Studies on κ-Casein of Bovine Milk. II. Heterogeneity of κ-casein.

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Summary Heterogeneity of κ-casein prepared by various methods was investigated using disc electrophoresis, starch-agar gel electrophoresis, agarose gel electrophoresis, starch-agarose gel electrophoresis, DEAE cellulose chromatography, Sepharose 6B gel filtration and isoelectric focusing. Results suggest that all the κ-caseins, regardless of their preparative methods, were more or less heterogeneous and that the heterogeneity depends upon at least two factors; molecular size and charge. The result of DEAE cellulose chromatography shows that κ-casein could dissociate into two components one of which seemed to be a para-like κ-casein. The para-like κ-casein had been impossible to identify by standard starch gel electrophoresis or by disc electrophoresis. Therefore, it does not seem to be a simple impurity, but seems to be a component which forms a κ-casein complex. Although random S-S bonds have been thought to be a unique cause for the heterogeneity of κ-casein, variation in the amount of para-like κ-casein may be another cause. The little deviation in the concentration of para-like κ-casein seems to indicate that it was in the original milk and was not an artifact produced during preparation. It was also found that the κ-casein molecules were the largest and the least heterogeneous when prepared by the urea sulfuric acid method. On the other hand, κ-casein prepared by the calcium ethanol method seemed to be somewhat unfolded into smaller units and to release para-like κ-casein most easily.

It is well known that the purest κ-casein does not appear as a sharp band in zone electrophoresis.[1] When reduced, however, it shows several sharp bands.[2] So, the κ-casein complex is believed to be composed of several distinctly different components crosslinked through S-S bonds in various ways so that molecules of different sizes are included.

Some researches[3] have focused on reduced κ-casein. But, since most of the κ-caseins reported so far were not entirely pure, containing various amounts of α_S-, β-, and γ-caseins, it is also necessary to reexamine the purity and the homogeneity of unreduced κ-casein in connention with its preparative methods. Some analytical data on κ-casein obtained using starch gel electrophoresis, standard method for investigating its purity, were previously reported.[4] In the present paper κ-caseins obtained by three different methods were subjected to zone electrophoresis, DEAE cellulose chromatography, gel filtration and isoelectric focusing. Results are discussed mainly from the viewpoints of molecular size and electric charge of κ-casein complexes.

Experimental methods

1. *Preparation of κ-casein.*

As previously described, κ-casein was prepared by the following three methods:

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the calcium ethanol method,[5] the Sephadex method,[6] and the urea sulfuric acid method.[7] Starch gel electrophoresis indicated that the κ-caseins obtained were reasonably pure.

2. Disc electrophoresis.

Polyacrylamide gel electrophoresis was performed according to the method of B.J. Davis with a modification where different concentrations of acrylamide, ranging from 4 to 6 percent instead of 7.5 percent, were used.

3. Starch-agar gel electrophoresis.

The general procedure and form of the apparatus used in all the present zone electrophoresis was similar to that described by R. G. Wake and R. L. Baldwin[1] for starch gel electrophoresis. Various amounts of partially hydrolyzed potato starch (0.8 - 8.0g per 35ml tris-citrate buffer, pH 8.6) were mixed with 1 g of agar and the mixture was heated to the boiling point. Then, urea (13.5g) was added. The sol obtained was defoamed in a vacuum and kept at 40°C for 3 hours. The gel formed at 5°C overnight. κ-Casein dissolved in TCU buffer (tris-citrate buffer, pH 8.6, containing 6M urea) was electrophoresised at 180 volts for 11 hours in a cold room.

4. Agarose gel electrophoresis.

A two percent agarose gel was formed in a way similar to that in starch agar gel electrophoresis. Electrophoresis was performed at 160 volts for 2 hours in a cold room.

5. Starch-agarose gel electrophoresis.

A gel consisting of 6.6 percent starch and 2 percent agarose was obtained in a way similar to that in starch-agar gel electrophoresis. Electrophoresis was performed at 100 volts for 12 hours in a cold room.

6. DEAE cellulose chromatography.

a) DEAE cellulose was washed with an appropriate amount of phosphate buffer (0.05M, pH8.3) containing 6M urea. κ-Casein (50mg/5ml starting buffer) was applied to a DEAE cellulose column (2×15cm). A linear NaCl gradient (0 to 0.2 or 0.5 M at pH 6.4 in 400ml) following a linear pH gradient, 8.3 to 6.4 in 100 ml, was used for the elution. All eluants contained 6M urea.

b) κ-Casein (50mg) was applied to a column of DEAE cellulose (2×8cm) equilibrated with 0.05M phosphate-citrate buffer, pH 7.4, containing 6M urea. The column was then washed with the starting buffer (80 ml). Elution was carried out with a pH and NaCl gradient consisting of 100 ml phosphate-citrate buffer (pH 7.4, containing 6M urea) and 100 ml of the same buffer (pH 4.5, containing 6M urea and 0.2M NaCl).

c) κ-Casein (30mg) was dissolved in phosphate buffer, pH 7.0, containing 6M urea, and was reduced overnight with 0.1 ml mercaptoethanol in nitrogen gas. Then, the reduced κ-casein was applied to a DEAE cellulose column (2 x 13cm) equilibrated with 0.05M phosphate buffer, pH7.0, containing 6M urea and 0.3 percent mercaptoethanol. After washing the column with 100 ml of the starting buffer, a NaCl gradient ranging from 0 to 0.2M in 400 ml of the above buffer was used for the elution. Finally, the column was washed with 100 ml of 0.2N

NaOH. Standard starch gel eletrophoresis was used according to R. G. Wake and R. L. Baldwin to characterize the κ-caseins fractionated by DEAE cellulose chromatography. The concentration of hydrolyzed potato starch prepared in our laboratory was changed to 17 percent.

7. Sepharose gel filtration.

κ-Casein (10 mg) was applied to a Sepharose 6B column (2×54cm) equilibrated with 0.1M TCU buffer, pH 8.6, then it was eluted with the same buffer at a room temperature. Fraction volume was 2.5ml and the flow rate was kept constant at 2.8ml per minute.

8. Isoelectric focusing.

To avoid precipitation of κ-casein, isoelectric focusing was performed in 6M urea at 5°C for 44 hours. Voltage was kept constant at 500V during the electrophoresis in which κ-casein (10 mg) was dissolved in the light solution for preparing the sucrose gradient (0 to 49.8%) throughout the ampholine column (3×25cm). The ampholine column was prepared by mixing 60 ml of light solution with 50 ml of concentrated solution in such a way that the above linear sucrose gradient was obtained. At the end of electrophoresis, the ampholine solution was fractionated into about 60 test tubes. The pH and OD at 280 mμ of each fraction were measured respectively. Other conditions are shown in Table 1,

Table 1. Composition of ampholine column for isoelectric focusing of κ-casein.

	Ampholine (ml)	Sucrose (g)	Urea (g)	Phosphoric acid (ml)	Ethylene diamine (ml)	*Mercapto-ethanol (ml)	Total (ml)
Conc. soln.	7.1	23.4	18.0	—	—	0.15	50
Light soln.	2.8	—	21.6	—	—	0.18	60
Anode soln.	—	25.0	18.0	0.8	—	—	50
Cathode soln.	—	—	—	—	0.4	—	25

Results and discussion

1. Disc electrophoresis.

Disc gel electrophoresis was carried out to find the concentration of acrylamide at which κ-casein completely enters into the small pore gel. Another purpose was to check whether the dyed pattern shows narrower bands as the acrylamide concentration decreases. We expected that the protein electrophoresised at a decreased concentration of acrylamide would give a sharper band if heterogeneity of molecular size is the only factor to cause the spread band of κ-casein at a normal concentration of acrylamide. Results in Fig. 1, however, did not satisfy our expectation. There was no significant difference in band widths between 4.0 percent and 4.6 percent concentration of acrylamide. All the κ-casein entered into the small pore gel when the concentration decreased to 4.0 percent. However, we found a distinct discontinuity between the protein which entered into the small pore gel and that which remained in the large pore gel. This discontinuity could not have been expected if the heterogeneity of κ-casein had been due only to molecules of continously varied size. This discovery suggested that the electric charge of κ-casein might be another cause for the heterogeneity. It is also possible that the huge κ-casein molecule may be asymmetrical; for instance, fiberous and rod-like.

2. Starch-agar gel electrophoresis.

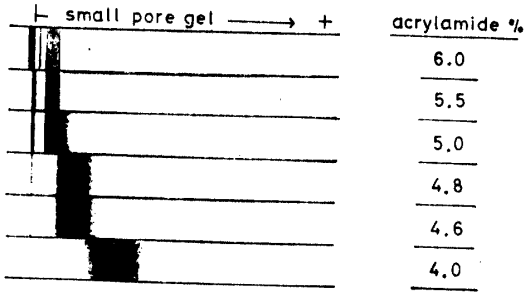

Fig. 1. Polyacrylamide-gel electrophoresis of κ-casein.

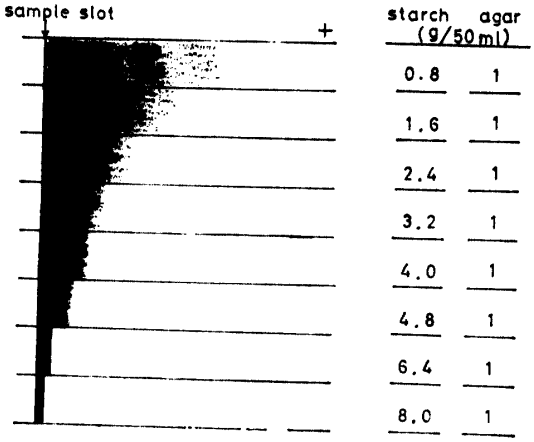

Fig. 2. Starch agar-gel electrophoresis of κ-casein. gel contained 4.5M urea.

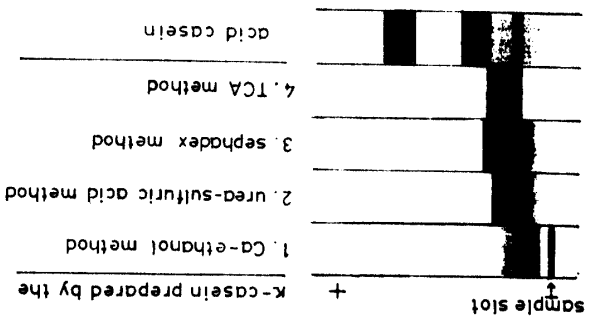

Fig. 3. Agarose-gel electrophoresis of various κ-caseins.
agarose: 2%
160V, 2hrs, pH 8.6.

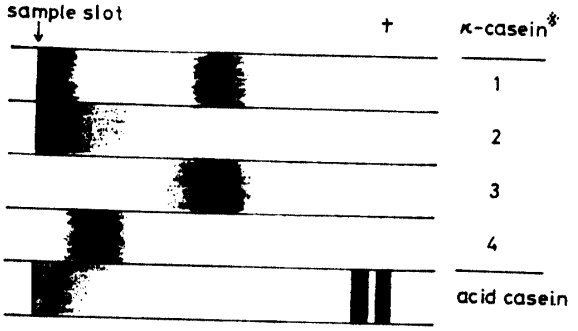

Fig. 4 Starch agarose-gel electrophoresis of various κ-caseins.
gel composition: 6.6% starch & 2% agarose.
※ see Fig. 3. 100V, 12hrs, pH 8.6.

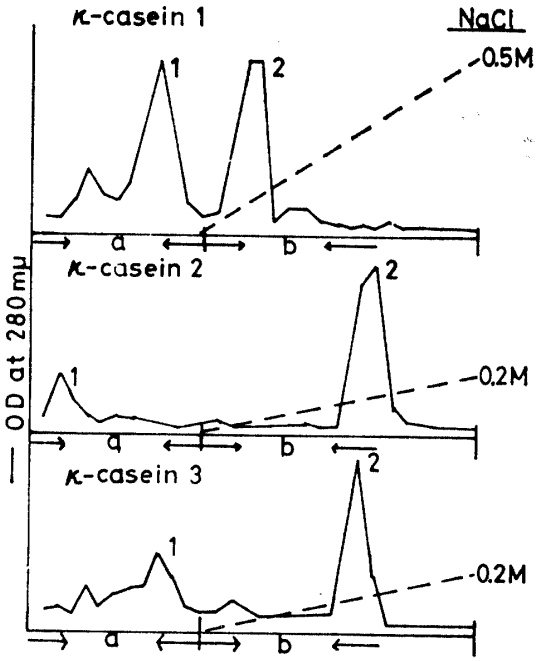

Fig. 5. DEAE cellulose chromatography of various κ-caseins in 6M urea.
κ-casein 1, 2, 3: see Fig. 3.
κ-Casein(50 mg)adsorbed by DEAE cellulose column (2×15) was eluted by:
(a) pH gradient 8.3—6.4.
(b) NaCl gradient 0—0.2 or 0.5M.

Figure 2 shows clearly that electrophoretic mobility and band width increased as starch concentration decreased. This supports the idea that κ-casein is composed of various components with continuously different molecular sizes. However, the fact that the most slowly mobile portion remained at the starting slot in every case indicates that molecular size is not the only cause for the heterogeneity of κ-casein. Some interaction probably occurs between starch or agar gel and κ-casein. Again in this experiment, there appeared no tendency for the electrophoretic band to become narrower as the starch concentration decreased to a level of 1.6 percent.

3. Agarose gel electrophoresis.

In agarose gel electrophoresis, the agarose gel pore is believed to be wide enough for even κ-casein to enter under electric pressure. So, broadening of the bands indicates the existence of heterogeneous charges on the molecules. Figure 3 shows that κ-casein prepared by the urea sulfuric acid method is the most homogeneous and that κ-casein prepared by the calcium ethanol method is the most heterogeneous. On the other hand, the average electrophoretic mobility was nearly the same for all the κ-caseins, indicating that all of them were of totally similar electric charge. The κ-casein prepared by the calcium ethanol method, however, split into two components; one of which hardly moved from the sample slot.

4. Starch-agarose gel electrophoresis.

As shown in Fig. 4, some κ-caseins moved well and far from the samle slot, especially the κ-casein prepared by the Sephadex method and the κ-casein prepared by the TCA method. The κ-casein isolated by the calcium ethanol method mainly split into two components, one of which remained near the starting slot just as found in the agarose gel electrophoresis. The κ-casein prepared by the urea sulfuric acid method also appeared near the sample slot. As differences in mobility in this experiment are ascribable mainly to differences in molecular size, κ-casein of the largest size would be that prepared by the urea sulfuric acid method.

5. DEAE cellulose chromatography of intact κ-casein.

Reduced or alkylated κ-casein has been examined by several researchers using DEAE cellulose chromatography, but no one has ever tried to examine the purity of intact κ-casein by this method. Results in Fig. 5, to our surprise, show at least two peaks in the present DEAE cellulose chromatography. The first peak was eluted by a pH gradient without NaCl. This may be an unadsorbed portion or a weakly adsorbed one. The second peak appeared when the NaCl gradient nearly reached 0.07 molarity. Figure 6 shows that the first portion was somewhat similar to para κ-casein in its starch gel electrophoresis in which most of that portion moved toward the cathode or otherwise remained at the sample slot; which is most charateristic of para κ-casein. The remainder, however, moved toward the anode as intact κ-casein does. These results indicate that the para-like κ-casein is not a simple impurity, but is one of the components which form a κ-casein complex. Not until κ-casein had been fractionated by DEAE cellulose chromatography was the para-like κ-casein found in the κ-casein complex. κ-Casein with the highest peak of the para-like κ-casein in DEAE cellulose chromatogram, however, does not always mean the most denatured

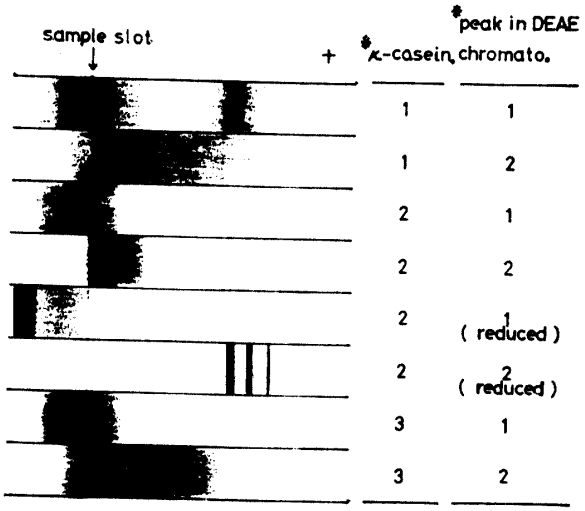

Fig. 6. Starch-gel electrophoresis of κ-caseins fractionated by DEAE cellulose chromatography.
※see Fig. 3, 5, & 7.
180V, 10hrs, pH 8.6, in 6M urea.

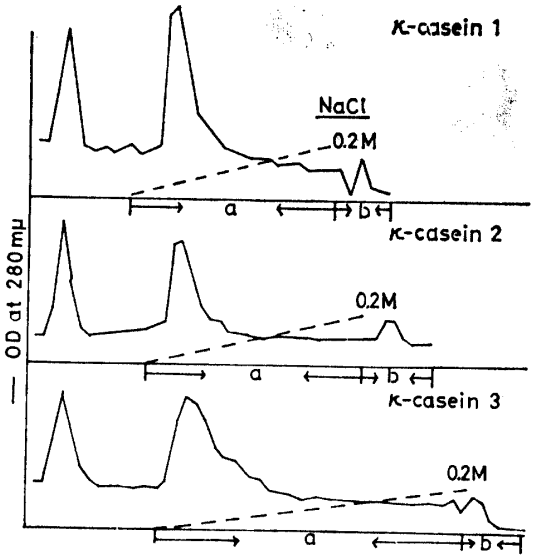

Fig. 7. DEAE cellulose chromatography of reduced κ-caseins in 6M urea.
κ-Casein 1, 2, 3: see Fig. 3.
κ-Casein (30mg) on DEAE cellulose column (2×13cm) was washed with phosphate buffer, pH 7, and eluted by:
(a) NaCl gradient 0—0.2M.
(b) 0.2N NaOH.
All the eluants contained 0.3% mercaptoethanol.

κ-casein because the para-like κ-casein contains a certain amount of intact κ-casein. Thus, it is impossible to estimate the exact amount of para-like κ-casein in the whole κ-casein complex by this method. DEAE cellulose chromatography of reduced κ-casein was used for this purpose.

6. DEAE cellulose chromatography of reduced κ-casein.

Reduced κ-casein also showed two peaks as seen in Fig. 7. The relative ratio of the first peak to the second one increased greatly when compared with that of intact κ-casein. This ratio suggests a practical ratio between the para-like κ-casein and intact κ-casein because the two components were clearly separated by the present method, so far as purity was determined by starch gel electrophoresis. The para-like κ-casein has been found in every paper reporting DEAE-cellulose chromatography of reduced κ-casein and it is simply regarded as an impurity.[2,3,9] The third peak, which was almost negligible, appeared when NaOH was used for the last elution. Although J. Pujolle et al.[3] reported that several components of reduced κ-casein were separable by the method in the present experiment, complete fractionation seemed to be very difficult.

7. Sepharose 6B gel filtration.

In a previous paper,[4] no differences were found in the gel filtration patterns of various κ-caseins with Sephadex G-150, in which a single peak appeared at a void volume so that it seemed as if κ-casein molecules had been homogeneous in size. Eigure 8 shows, however, different results that all the κ-caseins did not appear at a void volume of Sepharose column. Some κ-casein did not form a single peak and some formed a tailing skirt on the main peak. As far as molecular size is concerned, κ-casein isolated by the urea sulfuric acid method was the most homogeneous and

κ-casein isolated by the calcium ethanol method was composed of clearly different components, as was indicated in starch gel electrophoresis. The idea that the para-like κ-casein found through DEAE cellulose chromatography is not a simple impurity, but a component which forms a κ-casein complex is supported by Sepharose 6B gel filtration data of the κ-casein fractionated by DEAE cellulose treatment as seen in Fig. 9. Compared with unfractionated κ-casein, the fractionated components decreased in molecular size, showing that the κ-casein complex disaggregated in some measure to smaller fragments leaving the two components separated.

8. Isoelectric focusing.

Peak 1 in Fig. 10 is due to some impurity in the urea, while peaks 2 and 3 are believed to be caused by some components in carrier ampholine. The remaining broad peaks due to κ-casein are around pH 6. It is reasonable to assume that κ-casein consists of conti-

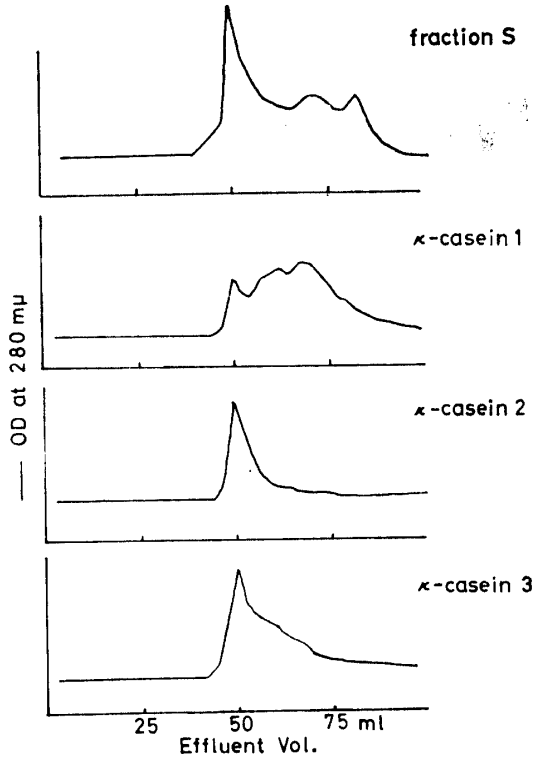

Fig. 8. Sepharose 6B gel filtration of various κ-caseins in 6M urea.
fraction S : soluble casein in 0.4M $CaCl_2$ solution.
κ-casein 1, 2, 3 : see Fig. 3.
Protein(10mg)in 1ml of 0.1M tris-citrate buffer, pH 8.6, was applied to a column (2×54cm).

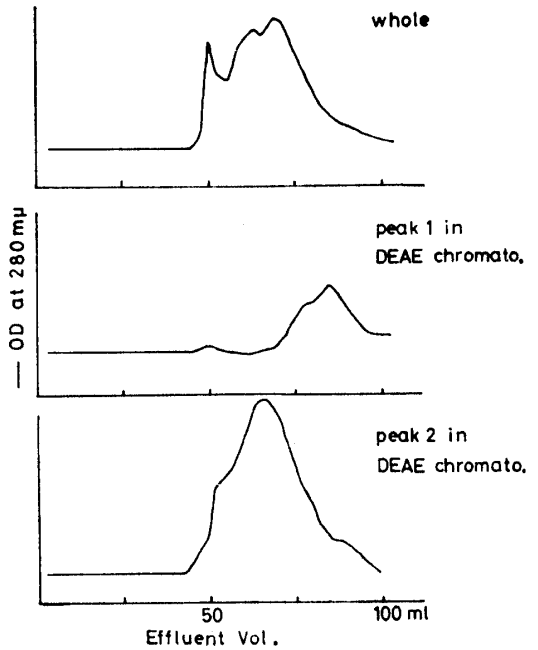

Fig. 9. Sepharose 6B gel filtration of κ-casein 1 and its components fractionated by DEAE cellulose chromatography.

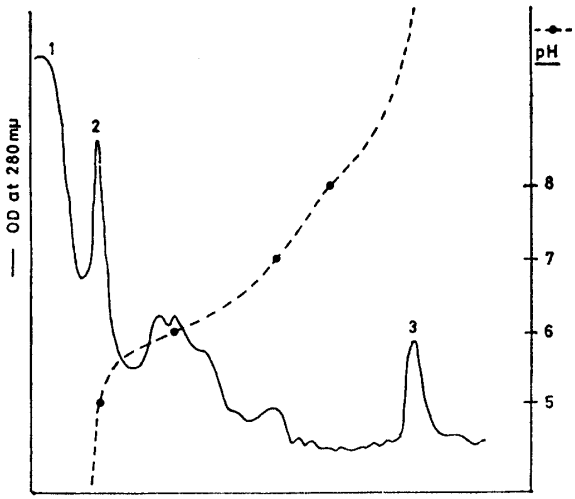

Fig. 10. Isoelectric focusing of κ-casein 1 in 6M urea.
pH range : 5—8

nously varied components rather than of several clearly different components. As the results of isoelectric focusing may be explained in terms of simple differences in the molecular charge of κ-casein, κ-casein proves to be not homogeneously charged.

要旨：3ないし4種類の方法で調製したκ-カゼインの不均一性を各種のゾーン電気泳動，DEAEセルロースクロマトグラフィー，セファロースゲルろ過，それに焦点電気泳動によって調べた。その結果，調製法のいかんによらず全てのκ-カゼインの不均一性は高く，その不均一性は少なくとも2つの要因，すなわち分子の大きさと分子の電荷の不均一性に帰せられることが判明した。

また，DEAEセカロースクロマトグラフィーの結果，κ-カゼインは少なくとも2成分に分画され，そのうちの1成分はパラκ-カゼイン様成分であった。この成分の存在は通常の電気泳動では認定し難く，単なる不純物というよりはκ-カゼイン複合体の1成分と推定される。したがって，κ-カゼインの不均一性をもたらす具体的な原因としてランダムS-S結合以外にパラκ-カゼイン様成分の存在も挙げられよう。また，尿素・硫酸法で調製したκ-カゼインが最も不均一性が低く，しかも分子が大きいこと，カルシュウム・エタノール法によるκ-カゼインはいくらか小さいユニットに解離しており，パラκ-カゼイン様成分を最も容易に遊離させることが判明した。

References

1. Wake, R.G. and R.L. Baldwin(1961) : Biochim. Biophys. Acta **47** : 225.
2. Mackinlay, A. G. and R. G. Wake (1965) : Biochim. Biophys. Acta **104** : 167.
3. Pujolle, J., B. Ribabadeau Dumas, J. Garnier and R. Pion (1966) : Biochem. Biophys. Res. Comm. **25** : 285.
4. Miyoshi M., F. Ibuki and M.Kanamori (1971): Sci. Rep. Kyoto Pref. Univ., Agr. No. **23**.
5. Mckenjie, H. A. and R. G. Wake (1961): Biochim. Biophys. Acta **47** : 240.
6. Yaguchi, M., D. T. Davies and Y. K. Kim (1968) : J. Dairy Sci. **51** : 473.
7. Zittle, C.A. and J.H. Custer (1963) : ibid **46** : 1183.
8. Davis, B.J. and L. Ornstein(1961) : in "Disc Electrophoresis" preprinted by Distillation Products Industries.
9. Mackinlay, A. G., R.J. Hill and R.G. Wake (1966) : Biochim. Biophys. Acta **115** : 103.