[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY,[1] PHILADELPHIA, PENNSYLVANIA]

Separation of α- and β-Casein

BY ROBERT C. WARNER

Casein was long considered to be one of the best examples of a pure, homogeneous protein, largely as a result of the contentions of Hammersten.[1a] Although there was some evidence to the contrary,[2] it was not until the solubility studies of Linderstrøm-Lang and Kodama[3] were published that casein was definitely shown to be a mixture of proteins. Since that time many attempts have been made to fractionate casein into its components. A number of workers have obtained fractions which differed from the original casein in solubility, reaction with rennet, or in some analytical respect. However, it has been characteristic of most of these results that those fractions which differed markedly from the original represented only a small percentage of the total protein and that entirely different fractions were obtained by the different procedures employed by various authors. While these results adequately confirm the idea that casein is a mixture, they do not offer any acceptable method of separation of the components.

The electrophoretic examination of proteins by the method of Tiselius[4] offers a more adequate criterion of homogeneity than the analytical data that previously were often relied on, although electrophoretic evidence alone cannot be considered as the final authority in establishing the homogeneity of a protein. This method also gives information on the number of components and their relative concentration in the fractions obtained from mixtures of proteins more readily and accurately than any other means. Casein has been examined electrophoretically by Mellander.[5] He reported the presence of three peaks, which he designated α, β and γ casein in the order of decreasing mobility in a phosphate buffer.

We have applied electrophoretic analysis to an investigation of the chemical fractionation of casein. It has been our aim in this work to employ methods of separation which would be as mild as possible and to obtain fractions which would be homogeneous or, if not, would show a definite relation to the electrophoretic pattern of casein.

Experimental

Preparation of Casein.—Unpasteurized cow's milk was collected at the time of milking, toluene was added, and the milk was immediately chilled to 2°. All subsequent operations were carried out in a refrigerated room at this temperature. The milk was skimmed and casein was prepared by precipitating with 0.1 *N* hydrochloric acid at *p*H 4.6. The acidified milk was diluted with ice water and the precipitate of casein was washed about six times by decantation. It was then dissolved using enough sodium hydroxide to attain a *p*H of 6.5. This solution was extracted twice with ether. It was then diluted to a protein concentration of 0.5% and precipitated with 0.01 *N* hydrochloric acid. The casein was thoroughly washed with water and dried with alcohol and ether. It was then spread on a glass plate at room temperature for twenty-four hours to remove the ether and to attain approximate moisture equilibrium.

For the fractionation experiments the drying with alcohol and ether was omitted, and the wet reprecipitated casein was used.

Methods.—Analytical data on two preparations of casein and on the casein fractions are given in Table I. The moisture and Kjeldahl nitrogen determinations were carried out according to the recommendations of Chibnall.[6] Phosphorus was determined by the Fiske and Subbarow[7] procedure after digestion with sulfuric and nitric acids. Ash was determined by heating in a muffle furnace to 700° after sufficient calcium acetate solution was added to bind all the phosphorus and provide a small excess. Blank values on the calcium acetate were subtracted from the casein ash. The theoretical ash calculated as phosphorus pentoxide[8] from the phosphorus determination is also given in the table. The difference between this and the actual ash may be taken as the ash arising from contamination with inorganic material; this value has been used in calculating the nitrogen and phosphorus on an ash-free basis.[9]

TABLE I

ANALYTICAL DATA ON CASEIN PREPARATIONS

Prepn.	% N[a]	% P[a]	% Ash[a,b]	Theoretical ash %[c]	True ash %[d]	% N (cor.)	% P (cor.)
Casein 1	15.62	0.848	2.30	1.94	0.36	15.68	0.85
Casein 2	15.62	.870	2.37	1.99	.38	15.68	.87
α-Casein	15.56	.982	2.64	2.25	.39	15.62	.99
α-Casein	15.44	.994	2.87	2.27	.60	15.53	1.00
β-Casein	15.39	.602	2.28	1.38	.90	15.53	0.61

[a] On a moisture-free basis. [b] Determined after adding calcium acetate and corrected for calcium oxide. [c] Calculated as P_2O_5 from % P. [d] Ash by difference, attributed to inorganic contamination.

The electrophoretic experiments were carried out in a Tiselius apparatus equipped with the schlieren scanning device of Longsworth.[10] The water-bath was regulated at 0.5°. A tall-form electrophoresis cell was used.[11]

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(1a) Hammersten, *Z. physiol. Chem.*, **7**, 227 (1883); **9**, 273 (1885).

(2) Mann, "Chemistry of the Proteids," The Macmillan Co., New York, N. Y., 1906, p. 403, and discussion in ref. 15.

(3) Linderstrøm-Lang and Kodama, *Compt. rend. trav. lab. Carlsberg*, **16**, No. 1 (1925).

(4) Tiselius, *Trans. Faraday Soc.*, **33**, 529 (1937).

(5) Mellander, *Biochem. Z.*, **300**, 240 (1939).

(6) Chibnall, Rees and Williams, *Biochem. J.*, **37**, 354 (1943).

(7) Fiske and Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

(8) The phosphorus appears as $Ca_2P_2O_7$ after the ashing. In the blank ash the calcium appears as CaO so that after subtraction of the blank the phosphorus is estimated as P_2O_5.

(9) The ash value reported for casein by Chibnall was determined without any calcium acetate. Under these conditions much of the phosphorus is lost, but usually not all of it. Chibnall's value is therefore probably too high. Assuming that no ash due to foreign inorganic material was present, his nitrogen value has been recalculated as 15.60%.

(10) Longsworth, THIS JOURNAL, **61**, 529 (1939). Apparatus made by Klett Mfg. Co., New York, N. Y.

(11) Longsworth, Cannan and MacInnes, *ibid.*, **62**, 2580 (1940).

Mobilities, reduced to 0°, were calculated for the descending boundaries from measurements made directly on the plates with a precision comparator using the methods devised by Longsworth[12] and employing conductivities determined at 0°. The areas of the peaks were determined with a planimeter from enlarged tracings of the patterns.

Fractionation of Casein.—The electrophoretic pattern obtained with casein in a phosphate buffer at *p*H 6.98 is shown in Fig. 1 (a). A fast- and a slow-moving boundary are present which, following Mellander, will be referred to as α- and β-casein, respectively. The mobilities found in this experiment are given in Table III. The relative amounts of α- and β-casein as determined from the areas on the descending pattern are 80.7 and 19.3%, respectively.

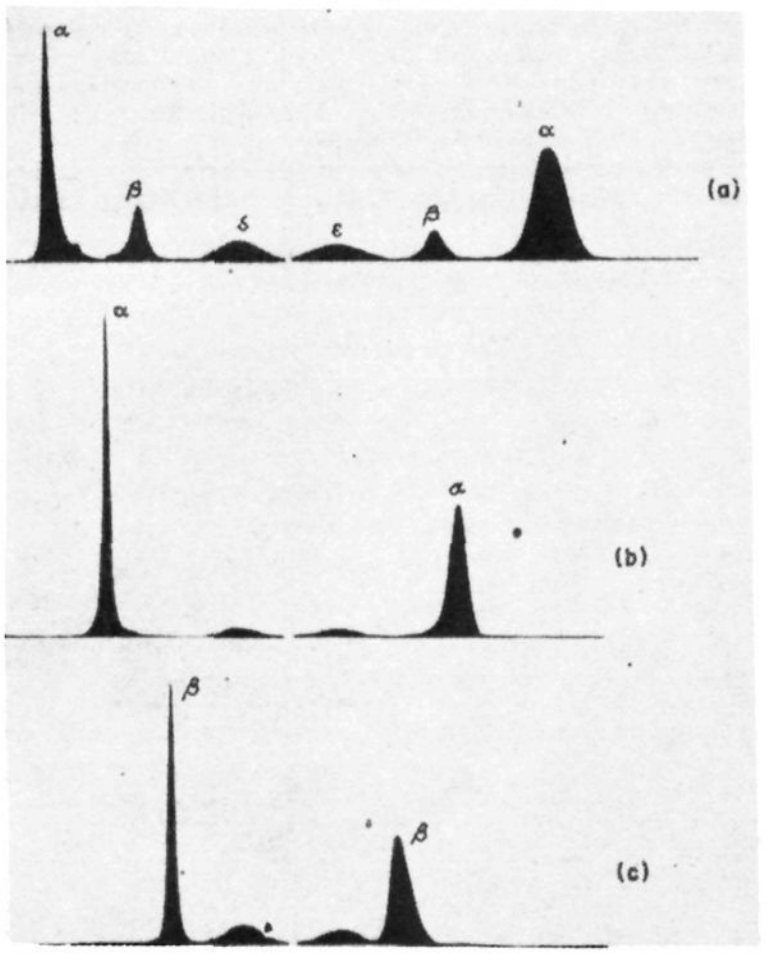

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Fig. 1.—Electrophoretic patterns obtained in phosphate buffer at a *p*H of 6.98, with an ionic strength of 0.1, and containing 0.05 *N* sodium chloride: (a) casein after electrophoresis for 9900 sec. at a field strength of 4.41 volts/cm.; (b) α-casein after 6000 sec. at 4.79 volts/cm.; (c) β-casein after 7800 sec. at 4.84 volts/cm.

A number of methods for fractionating casein were tried, and the fractions were examined electrophoretically in a phosphate or veronal buffer for comparison with the pattern of the original casein. In all, about 180 electrophoretic experiments were carried out. No evidence of fractionation was obtained when the following methods were used: (1) partial salting out from solutions in the *p*H range of 6 to 9; (2) precipitation in the presence of salt followed by the use of acetone, as suggested by the work of Cherbuliez and Meyer[13]; (3) partial precipitation at a *p*H slightly alkaline to the isoelectric point, either in the presence or absence of salt; (4) precipitation with oxalate at a *p*H alkaline to the isoelectric point. The method of Groh, *et al.*,[14] of precipitation from urea solution with alcohol produced some fractionation. The fraction called "γ2" by these authors contained more β-casein than the original but consisted chiefly of α-casein. Fraction "γ1" contained little β-casein but showed material not found in the electrophoretic pattern of casein.

Two other methods resulted in a partial separation of α- and β-casein. (1) Calcium caseinate was precipitated from solutions containing 2 *N* sodium chloride at a *p*H between 7 and 8 by adding calcium chloride. The least soluble calcium salt fraction contained less β-casein than the original. (2) Linderstrøm-Lang[15] found that the solubility of casein in the presence of chloride ion on the acid side of the isoelectric point is dependent on the amount of casein remaining undissolved. He showed that the precipitate had a higher P/N ratio than the filtrate and that the two fractions had different solubility characteristics. Experiments were carried out in this manner in the *p*H range 3.0 to 3.8. At the higher *p*H, the filtrate fraction contained more β-casein than the precipitate, in confirmation of Linderstrøm-Lang's data on the P/N ratio. However, the degree of fractionation attained in a single precipitation by either of these methods was too small to give any hope of obtaining purified fractions in a reasonable number of precipitations.

Analysis of the electrophoretic patterns obtained at various *p*H values on the alkaline side of the isoelectric point indicated that β-casein has a higher isoelectric point than α-casein. On the basis of this observation experiments were carried out to fractionate casein by partial precipitation on approaching the isoelectric point from the acid side. It was found that if such a precipitation was conducted at 2°, the protein in the filtrate consisted of 80 to 90% β-casein and 10 to 20% α-casein. A typical electrophoretic pattern of this crude β-casein fraction is given in Fig. 2 (e). Some β-casein remained in the precipitate, but on repeating the procedure a number of times on this fraction the β-casein could be removed completely, and a purified α-casein fraction obtained.

Preparation of α-Casein.—The original wet casein precipitate prepared as described is dissolved using sufficient sodium hydroxide (about 0.65 meq./g.) to give a 1% solution with a *p*H of 6.5. The solution is stirred vigorously with a motor stirrer, and sufficient 0.05 *N* hydrochloric acid (0.85 meq./g.) to bring the solution to *p*H 3.5 is added as rapidly as possible. The precipitate which forms on adding the hydrochloric acid dissolves readily yielding a slightly opalescent solution.[16]

This solution is chilled to 2° and diluted to a concentration of 0.2 to 0.3% protein. It is stirred by motor and cold 0.01 *N* sodium hydroxide is added dropwise. The solution becomes very cloudy and a precipitate appears at a *p*H of about 4.2. The sodium hydroxide addition is continued until a clear supernatant is obtained when a small sample of the mixture is centrifuged. This will occur at a *p*H between 4.4 and 4.5 on the first precipitation and at a slightly lower *p*H on subsequent precipitations. The precipitate is then dissolved with the use of sodium hydroxide and the above procedure is repeated. Usually six precipitations are required to remove the β-casein completely. After the last precipitation the α-casein is dissolved with sodium hydroxide and precipitated from a 0.2% solution at *p*H 4.5 and 2° by adding hydrochloric acid. The precipitate is thoroughly washed with water and dried with alcohol and ether.

An electrophoretic pattern of the purified α-casein is shown in Fig. 1 (b). A summary is given in Table II of an experiment in which nine precipitations were carried out. The amount of protein in the precipitate and filtrate

(12) Longsworth, THIS JOURNAL, **5**, 1755 (1943).

(13) Cherbuliez and Meyer, *Helv. Chim. Acta*, **16**, 600 (1933); Cherbuliez and Schneider, *ibid.*, **15**, 597 (1932).

(14) Groh, Kardos, Denes and Serenyi, *Z. physiol. Chem.*, **226**, 32 (1934).

(15) Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg*, **17**, No. 9 (1929).

(16) This method of preparing an acid solution of casein is similar to that used by Linderstrøm-Lang. Because of the extremely low rate of solution it is not feasible to dissolve isoelectric casein by the use of hydrochloric acid alone

fractions is shown in each case. The percentage of β-casein was calculated from the descending pattern after electrophoresis in a veronal buffer. After the fifth precipitation the amount of β-casein remaining in the precipitate was too small to detect in the electrophoretic pattern of this fraction. A small additional amount of β-casein was removed in the sixth precipitation, although the analysis of the filtrate shows that it consisted predominantly of α-casein. Insufficient material was obtained to analyze the subsequent filtrates electrophoretically, but the amount of protein becomes negligibly small in the last two filtrates and from the trend of the results on the first six, they were probably free of β-casein.

TABLE II

PREPARATION OF α-CASEIN

Precipitation no.	Protein in precipitate,[a] g.	Protein in filtrate, g.	β-Casein[b] in filtrate, % of total
0	74		
1	63	10.0	87.2
2	54	8.6	87.1
3[c]	46.2	3.9	82.0
4	42.2	2.8	58.9
5	40.2	0.9	37.5
6	38.0	.8	22.8
7	37.1	.8	
8	36.2	.2	
9	35.4	.2	

[a] All precipitations were made from a volume of 14 l. except the first, in which the volume was 26 l. [b] As pointed out later in this paper, this calculation gives only an approximate measure of the β-casein content, particularly in the presence of a large amount of α-casein. [c] An accidental loss of a few grams of protein from the precipitate fraction occurred after the second precipitation.

Preparation of β-Casein.—On warming the filtrates from the α-casein precipitations to room temperature and adjusting the *p*H to 4.9, the residual protein precipitates. The pattern in Fig. 2 (e) shows that this fraction contains in addition to α- and β-casein, some material with a lower mobility than β-casein. This may be a casein component which is present in too low a concentration to appear in the pattern of unfractionated casein or it may be a hydrolysis product of casein resulting from the fractionation procedure. In purifying the β-casein it is necessary to rid the fraction of this component and of the remaining α-casein. This can easily be accomplished since the β-casein is soluble at 2° and *p*H 4.9 in the absence of salt to the extent of 0.2 to 0.3 g./l. whereas α-casein has no appreciable solubility and the slow moving contaminant a much greater solubility under these conditions.

The crude β-casein residues from the first three or four filtrates are therefore dissolved with sodium hydroxide to give a 0.2% solution having a *p*H of 6. The solution is chilled to 2°, and cold 0.01 *N* hydrochloric acid is added dropwise while the solution is stirred. When a *p*H of 4.9 is reached, the precipitate is filtered off and dissolved with sodium hydroxide to give a 0.03% solution. This solution is brought to *p*H 4.5 at 2° with hydrochloric acid and the precipitate obtained (chiefly α-casein) is removed. The filtrate is warmed to room temperature and the precipitate which is obtained is dissolved with sodium hydroxide to give a 0.5% solution. This is precipitated as before at 2° at *p*H 4.9. The precipitate of β-casein is washed with water and dried with alcohol and ether. The first and third precipitations at 2° serve to remove the low mobility protein material which is more soluble than β-casein. The second precipitation at 2° removes α-casein which is much less soluble than β-casein under these conditions. Some β-casein is lost in each step and the over-all yield from the three precipitations is 40 to 50%. The residues can be reworked by the same method and some additional β-casein obtained.

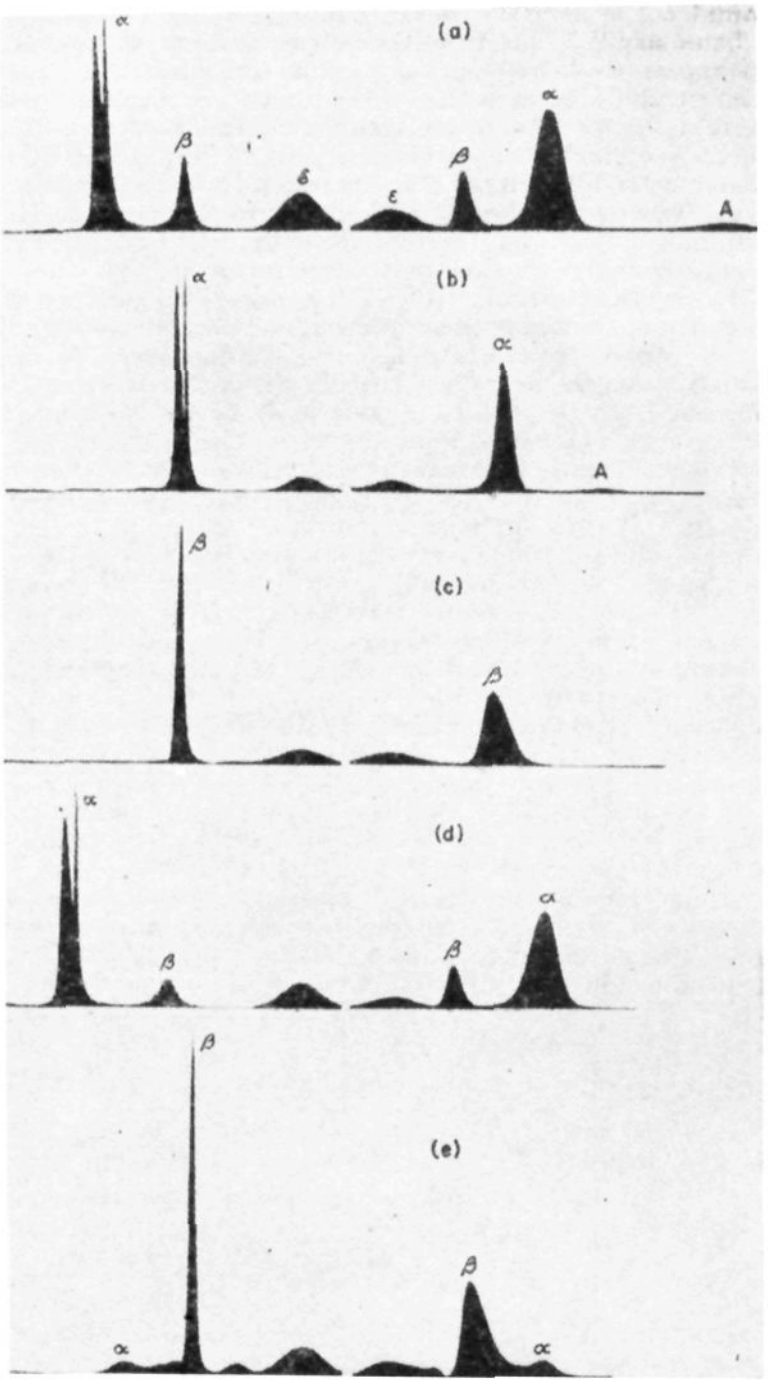

Fig. 2.—Electrophoretic patterns obtained in a veronal buffer at a *p*H of 7.78, with an ionic strength of 0.1, and containing 0.08 *N* sodium chloride: (a) casein after electrophoresis for 11,400 sec. at a field strength of 4.43 volts/cm.; (b) α-casein after 6600 sec. at 4.83 volts/cm.; (c) β-casein after 13,500 sec. at 4.82 volts/cm.; (d) reconstituted casein after 12,180 sec. at 4.90 volts/cm.; (e) crude β-casein fraction after 12,600 sec. at 4.49 volts/cm.

Electrophoretic Behavior of Casein.—The fractionation of casein has been considered with reference to the electrophoretic pattern obtained in a phosphate buffer at *p*H 6.98. This pattern (Fig. 1 (a)) is similar to that given by Mellander,[5] who studied the electrophoresis of casein using the schlieren band method of observing the boundaries. He reported, in addition to the α and β peaks, the presence of a third component, γ-casein, which did not migrate in any of the three buffers used. It seems probable that this stationary peak represents the well-established ε and δ boundary effects. However, in order to ascertain whether any protein component contributes to this peak, the solution above the β boundary was separated from the descending channel after electrophoresis in the phosphate buffer and was analyzed for nitrogen. The concentration of protein indicated by the nitrogen analysis

would not account for more than 4% of the area of the ϵ boundary.[17] This is too small an amount of protein to appear as a well-defined peak in the absence of the ϵ effect, and the existence of "γ-casein" is therefore uncertain. Some slowly migrating material is present in the β-fraction before purification (Fig. 2 (e)), but it has a high enough mobility to be separated from the ϵ boundary. Whether this material is present in casein as such is not known, but it may possibly contribute to the nitrogen found by analysis in the above experiment.

In veronal buffer at *p*H 7.78 the descending pattern of casein was similar to that obtained in phosphate buffer. At a protein concentration of 0.6%, however, the α boundary on the ascending pattern showed two peaks, as illustrated by Fig. 2 (a). The ratio of the mobilities of these peaks becomes lower as the protein concentration is increased until only one peak is evident at a concentration of 1%. The same effect is shown by purified α-casein. On all the patterns obtained in veronal buffer a small, diffuse boundary with a mobility of 13×10^{-5} was present on the descending side [*A* in Fig. 2 (a) and (b)] It had no clear counterpart on the ascending pattern, although there was usually a small disturbance moving in front of the α boundary, but with a mobility of not more than 9×10^{-5}. It was not observed in any other buffers and in these details recalls the extra boundary observed by Moore and Lynn[18] in the electrophoresis of serum with the same buffer. This boundary was present with casein or purified α-casein, but not with purified β-casein. This latter was homogeneous in veronal buffer, giving the pattern shown in Fig. 2 (c).

Krejci, Jennings and Smith[19] noted that in the electrophoresis of casein in a phosphate buffer, the area of the β peak was greater on the ascending pattern than on the descending, in spite of the dilution which occurs at the

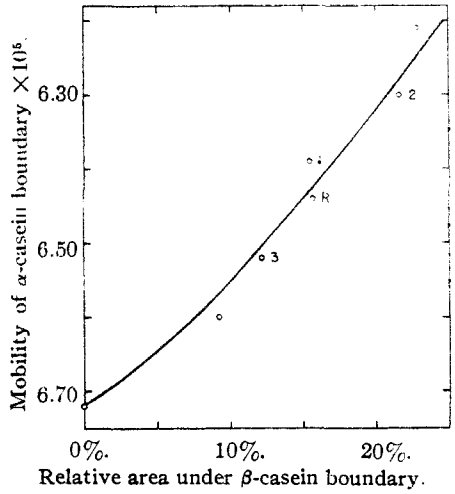

Fig. 3.—Change in mobility of α-casein in the presence of different amounts of β-casein in veronal buffer. Experiments 1, 2 and 3 were made on three different samples of unfractionated casein and the differences in the relative areas of the peaks in these experiments indicate the range of normal variation in the composition of casein. Experiment R refers to reconstituted casein.

δ boundary. These authors concluded that this was a result of complex formation between α- and β-casein and presented calculations on the extent of this reaction based on an area analysis of their patterns. This abnormal distribution of area is qualitatively confirmed in this investigation by the patterns in both phosphate and veronal buffers. However, the β peak on the ascending side was never more than 10% larger than on the descending side, whereas in Krejci's patterns it averaged 62% greater in area. If the two components migrated independently, the β peak on the ascending pattern would be about 10% smaller, as calculated from the dilution occurring at the δ boundary. Information on the interaction between α- and β-casein is given by an experiment on "reconstituted" casein prepared from 21.6% purified β-casein and 78.4% purified α-casein in veronal buffer. Here the areas on the patterns indicated 15.7 and 16.4% β-casein, as calculated from the descending and ascending patterns, respectively. Since even on the ascending side this is less than the β-casein taken in preparing the solution, it is evident that the interaction cannot be described as being of a type similar to that occurring between ovalbumin and nucleic acid.[20] As Krejci's calculations were based on this assumption, they can yield no significant information on the electrophoretic behavior of casein.[21]

The complex formation between the components of casein which seems probable from these data is given further support by an analysis of the mobilities calculated from a series of experiments in veronal buffer. In this series the relative amounts of α- and β-casein were varied while the total protein concentration was held roughly constant at about 0.8%. The results are summarized in Fig. 3, where the mobility of the descending α boundary is plotted against the relative area of the β-casein peak on the descending pattern. The mobility decreases regularly as the area increases, the mobility of purified α-casein forming an upper limit to the series. It is, of course, evident from the experiment on reconstituted casein that the area of the β boundary does not give a measure of the total amount of this component, but this area may be expected to be functionally related to the β-casein concentration.

While these experiments do not define the mode of interaction, some β-casein was apparently combined with α-casein in both the ascending and descending channels. None of the five types of complex formation considered by Longsworth and MacInnes[20] appears to be applicable to this case. This behavior may be related to the demonstrated electrophoretic complexity of both fractions.

The mobility of β-casein in the experiments illustrated in Fig. 3 was essentially constant (2.89 to 3.08×10^{-5}). The moblity of purified β-casein in two experiments was 3.27×10^{-5}. The apparent lower value in the presence of α-casein is probably due to the fact that the conductance of the solution in which this boundary moves cannot be directly determined, but it will, in general, be higher than that of the protein solution below the α boundary which of necessity is used in calculating the mobilities.[22]

The mobility of α-casein in veronal and other buffers shows a marked decrease with increasing protein concentration. This can be attributed to the higher viscosity of the more concentrated solutions, and Longsworth and MacInnes[22] have suggested that it may be corrected for by multiplying the value for the mobility by the value for the relative viscosity of the solution. Casein offers a more critical test of this assumption than the ovalbumin used by these authors because of its higher viscosity increment. The mobilities found in two experiments on purified α-casein in veronal buffer were: 6.98×10^{-5} at a protein concentration of 0.43% and a viscosity relative to the buffer at 0.5° of 1.077; and 6.62×10^{-5} at a

(17) This is not necessarily in contradiction to Mellander's similar experiment. He isolated 18 mg. of protein after electrophoresis of a 5% solution in a quantity electrophoresis cell. There are insufficient data to make an exact comparison with the experiment reported here, but the two results are probably of the same order of magnitude.

(18) Moore and Lynn, *J. Biol. Chem.*, **141**, 819 (1941).

(19) Krejci, Jennings and Smith, *J. Franklin Inst.*, **232**, 592 (1941); Krejci, *ibid.*, **234**, 197 (1942).

(20) Longsworth and MacInnes, *J. Gen. Physiol.*, **25**, 507 (1942).

(21) In addition, Krejci's calculations are complicated by the assumption that the major portion of the ϵ and δ boundaries is due to "γ-casein."

(22) Longsworth and MacInnes, THIS JOURNAL, **62**, 705 (1940).

protein concentration of 0.95% and a relative viscosity of 1.211. In the first case the mobility multiplied by the relative viscosity gives 7.52×10^{-5} and in the second case 8.02×10^{-5}. It is evident that this procedure gives an overcorrection and that the macroscopic viscosity of the solution is not the function that should be applied in correcting the mobility. This overcorrection is also evident in the data of Longsworth and MacInnes, although because of the low relative viscosities of their solutions it is not great.

No correction for viscosity has been applied to any of the data in this paper, but where comparisons are made, solutions of approximately the same protein concentration have been used.

In 0.1 *N* sodium acetate buffer at *p*H 5.63, the descending patterns of casein and α-casein were similar to those obtained in phosphate and veronal buffers. A small additional peak was present on the ascending pattern in both cases, as shown in Fig. 4, (a) and (b). The β-casein pattern in this buffer showed two peaks on both sides [Fig. 4 (c)]. The faster boundary was small and diffuse on the descending pattern, but produced an exceptionally sharp spike on the ascending pattern. Reconstituted casein yielded the same pattern as the unfractionated material and as in the veronal buffer, the area of the β peak was smaller than could be accounted for by the amount of β-casein taken (7.5% of the area as compared with 21.6% β-casein taken). The patterns obtained in acetate and phosphate buffers were also similar to those in veronal buffer in that the mobility of the α-casein boundary was lower in the presence of β-casein than in its absence. This effect was especially pronounced at *p*H 5.63 and, together with the results of the experiment on reconstituted casein at this *p*H, it indicates that more β-casein was combined with α-casein in this buffer than at higher *p*H's. This is confirmed by the area distribution on patterns obtained with the same casein sample in these three buffers. The relative area of the β boundary was 21.6% at *p*H 7.78, 19.3% at *p*H 6.98, and 12.4% at *p*H 5.63. On raising the protein concentration in the phosphate buffer from 0.61 to 1.10%, the relative area of the β boundary decreased from 19.3 to 16.7%.

On the acid side of the isoelectric point it is not possible to obtain patterns at a *p*H greater than about 3.1 when the ionic strength is 0.1 because the solutions become cloudy or the protein precipitates. The patterns obtained in various buffers in this *p*H region showed marked differences, depending on the *p*H and ionic strength of the solution. Those obtained in a lactate buffer at *p*H 3.07 and an ionic strength of 0.1 are shown in Fig. 5. β-Casein is not sufficiently soluble in this buffer to give a satisfactory pattern. The one in Fig. 5 was obtained in the same buffer, but with the ionic strength reduced to 0.05. Neither of the casein fractions was homogeneous under these conditions, and the relation between the peaks in the patterns of the fractions and those in the casein pattern was not clear in all cases. In spite of this, reconstituted casein gave a pattern identical with that of unfractionated casein. The mobilities are summarized in Table III.

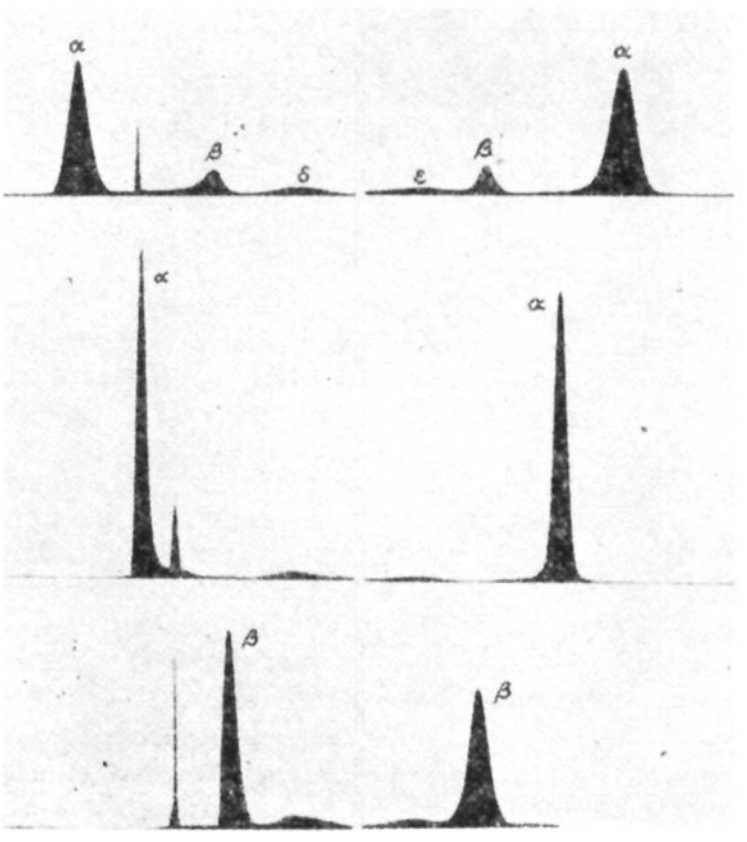

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Fig. 4.—Electrophoretic patterns obtained in 0.1 *N* sodium acetate buffer at a *p*H of 5.63, and with an ionic strength of 0.1: (a) casein after electrophoresis for 12,900 sec. at a field strength of 4.80 volts/cm.; (b) α-casein after 7800 sec. at 4.79 volts/cm.: (c) β-casein after 13,320 sec. at 4.84 volts/cm.

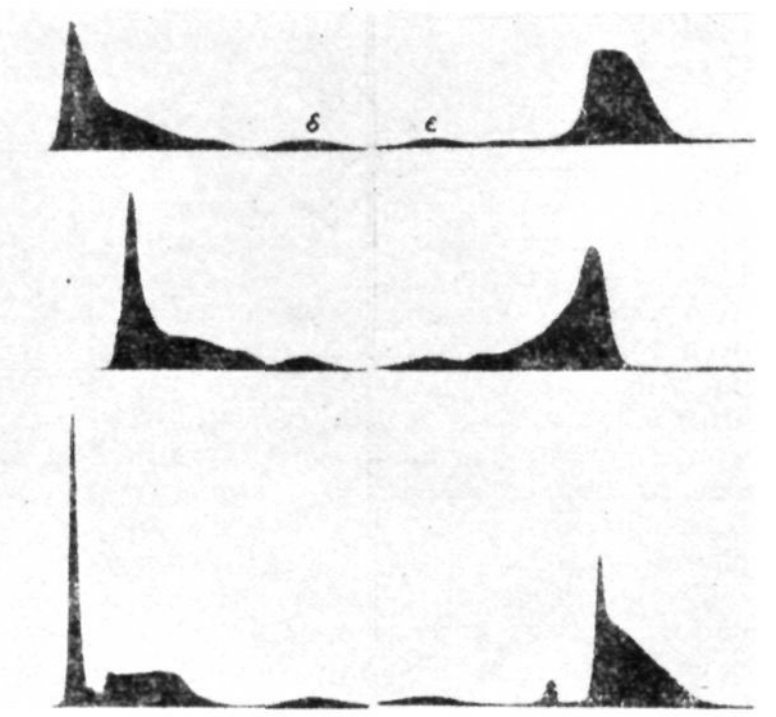

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Fig. 5.—Electrophoretic patterns obtained in a lactate buffer at a *p*H of 3.07, with an ionic strength of 0.1, and containing 0.05 *N* sodium chloride: (a) casein after electrophoresis for 11,400 sec. at a field strength of 4.75 volts/cm.; (b) α-casein after 7800 secs. at 4.77 volts/cm.; (c) β-casein after 13,020 sec. at 4.87 volts/cm. In this case the ionic strength was reduced to 0.05.

Isoelectric Point of Casein.—The data in Table III permit the determination of isoelectric points for the two casein fractions by interpolation to the point of zero mobility. The *p*H interval over which the interpolation must be made is large, especially in the case of α-casein, so that the isoelectric points found are only approximate. Values of 4.0 to 4.1 for α-casein and 4.5 for β-casein were obtained at an ionic strength of 0.1. These values are both lower than the commonly accepted isoelectric point of casein. However, they are confirmed by Moyer's[23] study of the isoelectric point of casein particles as a function of the ionic strength by the method of microelectrophoresis. He found that the isoelectric point decreases from 4.6 in the absence of salt to 4.35 at an ionic

(23) Moyer, *J. Biol. Chem.*, **133**, 29 (1940).

TABLE III

MOBILITIES OF CASEIN PREPARATIONS AT 0° AND AT AN IONIC STRENGTH OF 0.1, MOBILITY IN CM.^{2} VOLT^{-1} SEC.$^{-1}$ × 10^5

*p*H	7.78	6.98	5.63	4.92	3.07
Buffer	veronal	phosphate	acetate	acetate	lactate
α-Casein (0.4–0.5%)[a]	−6.98	−7.52	−6.16		+5.82[b]
β-Casein (0.39–0.45%)	−3.27	−3.00	−1.60	−0.74[c]	+3.78[c]
Casein 2	(0.89%)[a]	(0.61%)	(0.50%)		(0.58%)
α Boundary	−6.30	−6.81	−5.17		+5.0[b]
β Boundary	−2.92	−2.83	−1.53		

[a] Figures in parentheses give the protein concentration. [b] Calculated from the bisecting ordinate of the entire pattern cepting the ε boundary. [c] Protein concentration was 0.1% in these two experiments (limited by the solubility of the otein).

strength of 0.1. In the absence of salt the isoelectric point of β-casein is increased to about 4.9, as judged by the point of minimum solubility.

Discussion

In the work on the fractionation of casein reported in the literature, little evidence has been presented for the homogeneity of the fractions or for the completeness of the separation into distinct components, and usually no such claims have been made. Linderstrøm-Lang succeeded in producing fractions with markedly different phosphorus contents and solubility properties, but concluded that while his experiments demonstrated the existence of several components, his fractions were chiefly mixtures. Linderstrøm-Lang's analytical results in his fractionation with sodium chloride and hydrochloric acid have been confirmed, but the degree of fractionation indicated electrophoretically was slight. The other main type of fractionation employed by this worker, extraction with acidified alcohol at an elevated temperature, yields a fraction containing both α and β casein, as is evident from the electrophoretic patterns of Krejci, *et al.*[19]

There were also considerable differences in phosphorus, tyrosine or tryptophan content in fractions obtained by Cherbuliez[13] and Groh[14] and their co-workers and by Osborne and Wakeman.[24] Such of these fractions as we have examined electrophoretically contained both α- and β-casein, although the reported phosphorus contents cannot be accounted for by mixtures of these two components. Products of this type may arise in several ways. (1) Both α- and β-casein have composite nature, and some of the procedures may result in a fractionation of one or both of these components. (2) Methods have sometimes been employed which are rather destructive and may result in slight hydrolysis of the proteins.[25] (3) All preparations of casein except those which have been exposed to heat or to extensive treatment with alcohol contain a proteolytic enzyme which slowly hydrolyzes casein, with the production of a large number of non-dialyzable fragments which are electrophoretically distinct.[26]

Linderstrøm-Lang[15] also reported some experiments which he considered to be on the border line between purification and fractionation. He found that the small amount of protein recovered from the filtrate of an isoelectric precipitation of casein at a low temperature consisted of several components with an overall P/N ratio of 0.046. Considering the method of preparation and the P/N ratio, this fraction conceivably contained some β-casein. It is therefore possible that in the reprecipitation employed in purifying casein some of the β-fraction is lost. The amount of protein recovered by this method increases, however, when casein is allowed to stand either as a wet precipitate or as a solution, even at refrigerator temperatures. This increase is presumably a result of the activity of the enzyme mentioned above. A series of hydrolysis products, isolated by this method and fractionated by salting out with ammonium sulfate, had P/N ratios of 0.034 to 0.078. They were to some extent soluble in alcohol.

In view of these considerations, it can be concluded that, while quantitatively minor fractions can arise in a number of ways during fractionation, the presence in casein of a protein of low phosphorus content, such as that of Osborne and Wakeman, is not excluded.

The two fractions into which casein has been separated in this research, while not electrophoretically homogeneous at all *p*H's, are distinct fractions, each of which has been purified by removing all traces of the other. By mixing these two fractions in suitable proportions, the electrophoretic pattern of casein can be reproduced even though it is of a complex nature in some buffers. The completeness of the separation is shown, not only by the electrophoretic evidence, but also by the data in Table II, in which β-casein is seen to be a component removed in a definite amount by repetition of the same procedure. This finally results in an α-fraction yielding no more β-casein on further treatment. The procedure by means of which these fractions have been separated consists of a mild treatment from the standpoint of possible decomposition of the proteins. It does not involve more extreme *p*H's than 3.5 and 6.5 and is for the most part carried out at 2°.

(24) Osborne and Wakeman, *J. Biol. Chem.*, **33**, 243 (1918).

(25) Some information on the stability of casein under various conditions is given by Carpenter, *J. Biol. Chem.*, **67**, 647 (1926); Howat and Wright, *Biochem. J.*, **28**, 1336 (1934); and Laqueur and Sackur, *Hofmeister's Beitrage*, **3**, 193 (1902).

(26) The presence of a proteolytic enzyme in milk has been noted by Babcock and Russell, 14th Ann. Rept. Wis. Agr. Expt. Sta., 161 (1897), and by others, but has not been previously reported in purified casein. Work on this subject will be published elsewhere.

Acknowledgment.—The author is indebted to Edith Polis for performing most of the chemical analyses and to T. L. McMeekin and R. W. Jackson for their advice and interest in the investigation.

Summary

1. Casein has been separated into two fractions, α- and β-casein, which represent the two peaks in the electrophoretic pattern of casein at *p*H 7.

2. The fractions are not electrophoretically homogeneous under all conditions, but they have been purified so that neither fraction contains any of the other.

3. The electrophoretic behavior of casein and the existence of complex formation between α- and β-casein are discussed.

RECEIVED JULY 13, 1944

[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 972]

The Serological Properties of Simple Substances. VIII. The Reactions of Antiserum Homologous to the *p*-Azobenzoic Acid Group

BY DAVID PRESSMAN, STANLEY M. SWINGLE, ALLAN L. GROSSBERG, AND LINUS PAULING

Our earlier studies of the reactions of antibodies with simple substances have for the most part been limited to antisera homologous to the *p*-azophenylarsonic acid group[1,2,3,4,5] and the *p*-(*p*-azophenylazo)phenylarsonic acid group[5,6]; recently we have investigated also the reactions of antiserum homologous to the *p*-azobenzoic acid group, with the results described in the present paper.[7] These investigations include quantitative studies of the specific precipitation of antiserum homologous to the *p*-azobenzoic acid group (hereafter called anti-X serum) by ovalbumin coupled with diazotized *p*-aminobenzoic acid (X-ovalbumin) and by the simple dihaptenic substance made by coupling diazotized *p*-(*p*-aminophenylazo)benzoic acid with chromotropic acid, of the effect of change of hydrogen-ion concentration on these precipitation reactions, and of the inhibition of precipitation by haptens. The data on inhibition by haptens have been interpreted in terms of the recently developed theory of heterogeneity of antisera.[5]

The inhibition by a great number of haptens of the precipitation of antisera homologous to the *p*-azobenzoic acid group with an azoprotein antigen was investigated qualitatively by Landsteiner[8] and Landsteiner and van der Scheer[9] and to a smaller extent by Hooker and Boyd.[10,11] That simple polyhaptenic substances can act as precipitating antigens was discovered by Landsteiner and van der Scheer,[12] who, however, did not include substances containing azobenzoic acid groups among those studied in this way. A study of these substances was first carried out by Hooker and Boyd,[11,13] who reported their failure to obtain precipitates with anti-benzoic acid sera and certain homologous polyhaptenic substances. In our work precipitation with anti-benzoic acid serum was observed for one of seven polyhaptenic substances studied.

Experimental Methods

Simple Test Antigens.—The polyhaptenic simple substances tested as precipitating antigens are listed in Table I. Their preparation is discussed in the following section.

Haptens.—The haptens used either have been described elsewhere[1,5] or were commercial products purified to the correct melting points.

Protein Antigens.—The immunizing antigens used for inoculation were prepared by coupling diazotized *p*-aminobenzoic acid with whole beef serum by the method of Landsteiner and van der Scheer.[14] Two preparations were made, with, respectively, 0.9 g. and 1.8 g. of *p*-aminobenzoic acid coupled with 200 ml. of serum and made up to 600 ml. These preparations were used interchangeably after it was found that they gave rise to antisera of similar titer.

The azoprotein test antigen was prepared by Mr. Carol Ikeda by diazotizing 0.03 g. of *p*-aminobenzoic acid and coupling the product with 2.0 g. of ovalbumin at *p*H 8. The antigen was dialyzed against distilled water for ten days, after which time the rate of passage of colored material through the membrane was negligible.

Antisera.—Antisera were prepared by injecting eight rabbits with beef serum coupled with *p*-aminobenzoic acid; the method was similar to that described previously for the preparation of anti-phenylarsonic acid sera.[1] The sera were pooled according to titer. A single pool of antiserum, which was obtained after nine months of inocula-

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(2) L. Pauling, D. Pressman, D. H. Campbell, and C. Ikeda, *ibid.*, **64**, 3003 (1942).

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(4) D. Pressman, D. H. Brown, and L. Pauling, *ibid.*, **64**, 3015 (1942).

(5) L. Pauling, D. Pressman, and A. L. Grossberg, *ibid.*, **66**, 784 (1944).

(6) D. Pressman, J. T. Maynard, A. L. Grossberg, and L. Pauling, *ibid.*, **65**, 728 (1943).

(7) An account of experiments carried out with mixtures of these antisera has already been published: L. Pauling, D. Pressman, and D. H. Campbell, *Science*, **98**, 263 (1943); THIS JOURNAL, **66**, 330 (1944).

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(10) S. B. Hooker and W. C. Boyd, *Proc. Soc. Exptl. Biol. Med.*, **47**, 187 (1941).

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