ampholytes, proved to be very suitable for this purpose. From works by Theorell and Åkeson [4-6] and others, it is well known that

* The new system of carrier ampholytes, electrolytically fractionated, will be commercially produced by *LBK-Produkter AB*, Stockholm-Bromma.

horse myoglobin shows microheterogeneity. By ion exchange chromatography and front electrophoresis, they have separated four myoglobins called Mb I, Mb II_1, Mb II_2, and Mb III. Among these, Mb II_1 and Mb II_2 were very similar and could be separated only with difficulty. Any difference in pI could not be detected, but in alkaline media the two proteins showed diverging mobility curves. This was interpreted by Åkeson as depending on the presence in Mb II_2 of a protolytic group with a pK value of about 8.6. This means that there is a charge difference of $\frac{1}{2}$ electronic unit at this pH; at the pI found by Åkeson,[6] 6.58, such a protolytic group would retain a charge difference of only 0.01 electronic unit, which explains that a difference in pI between the two proteins could not be detected by the use of front electrophoresis. In the present report it will be shown that these two very similar proteins can be completely resolved by isoelectric focusing, a result which we attribute not only to the higher resolving power of stationary electrolysis compared to front electrophoresis, but also to the shift in pI of proteins that occurs on removal of salts. The relative importance of these effects will be clarified in the discussion.

EXPERIMENTAL

Apparatus. An electrolysis column of 110 ml capacity with cooling mantle and platinum electrodes was purchased from *Ingenjörsfirman Consulta.** This column is a technically improved variant of the column shown in Fig. 2 in Ref. 1. Cold water from a thermostated bath was circulated through the mantle during electrolysis.

The electric power supply (type No. 3371 B) was purchased from *LKB-Produkter*, Stockholm-Bromma, and could give up to 1200 V and 60 mA.

Extinction measurements were made in a Beckman spectrophotometer, model DK 2, at 23°C.

pH measurements were made with a Radiometer (Copenhagen) pH meter, model 25 SE, and a glass electrode Radiometer type GK 2021 C.

Chemicals. The synthetic ampholytes mentioned in the introduction had isoelectric points distributed between pH 3 and 10. The mixture was subjected to electrolytic fractionation as previously described for the peptides. A fraction isoelectric between pH 6.5 and 7.5 was selected for use in the present investigation.

For preparation of the density gradient in the column, Mallinckrodt's sucrose, analytical reagent grade, was used.

The myoglobin preparations were kindly supplied by Dr. Åkeson at this laboratory. Relatively pure ferrimyoglobin preparations of Mb II_1 and Mb II_2 from horse skeletal muscle were supplied in the form of crystals kept in saturated ammonium sulphate solutions at low temperature. The crystals were centrifuged down and dissolved in a small amount of distilled water. This solution was dialyzed, with several changes of the dialyzate, for 24 h at +4°C against an 0.5 % water solution of the carrier ampholytes of pH 7. Quantitative determination of myoglobin was done spectrophotometrically at a wavelength of 409 nm, using a molar extinction coefficient of 171 000 and a molecular weight of 18 800 (*cf.* Theorell and Åkeson [4]).

Preparation of the column. In order to prevent thermal convection, a density gradient was arranged in the column by the use of one dense and one less dense solution. The dense solution was obtained by dissolving 3/4 of the total amount of carrier ampholytes in 60 ml of a stock solution containing 500 g sucrose per liter. The less dense solution was 60 ml of distilled water containing 1/4 of the carrier ampholytes. The greater concentration of ampholytes in the dense solution was used in order to balance the higher viscosity of this solution, tending to substantially decrease the conductance. The two solutions were transferred to burettes.

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Table 1. A volumetric aid for the preparation of a constant density gradient in a 110 ml column.

Fraction No.	Dense solution		Less dense solution	
	ml	sum ml	ml	sum ml
1	4.6	4.6	0.0	0.0
2	4.4	9.0	0.2	0.2
3	4.2	13.2	0.4	0.6
4	4.0	17.2	0.6	1.2
5	3.8	21.0	0.8	2.0
6	3.6	24.6	1.0	3.0
7	3.4	28.0	1.2	4.2
8	3.2	31.2	1.4	5.6
9	3.0	34.2	1.6	7.2
10	2.8	37.0	1.8	9.0
11	2.6	39.6	2.0	11.0
12	2.4	42.0	2.2	13.2
13	2.2	44.2	2.4	15.6
14	2.0	46.2	2.6	18.2
15	1.8	48.0	2.8	21.0
16	1.6	49.6	3.0	24.0
17	1.4	51.0	3.2	27.2
18	1.2	52.2	3.4	30.6
19	1.0	53.2	3.6	34.2
20	0.8	54.0	3.8	38.0
21	0.6	54.6	4.0	42.0
22	0.4	55.0	4.2	46.2
23	0.2	55.2	4.4	50.6
24	0.0	55.2	4.6	55.2

24 test-tubes of 6 ml capacity were numbered and placed in a rack. They were charged with less dense and dense solutions from the burettes in the proportions given in Table 1, columns 2 and 4. To facilitate this, the successive burette readings are given in columns 3 and 5, containing the cumulative sums of the volumes in columns 2 and 4. During this procedure, the sample solution of dialyzed myoglobin was allowed to replace the less dense solution in a few of the middle fractions. The proper volumes of the sample solution were then taken from a graduated pipette, while the corresponding quantities from the burette were discarded. The liquid fractions 1 and 24 were treated separately in that 0.05 ml of ethylene diamine was added to tube No. 1 and 0.05 ml of concentrated phosphoric acid to tube No. 24. During electrolysis, this base and this acid are pulled to the respective electrodes. The acid at the anode gives a positive net charge to the carrier ampholytes, which are thus repelled from the anode. Similarly, the base at the cathode renders the ampholytes negative there, causing them to keep away from the cathode. By these means, the carrier ampholytes are protected against anodic oxidation and cathodic reduction.

The 24 liquid fractions were then completely homogenized by gently turning the tubes (with a rubber stopper) upside down a couple of times. With cold water (+4°C) running through the mantle of the column, the liquid fractions were then transferred to the latter simply by pouring them, in succession and in their number order, through a funnel and a piece of capillary plastic tubing, in such a way that the liquid ran along the internal wall of the column. During this operation, the central tube of the column was kept closed; it was subsequently filled with dense solution containing only sucrose and ethylene diamine. The pouring of liquid fractions into the column was undertaken without precautions; an extensive mixing of each fraction with the preceding one in the

column is desirable because it gives rise to a smooth density gradient without steps. The gradient is further improved by diffusion during the run.

The procedure described above gives a constant density gradient (a linear density course). An automatic gradient mixer, specially devised to give constant gradients, is under construction. With such a device, the preparation of the column will be much simplified.

Isoelectric focusing by electrolysis. By the procedure described above, the protein sample becomes localized to a diffuse zone at a safe distance from the electrodes, and the initial conductance becomes essentially constant from anode to cathode. This makes it possible to use a rather strong current during the first period of electrolysis without any risk for local thermal convection. This minimizes the time necessary for reaching the steady state, characterized by a stable pH gradient with focused isoelectric protein zones.

When all liquid fractions had been introduced and the central tube had been opened, the meniscus was about 1 cm above the upper platinum electrode. The electric current was switched on with this electrode as the anode. The electrolysis was performed at a maximum load of about 1 watt. In the course of the process, the resistance of the column increases gradually, and the voltage can be increased from time to time. The final steady state is indicated by a constant ohmic resistance, which implies a constant current at a constant voltage. Normally this occurs after 24—28 h, but these experiments were extended to 48 h, with a final electric tension of about 1000 V. At this time, brown zones of myoglobin had separated and focused beautifully at their respective isoelectric points in the column. The latter was photographed, and the positions of the zones were read by a ruler and noted in order to facilitate the localization of the isoelectric zones in the fractions taken after electrolysis.

Fractionation and measurements. One great advantage of this method lies in the fact that the contents of the column can be cut into fractions at any time that is convenient after the steady state has been reached. This is a simple consequence of the focusing character of the method, the final steady state being an equilibrium between electric migration and diffusion (Svensson [7]) for the proteins as well as for the carrier ampholytes. No blurring of the protein zones occurs if the electrolysis is allowed to go on longer than necessary.

The electrolysis experiments were carried out at a temperature of +4°C. In some experiments, however, the temperature was raised to +25°C after the steady state had been reached, and the electrolysis was continued for 2—3 h at this temperature.

When the electrolysis was completed, a series of numbered test-tubes were placed in a rack. The current was broken, and the central tube was closed from the rest of the column. This is an absolutely necessary step in the procedure because otherwise the highly alkaline liquid in this tube would run out and mix with the fractions taken from the column.

For taking fractions, the stop-cock at the bottom of the column was opened very cautiously until a flow speed of about 2 ml/min (about 2 drops in 3 sec) was obtained. With this rate of flow, the column could be emptied in less than one hour, and the use of an automatic fraction collector was found superfluous. Manual fractionation also has the advantage that the size of fractions can be varied according to the distribution of protein zones in the column. To secure a very good resolution in the region of the myoglobin zones, these fractions were chosen as small as 0.6 ml, whereas from other regions of the column 2 ml fractions were taken. The pH of each fraction was measured as accurately as possible with the available instrument, the uncertainty of which can be estimated at ±0.01 pH unit. The measurements, as well as the calibration of the pH meter with standard buffer solution, were carried out both at +4 and at +25°C.

The light absorption of fractions containing myoglobin was recorded between 400 and 700 nm. Myoglobin concentrations were calculated from the extinction value at 409 nm.

A great number of experiments with various, even freshly prepared, myoglobin samples have been carried out during the last two years, using peptides as well as the new system of carrier ampholytes for the creation of pH gradients. However, only experiments having a bearing upon the resolv-

ing power of the method will be reported in this article. A fuller account of the electrochemistry of myoglobin fractions will be given in another connection.

Experiment 1 was carried out with a mixture of Åkeson's preparations "Mb II_1" and "Mb II_2", about 3.5 mg of each. These preparations were not 100 % pure Mb II_1 and Mb II_2, respectively, but contained them as main components with various other myoglobin fractions as impurities. Experiments not reported in this article have shown Mb II_2 to be slightly more electronegative than Mb II_1, in agreement with Åkeson's [6] mobility measurements. The said quantity of myoglobins was dialyzed against a solution of carrier ampholytes and introduced into the column as described in the section "Preparation of the column". The carrier ampholyte concentration was 0.3 %. After focusing of the zones at a temperature of +4°C, a voltage of 900 V and a current of 0.3 mA, the zones were photographed, the current still flowing, in ordinary light against a white background. This photograph is shown in Fig. 1. From the negative, a diapositive was also prepared, and a densitometric record thereof (obtained with a Joyce-Loebl microdensitometer) is shown in Fig. 3a. After photographing, the current was broken, and the contents of the column were cut into fractions as already described, but with special precautions against possible absorption of carbon dioxide from the atmosphere or from the experimentator's breath. To that end, a slow stream of argon

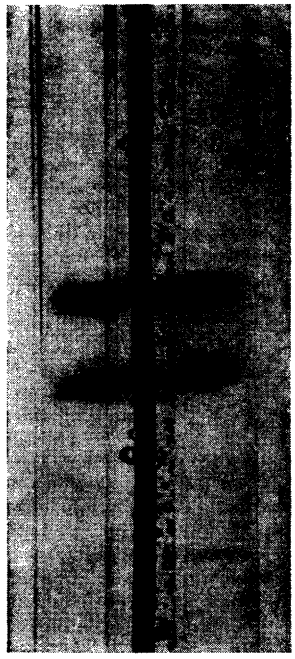

Fig. 1. Isoelectric spectrum from Experiment 1. pH increases downwards in the column. The two zones represent Mb II_2 (above) and Mb II_1 (below).

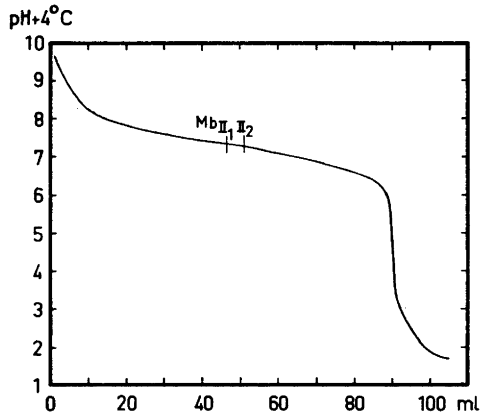

Fig. 2. pH course recorded from Experiment 1. The cathode to the left, the anode to the right. The shallowness of the curve around pH 6.9 made a clear-cut separation of the two proteins possible.

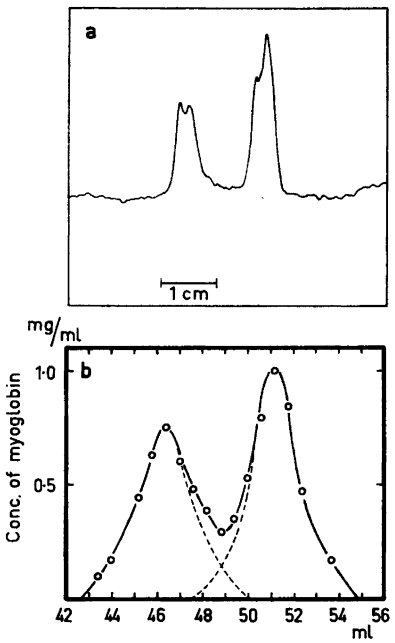

Fig. 3. a) Densitometric record of a diapositive of Fig. 1. b) Protein concentration, as measured spectrophotometrically on fractions from Experiment 1, as a function of the volume of the effluent.

was directed into the test-tubes, hitting the drops just as they emerged from the column. The pH of all fractions were measured at +4.0 and at +25.0°C. Their conductances were measured at +4.0°C. Finally, they were analyzed for myoglobin content spectrophotometrically at room temperature. The pH course for the temperature prevailing during the experiment is shown in Fig. 2. The concentration of myoglobin in the fractions as a function of the effluent volume is shown in Fig. 3b.

Experiment 2 was conducted with the same material as in Experiment 1, the fractions from that experiment being retransferred to the column in the order of decreasing density. The zones were refocused under the same conditions as in Experiment 1, and fractions were taken in the same manner, yet without protection by argon against carbon dioxide absorption. The pH of the fractions were measured at +4.0 and at +25.0°C, and their myoglobin contents were determined spectrophotometrically. The pI values of the components were, as usual, identified with the pH of the fraction having a maximum concentration of the component in question. Conductance measurements were not carried out on these fractions.

Experiments 3a and 3b were carried out with 7 mg of the same mixture of the preparations "Mb II_1" and "Mb II_2" as that used in the previous experiments. The zone focusing temperature was also the same, +4°C, but the carrier ampholyte concentration was 1 %.

Experiments 4a and 4b were performed as Experiments 3a and 3b, with the only difference that the final focusing of the zones was done at +25°C.

The pI measurements of Mb II_1 and Mb II_2 that resulted from these experiments have been collected in Table 2.

Table 2. The isoelectric points of the myoglobins II_1 and II_2.

Experiment No.	Myoglobin component	Zone focusing temperature °C	pI measured at the temperature		Carrier ampholyte concentration, %
			+4.0°C	+25.0°C	
1	Mb II_1	+ 4	7.34	6.94	0.3
	Mb II_2		7.28	6.88	
2	Mb II_1	+ 4	7.35	6.94	0.3
	Mb II_2		7.29	6.89	
3 a	Mb II_1	+ 4	7.32	6.95	1.0
	Mb II_2		7.27	6.90	
3 b	Mb II_1	+ 4	7.33	6.98	1.0
	Mb II_2		7.27	6.92	
4 a	Mb II_1	+25	7.35	6.98	1.0
	Mb II_2		7.28	6.92	
4 b	Mb II_1	+25	7.34	6.97	1.0
	Mb II_2		7.28	6.92	

DISCUSSION

General aspects. A closer inspection of the data given in Table 2 gives reason for the following comments.

In the first place, a comparison between Experiments 1 and 2, which differ only with respect to the possible interference of carbon dioxide, reveals that such an interference is not a source of error in the present investigation. This result can of course be extended to protein systems more acidic than myoglobins, but not to less acidic ones. As a matter of fact, the suspicion of carbon dioxide interference arose in experiments with cytochrome *c*, being isoelectric in the pH region 10—11. Whereas it is quite certain that isoelectric protein fractions emerging from the column are completely carbonate-free, the possible error due to carbon dioxide interference must be assumed to increase with the pH of the fractions and to decrease as their buffering capacity increases. The experiments 1 and 2 show that an interference of carbon dioxide need not be feared for neutral and acidic proteins, even if the buffering capacity of the fractions is very low (the carrier ampholyte concentration in these experiments having been only 0.3 %). An interference by carbon dioxide in the experiments 3 and 4 can therefore also be excluded.

In the second place, a comparison between Experiments 1 and 2 on the one hand and Experiments 3a and 3b on the other reflects the possible dependence of pI of the proteins on the carrier ampholyte concentration. According to Table 2, the pI for Mb II_1 is, at the zone focusing temperature, 7.34_5 for the lower and 7.32_5 for the higher carrier ampholyte concentration. For Mb II_2 the corresponding figures are 7.28_5 and 7.27, respectively. Whereas we have estimated the reproducibility of our pH meter at ± 0.01 pH unit,

pertaining to repeated measurements on one and the same solution, we must admit that the uncertainty in pI measurements, involving pH measurements on a series of fractions from different experiments, is greater than this and may amount to ± 0.02 pH units. Our conclusion is consequently that the pI of a protein, as determined by this method, is independent of the carrier ampholyte concentration.

In the third place, we compare the results of Experiments 3a and 3b on the one hand and Experiments 4a and 4b on the other, the only difference being the zone focusing temperature. The table reveals that the true isoelectric points of Mb II_1 and Mb II_2 at $+4$°C are 7.32_5 and 7.27, respectively (pI measurements being performed at the temperature of zone focusing), whereas the true isoelectric points at $+25$°C are found to be 6.97_5 for Mb II_1 and 6.92 for Mb II_2 (these pI measurements pertaining to the zone focusing temperature of $+25$°C). It is of prime interest, however, to compare the values obtained for the isoelectric points by pH measurements at a "wrong" temperature (compared to the zone focusing temperature). The relationships thus found are shown in Table 3. With due regard to the possible uncertainty in absolute pI measurements of ± 0.02 pH units, this table shows that the pI value obtained refers to the temperature of the pH measurements, irrespective of the zone focusing temperature. We regard this as a practically important result because an isoelectric point pertaining to $+25$°C is of special interest for the reasons that most pK values in the literature are recorded at this temperature and that pH 7, in water solutions, represents neutral reaction only at this temperature. The thermal instability of proteins in many cases requires that experiments be performed at a much lower temperature. Our results collected in Table 3 indicate that isoelectric points pertaining to $+25$°C are obtainable simply by making the pH measurements at this temperature, irrespective of the zone focusing temperature.

We are, however, for the present unable to present rigorous physicochemical evidence for the general validity of this conclusion. It is well known that different protolytic groups in proteins display widely different degrees of temperature dependence in their dissociation constants, and consequently the relative acidity of a protein should be expected to depend on the temperature. The experimental fact that Table 3 indicates an independence of the pI

Table 3. pI values as dependent on zone focusing temperature and on the temperature for pH measurements.

Protein	Zone focusing temperature °C	pI measured at the temperature	
		+4.0°C	+25.0°C
Mb II_1	+ 4.0	7.32_5	6.96_5
	+25.0	7.34_5	6.97_5
Mb II_2	+ 4.0	7.27	6.91
	+25.0	7.28	6.92

of a protein on the zone focusing temperature may possibly be explained by the fact that the acid-base balance of the protein as well as that of the carrier ampholytes is largely dependent on the same types of protolytic groups — amino groups and carboxyl groups. If this is true, the conclusions that seem evident from Table 3 could be regarded as a general approximate, but not as a general absolute, rule.

As pointed out in part I of this series (Svensson [7], pp. 338—339), pI measurements by other methods require rather large amounts of protein, are laborious, and not very accurate. For these reasons, pI values of proteins have not come into general use for characterization. For identification purposes, the great majority of workers have been content with a mobility value at specified pH, salt medium, and temperature. The possibility of direct and accurate pI measurements on milligramme quantities of proteins that is offered by the present method can therefore be expected to become of great importance. Since the method is at the same time a powerful purification tool, the results are independent of impurities in the protein preparation.

Table 2 gives, as over-all averages for the 6 reported experiments, a pI value of 7.34 for Mb II_1 and 7.28 for Mb II_2, both pertaining to +4.0°C. Åkeson [6] found for both components a pI = 6.58 by moving boundary experiments at an ionic strength of 0.1 and at 0.0°C. (Åkeson's pH measurements were also performed at 0.0°C.) Since the temperature correction between 0 and +4°C is not more than a few hundredths of a pH unit, a great discrepancy between Åkeson's and our pI values remains. A similar great discrepancy in the same direction was reported by Svensson [1] for hemoglobins when pI values obtained by moving boundaries and steady state electrolysis were compared. This phenomenon seems to be quite universal: every protein shows an appreciably higher pI value in an electrolysis than in a moving boundary apparatus. The electrolytic procedure thus increases the positive charge of a protein, which also can be described as a deacidification. This is in harmony with the generally accepted view, corroborated by many investigations, that proteins form complexes with anions in the presence of salts. Whereas salts are necessarily present in moving boundary experiments, they are effectively removed by electrolysis.

The question then arises whether there is a complex formation between proteins and carrier ampholytes. Although such a suspicion cannot be excluded, there is at least no direct indication in its favour in Table 2. Electrolytically determined pI values of proteins seem to be independent of the type and concentration of carrier ampholytes. This is reasonable even if it be admitted that proteins do form complexes with carrier ampholytes. When the proteins have been focused to their isoelectric points, they are in a medium of carrier ampholytes which are also essentially isoelectric. A possible complex formation thus cannot be expected to show up as a shift in the isoelectric point.

It follows from the above that pI values derived by stationary electrolysis pertain to the pure, non-complexed protein, whereas a pI obtained from moving boundary experiments varies with the salt concentration and even with the kind of ions present. This is of course another strong argument in favour of the electrolytic method. An electrolytically derived pI of a protein expresses a fundamental and characteristic property and can be regarded as

a true measure of the intrinsic acidity of the pure protein. It shares this property with *the isoionic point* which is, by definition, the pH of a solution which does not change its pH on addition of a small quantity of the pure protein. This definition is directly applicable to an electrolysis column with a focused protein zone. If more pure protein is added to the zone, its pH remains unchanged, but if the pure protein is added to any point outside the zone, the local pH will change since the protein will either bind or release protons from or to the medium. Consequently, the pI of a protein obtainable by steady state electrolysis also represents its isoionic point, which is identical with the isoelectric point in the absence of complex-forming ions. The isoionic point can otherwise only be measured by acidimetric titration on fairly large amounts of pure proteins.

Since a density gradient is necessary in the present technique, a dependence of pI on the sucrose concentration can of course also be suspected. Although the myoglobin zones have been focused at different levels in different experiments, we have not found any systematic variation of pI with this level. The matter is, however, subject to further investigation using greater level differences, that is, greater differences in sucrose concentration. For theoretical reasons, a slight dependence can be anticipated.

Resolving power. The experiments reported above, and others of the same kind, reveal without doubt that the proteins Mb II_1 and Mb II_2, having a pI difference of only 0.06 units, can be nicely separated and analyzed by steady state electrolysis. This is indeed a very stimulating result of great promise to protein chemistry in general. The high resolving power, which is of course due to the focusing character of the method, makes it superior in this respect to conventional electrophoretic methods, working at a constant pH.

Svensson [7] has derived the following expression for the distance between the top and one inflexion point (the standard deviation) in an isoelectrically focused protein zone with a Gaussian concentration distribution:

$$x_i = \pm \sqrt{\frac{-D}{E(\mathrm{d}u/\mathrm{dpH})\ (\mathrm{dpH}/\mathrm{d}x)}} \qquad (1)$$

where E is the field strength, D the diffusion coefficient, $\mathrm{d}u/\mathrm{dpH}$ the (negative) slope of the pH-mobility curve at the isoelectric point, and $\mathrm{dpH}/\mathrm{d}x$ the value of the pH gradient in the direction of the current. It is of some interest to compare this theoretical result with a zone breath measurable in Fig. 3.

For the diffusion coefficient of myoglobin at 20°C, the value 1.13×10^{-6} cm²/sec has been reported (Svedberg and Pedersen [8]). At +4°C it is much lower, and an approximate calculation by the use of Stokes-Einstein's relation:

$$\frac{D_4 \eta_4}{277} = \frac{D_{20} \eta_{20}}{293} \qquad (2)$$

where η denotes the viscosity of the solvent, gives the value 6.85×10^{-7} cm²/sec for +4°C. A further correction for the great viscosity increase due to the presence of sucrose affects u in the denominator as much as D in the numerator and can thus be omitted for both. The pH difference between the zones was 0.05_5, and the linear separation between the peaks was 1.40 cm (as measured in Fig. 2a), hence the value of the pH gradient becomes 0.055/1.40 = 0.039 pH units per cm.

The slope of the mobility curve can be taken from Fig. 1 in Åkeson's paper,[6] which gives a value of -1.3×10^{-5} cm²/V sec at 0°C and ionic strength 0.1. A viscosity correc-

tion up to +4°C increases this value to 1.49×10^{-5}, but a further correction due to the ionic strength effect is required. This is difficult because no data are available concerning the ionic strength dependence of the mobility of myoglobin. The importance of the ionic strength effect is, however, evident from an old investigation by Tiselius and Svensson,[9] from which it may be deduced that the mobility slope at the isoelectric point increases by 55 % when the ionic strength is decreased from 0.1 to 0.01 for egg albumin. The ionic strength in our experiments is unknown, but, to judge from our conductance data, it is still smaller than 0.01. On the other hand, myoglobin molecules are smaller than egg albumin molecules, and the ionic strength effect on mobility can therefore be expected to be less pronounced. Lacking exact information on this point, we accept a 55 % correction for the mobility slope as a reasonable correction from Åkeson's experimental conditions to our own. This gives a numerical value of $du/dpH = -2.31 \times 10^{-5}$ cm²/V sec for myoglobin at our experimental conditions.

The electric field strength is calculated by the formula:

$$E = i/q\varkappa \tag{3}$$

where i is the electric current, q the cross-sectional area of the column, and $\varkappa$ the conductance. The final current was 0.29 mA, and q was 3.75 cm². Concerning the conductance, we ran into great difficulties, since it turned out that the conductances of the fractions were far too high to be compatible with the electric current, the cross-sectional area, and the total voltage. This must be explained by the rapid diffusion of the carrier ampholytes as soon as the current is broken prior to the fractionation. Whereas the carrier ampholytes, just as the proteins, are concentrated into isoelectric zones while the current is still flowing, thereby acquiring their minimum conductances (*cf.* Svensson [2]), they start diffusing into one another as soon as the current is broken. A less acidic carrier ampholyte then diffuses into the region of a more acidic one, and *vice versa*, and the ionization and the conductance of both ampholytes thereby increases. This is the reason why we could not use the conductances of the fractions.

Instead, a catheter was submerged from the top of the column down to the Mb II_2 zone while the current was still flowing, and, immediately after it was broken, two 1.5 ml fractions were taken, the first from the upper part, the second from the central part of the zone. The conductances of these fractions were measured at +4.0°C and were found to be 3.76 and 2.87×10^{-6} ohm^{-1} cm^{-1}, respectively. The conductances of corresponding fractions taken in a parallel experiment by draining the whole column were 3—4 times as high. The conductances of the catheter fractions correspond to field strengths of 27.0 and 20.6 V/cm, respectively. Although a variable conductance within a protein zone necessarily involves a non-Gaussian concentration distribution and thus a limited quantitative validity of eqn. (1), we accept the mean value of 23.8 V/cm for the field strength within the zone in order to arrive at a correct order of magnitude of the theoretical zone breadth.

If the numerical values deduced above are introduced into eqn. (1), a theoretical zone breadth of $2x_i = 0.36$ cm is obtained. This is to be compared with actually measured zone breadths in Fig. 3. Although the curves are not Gaussian in shape, we have measured their breadths at theoretical ordinates of $e^{-\frac{1}{2}} = 0.61$ of the peak heights. This gives a zone breadth of 0.37 cm for both curves, in excellent agreement with the calculated value.

We have also measured the zone breadths in Fig. 3b, that is, after fractionation. The result was 0.48 cm for Mb II_1 and 0.59 cm for Mb II_2. Some zone blurring is of course unavoidable in a fractionation procedure, but it is remarkable that the blurring can be kept as slight as these figures indicate. The great stabilizing effect of the density gradient is certainly operative in the fractionation as well as during electrolysis.

According to eqn. (1), the zone thickness should be independent of the amount of protein contained in the zone. One finds experimentally, however, that the zone thickness tends to increase with the amount of protein in the

zone. This discrepancy between theory and experiment is, however, only apparent. It was clearly pointed out in Ref. 7 that eqn. (1) is strictly valid only if the field strength and the pH gradient can be regarded as constant within the zone, and if the protein concentration is too small to substantially alter these variables. With increasing amounts of protein, its buffering capacity is no longer negligible in comparison with that of the carrier ampholytes. The protein itself will then influence the pH course in the steady state. It is altogether possible for proteins to act as carrier ampholytes in separations of still larger particles such as viruses, cell fragments, and cells. There is consequently no fundamental difference in the behaviour of proteins and carrier ampholytes. Both classes of ampholytes are focused by steady state electrolysis, and good carrier ampholytes are better focused than poor ones. The appreciable variation in conductance that we found experimentally within the Mb II_2 zone indicates a focusing of the carrier ampholytes that is comparable to that of the proteins.

The complete separation of the proteins Mb II_1 and Mb II_2 demonstrates that the resolving power of the method is better than 0.06 pH units. One can ask where the ultimate limit lies, and, having access to a theoretical equation for the zone breadth, whether a theoretical expression for the resolving power is conceivable. This necessitates, however, a mathematically strict definition of what is really implied by a resolving power.

For simplicity, let us consider two identical Gaussian curves, representing the concentration distributions of equal amounts of two very similar proteins, at a distance of Δx from each other. Mathematical analysis then shows that the sum of these two curves has only one maximum if Δx is smaller than $2x_i$. This distance is thus insufficient for plainly visible separation of the proteins. For Δx values greater than $2x_i$, the summation curve displays two maxima and, between them, one minimum, and if this minimum is detectable by the eye or by optical means, one can speak of a detectable partial separation. As the value of Δx increases, the depth of said minimum increases too, at first very slowly, then more rapidly. For $\Delta x = 3.07\ x_i$, the minimum has an ordinate of $e^{-\frac{1}{2}} = 0.61$ of the two maxima, that is, at this separation the minimum has the same relation to the maxima as the ordinate at the inflexion points has to the peak height in a single Gaussian curve. A concentration minimum of 61 % of two surrounding maxima is measurable with a fair precision by an optical instrument and is well visible to the naked eye in the case of coloured proteins. Although the two zones are still contaminated if taken out as fractions, the separation will allow a certain accuracy in an analytical concentration analysis by optical means in the cell. One can thus say that a separation of $\Delta x = 3.07\ x_i$ represents an acceptable analytical separation, and this criterion will therefore be used in order to define the resolving power.

The pH difference across a distance of $3.07\ x_i$ will consequently be calculated. If a constant pH gradient is postulated, it becomes:

$$\Delta\mathrm{pH} = \frac{\mathrm{dpH}}{\mathrm{d}x}\Delta x = \frac{\mathrm{dpH}}{\mathrm{d}x} 3.07\ x_i = 3.07\sqrt{\frac{D(\mathrm{dpH}/\mathrm{d}x)}{-E(\mathrm{d}u/\mathrm{dpH})}} \qquad (4)$$

By inserting the numerical figures derived earlier for myoglobin and for our experimental conditions, one gets a resolving power of 0.02 pH units. This is 10 times better than what was proved by the early experiments with hemoglobins reported by Svensson.[1]

As is evident from eqn. (4), the resolving power can be influenced by the experimentator only by way of the pH gradient and the field strength since D and du/dpH are fixed by the chemical nature of the proteins. The pH gradient in our experiments was very small indeed, but there is no theoretical limit for the shallowness of the pH course obtainable by electrolysis. The field strength can also be increased by suitable modification in the design of apparatus. A reduction of the cross-sectional area (present value 3.75 cm^2, to be compared with 0.75 cm^2 in a conventional moving boundary apparatus) will make a more efficient cooling possible, and thereby an increased field strength. It should finally be pointed out that myoglobins belong to the smallest class of all proteins and consequently diffuse comparatively rapidly. For larger proteins, with smaller D values, the resolving power is still better. (Due to the square root sign in eqn. (4), and due to the third-root dependence of D on molecular size, the resolving power varies, however, only with the sixth root of molecular size. Consequently, viruses with an average molecular weight of 20×10^6 can be expected to be isoelectrically resolvable only about three times more effectively than myoglobins if the mobility slopes are equal.) To sum up, it seems possible to reach, but technically difficult to surpass, a resolving power of 0.01 pH unit. Thus, if the pI difference between two proteins is great enough to be picked up by a good pH meter, the proteins can be resolved by steady state electrolysis.

The limitation of this method certainly does not lie in its resolving power; it has to be sought in other factors. One such factor is concerned with solubility. Isoelectrically insoluble proteins are not so easily handled by the method for the present, but there are certain possibilities of adapting apparatus construction and experimental procedure to the requirements of such proteins. On the other hand, proteins that undergo irreversible denaturation on removal of their natural salt medium can never be treated by steady state electrolysis. Thus every attempt to fractionate serum lipoproteins by this method is *a priori* deemed to failure.

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