MODIFICATION OF HISTIDINE IN κ-CASEIN BY 2-PHENYL-1,4-DIBROMOACETOIN

by

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ABSTRACT

The main object of this thesis was to specifically modify histidine residues in κ-casein and KA1 and KA2 with the reagent 2-phenyl-1,4-dibromoacetoin (PDA) for the overall purpose of testing the hypothesis that histidine plays an important role in the stabilizing ability of κ-casein. Experiments designed to test the hypothesis that PDA causes κ-casein to aggregate by cross-linking were also carried out and these included the preparation of 2-phenyl-4-bromoacetoin (PMA) and reacting this with κ-casein and KA1 and KA2, as well as reacting PDA with histidine. Preliminary objectives of this thesis were to purify large amounts of κ-casein by scaling up the method of Zittle (66), to improve the electrophoresis technique described by Perrin (41) and Beveridge (2) so that κ-casein subfractions could be seen, and to purify the subfractions by DEAE cellulose chromatography using the method of Mercier (28).

The lowest yield of κ-casein during four attempts of scaling up Zittle's method was 6.0% and the highest yield was 7.5%. Two preparations of κ-casein resembled those published by McKenzie (23) both in amino acid composition and electrophoretic heterogeneity.

Modifications in the electrophoresis technique which gave promising results included steps to increase the voltage through the gel equilibrating the gels by carrying out preliminary runs without samples, lowering the ionic strength of the system, changing the bridges, and increasing the concentration of urea in the gel.

Suggestions by Mercier (28) which proved to be important in the separation of the subfractions of κ-casein by DEAE cellulose chromatography included sifting the cellulose before use, recrystallizing the urea, and using concentrations of NaOH and HCl below 1.0 molar during regeneration of the cellulose.

Reactions of PDA and PMA with κ-casein, KA1 and KA2 did not support the hypothesis that histidine plays an important role in the stabilizing ability of κ-casein because 1) PDA κ-casein was prepared in low ionic strength buffer, which dissolved normally and had the same stabilizing ability as untreated κ-casein, even though 0.6 histidine residue was modified; and 2) PMA KA1 and PMA KA2 were prepared which, although having 1.05 histidine and 1.60 lysine residues less than untreated KA1 and KA2, nevertheless had the same stabilizing ability as the untreated protein. Rather, these results support the contentions that 1) the low stabilizing ability of PDA-κ-casein results from aggregation caused by cross-linking of histidine residues on separate κ-casein molecules; and 2) the cross-linking may be dependent upon the ionic strength of the reaction.

Removing one bromine from PDA to make PMA seemed to result in a change of selectivity from histidine to lysine. PMA did not react on κ-casein in the same way as it did on KA1 and KA2 and this supports the statements that future work on the modification of amino acids in κ-casein and studies on the interaction of this protein with α_s-casein should be carried out on the subfraction rather than on κ-casein.

Reactions involving PDA and histidine did not lead to an explanation of the mechanism of action of PDA because the yields of the products were too low to allow chemical analysis. Products having Rf's of 0.62, 0.58, and 0.40 were produced and purified by cellex and paper chromatography in Jones' solvent (33) for the thin layer chromatographic separation of amino acids. These products reacted positively to Pauley's reagent, indicating that they were histidine addition products.

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INTRODUCTION

It has been known for some time now that κ-casein has the ability to interact with α_s - and β -caseins and form a stable micelle in the presence of calcium. Thus κ-casein is important in the over-all stability of the milk system. This is manifested when milk is processed in various ways. For instance, when milk is heated at 85°C for 5 minutes κ-casein interacts with α-lactoglobulin to form a complex. When steps are taken to interfere with this interaction, for example, by stopping the disulfide interchange reaction, then the heat stability of the milk is increased because the κ-casein is available to stabilize the system (29, 45). Rennin acts on κ-casein at least 1000 times more rapidly than it does on the other caseins (23). The enzyme splits a glycomacropeptide from the κ-casein and thus renders the protein unable to protect α_s - and β-caseins. Heat processing and enzymatic action are preliminary steps in the formation of many dairy products. Thus, it is important to know the factors which govern the ability of κ-casein to stabilize the other caseins.

It has been known since the early 1960's that there are two genetic variants of κ-casein. These have been classified as κ-casein types A or B according to their electrophoretic mobility on starch and polyacrylamide gels containing 2-mercaptoethanol. It is possible to obtain a single genetic variant from the milk of cows which are homozygous for the desired type.

Early experiments involving the electrophoresis of κ-casein indicated that this protein consists of at least six distinct subfractions joined together by disulfide bonds. Each subfraction has been partially purified and characterized. Schmidt in 1966 found that N-acetyl neuraminic acid increased as the fraction mobility increased and that the cystine and phosphorous content did not differ between the subfractions. Further, it

was found that the ability to stabilize α_s-casein against precipitation by calcium did not vary between the subfractions. The electrophoretic patterns of κ-casein as published in the literature have not been consistent in that the subfractions have not appeared in a very distinct way. In many cases the subfractions cannot be seen. What appears is a large stained area which shows the heterogeneity of the κ-casein molecule. Attempts to purify the individual subfractions have been partially successful and probably for this reason most of the work that has been done to explain the mechanism of interaction of κ-casein with α_s-casein has been carried out on whole κ-casein.

It was stated above that each subfraction of κ-casein can stabilize α_s-casein equally as well as whole κ-casein. This indicates that the carbohydrate moiety on the molecule is not responsible for the stabilizing ability. This hypothesis was supported by experiments which involved the removal of neuraminic acid from κ-casein by neuraminidase prior to carrying out the interaction with α_s-casein (40). It was found that the κ-casein so treated did not lose its stabilizing ability. Photooxidation studies on κ-casein indicated that histidine might be an important amino acid in the stabilizing ability of κ-casein (67). However, Nakai's work (32) did not support this hypothesis. No attempt was made by the above workers to check the specificity of their modification reactions. Therefore, they could draw no definite conclusions as to the role of histidine in the ability of κ-casein to stabilize α_s-casein.

The object of this thesis was to specifically modify histidine residues in κ-casein with the reagent 2-phenyl-1,4-dibromoacetoin (PDA), and to identify the products of the reaction of PDA with histidine for the over-all purpose of testing the hypothesis that histidine plays an important role in the stabilizing ability of κ-casein. Since κ-casein is a heterogeneous

protein, it was also decided to react subfractions one and two with this reagent. Preliminary objectives, therefore, were to purify κ-casein, to improve the electrophoresis technique to the point where the subfractions of κ-casein could be readily seen, and also to separate the subfractions of κ-casein by DEAE cellulose chromatography.

Early in these studies it was observed that PDA caused κ-casein to aggregate and for this reason tests were carried out that involved changing the reaction conditions to see if a workable derivative of PDA-κ-casein could be obtained. In order to test the mechanism of aggregation the monobromo derivative 2-phenyl-4-bromoacetoin (PMA) was prepared and reacted with whole κ-casein and subfractions one and two; also, PDA was reacted with the amino acid histidine and attempts were made to purify and identify the reaction products.

LITERATURE REVIEW

A. Definitions

In this thesis the main components of casein are called fractions and are referred to by their proper names, e.g. α_s - and β -casein. The genetic variants are designated as "types", for example, κ-casein type A. This is the fastest moving major κ-casein band that appears following polyacrylamide or starch gel electrophoresis in the presence of urea and 2-mercaptoethanol of whole casein prepared from pooled milk. The term subfraction applies only to κ-casein. Subfractions are the macromolecules which result after reduction of whole κ-casein and chromatography on DEAE cellulose columns in the presence of urea. According to Hill and Wake (17) "subfractions are intact κ-casein molecules which differ from each other largely in their carbohydrate content". The operational definition of casein used in this thesis is that quoted from Nakahori (30): "Casein is a heterogeneous group of phosphoproteins precipitated from milk at pH 4.6 and 20°C".

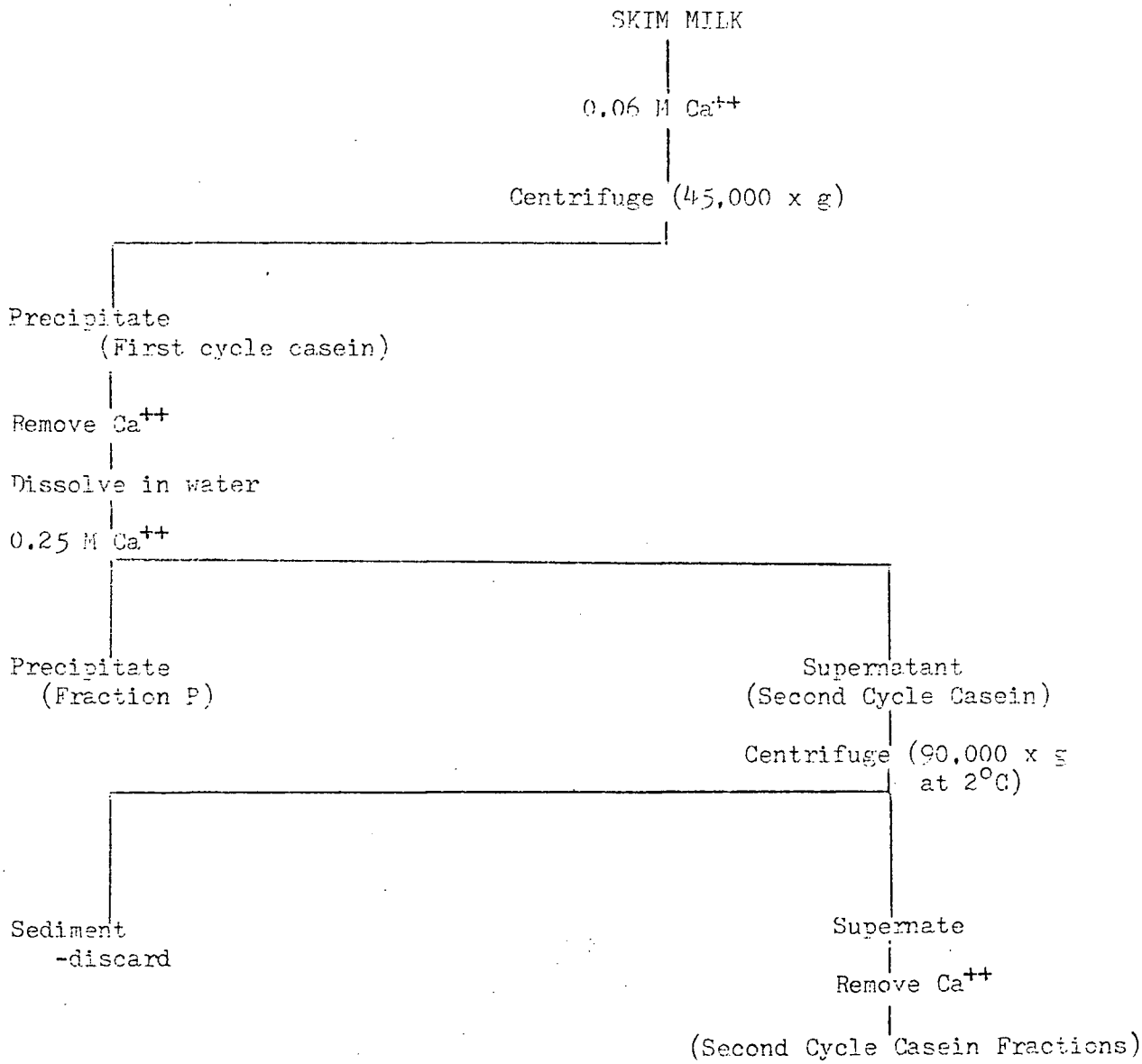
In 1939 Melander first observed the resolution of acid casein by moving boundary electrophoresis at pH 8.6 into three peaks which he named α-, β-, and γ- in order of decreasing mobility (27). It was originally thought that α-casein was a single protein and that it was the fraction upon which the enzyme rennin acted. However, it was found in 1956 that part of the α-complex was calcium insoluble and part of it was calcium-soluble. The enzyme rennin acted at least one thousand times more rapidly on the latter than it did on the other caseins. At this time the term α_s-casein was applied to the calcium-sensitive fraction and the term κ-casein was applied to the rennin-sensitive, calcium-soluble portion. The discovery and purification of the latter will now be briefly discussed.

B. The Discovery and Purification of κ-casein

The idea that one of the caseins acts as a protective colloid to stabilize the milk system dates back to the work of Hammerstein in 1872-77 (13). This view was supported by Linderstrøm-Lang in 1923 (21). He stated that casein consisted of at least two components, one insoluble and the other soluble, in the presence of calcium (II). This latter component or protective colloid interacted with the calcium-insoluble component to prevent its precipitation. This protective colloid was demonstrated by Waugh and von Hippel in 1956 (60). These workers centrifuged (45,000 x g) skim milk after addition of 0.06 M calcium chloride and obtained calcium caseinate gel (first cycle casein fraction). After removal of calcium ions from first cycle casein and dissolution in water, the calcium concentration was brought to 0.25 M with calciun chloride. A precipitate was obtained, designated second cycle casein - fraction P. The supernatant of second cycle casein was then centrifuged (90,000 x g at 2°C) and the sediment discarded. Calcium was removed from the supernatant and this fraction was called second cycle casein - fraction S. The procedure is shown in Figure 1.

It was found that the addition of calcium to the second cycle casein fraction P at concentrations markedly lower than those found in milk, led invariably to the formation of a coarse, heavy precipitate. Addition of calcium to the first cycle casein led to the formation of stable micelles of skim milk. It was reasoned, therefore, that a stabilizing factor was removed during the separation of the secondary cycle fraction P. Support for this possibility was obtained when it was demonstrated that stable micelles formed when appropriate amounts of fraction P and the corresponding supernatant, freed of calcium, were combined in the presence of calcium levels similar to those found in

Figure 1. The preparation of casein fractions according to Waugh and von Hippel.



skim milk. Thus it was at this time that the α-casein fraction as named by Mellander (27) was shown to consist of a calcium-sensitive fraction called α_s - and a calcium-insensitive fraction called κ-casein. Since then many methods have been suggested for the purification of κ-casein.

By 1952 several workers had succeeded in fractionating whole casein into the three major electrophoretic components mentioned earlier. Wake (23) succeeded in the isolation of highly pure κ-casein from second cycle casein fraction S. McKenzie and Wake (24) improved upon this method by isolating α-casein by the alcohol fractionation method of Hipp et al (23), followed by removal of α_s -casein by calcium precipitation. Swaisgood and and Brunner (1962) (49) dissolved whole acid casein in 4.6 M urea and prepared κ-casein by preliminary removal of the other caseins with 12% trichloroacetic acid. Hill (1963) (14) combined Waugh and von Hippel's method with a DEAE cellulose chromatography step for the purification of κ-casein.

These and several other methods (22, 55, 56) have been proposed and each one has its advantages and disadvantages. The main disadvantage of all of these methods is that they are tedious and time-consuming and do not give high yields. Zittle and Custer (1963) (66) proposed a method that has had the widest use because it is relatively simple to perform and gives high yields. Its main disadvantage is the harsh chemical treatment that the method entails. Whole casein dissolved in 6.6 M urea is brought to pH 1.3-1.5 with sulfuric acid. Reduction of the urea concentration by the addition of two volumes of water results in a precipitate containing α_s - and β -caseins. The κ-casein in the supernatant is removed by ammonium sulfate, and this is followed by two alcohol fractionation steps. Experiences with large scale preparations of κ-casein using this method are given later in this thesis. Since a

necessary preliminary aspect of the present work involved the improvement of the electrophoretic technique to the point where the subfractions of κ-casein could be readily observed, the subject of the electrophoresis of caseins will now be reviewed.

C. Electrophoretic Analysis of Caseins

For many years following Mellander's work on moving boundary electrophoresis of casein, difficulties regarding the interpretation of the electrophoretic patterns existed. It became increasingly apparent that the caseins interacted with themselves and with one another (23). Many workers demonstrated that considerable interactions took place in the α-peak (5,6,48,54,59). Moreover, the results of Krecji et al (1941) (20) indicated that interactions between α- and β - peaks took place. These early studies showed the heterogeneity of casein in that inclusion of calcium in the buffer systems resulted in a decrease in intensity of the α peak and an increase in material remaining at the origin. It became more and more apparent that, if reliable information on the heterogeneity of casein was to be obtained, a method of resolution would have to be used in which the caseins were dissociated to their monomeric forms.

A careful study of this problem was conducted by Wake and Baldwin in 1961 (58). They carried out electrophoresis of whole acid casein in starch gels using tris-citrate buffer (pH 8.6) containing 7 M urea. They found over twenty bands. A number of α-casein fractions were examined and found to have a major band identifiable with the major bands in whole casein, but they also exhibited a number of minor bands. Preparations of β- casein showed considerable variation in heterogeneity, all giving one major band in common with the second most pronounced band in the whole casein, but a number of preparations showed other bands as

well. A smeared zone was observed for κ-casein with some sharp bands superimposed on the smeared bands.

Neelin et al (1963) (36) added to this work by examining the effect of urea concentration on causing dissociation and of pH and buffer type on resolution. Mobilities increased with increasing pH, but above pH 7.0 the separation of major zones diminished. Neelin et al preferred pH 7.0-7.2 (cacodylate buffer, 5.5 M urea) for comparison of more mobile bands, but pH 8.2-8.4 (veronal, 4.8 M urea) for slower moving bands. The problem of "smearing" and lack of complete reproducibility of κ-casein behavior still remained.

Peterson (1963) (42) obtained good resolution of α- and β-caseins by using polyacrylamide gel with a 4.5 M urea, Tris-EDTA-boric acid (pH 9.0) mixture. This has been widely used and Aschaffenburg (1964) (1) used it for typing α_s- and β-caseins by electrophoresis of whole milk samples.

Thompson (1965) (53) carried out a collaborative survey of the reproducibility of typing α_s- and β-caseins by the various gel electrophoretic procedures used in a number of laboratories. On the whole the agreement between the various workers has been satisfactory. However, according to McKenzie (23), the present procedures are empirical and fundamental studies of resolution in zone electrophoresis is necessary before the heterogeneity of the caseins can be more objectively understood.

Several workers concentrated on solving the smearing problem that was evident when electrophoresis was carried out on κ-casein, even in the presence of 7 M urea. MacKinley and Wake (1964) (25) treated κ-casein prepared by the method of McKenzie and Wake (1961) (24) with sodium sulfite to break any S-S bands that might be present.

They then examined the product by the urea-starch gel electrophoretic method of Wake and Baldwin (1961) (58). The S-sulfo- κ-casein showed two major sharp bands and three minor sharp bands. Likewise Neelin (1964) (36) found two major sharp bands in κ-casein which had been prepared from pooled milk by the method of Zittle and Custer (1963) (66) and reacted with 2-mercaptoethanol to reduce S-S bonds. On the other hand, reduced κ-casein preparations from milk of individual cows showed the two main bands or either one of them. Woychik published almost identical results at about the same time (1964) (61) and suggested that the presence or absence of these bands was under genetic control. Minor bands were also present, the fastest of which migrated slightly slower than the β-casein band. The notion that these two major bands were under genetic control was supported by Schmidt (1964) (46) and MacKinley and Wake (1965) (26). The latter workers (23) also prepared S-carboxymethyl κ-casein (SCM- κ-casein) purified from pooled milk. The κ-casein showed 5 bands on urea-starch gel electrophoresis. This indicated that κ-casein consists of subfractions joined together by disulfide bonds. The κ-casein samples used were a mixture of types A and B and it remained for other workers to show that each type consists of disulfide-linked subfractions.

D. The purification of κ-casein Subfractions

Dumas (1961) (9) achieved partial fractionation of whole casein on DEAE cellulose columns using 0.02 M imidazole-HCl-3.3 M urea buffer, pH 7.0, and a linear sodium chloride gradient from 0 to 0.6 M. Rose (1963) (43) unsuccessfully applied this technique to the fractionation of κ-casein. Thompson (1966) (2), recalling Neelin's use of 2-mercaptoethanol in the electrophoresis of κ-casein (36), incorporated this component into the chromatographic buffer system and obtained

fairly extensive fractionation of whole casein. In the same year Schmidt et al (47) reported an attempt to fractionate κ-casein on DEAE cellulose using an imidazole-HCl buffer system and stepwise elution with sodium chloride. Five peaks were obtained from the homozygous κ-casein samples applied to the column. Only one subfraction from each of the K-A and K-B was obtained which was homogeneous on starch gel electrophoresis. This degree of separation was good enough, however, to show that N-acetyl neuraminic acid increased as the fraction mobility increased and that the cystine and phosphorous content did not differ between the subfractions. Further, it was found that the ability to stabilize α_s-casein against precipitation by calcium did not vary between the subfractions. Amino acid analysis of K-A and K-B revealed that K-B contains one aspartic acid less and one proline, alanine and isoleucine more than K-A. This work supported the theory of Wake and Baldwin (23) that the major difference between κ-casein subfractions lies in their sialic acid content while K-A and K-B differ in peptide backbone and are therefore under genetic control.

Woychik et al (1966) (62) chromatographed reduced alkylated (carboxamidomethyl-) and reduced unalkylated κ-casein on DEAE cellulose columns in the buffer system of Dumas (9) using a linear gradient of sodium chloride from 0 to 0.15 M. They obtained a major peak and four or five minor peaks from each of κ-casein types A and B. Amino acid analysis showed that each of the subfractions had approximately the same composition; the carbohydrate content was larger in the minor components and this was shown to be the main reason for their higher charge. The subfractions obtained from the reduced, unalkylated κ-casein again aggregated due to intermolecular disulfide bonding.

Sedimentation analysis showed that the molecular weights of the major components, designated A-1 and B-1, were around 20,000. These findings supported the hypothesis that κ-casein is an aggregate that results from intermolecular disulfide bonding and that the basic monomer unit is obtained by reduction. All of the functional ability of κ-casein resides in the polypeptide unit since all components stabilized α_s-casein equally well.

It is important to add at this time that Hill and Wake (1969)(18) feel that the strength of the interaction of each subfraction with α_s-casein should be tested in order to see if the hydrophilic carbohydrate portion of κ-casein is important in its stabilizing ability. Another reason why it is necessary to use subfractions of κ-casein to test the mechanism of interaction is that the subfraction is the monomer unit (18). Nakai and Clarke (34) have obtained conflicting results in the reaction ratio of κ- and α_s-casein as measured by ultracentrifugation and spectrofluorometry. Other workers (12,38,39,60) have reported various reaction ratios ranging from unity to four α_s molecules to one κ-casein molecule.

Experiments involving the modification of particular amino acids in the protein have been carried out on whole κ-casein. The results using whole κ-casein are difficult to interpret because of the heterogeneity of the protein. It is very difficult, for instance, to separate unreacted molecules from reacted ones as has been done with other proteins that have been modified at a single point on the primary structure (37). These difficulties have shown up in the variation between results of different workers. Talbot and Waugh (50) found a decrease in stabilizing ability after one lysine was modified, whereas

Pepper et al (40) found it necessary to modify five residues of lysine in κ-casein before there was any loss in stabilizing ability. Hill (1970) (16) reported that arginine residues were easily modified by the reagent glyoxal whereas Clarke could not agree (7). It is clear that one cause for conflicting results in the same modification experiments performed in different laboratories is the heterogeneity of the κ-casein molecule. This accentuates the importance of working with the subfractions of κ-casein in order to better understand how this protein stabilizes α_{s}-casein.

Woychik's work on this problem (62) was the most successful at the start of this present thesis. The subfractions were not absolutely pure even after rechromatography. Approximately one year after the start of the present work, a paper was published from France (28) which reported much more success at obtaining subfractions of κ-casein. From that point on, attempts were made to repeat their work. Pure subfractions were obtained and the first two of these were used to modify histidine with the reagent 2-phenyl-1, 4-dibromoacetoin (PDA). Histidine was chosen because it had been implicated as playing an important role in the stabilizing ability of κ-casein. The work which has led to this hypothesis will now be mentioned.

E. The Modification of Histidine Residues in κ-casein

Zittle (67) photooxidized κ-casein with methylene blue in the solution in illuminated Warburg flasks with an atmosphere of air. The respective decreases in tyrosine, tryptophan, and histidine were 35, 80 and 100% when κ-casein was oxidized by an uptake of 15 umoles of oxygen per mole of κ-casein. The stabilizing ability of the oxidized κ-casein was greatly reduced and at this level, a product resulted which was unable to stabilize α_{s}-casein. Rennin did not hydrolyze

this product and it also did not act on a mixture of untreated and oxidized κ-casein. It was reasoned that, whereas the oxidized κ-casein could not react with α_s-casein it is possible that it could react with native κ-casein. Since histidine was the amino acid most affected by photooxidation, it was postulated that the amino acid plays an important role in the stabilizing ability of κ-casein. Nakai added support to this contention when he demonstrated that modifying tryptophan residues with N-bromosuccinimide resulted in no decrease in stabilizing ability (31).

Later, Nakai (31,32) found that alkylation of κ-casein with 2-phenyl-1,4-dibromoacetoin for 24 hours at pH 7.5 resulted in a modification of 1.5 to 2 residues of histidine per molecular weight 28,000 and a decrease in stabilizing ability of 55%. The product(s) of alkylation at pH 6.6 eluted with a Kav of 0 and 0.19 on Sephadex G200, whereas that at pH 7.5 eluted at only one peak with a Kav of 0. The optical density ratio of the latter at 280 nm, pH 12.0 to 220 nm, pH 7.0 was 0.136 as opposed to 0.103 and 0.091 for the Kav 0 and 0.19 peaks of the pH 6.6 modification. This indicated that more alkylation occurred at pH 7.5. Nakai later showed that the reagent is less specific at the higher pH (33). The higher optical density ratio of the pH 6.6 Kav 0 peak as opposed to that of the pH 6.6 Kav 0.19 peak indicated that more alkylation occurred on the aggregated peak and that aggregation followed histidine modification.

Treatment of κ-casein with iodoacetic acid and with diisopropyl fluorophosphate respectively decreased the histidines by 1.5 and 0.5 residues in 5 per molecular weight 28,000. Aggregation was not observed, nor was there any decrease in stabilizing ability. The difficulty with these reagents, however, is that they are not specific (33).

The stabilizing ability of κ-casein alkylated with PDA at pH 6.6 was restored by treatment with base, whereas that of κ-casein alkylated at pH 7.5 was not. Nakai has shown that cystine is decreased during alkylation at the latter pH (33). Treatment with 0.2N NaOH at 25°C for 30 minutes restored most of the stabilizing ability of sterilized and stored κ-casein, similar to the PDA-alkylated casein at pH 6.6. Reoxidation of PDA-alkylated κ-casein at pH 6.6 after reduction with 0.03 M mercaptoethanol in 6 M urea and dialysis (32) restored stabilizing ability; whereas 0.06 M mercaptoethanol restored only one-half of the stabilizing ability of κ-casein stored for 90 days. The elution patterns did not change regardless of how much the stabilizing ability was restored. Furthermore, both peaks for PDA-alkylated κ-casein at pH 6.6 showed decreased stabilizing abilities regardless of their differences in aggregation. Sulfo-compounds in stored κ-casein may be implicated in decreased stabilizing ability. Nevertheless, it is felt that histidine could be involved in that aggregation follows the modification of these residues, possibly due to a strengthening of the hydrophobic bonds, since histidine in its native form takes part in hydrogen bonding in proteins (32). Nakai found that the total amount of histidine residues decreased after dialysis or gel filtration of stored κ-casein and concluded that "the sum of free and masked histidine residues were responsible for decreased stabilizing ability and conceivably deeply masked residues may not be available for maintaining stabilizing ability" (32). Later, Tatto and Nakai (51) identified low molecular weight amino compounds derived from sterilized and stored κ-casein as NH_3 and cysteic acid. They attributed the presence of NH_3 to the heat decomposition of amide and histidine

residues in the κ-casein and the cysteic acid to the oxidation of cysteine. Again it was suggested that histidine may play a role in the stabilizing ability of κ-casein and the destruction of this amino acid may be one of the causes of gelation of stored milk products, such as ultra high temperature pasteurized milk, condensed milk, and chocolate milk.

The major purpose of this thesis was to specifically modify histidine residues in κ-casein and two of its subfractions. Much preliminary work had to be done in order to get at the major problem, and it is felt that important observations can be made from experience at each preliminary step.

CHAPTER II

Practical Experience in Large Scale Production of Zittle's κ-casein

The purpose of the present chapter is to report experiences with attempts to scale up the method of Zittle (66) so that approximately 60 grams of κ-casein could be purified at one time.

MATERIALS

The starting material for 60 grams of κ-casein was 600 g of whole casein. This was obtained from approximately 6 gallons of whole milk from a cow known to be homozygous for κ-casein type A. The milk for the first three batches of κ-casein was obtained from a cow named "Ideal Model" and that for the fourth batch was obtained from "Tune". Both cows were free of mastitis. Other materials required were:

(1) A container to hold at least 20 litres of liquid. In the present work one 5-gallon, two 10-gallon, and one 17-gallon plastic buckets were used.

(2) A cream separator. Alternately, milk may be skimmed by centrifuging (800 rpm) for 20 minutes at room temperature, followed by centrifugation at the same speed and time at 4°C. The skim milk is simply poured off. This is a very effective procedure but not practical for large quantities of milk.

(3) A detachable electric mixer.

(4) An electrophoresis apparatus. This was used to test the purity of the final product and to test the efficiency of the fractionation steps. The apparatus used were those described by Perrin (41) and by Beveridge (2).

(5) A pH meter or pH Hydrion papers.

(6) Chemicals. The following quantities of chemicals were required for the preparation of 40-60 grams Zittle's κ-casein.

3 gallons of 95% ethanol

2 litres of concentrated hydrochloric acid

400 ml of concentrated sulfuric acid

52.3 pounds of urea

5.5 pounds of ammonium sulfate

10.0 grams of ammonium acetate

METHODS

A. Typing of Cows

Ten cows were typed by a modification of the polyacrylamide gel electrophoresis technique of Thompson (53). Two tenths millilitre of whole milk were dissolved in approximately 0.1 ml of a solution consisting of 0.5 ml 2-mercaptoethanol and 5.0 ml with tris-glycine buffer, pH 9.1. Alternately, sufficient urea was added to 0.2 ml of milk to disperse the casein, then 1 drop of 2-mercaptoethanol and 1 drop of N NaOH were added.

B. Purification of κ-casein by Zittle's Method

Each step will be commented on during the Results and Discussion section.

RESULTS AND DISCUSSION

Observations and comments on each step of the procedure are as follows.

A. Skimming the milk

It took two full days to collect enough milk to start the separation. During this time the milk was stored at 4°C. It was

found that when milk was run through the separator at this temperature it did not separate at all. When it was run through at room temperature it had to be recycled four times in order to achieve sufficient separation.

When the milk was heated to 45°C excellent separation was obtained by one passage through the separator.

B. Preparation of Whole Casein

During the first trial it was found that a poor yield of casein was obtained. The reason for this is that the milk was at the low temperature of 15-20°C during the separation of casein. It was found during all four trials that the amount of 0.1 N HCl required to bring the pH of the milk to 4.5 was exactly half the volume of skim milk.

The speed at which the acid is added to the skim milk is very important and thorough mixing must be done throughout the addition; otherwise isolated areas of coprecipitation occur which are not readily redissolved.

C. Filtering Casein

It is recommended that close knit material such as silk be used instead of cheesecloth because too much casein is lost using the latter. The filtration and washing processes are very slow using the former but very little casein is lost through the material.

D. Washing Casein

It is recommended that the filtered casein be placed in a large bucket and approximately 20 volumes of distilled water added. The casein should be washed thoroughly by mixing. The casein should not be left standing in the wash water but all three washings should be done as soon as possible. This is a tedious part of the operation and requires approximately eight hours to complete.

E. Redissolving Casein

The casein should be made up to the concentration it was in the skim milk. For instance, if 24 litres of skim milk is the starting amount then the casein precipitated from this should be redissolved in a total of 24 litres of water.

Upon adding the water to the casein the latter should be broken up into very small pieces and then stirred rapidly. The pH can be maintained at 7.5 by attaching the electrodes of an automatic titrator to the sides of the bucket with masking tape and allowing 1N NaOH to drop in through the automatic switching unit. The casein takes at least three hours to dissolve. During the first three trials the pH of the solution was measured by pH Hydrion papers and 1N NaOH added as needed. The use of the titrator therefore speeded up the process and also saved a great deal of labor.

F. Reprecipitating Casein

This was done as before and again half the volume of 0.1N HCl was used to precipitate all the casein. The HCl again was added slowly, the casein collected as before and then squeezed by winding the cloth around it. It was then hung up and allowed to drain in the cold room. Before discussing the next step there are several things which should be kept in mind about the scheme followed up to this point.

The practice of collecting the milk over a two-day period, warming it up to 45°C, allowing it to cool to room temperature or lower is not a good practice from a bacteriological standpoint. It meant that by the time the separation of casein was started part of the milk was 72 hours old, and that five days elapsed before commencing purification of the casein. It is recommended that the casein be separated from several lots of afternoon milk from the desired cow and frozen in blocks

until enough is collected. This procedure would be safe from a bacteriological point of view and also allow skimming and casein precipitation while the milk is still warm, which will result in higher yields of casein. If the temperature of the milk decreases markedly during the skimming, the milk should be carefully warmed to 30°C before precipitating the casein.

G. Dissolving the casein in 6.6M urea

Zittle's original procedure (66) called for the dissolution of from 60 to 95 grams of dry casein in 1 litre of 6.6M urea. If the casein used is frozen it is good to know the moisture content so that the correct final volume of solution is obtained.

The urea (52.3 lbs.) was added directly to the casein and mixed thoroughly. At first a very viscous paste was formed. The casein was broken up into small pieces by crushing it against the sides of the container with a large stirrer. Eventually the casein was completely dissolved and water was added to make the total volume up to 6.0 litres. This usually required approximately 1.5 litres of water. It took approximately two hours for the casein to completely dissolve during this step. After learning how much water it would take to make up the final volume, during all other batches the water was added in with the urea so as to limit the concentration of the latter to as close to 6.6M as possible.

H. Decreasing the pH to 1.3-1.5

This step was accomplished by dropwise addition of 1,200 ml of 7N H_2SO_4. The solution was stirred very thoroughly with a detachable electric stirrer. The addition of acid was made by placing a separatory funnel on a ring stand and allowing the acid to drop into the solution.

I. Diluting with water

Twelve litres of water were then added by allowing the water to run in very slowly with thorough stirring throughout the addition. It is important to decrease the pH before adding the water, otherwise the casein will precipitate simply by acid precipitation.

As the addition of the water nears completion a precipitate forms which becomes more and more flocculent. The solution was allowed to stand for another $1\frac{1}{2}$-2 hours and the precipitate was separated.

J. Separation of the Precipitate

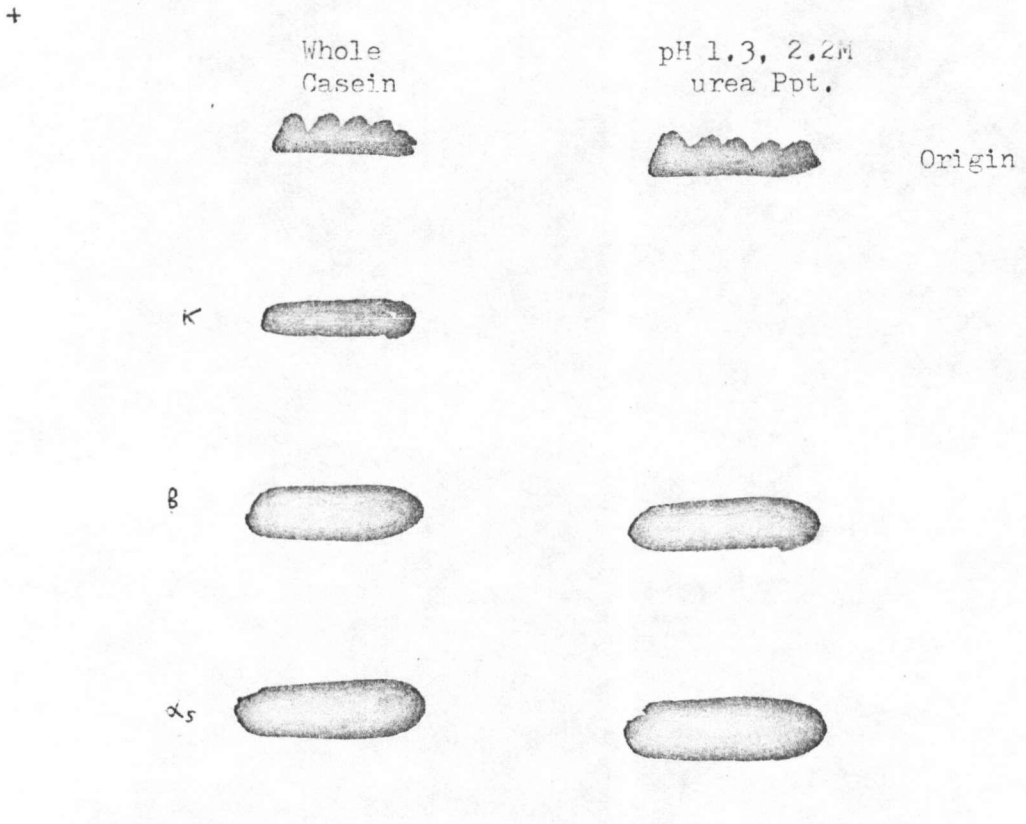
The procedure recommended by Zittle was to filter the precipitate. This was attempted but found to be unsuccessful because it was a very slow process and also because the filtrate was still very milky after filtration. Better separation was obtained when centrifugation was done at $4^{\circ}C$ at 2,500 rpm for 10 minutes in a Sorvall refrigerated centrifuge. Figure 2 shows that the precipitate contained no κ-casein. This indicated that this step in the procedure was successful. The precipitate was frozen for later use in the purification of α_s- and β-casein.

It was found that during the centrifugation period, which lasted 20 hours, that the mixture of the precipitate and supernate should be stored at room temperature as the α_s - and β-caseins are soluble at refrigeration temperature (3). A difficult question and perhaps a source of error at this point is the wisdom of holding the caseins in this amount of urea (2.2M) for such a long period. There is one theory which states that the exposure of κ-casein to urea during the purification procedure could give rise to the subfractions through the process of carbamylation (17).

K. Ammonium Sulfate Precipitation

The volume of the filtrate was measured and ammonium sulfate was

Figure 2. Electrophoretic analysis of the pH 1.3, 2.2 M precipitate in Zittle's method.



added to make a final concentration of 1 molar. For 16 litres of liquid this amounted to 2,112 kilograms. Stirring was not done during addition of the ammonium sulfate, the solid material was simply added slowly and allowed to sink to the bottom of the liquid. After all the ammonium sulfate was added the mixture was stirred slowly with a long knife and the precipitate separated by filtration.

Figure 3 shows the results of electrophoresis analysis of the precipitate obtained. Very little β-casein was present and no α_s-casein could be seen. Material at this stage could be used for the chromatographic separation of subfractions. But when it was desired to use whole κ-casein, the impurities were removed by alcohol fractionation.

L. Alcohol Fractionation

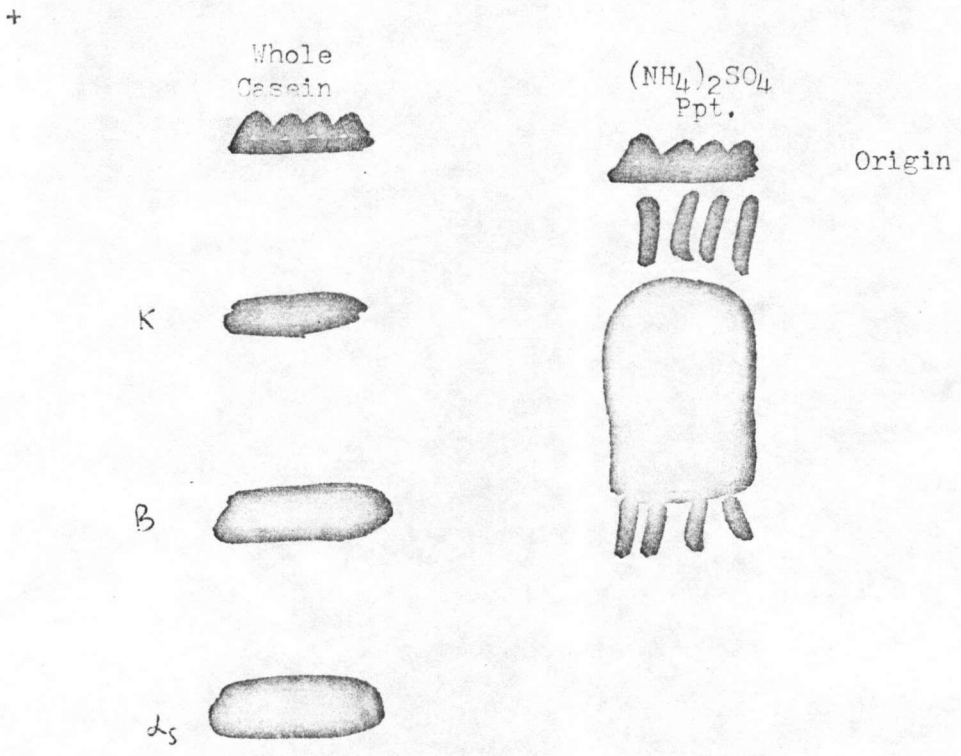
The precipitate obtained by ammonium sulfate fractionation was dissolved in water at a concentration of approximately 1% protein and dialyzed for 72 hours in the cold room against 80 litres of water. The final volume of dialyzed solution was four litres.

The concentration of protein was checked by the optical density method of Nakai (33) using the conversion factor of 11.69 as being equivalent to 1% κ-casein.

The solution was diluted with the necessary amount of water to adjust the final protein concentration to 1%. Two volumes (usually 8-10 litres) of 95% ethanol were slowly added and then 2M ammonium acetate was added dropwise until a dry precipitate formed. The precipitate was mucilaginous and gathered at the bottom of the container.

A mistake was made during the preparation of a small batch of κ-casein in that the mixture was stirred during the dropwise addition of ammonium acetate. The precipitate was very gummy - like thick glue. The stabilizing ability of this κ-casein turned out to be as good as

Figure 3. Electrophoretic analysis of the ammonium sulfate precipitate in Zittle's method.



the other batches and the subfractions obtained from this batch of κ-casein were like those from the other batches; but this indicates a difficulty that can occur during alcohol fractionation. Stirring in some way leads to greater aggregation of the protein.

The precipitate obtained from this step was dissolved in water by breaking it up and blending small amounts in a blender jar. The pH was raised to 7.5 by the addition of N NaOH. Blending, admittedly, could lead to further aggregation but the precipitate could not be handled in any other way. The solution was dialyzed in the same way as was the ammonium sulfate precipitate.

Following dialysis the concentration of protein was again adjusted to 1% and the alcohol fractionation procedure repeated. The sticky precipitate was again dissolved, dialyzed, and this time freeze-dried. The desired yield from this amount of starting material was 60 grams of κ-casein. This would be 10% of the starting material. Zittle's original paper (66) stated yields of 7 to 10% of the total starting material.

The highest yield that was obtained during the present studies was 45 grams of κ-casein. This was 7.5% of the starting material. The lowest yield was 36 grams or 6.0% during the first attempt.

It is felt that one suggestion that will increase the yields is that the casein be prepared from several small batches of milk that can be carefully warmed to 30°C unless an automatic system for the continuous preparation of casein is used at a dairy. The pilot plant pasteurizer could be used for large scale production with the only difficulty being that it would take a long time to collect all the milk and a storage problem arises.

Two people should work on this together because it is too long a job for one person, especially such stages as the washing of the casein and the centrifugation of the α_s- and β-casein precipitate.

Properties of the Purified κ-casein

Figure 4 shows that the κ-casein purified by the above method was free of impurities. Electrophoretic analysis agrees with that of MacKinlay and Wake (25) which showed κ-casein to be a heterogeneous protein.

The κ-casein from the first batch had lower stabilizing ability than the other preparations. It was also difficult to dissolve this κ-casein.

Treatment of this κ-casein with urea and mercaptoethanol according to Nakai (32) resulted in a complete restoration of stabilizing ability and solubility.

It is felt that the reason for these observations is that there was greater aggregation of the first preparation of κ-casein, probably due to lack of experience with the method and because the alcohol precipitation step was accompanied by too vigorous stirring throughout the procedure. Again it is strongly recommended that the very minimum of stirring be carried out during the alcohol fractionation step.

Table I gives the amino acid composition of two batches of κ-casein. These results agree very closely with those published in a recent review by McKenzie (23).

It will be noticed that the subfractions of κ-casein cannot be seen in the electrophoretic analysis (Figures 3,4).

Attempts to modify the method of electrophoresis to the point where the subfractions can be readily seen will be the subject of the next chapter.

Figure 4. Electrophoretic analysis of the first preparation of κ-casein.

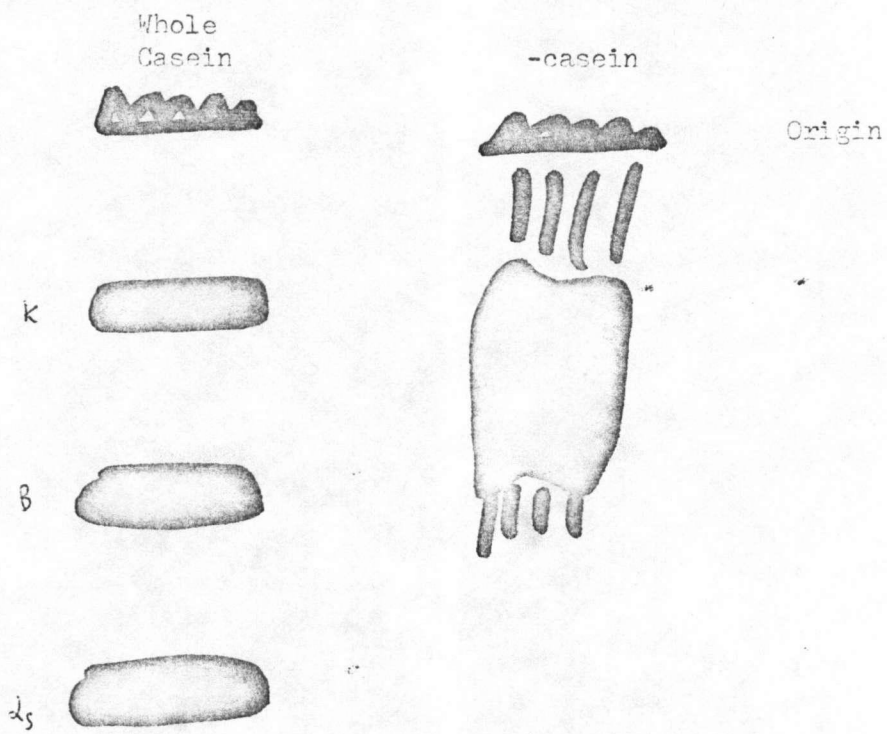


Table I. Amino acid composition of two preparations of k-casein purified by the method of Zittle.

Amino Acid	k-casein	
	First Preparation	Second Preparation
	Residues per 20,000 g*	
Aspartic acid	14.9	15.0
Threonine	16.5	16.5
Serine	14.6	14.4
Glutamic acid	31.0	31.5
Proline	22.6	23.0
Glycine	2.9	2.9
Alanine	16.6	16.6
½ Cystine	2.5	2.5
Valine	12.9	12.9
Methionine	2.0	2.0
Isoleucine	13.9	13.9
Leucine	10.4	10.4
Tyrosine	8.0	8.0
Phenylalanine	4.5	4.5
Lysine	9.6	9.6
Histidine	3.0	3.1
Arginine	5.9	5.9

* Statistics based on earlier analyses done by Nakai (33).

CHAPTER III

Attempts to Improve the Electrophoresis of κ-casein

A large number of attempts have been made by various workers in this and other laboratories to obtain the distinct separation of subfractions of κ-casein by starch and by polyacrylamide gel electrophoresis. The latter was chosen in these studies because it was felt that it was easier to prepare and handle. Several discouraging initial runs showed that results were very inconsistent and therefore it was decided to thoroughly test at least three factors known to be important in determining the success of an electrophoretic run. Attempts were then made to control these parameters closely. These variables included

(1) the type of bridges used;

(2) the voltage through the gel; and

(3) the ionic strength of the buffers.

MATERIALS

The electrophoresis apparatus used in this study was the one described in detail by Perrin (41) and by Beveridge (2). A paper electrophoresis apparatus was also adapted to test the effect of high voltage (maximum 250 volts) through the polyacrylamide gel. This apparatus was equipped with a water jacket for cooling the system.

Tris-glycine buffer was prepared by dissolving 207.4 grams of glycine in water and bringing the total volume up to 6 litres with distilled water. The pH was adjusted to 9.1 by the addition of 2.4 ml of 30% NaOH.

For gels of 10% strength 10 grams of acrylamide and 0.5 grams of N, N^1-methylene-bis acrylamide as well as 32 grams of urea were dissolved in tris-glycine buffer diluted 1:1. The final volume was made up to 100 ml with this buffer. Then 1 ml each of 10% (w/v) ammonium persulfate, 30% (v/v)

tetramethylethelenediane (TMED) and 0.34 ml of 2-mercaptoethanol were added. The gel was then poured into a mold made of plexiglass and a cover made of the same material was placed on top of the gel. After the gel had solidified the cover of the gel was replaced by saran wrap.

Special bridges were constructed and tested during the first phase of the study. These are listed below.

(a) Cheesecloth and Filter Paper Bridges

Listed in Table II are the words Cheesecloth and Filter Paper with various subscripts. These subscripts represent the thickness or number of folds of the respective materials. The filter paper used was Whatman No. 3.

(b) Agar Bridges

These were prepared by placing 2% Difco Flake Agar dissolved in 5% KCl into glass U-tubes. On two occasions this agar was poured onto three thicknesses of filter paper and in Table II this is designated as Filter Paper & Agar.

(c) Polyacrylamide Gel Bridges

These were constructed by preparing a 10% gel as described before but leaving out the urea and mercaptoethanol.

(d) Sponge Bridges

This type of bridge consisted simply of strips of the sponge used to line the drawers in the laboratory.

METHODS

A. Preliminary studies

Voltage measurements were taken at various points on the electrophoresis apparatus (2,41) equipped with different bridges and buffers.

B. Changes in the technique

The following modifications in the technique were applied:

1. Based on a suggestion by Dr. Yaguchi (63), 10% gels were prepared without urea and mercaptoethanol and equilibrated for one day in deionized water and for at least two days in 8M urea/0.0875M Tris-glycine buffer, pH 9.1/0.001M 2-mercaptoethanol. Mercaptoethanol was added daily. Following equilibration the gels were dried off with paper towels before being used for electrophoresis.

2. The high voltage paper electrophoresis apparatus was adapted as follows:

> Each terminal has three separate buffer compartments so that paper strips can be run. Three layers of filter paper were cut to act as one bridge connecting the three buffer compartments on each side to the gel. A cover was placed on the gel and water run through the cooling jacket during the electrophoresis run. The electrophoresis was run for 24 hours with a voltage of up to 265 passing through the gel.

3. The modification based on the suggestion of Yaguchi was combined with the use of the adapted high voltage paper electrophoresis apparatus described above. The run was for only 4 hours.

4. Ten per cent gels containing 4.5M urea were prepared by bringing the final volume up to 100 ml with 0.0875M or 0.0175M Tris-glycine buffer and dissolving the ingredients while warming to 25°C. One-half millilitre of 2-mercaptoethanol was added, followed by 1.0 ml each of 10% (w/v) ammonium persulfate and 30% (v/v) tetramethylethylenediamine (TMED). The gels were poured into the molds and plexiglass covers placed on them. After the gels were set (40-60 minutes) the plexiglass covers were replaced by saran wrap and the gels run for 24 hours without samples. Next day the samples were applied and the electrophoresis run for 24 hours with a gel voltage of 100 volts and a current of 20-25 milliamperes.

RESULTS AND DISCUSSION

A. Preliminary studies

Table II shows the results of voltage measurements taken through different points on the electrophoresis apparatus as affected by different combinations of bridge and buffer systems. The final system that was chosen on the basis of these experiments was the one consisting of 0.1M NaCl in the outer chambers, 0.0875M Tris-glycine buffer, pH 9.1 in the inner chamber and gel, and bridges consisting of ten thicknesses of cheesecloth.

Cheesecloth bridges were chosen over filter paper bridges because they are less expensive and can be used longer.

Polyacrylamide gel, agar, and sponge bridges were not chosen because they were found to be difficult to handle and offered no advantage in regard to electrical conduction over the cheesecloth bridges.

B. Modification in the technique

Figure 5 is a drawing of electrophoresis runs that show good separation of κ-casein subfractions. This type of separation was obtained when i) the modification based on Yaguchi's suggestion, ii) the combination of the adapted high voltage apparatus with Yaguchi's suggestion and iii) the technique based on suggestions of Nakahori and Nakai were used.

Use of the high voltage apparatus on gels which were not previously equilibrated gave good migration but no separation of the subfractions. It is suggested that this modification could be used successfully if the equilibration step was included.

The one disadvantage of treating the gels according to Yaguchi is that they swell up and become very brittle.

Table II. The effects of buffer and bridge systems on voltage measurements taken at various locations on the electrophoresis apparatus.

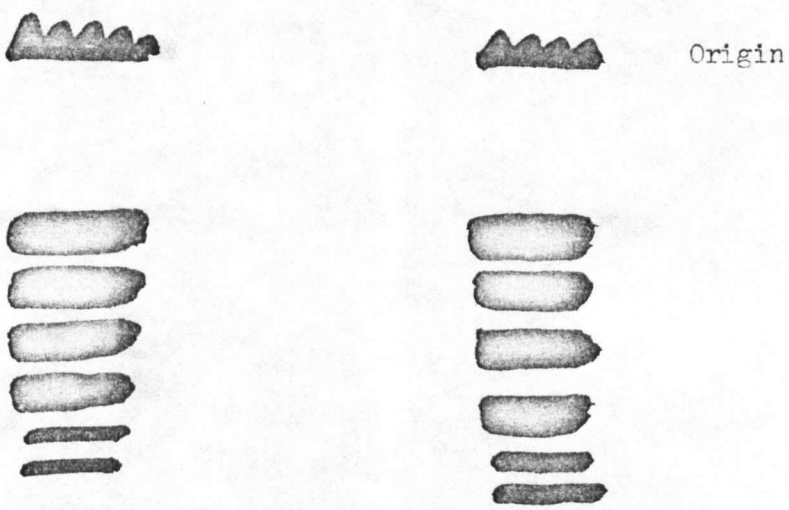
Expt. No.	Buffer used Outer	Inner	Gel	Bridge System Outer	Inner & Gel	mA	VOLTAGE Initial	Outside Ter'l.	Outer Chamber	Inner Chamber	Gel
1	0.1 M NaCl	0.35 M Tri-gly	0.35 M Tri-gly	F.P.$_1$	F.P.$_1$	30	340	340	325	325	100
2	"	"	"	F.P.$_2$	F.P.$_2$	30	270	268	258	190	100
3	"	"	"	F.P.$_3$	F.P.$_3$	30	197	197	188	148	83
4	"	"	"	F.P.$_3$+Agar	"	30	180	180	169	145	76
5	"	"	"	Agar	"	30	222	220	210	168	80
6	"	"	"	"	"	30	262	260	252	165	80
	----------Repeated----------					30	259	259	240	162	80
7	0.1 M NaCl	0.35 M Tri-gly	0.35 M Tri-gly	F.P.$_3$+Agar	"	30	180	178	169	145	75
8	"	"	"	F.P.$_3$	"	30	192	190	190	149	78
9	5% KCl	"	"	"	"	30	262	261	252	172	82
	----------Repeated----------					30	255	252	252	170	90
10	5% KCl	0.35 M Tri-gly	0.35 Tri-gly	Agar	"	30	180	180	175	150	90
11	0.1 M NaCl	"	"	ChC$_1$*	ChC$_1$*	30	92	89	82	72	10
12	"	"	"	ChC$_2$*	ChC$_2$*	30	99	95	90	79	18
13	"	0.35 M/4 Tri-gly	0.35 M Tri-gly	ChC$_3$*	ChC$_3$*	30	102	98	90	75	22
14	"	"	"	ChC$_{10}$*	ChC$_{10}$*	30	195	195	185	160	90
15	"	"	"	ChC$_{20}$*	ChC$_{20}$*	30	170	170	160	132	85
16	"	"	"	ChC$_{15}$*	ChC$_{15}$*	30	180	180	174	140	80

Table II. (Cont'd.)

Expt. No.	Buffer used Outer	Inner	Gel	Bridge System Outer	Inner & Gel	mA	VOLTAGE Initial Voltage	Outside Ter'l.	Outer Chamber	Inner Chamber	Gel
17	0.1 M NaCl	0.35 M Tri-gly	0.35 M/4 Tri-gly	ChC_{10}*	ChC_{10}*	30	225	225	215	180	140
18	"	0.35 M/4 Tri-gly	"	ChC_{10}*	ChC_{10}*	30	270	270	255	225	168
19	"	0.35 M Tri-gly	0.35 M Tri-gly	F.P.3	F.P.3	30	190	190	178	169	138
20	5% KCl	"	"	"	"	30	218	218	210	188	118
21	0.1 M NaCl	0.35 M/4 Tris-gly	0.35 M/4 Tris-gly	"	"	30	190	190	180	170	99
22	5% KCl	0.35 M Tris-gly	0.35 M/4 Tri-gly	KCl Agar	"	30	300	298	290	188	110
23	"	"	"	"	ChC_{10}*	30	260	259	249	150	110
24	"	"	0.35 M/4 Tris-gly	ChC_{10}*	"	30	207	207	200	190	130
25	"	0.35 M/4 Tris-gly	0.35 M/4 Tri-gly	"	"	30	159	159	149	130	100
26	0.1 M NaCl	0.35 M T-gly	"	F.P.3	PAG in 5/4	30	172	171	165	150	100
27	"	"	"	ChC_{10}*	PAG in 0.35 M/4 T-gly	30	170	168	155	140	90
28	"	"	"	2% KCl Agar	"	30	215	215	208	150	99
29	"	0.35 M/4 T-gly	"	F.P.3	"	30	165	165	155	140	100

* Cheesecloth

Figure 5. Electrophoresis of κ-casein showing good resolution of subfractions.



SUMMARY

Several modifications of the electrophoresis technique described by Perrin (41) and Beveridge (2) gave promising results in the separation of the subfractions of -casein. These changes included steps to increase the voltage through the gel, equilibration of the gels with the buffers, increasing the urea concentration, decreasing the ionic strength of the buffer system, and changing the bridges.

CHAPTER IV

Separation of Subfractions of κ-casein by DEAE Cellulose Chromatography

As mentioned in the Introduction and Literature Survey, several workers have attempted to fractionate κ-casein by various modifications of DEAE cellulose chromatography. The first attempt in this laboratory was reported by Beveridge in 1968 (2). His thesis stated quite a number of serious difficulties involved in achieving good separation. The purpose of the present chapter is to describe in detail several important modifications of the method followed by Beveridge which have resulted in the successful purification of κ-casein subfractions.

Experimental Plan

Series one

The first series of experiments was a direct repeat of the method described thoroughly by Beveridge, with the only modification being that a micro pump was used to pump the gradient elution buffer onto the column. Several successful runs were obtained using this method but success was not consistent.

Series two

The second series of experiments was a slight modification as suggested by Mercier in 1968 (28). This method gave consistently good separation once experience with the method was obtained.

After achieving good separation on a small column using 300 mg of κ-casein, a large column was used on which 8.8 grams of κ-casein was successfully fractionated into six subfractions.

Series three

The third phase of this study was to rechromatograph subfractions obtained from the first fractionation. An attempt was made to completely purify subfractions which were not well separated on the first run as well as those that were very pure from the first run.

MATERIALS

Some of the κ-casein applied to the column was not carried through all of the purification steps mentioned in chapter two but was κ-casein which was carried only to the ammonium sulfate precipitation stage. Thus the κ-casein contained some α_s - and β -casein. During several of these runs the NaCl gradient was stepped up at the end of the κ-casein subfraction separation so that the buffer would also elute the α_s - and β-caseins. This provided a stock of α_s -casein which was used to carry out stabilizing ability determinations.

The DEAE cellulose was obtained from Whatman Chemical Company. Purified grade urea, imidazole and sodium chloride were obtained from Fischer Chemical Company. Reagent grade mercaptoethanol was obtained from Eastman Kodak Company.

The chromatographic buffer used in the first series of experiments was as follows: 0.01 M imidazole, 4.0 M urea, 3 ml. of 2-mercaptoethanol per litre, pH adjusted to 7.0 by the addition of 1.05 ml. of concentrated HCl per litre of buffer. The buffer was made up fresh each time and filtered before being used. In the second and third set of experiments the buffer was 0.02 M imidazole, 3.3 M urea, 3 ml. of 2-mercaptoethanol per litre, pH 7.0.

METHODS

Series one

The first set of trials was done according to the method described by Beveridge (2) with the only difference being that a micropump was used to pump the buffer onto the column.

Series two

Several important modifications suggested by Mercier in 1968 (28) were incorporated into the procedure. The treatment of the DEAE cellulose is the first major change.

The powdered cellulose is first of all sifted overnight on a shaker and only that between 120 and 200 mesh size is used. This is placed in approximately 10 volumes of 1N NaOH and soaked therein for 30 minutes. This is then washed with deionized water on a filter pad in a Buchner funnel until the wash water reaches approximate neutrality. If the water is still yellow then a repeat of the NaOH soaking and water wash is suggested. The cellulose is then held for 30 minutes in approximately 5 volumes of a solution of 0.1N HCl/25% NaCl followed by washing with 10 volumes of water and suspension into the pH 7.0 buffer described above. A 20 cm x 7 cm column requires approximately 100 grams of sifted DEAE cellulose.

After each chromatographic run the 20 cm x 7 cm column is regenerated by the successive passage of the following three solutions:

1.5 to 2 litres of 0.25N NaOH (time of passage approximately 3 hours)

2.5 to 3 litres of 0.1N HCl/25% NaCl (time of passage, approximately 4 to 5 hours)

3.5 to 4 litres of deionized water

3.5 to 4 litres of pH 7.0 buffer (the flow of buffer is stopped when the column reaches neutral pH).

In the present studies it was found that the last step could be done with a preliminary passage of 3.5 litres of 0.02M imidazole buffer, pH 7.0 without the urea and mercaptoethanol followed by approximately 1 litre of the complete buffer. This cut down somewhat on the amount of urea required.

The use of low molarity NaOH and HCl compared to the procedure used in the first series of experiments is believed to be an important step which would greatly lengthen the life of the DEAE cellulose.

Another suggestion of Mercier was to recrystallize the urea before using it in the buffer. Several runs were attempted without following this step but proved to be unsuccessful. The recrystallization procedure used was as follows:

> 600 grams of Fischer reagent grade urea was dissolved at 70°C in 600 ml of 95% ethanol and 216 ml of deionized water. This was filtered through Whatman No. 1 filter paper and stored in the cold room for 24 hours. The recrystallized urea was collected on a large Buchner funnel and the filtrate was stored in the deep freeze for 24-72 hours after which time more urea had crystallized. This again was collected on a Buchner funnel and washed with cold absolute ethanol. Suction was continued until almost all of the liquid had passed through the urea, and the recrystallized urea was placed in tin foil at room temperature and allowed to dry. The yield of urea was 90-95%.

Preliminary studies were carried out on a column 14 cm x 2.0 cm in size prepared as follows:

> A one-holed rubber stopper of appropriate size was placed on the bottom end of the glass tubing. One layer of cheesecloth was then placed on the bottom followed by a small layer of glass wool, a one-half inch layer of aquarium gravel and one thickness

of Whatman No. 3 filter paper cut to fit the tubing. De-gassed buffer or water was placed to a level of approximately one third of the length of the tube and to this was added the total amount of de-gassed cellulose as would be required to fill the column. One thickness of Whatman No. 3 filter paper was placed on the top of the resin. The cellulose was then washed with successive amounts of the above materials in proportion to the size of Mercier's column. Thus for a 14 cm x 2.0 cm column the following amounts of the four materials were used:

100-150 ml of 0.25N NaOH

170-240 ml of 0.1N HCl/25% NaCl

240-400 ml of 0.02M imidazole buffer, pH 7.0

Approximately 200 ml of complete buffer, pH 7.0.

When first using the dry, sifted DEAE cellulose, an appropriate amount is suspended in a solution of 0.1N HCl/25% NaCl for 30 minutes, washed thoroughly with water and then placed in imidazole buffer, pH 7.0..

Following preliminary studies with the small column, a large column (20 cm x 7 cm) was packed. The bottom of the column contained a sintered glass filter disc and was joined with a very heavy clamp to the upper part of the column. One thickness of Whatman No. 3 filter paper was placed on top of the sintered glass. This was followed by a $\frac{1}{2}$ inch layer of aquarium gravel and either a small piece of glass wool or another layer of Whatman No. 3 filter paper. The column was packed using the same precautions as mentioned above.

Samples were prepared as follows: κ-casein (approximately 7%) was dissolved in the complete buffer and nitrogen was bubbled into the solution for 30 minutes. Then the 2-mercaptoethanol was added in the same concentration as that in the whole elution buffer (3 ml/litre) and the sample stirred a further 15 minutes with bubbling nitrogen gas. The samples (0.3 to 1.0 gram of protein) were placed on the small column with a 5 ml long tip

delivery pipette altered so that the tip was shaped like a fishhook so as not to disturb the top of the cellulose when the sample was added. The sample (5-10 grams of protein) was pumped onto the large column at a rate of 200 ml/hour.

After applying the sample to the large column, the latter was washed with 1 litre of complete buffer containing 0.025M NaCl. The linear gradient gradient 0.025 to 0.135M NaCl was then set up by connecting two 2,500 ml bottles in an open system. The buffer which was pumped directly to the column contained the 0.025M NaCl and was stirred with a magnetic stirrer throughout the operation. The other bottle contained buffer with 0.135M NaCl. Both bottles were of exactly the same size and shape and were level with each other. Preliminary experiments were carried out to see what system would give a suitable gradient. The chloride concentration of every fifth tube was measured by the following procedure:

> One half millilitre of sample was diluted to 20 ml with deionized water. Three drops of 10% $K_2Cr_2O_7$ was added and the mixture titrated with 0.1M AgNO to the first appearance of the reddish-brown precipitate of silver chromate.

Series three

Fractions obtained from the first chromatographic run were rechromaographed on the small columns described above. Two hundred milligrams of sample was applied and treated exactly as above.

Several attempts were made to separate very impure fractions by rechromatography.

All fractions collected were dialyzed against running tap water overnight followed by 72 hours against distilled water. They were then pervaporated to one-half the volume and freeze-dried.

RESULTS AND DISCUSSION

Series one

The first series of experiments gave successful separation but not consistently.

Series two

Figure 6 shows part of a successful run of whole casein. The α_s-casein obtained from this and several other runs was used for stabilizing ability tests of κ-casein.

Figure 7 shows one of the first runs of κ-casein. It is felt that the poor separation evident in this run emphasized the importance of uniform column packing. All of fractions I, II and III denoted by the arrows belonged to subfraction number one as shown by polyacrylamide gel electrophoresis. Fraction IV was a mixture of subfractions two and three, and the rest of the fractions were mixtures. Fraction VI and the rest contained β-casein which was expected since the purification of κ-casein was carried out only to the ammonium sulfate step.

Figure 8 shows one of several successful runs of whole κ-casein. 8.8 grams was applied to the column and the following yields were obtained. KA1, 502 mg; KA2, 343.5 mg; KA3, 565.5 mg; KA4, 327 mg; KA5, 56.7 mg; and KA6.60 mg.

Figure 9 shows a run of whole κ-casein where the gradient was allowed to run by gravity onto the column. The separation was poor and it is believed that the gradient system was the cause for this. Even though separation was not good, rechromatography of the shaded area gave 60 mg of pure subfraction number one.

Series three

The three fractions denoted by the arrows in Figure 7 were combined and rechromatographed, with the results shown in Figure 10. The shape of the

Figure 6. Part of a successful chromatographic fractionation of whole casein on DEAE cellulose using the method described by Mercier.

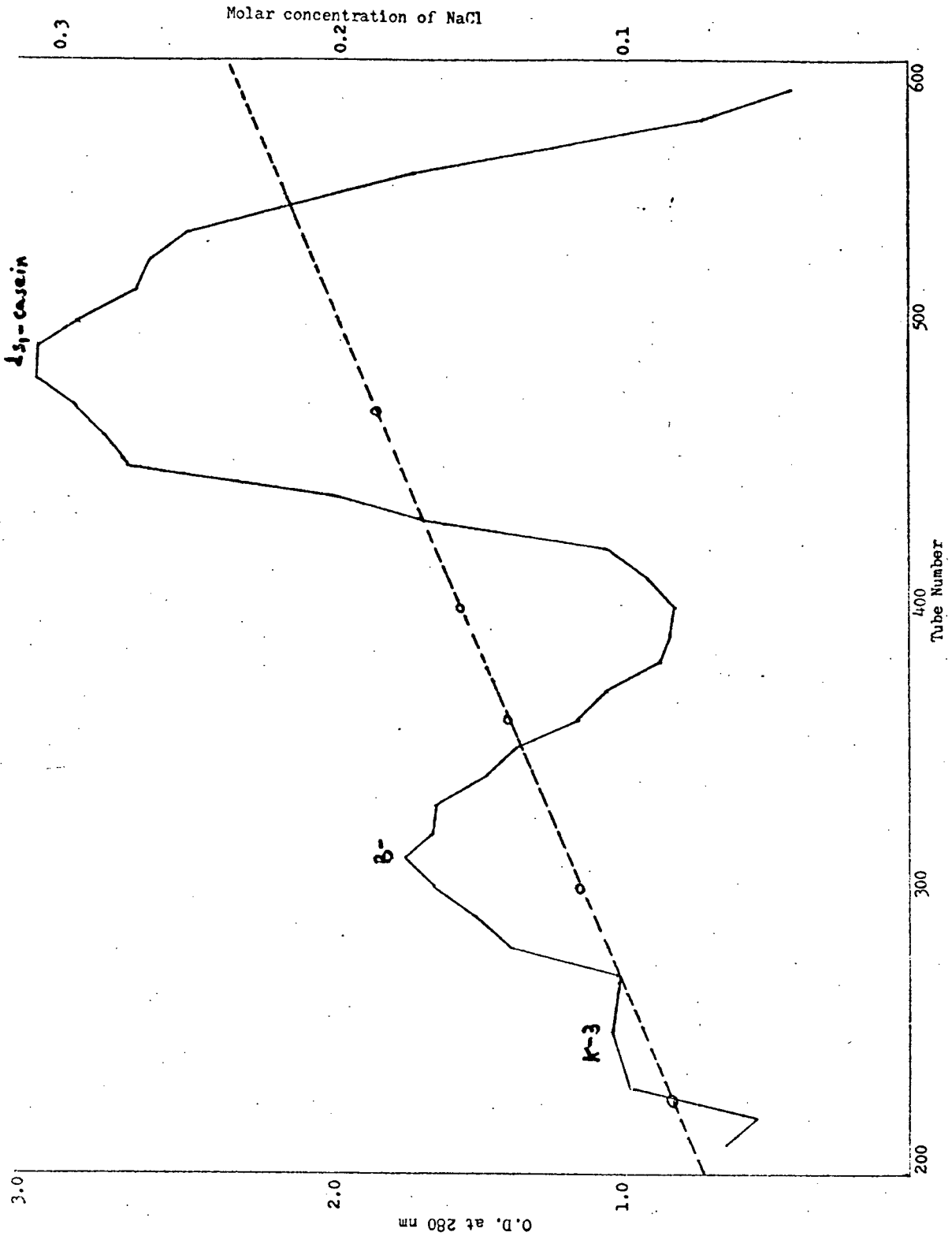

Molar concentration of NaCl
0.3
0.2
0.1
α_{s_1}-casein
β-
κ-3
O.D. at 280 nm
3.0
2.0
1.0
200
300
400
500
600
Tube Number

Figure 7. Elution profile of one of the first attempts to fractionate K-casein on DEAE cellulose using the modifications of Mercier et al.

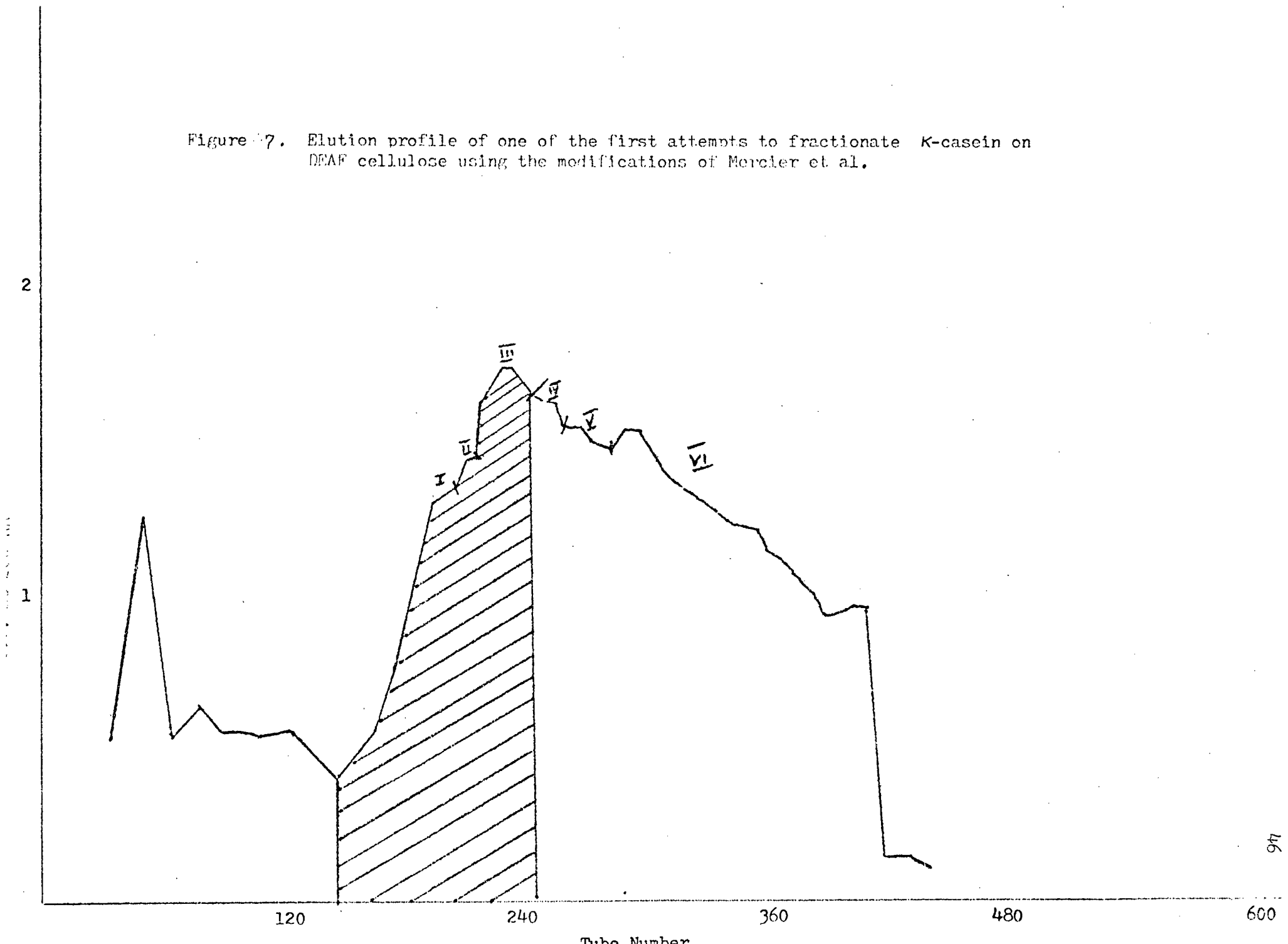


Figure 13. Successful fractionation of κ-casein on DEAE cellulose.

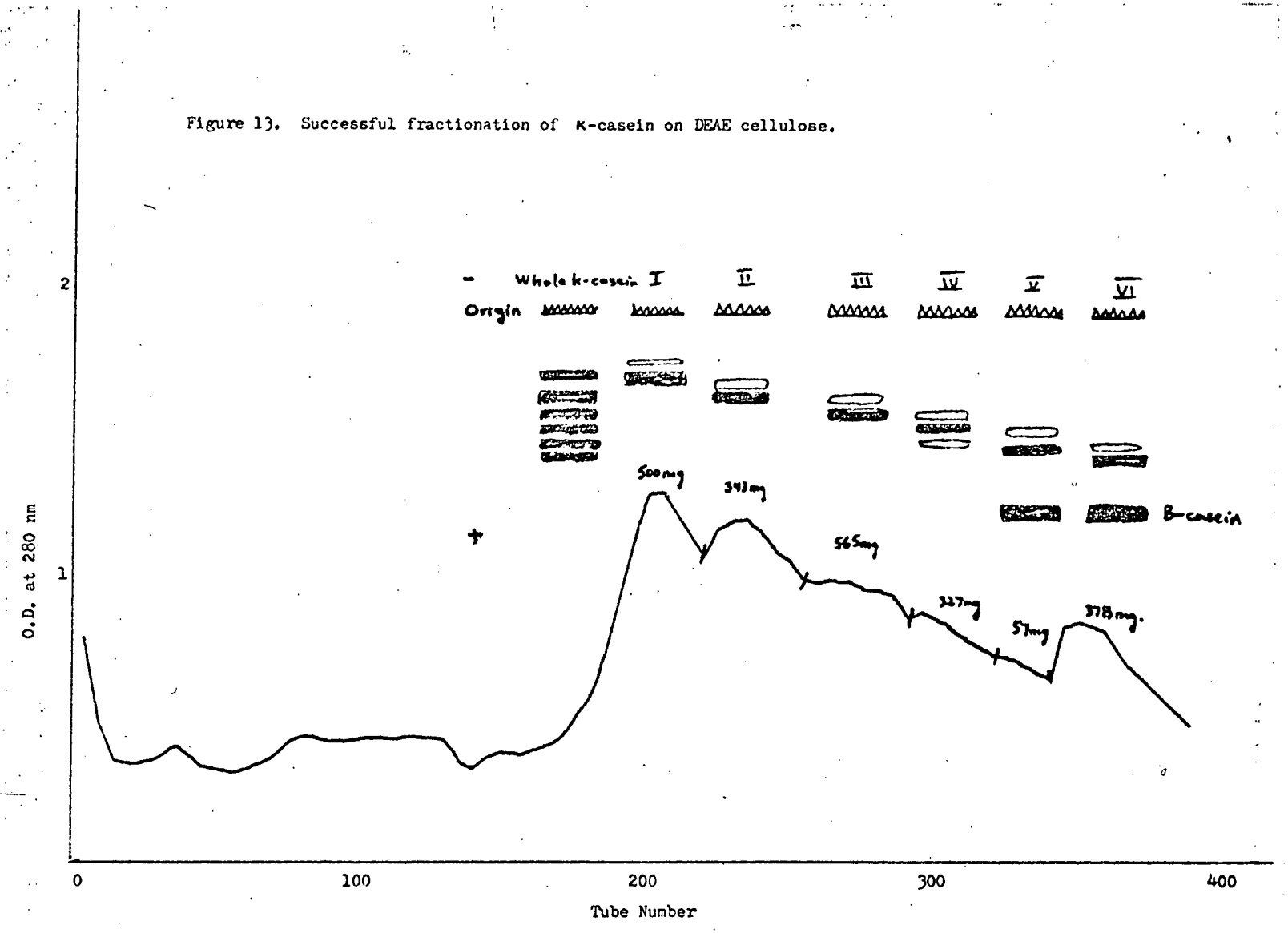


Figure 9. Fractionation of κ-casein during which gradient was applied by gravity.

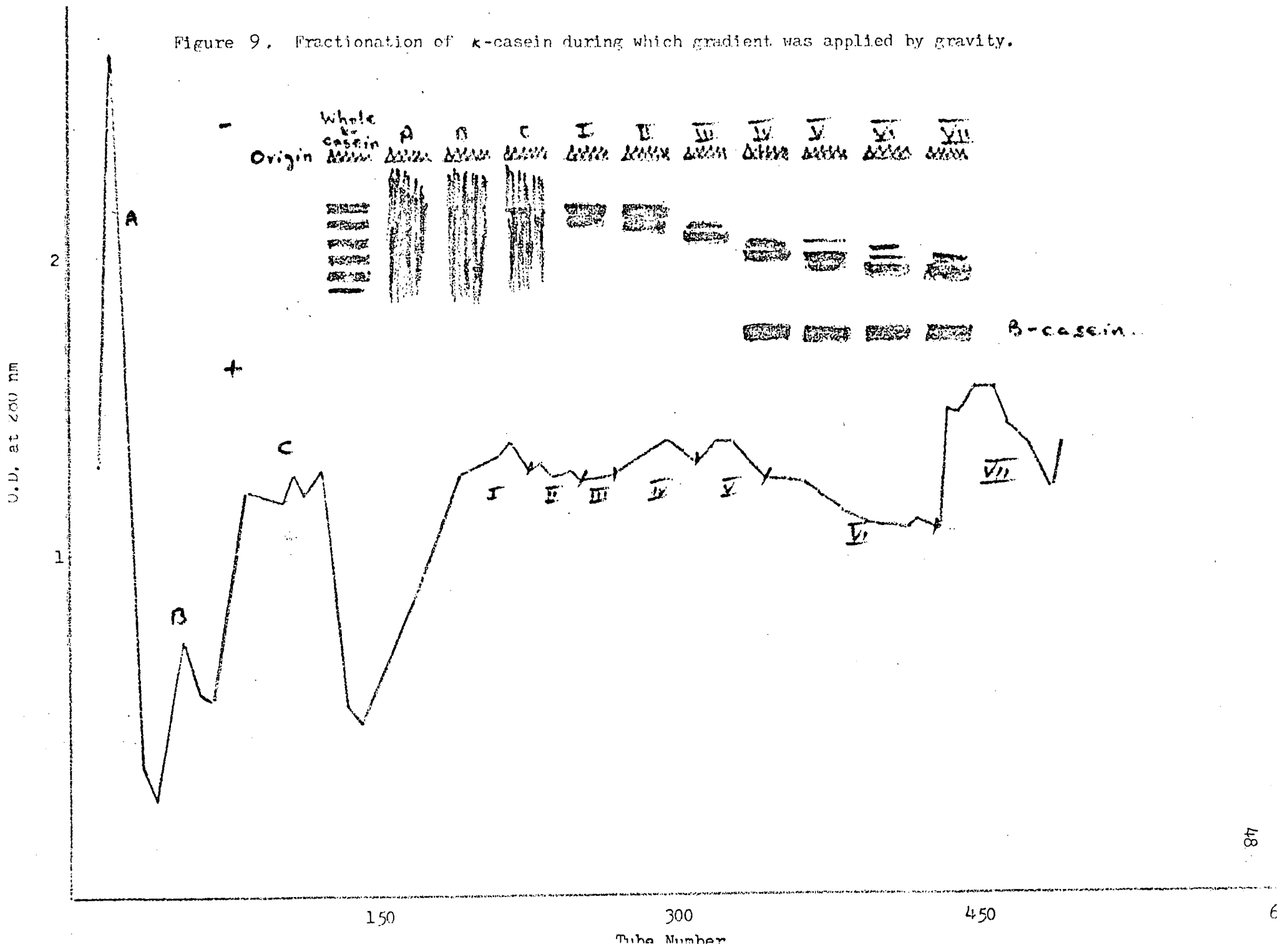


Figure 10. Rechromatography of shaded areas of Figure 7.

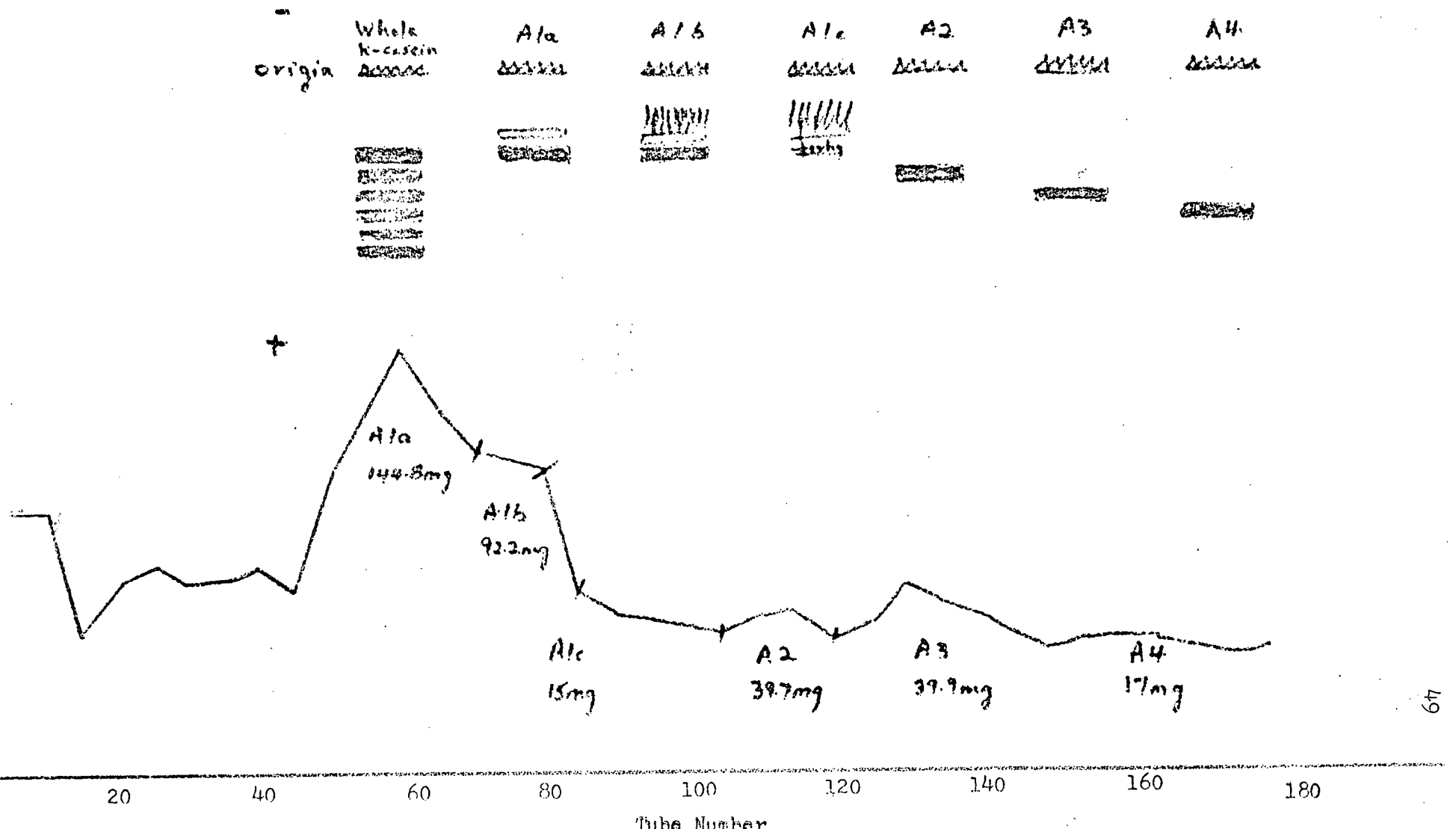
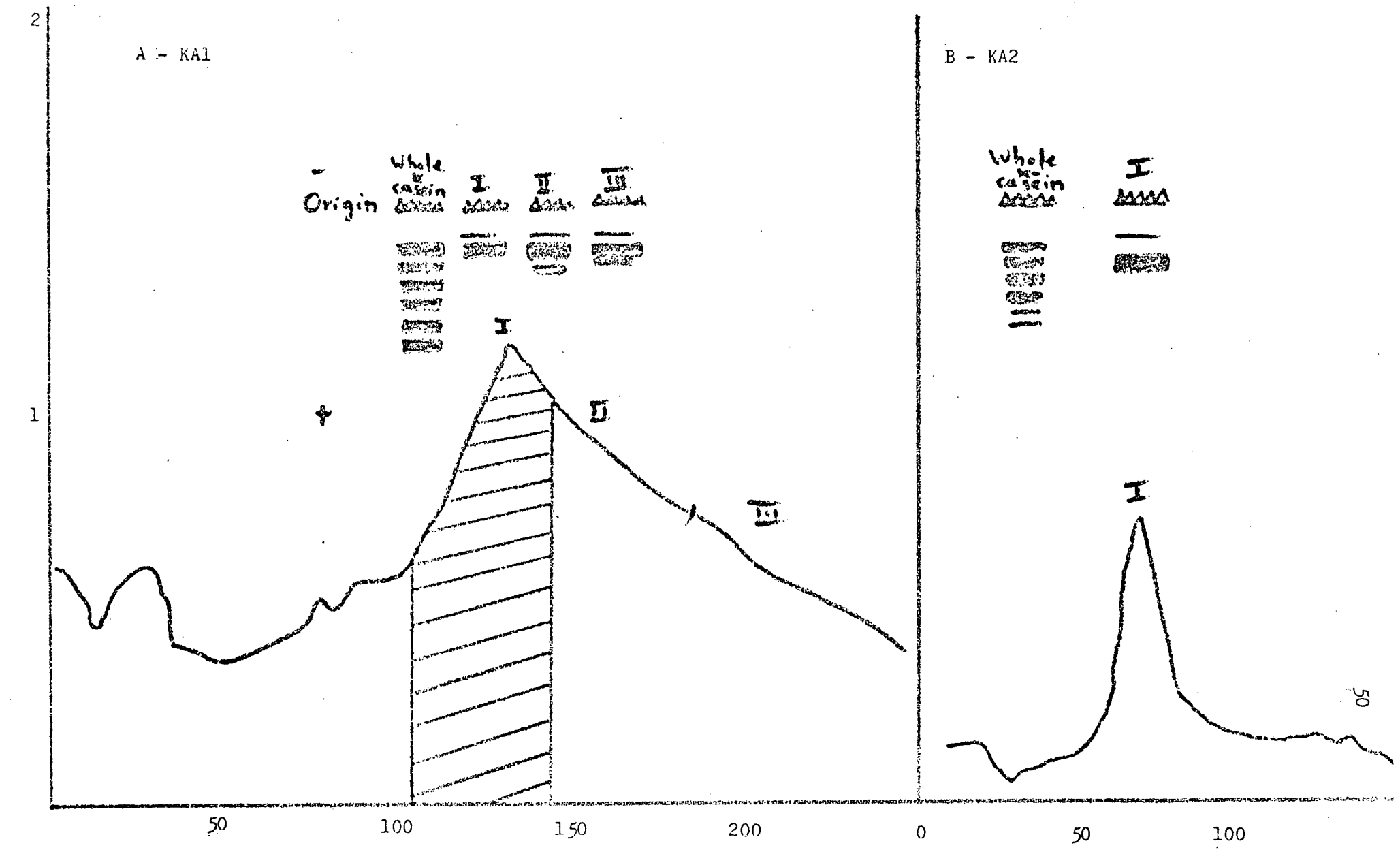


Figure 11. Rechromatography of KA1 and KA2.



curve is almost exactly the same as the rechromatograph of subfraction one shown by Mercier in 1968 (28). Better results were obtained when the fractions of Figure 8 were rechromatographed. The rechromatographs of KA1 and KA2 are shown in Figure 11. It was noticed that a small band just before subfraction one persisted even after rechromatography. This is in agreement with earlier work (62) and with that of Nakai (32).

SUMMARY

The results of this work supported the work of Mercier in that highly pure subfractions were obtained. Certain observations which are important are:

(1) The DEAE cellulose must be sifted; this affords more uniform column packing by removing a large amount of small and large material. In a typical batch of Whatman DEAE cellulose approximately 80% was of the 120-200 mesh size. Thus there is the possibility of large porosity gradients throughout a particular batch.

(2) The urea must be recrystallized or purified by ion exchange. Several runs (results not shown) on a column which gave good separation with recrystallized urea failed to give good separation with unrecrystallized urea. In recrystallizing urea it is suggested that small amounts be used in lieu of very large batches because the recrystallization procedure is more readily controlled.

(3) The DEAE cellulose must not be left in strong base any longer than prescribed in the above method. Old DEAE cellulose was found to have an exchange capacity of 0.65 whereas a newly purchased one had an exchange capacity of 0.95 milliequivalents of Cl^- per gram.

(4) The urea buffer must be filtered just before use. If ion exchange columns are used to treat urea then filtration is not necessary providing the buffer is used right away.

(5) At the end of the first run the column will have dark brown or black material on the top of it. This does not present a problem. Following the wash with 0.25N NaOH most of the discoloration disappears. Dark yellow to brown material remains even after complete regeneration, but this is not a problem. The next run can be carried out on the column. Once it is established that a particular column gives good separation then it is recommended that this column be used for several more runs. If all the runs are successful then this column can be used also for the rechromatography of a combination of one subfraction from several runs.

CHAPTER V

Modification of Histidine Residues with PDA and PMA

As mentioned in the Literature Survey several workers obtained evidence that supports the contention that histidine residues are important for the stabilizing ability of κ-casein. Nakai (32) alkylated κ-casein at pH 6.6 and 7.5 with 2-phenyl-1,4-dibromoacetoin and obtained a decrease of 1.5 to 2 out of a total of 4 histidine residues per molecular weight 28,000, accompanied by a decrease in stabilizing ability of 55%. Zittle (67) photooxidized κ-casein and concluded that the resulting decrease in stabilizing ability occurred as a result of destroying the histidine residues and therefore reasoned that histidine is an important functional amino acid in κ-casein. Neither of these workers tested the specificity of the modifications. The purpose of the present work was to modify whole κ-casein with PDA and to test the specificity of the reaction and also the effect of the reaction. KA1 and KA2 were also reacted with PDA. Early in these studies it was found that PDA caused aggregation of κ-casein and of KA2. It was postulated that aggregation occurred by the PDA cross-linking with single histidine residues on two molecules of κ-casein. For this reason the monobromo derivative (PMA) was prepared and reacted with whole κ-casein and with KA1 and KA2. The specificity and the effect of this reagent on the proteins were then tested. At the same time, the reaction conditions were changed to see if a more usable derivative of PDA-κ-casein could be obtained.

MATERIALS

κ-Casein, subfractions one and two and α_s-casein were prepared as described in chapters II and IV of this thesis.

The sulphenyl sulphonate derivatives of κ-casein and the two subfractions were prepared in the following way:

One hundred thirty mg of protein was dissolved in 14.0 ml of 0.02 M phosphate buffer pH 6.7, 8 M urea and 0.001 M EDTA (the urea was freshly recrystallized). Nitrogen was bubbled into the solution for 15 minutes, then 20 ul of 2-mercaptoethanol was added and the reaction mixture incubated at 37°C for 30 minutes after which 200 mg of sodium tetrathionate ($Na_2S_4O_6.2H_2O$) was added. After 5 minutes the pH was adjusted to 6.7 with 1N NaOH and the protein was then desalted by passage through a 2.4 x 52 cm Sephadex G-25 column equilibrated with 0.0008 M phosphate buffer, pH 7.6.

PDA was prepared according to the method of Ruggli (44) by Dr. S. Nakai. PMA was prepared from PDA by the following method:

Two grams of PDA was dissolved in 25 ml of 95% ethanol and 2 ml of distilled water. Two grams of aluminium amalgum (20-40 mesh) was added and the mixture was refluxed at 60-70 C for 5 hours. The aluminium amalgum was removed by centrifugation and the supernatant was placed in 100 ml of ice water and allowed to crystallize in the cold room. The white material which resulted during the crystallization was stored at 4°C in the mother liquor and collected and dried before use.

The aluminium amalgum was obtained from Fischer Scientific Company and was treated in the following way before use.

10 grams alumium granule, 20 mesh, was washed with 1N NaOH, then with water. It was then soaked in 100 ml of 0.5% mercuric chloride for 2 minutes

and washed once or twice with water. The 1N NaOH wash was then repeated and followed by another water wash. It was soaked again in 0.5% mercuric chloride for 1.5 minutes, and washed successively with water, alcohol, anhydrous ether, and stored in Petroleum ether.

METHODS

Amino acid analysis of the native and modified proteins was carried out in a Phoenix Model M6800 Moore-Stein system (32), three runs each after 24, 72, 96 hour hydrolyses with glass-distilled 6N HCl.

Nitrogen determinations were carried out by the micro-kjeldahl method (33). Stabilizing ability tests were carried out by the centrifugation method (33). Preliminary experiments involved the reaction of PDA with κ-casein as follows:

> 50 mg of κ-casein was dissolved in 16.0 ml of 0.01M phosphate buffer, pH 6.6. A solution containing 18 mg of PDA in 20 ml of absolute methanol was added and the reaction allowed to proceed in the dark for 16 hours at room temperature. At the end of the 16 hours the reaction mixture was either dialyzed against a total of 80 litres of distilled water for 72 hours in the cold room or placed on a Sephadex G-25 column equilibrated with 0.0008M phosphate buffer, pH 6.8 and eluted with the same buffer at room temperature. This is the same treatment used earlier by Nakai (32). The desalted modified protein was then frozen and freeze dried and analyzed by electrophoresis, amino acid analysis and for stabilizing ability against κ-casein. During the first reaction a negative control was run during which no PDA was added to the casein solution.

Preliminary studies of the reaction of κ-casein A1 with PMA were done as follows:

> 10 mg of protein was dissolved in 4.0 ml of 0.02M phosphate buffer,

pH 6.8. To this was added 3.5 mg of PMA dissolved in 1.5 ml of absolute methanol. The reaction was allowed to prodeed in the dark at room temperature for various times up to 48 hours. Before analysis the mixtures were desalted either by Sephadex G-25 chromatography as described above or by dialysis at 4°C for 48 hours against several changes of demineralized water.

Electrophoresis was carried out according to the method described in Chapter III where the gels were equilibrated for 24 hours by running the electrophoresis overnight without samples, followed by electrophoresis of the samples for 24 hours with a current of 20-25 mA and a voltage of 100 volts through the gel.

RESULTS AND DISCUSSION

A. Effect of PDA alkylation of κ-casein

Table III gives the results of amino acid analysis of κ-casein reacted at pH 6.6 with a thirty fold excess of PDA for 16 and 24 hours. The statistics of the analyses were based on work done by Nakai (33) which showed that certain amino acids remain constant during PDA alkylation of whole κ-casein. Thus, the calculation of the basic amino acids was based on a constant arginine value of 5.1 residues per molecular weight 20,000 and that of the neutral and acidic amino acids was based on a constant leucine value of 9.3 residues per molecular weight 20,000.

The results confirm Nakai's work (33) in that alkylation of κ-casein with PDA for 16 hours at pH 6.6 resulted in a decrease in histidine of 0.60 residue and in lysine of 0.20 residue per molecular weight 20,000. Treatment under the same conditions for 24 hours resulted in respective decreases of 0.86 and 0.20 in histidine and lysine residues. No new peak was seen on the analyzer as a result of the alkylation. Studies on the mechanisms of PDA modification are described in the next chapter.

Nakai also found that alkylation at pH 7.5 for 16 hours caused a

Table III. Amino acid analysis of κ-casein and PDA κ-casein.

Amino Acid	κ-Casein	PDA- κ-Casein (pH 6.6) 16 hour	PDA-κ-Casein (pH 6.6) 24 hour
		Residues per 20,000 g	
Aspartic acid	14.9	14.9	14.9
Threonine	16.5	16.5	16.5
Serine	14.6	14.9	14.6
Aspartic acid	31.0	31.0	31.0
Proline	22.6	22.6	22.6
Glycine	2.9	2.9	2.9
Alanine	16.6	16.6	16.6
½ Cystine	2.5	2.5	2.5
Valine	12.9	12.9	12.9
Methionine	2.0	2.0	2.0
Isoleucine	13.9	13.9	13.9
Leucine	10.4	10.4	10.4
Tyrosine	8.4	8.4	8.4
Phenylalanine	4.5	4.5	4.5
Lysine	9.6	9.4	9.4
Histidine	3.0	2.4	2.2
Arginine	5.1	5.1	5.1

decrease of 3 lysine, 0.8 residue of tyrosine along with 0.5 residue of histidine per molecular weight 20,000. The loss in stabilizing ability of the κ-casein modified at pH 7.5 was not reversible, whereas that at pH 6.6 was restored by treatment with urea or 0.2N NaOH. It was postulated by these findings that the decrease in lysine in combination with histidine might be responsible for the decrease in stabilizing ability of the κ-casein. At the same time the possibility of disulfide linkages playing a role in the stabilizing ability restoration phenomenon could not be ruled out. Experiments to test the latter were carried out by using κ-casein subfractions A1 and A2 and will be described later in this chapter.

Special steps had to be taken to dissolve the modified protein. One method was to suspend the dry material in water and raise the pH to 12.0 with 0.1N NaOH while maintaining a low temperature by carrying out all operations in an ice bath. The dissolved protein was then immediately neutralized by the addition of 0.1N HCl. An alternate method was to dissolve the protein in 0.001N NaOH (pH 10.0) and then neutralize by the addition of 0.001N HCl.

Figure 12 shows the electrophoresis of κ-casein and PDA modified κ-casein. The results confirm Nakai's work in that a smeared band was obtained.

B. Alkylation of κ-casein A1 and A2

It was postulated that PDA caused aggregation through cross-linkage of two histidines on separate κ-casein molecules. One way in which this was tested was by preparing the mono-bromo derivative, 2-phenyl-4-bromo-acetoin (PMA). This was then reacted with subfractions KA1 and KA2 as described in the methods.

Figure 12. Electrophoresis of κ-casein and PDA- κ-casein.

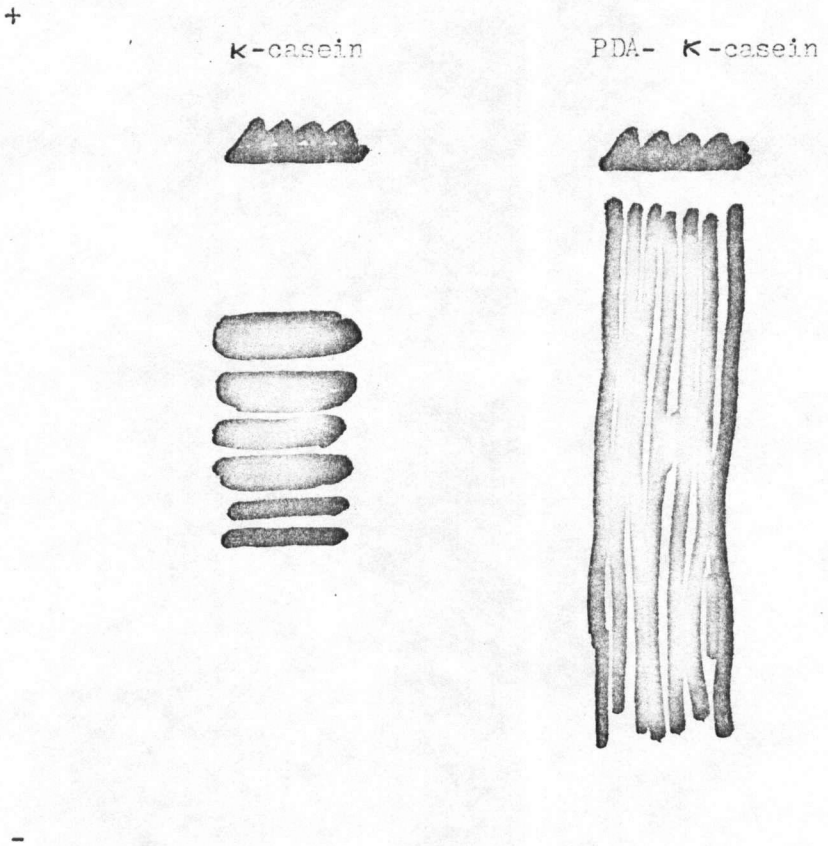


Figure 13 shows the decrease in histidine residues with reaction time. A total of 1.05 residues of histidine and 1.6 residues of lysine were modified after a reaction time of 20 hours. Figure 14 shows that the electrophoretic pattern of subfraction KA1 was not changed at all during the alkylation. Figure 15 shows that the stabilizing ability of PMA-κ-casein A1 and PMA-κ-casein A2 are the same as 'native' κ-casein and subfractions. SSS-κ-casein and KA1 showed a slightly higher stabilizing ability, possibly due to the increased negative charge.

These results support the theory that PDA causes aggregation of κ-casein by cross-linking and show that the modification of one histidine residue does not cause a decrease in stabilizing ability. This was also found by Nakai when he modified one residue in subfraction KA2 with the reagent N-bromoacetyl-l-arginine methyl ester (BAA) (33). It appears from these results that the reagent 2-phenyl-4-bromoacetoin (PMA) reacts more rapidly with lysine in κ-casein A1 and A2 than does PDA, as 1.6 residues was modified by reaction for 20 hours with the former, whereas only 0.2 residue was decreased by the latter.

C. Testing the role of SS-SS Interchange in the loss of stabilizing ability due to Reaction of κ-casein and Subfractions with PDA

Nakai found that the alkylation of whole κ-casein with PDA for 16 hours at pH 6.6 resulted in a decrease of 0.2 residue of cystine and that the decreased stabilizing ability was restored by dissociation with 0.2N NaOH. There is a possibility of involvement of the SS-SS interchange to explain the decreased stabilizing ability due to aggregation.

The normal procedure used to remove the blocking group, S-sulfenylsulfonate, after alkylation was to add 22 ml of 0.1M cysteine solution in 0.1M phosphate, pH 7.6 and to incubate the mixture at 37°C for 20 minutes.

Figure 13. Change in histidine residues in KA1 with time or reaction with 2-phenyl-4-bromoacetoin (PMA).

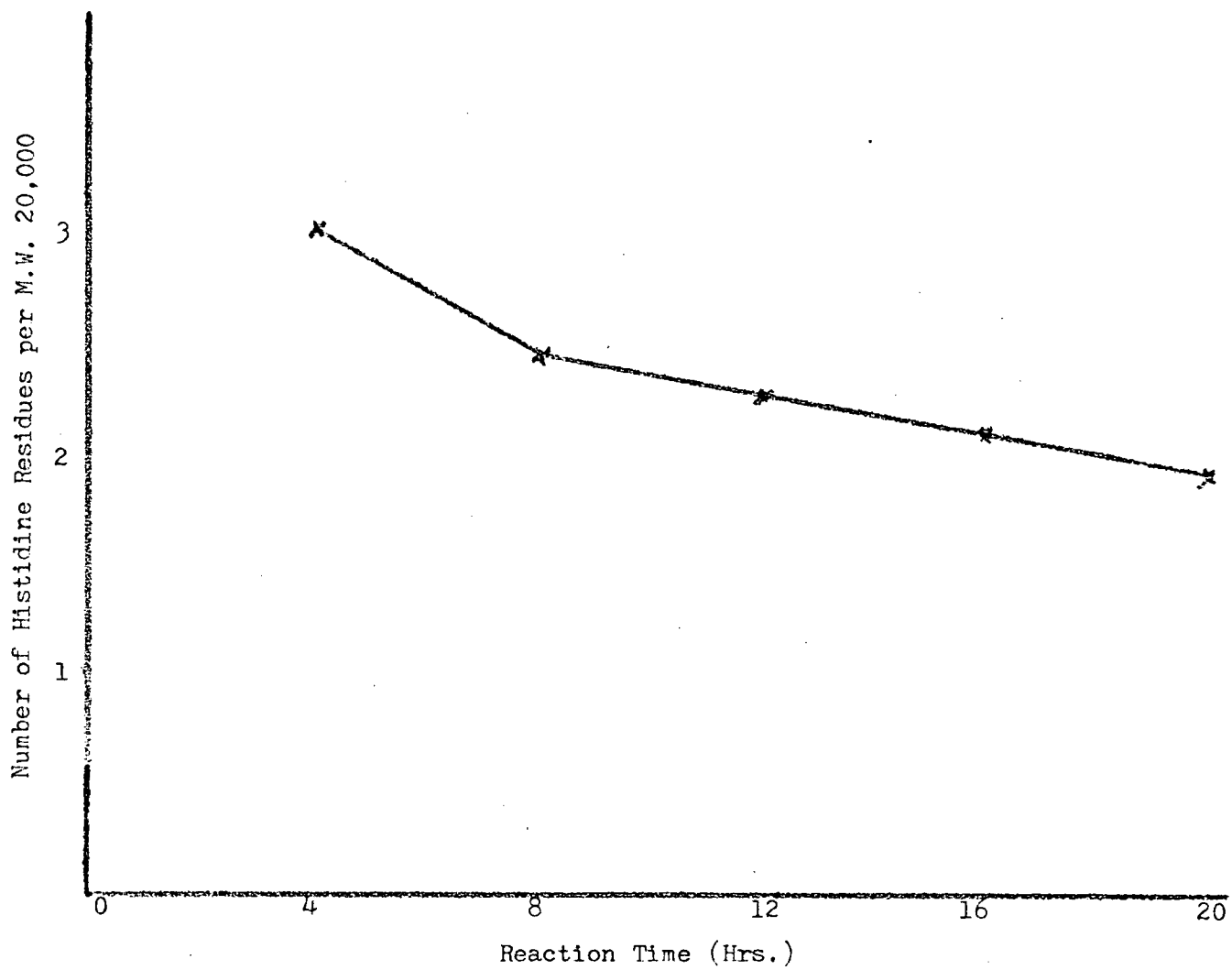


Figure 14. Electrophoresis of KAl and PMA-KAl.

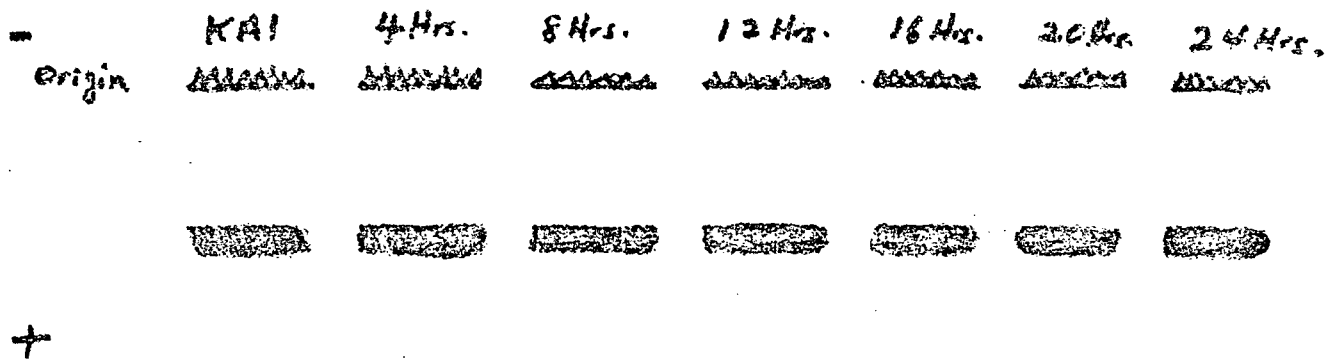


Figure 15. Stabilizing ability of KA1, KA2, PMA-KA1, κ-casein and SSS- κ-casein. (Closed triangles - KA1 and KA2; Closed squares - PMA-KA1; Open circles - κ-casein; Open squares - SSS- κ-casein)

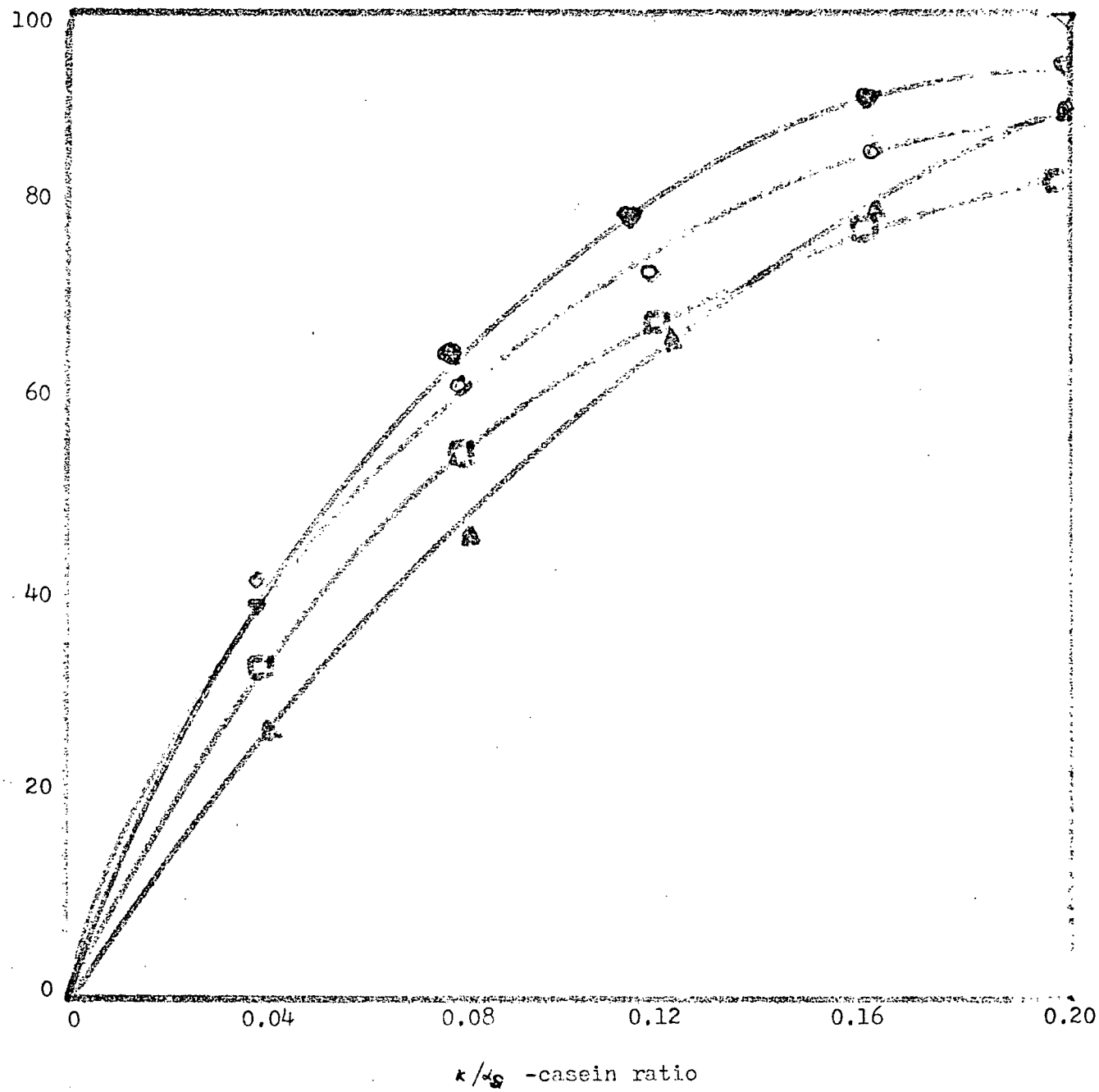


Table IV. Amino acid analysis of κ-casein and PMA-κ-casein.

Amino Acid	κ-Casein	PMA-κ-Casein (pH 6.8) 24 hour	PMA-κ-Casein (pH 6.8) 48 hour
	Residues per 20,000 g		
Aspartic acid	14.9	14.9	14.9
Threonine	16.5	16.5	16.5
Serine	14.6	14.6	14.6
Glutamic acid	31.0	31.0	31.0
Proline	22.6	22.6	22.6
Glycine	2.9	2.9	2.9
Alanine	16.6	16.6	16.6
½ Cystine	2.5	2.5	2.5
Valine	12.9	12.9	12.9
Methionine	2.0	2.0	2.0
Isoleucine	13.9	13.9	13.9
Leucine	10.4	10.4	10.4
Tyrosine	8.4	8.4	8.4
Phenylalanine	4.5	4.5	4.5
Lysine	9.6	8.6	8.6
Histidine	3.0	2.7	2.7
Arginine	5.1	5.1	5.1

To test the role of the SS-SS interchange reaction in the decrease in stabilizing ability due to aggregation, the blocked SH groups of KA1 were not reduced after alkylation with PDA, thus leaving the modified protein in its S-sulfenylsulfonate form. The stabilizing ability of this protein was the same as the reduced modified protein, thus indicating that the role of the sidulfide interchange was remote.

D. The Reaction of PMA with Whole κ-casein

Several experiments were carried out to see if the reagent 2-phenyl-4-bromoacetoin reacted with κ-casein in the same way as it did with the subfractions KA1 and KA2.

Table IV shows that 1.0 lysine residue and only 0.3 histidine residues were modified after a 24 hour reaction time. Increasing the reaction time to 48 hours (Table IV) did not show any increase in reactivity. There was no change in stabilizing ability or electrophoretic pattern.

These results could be due to the possibility that the subfractions of κ-casein were less aggregated than was the whole protein and support the statement made above that studies on the role of important amino acids in the stabilizing ability of κ-casein should be carried out on the subfractions for these are probably closer to the monomer unit of κ-casein than is the heterogeneous molecule.

The results again show a possible change in selectivity of the reagent PDA from histidine to lysine as one of the bromines is removed. It may be possible to use this reagent for the specific modification of lysine in other proteins.

Another aspect of this work is that it does not support the results of Waugh and von Hippel (50) who reported that the modification of one lysine residue by acetic anhydride and six other reagents caused a decrease in stabilizing ability of κ-casein. This could be due both to the different

reagents used and again due to the heterogeneity of the different preparations of κ-casein. Also, the specificity of their reaction was not checked.

E. Alterations in the reaction of PDA with κ-casein

To see if a more workable derivative of PDA-κ-casein could be obtained, the reaction mixture was changed mainly by decreasing the ionic strength of the buffer used in the reaction mixture. Instead of using 0.1M phosphate buffer, the following buffer was used: 0.01M imidazole-NaCl, pH 6.6.

The reaction was allowed to proceed for 16 hours in the dark and was then tested for stabilizing ability and amino acid analysis. Table V shows shows that 0.6 residue of histidine was modified. Figure 16 shows that the stabilizing ability of the modified protein was the same as the unmodified protein.

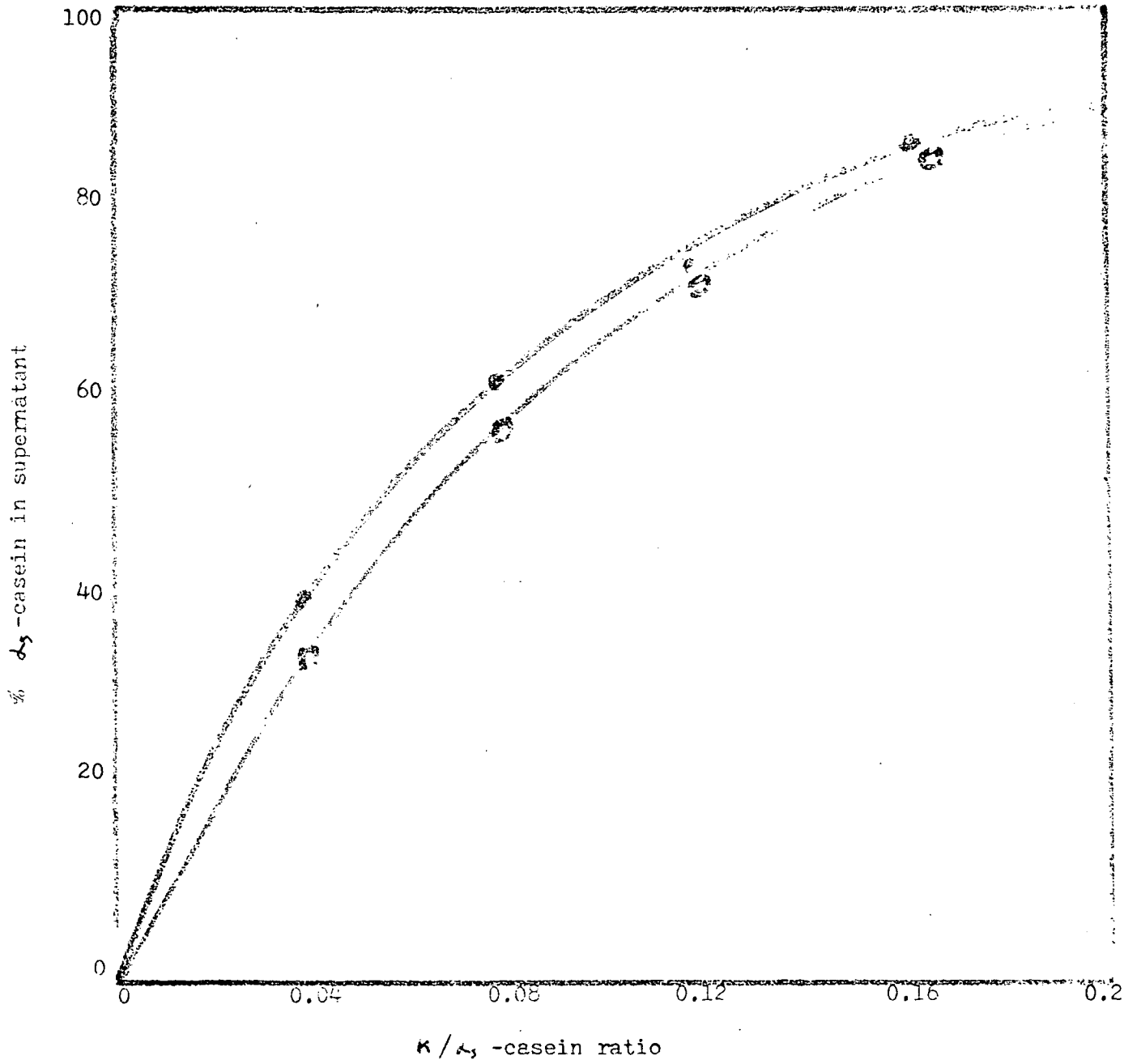
This work was repeated using another preparation of κ-casein. It was found that the reaction of PDA with this preparation for 24 hours at the low ionic strength resulted in complete aggregation and precipitation of the protein. The pH was 6.6 through the reaction. Time did not permit further investigation of this phenomenon but certain possibilities should be mentioned.

1. Clarke (7) concluded that increasing the ionic strength of solutions of κ-casein resulted in increased compactness of the molecule. Thus the reaction of this protein with specific modifying reagents could be dependent upon the ionic strength of the reaction mixture. It would appear from Clarke's results that the lower the ionic strength the easier will certain reagents combine with specific amino acids. A decrease in selectivity for certain amino acids could accompany the increased reactivity of the reagent(s) with decreasing ionic strength. Nakai (33) found that PDA became less specific when it was reacted with κ-casein in the presence of increasing concentrations of urea. A similar dependency

Table V. Amino acid analysis of PDA-κ-casein reacted in low ionic strength buffer

Amino Acid	κ-Casein	PDA-κ-Casein (pH 6.8) 16 hour
	Residues per 20,000 g	
Aspartic acid	14.9	14.9
Threonine	16.5	16.5
Serine	14.6	14.6
Glutamic acid	31.0	31.0
Proline	22.6	22.6
Glycine	2.9	2.9
Alanine	16.6	16.6
½ Cystine	2.5	2.5
Valine	12.9	12.9
Methionine	2.0	2.0
Isoleucine	13.9	13.9
Leucine	10.4	10.4
Tyrosine	8.4	8.4
Phenylalanine	4.5	4.5
Lysine	9.6	9.4
Histidine	3.0	2.4
Arginine	5.1	5.1

Figure 16. Stabilizing ability of untreated κ-casein and PDA-κ-casein reacted in low ionic strength buffer. (Closed circles - Untreated κ-casein; Open squares - low ionic strength PDA-κ-casein)



is possible with the ionic strength of the reaction mixture, and this factor should be taken into account when modifications of specific amino acids in κ-casein are attempted.

2. The difficulty in obtaining the same results with two different preparations of κ-casein could accentuate the problem mentioned in Chapter II of this thesis, that is, Zittle's method is a harsh method which must be standardized, especially when large amounts are prepared at one time.

3. If PDA caused aggregation of κ-casein by cross-linking and the structure of κ-casein opens up with decreasing ionic strength, then PDA should cause more rapid cross-linking as the ionic strength is lowered. The results with one preparation of κ-casein corroborate this hypothesis whereas those with another do not.

A sample of the former was examined in the spectrofluorometer and found to have a higher fluorescent peak than did the κ-casein which did not completely aggregate during a 24 hour reaction with PDA. It was also found that the former had a lower stabilizing ability (82%) and solubility than the latter. This indicated that the κ-casein preparation which did precipitate was in a more aggregated state before reaction with PDA.

Lowering of the ionic strength of a solution of aggregated κ-casein could serve to push the aggregation to completion once PDA is reacted with the protein. Thus, the state of aggregation of the native protein could be a determining factor in making use of possible higher reactivity of the protein in decreasing ionic strength toward modifying reagents.

SUMMARY

The finding that a PDA-κ-casein derivative could be prepared which dissolved normally and also showed the same stabilizing ability as untreated κ-casein indicates that changing one histidine residue does not cause a decrease in stabilizing ability. This work and that with PMA which modified 1.05 residues of histidine and 1.60 residues of lysine in KA1 and KA2 without any change in stabilizing ability does not support the hypothesis that histidine is an important amino acid for maintaining the stabilizing ability of κ-casein and supports the contention that the decrease in stabilizing ability observed earlier (32) resulted from aggregation caused possibly by cross-linking, the extent of which could be dependent upon ionic strength. Subtracting one bromine from 2-phenyl-1,4-dibromoacetoin (PDA) to produce 2-phenyl-4-bromoacetoin (PMA) seems to result in a transfer of selectivity from histidine to lysine. The reagent PMA does not react with whole κ-casein in the same way as it does with KA1 and KA2 and this could be due to the latter being more homogeneous proteins than the former. This finding supports the contention that future work on the modification of amino acids in κ-casein and studies on the interaction of this protein with α_s-casein should be carried out on the subfractions rather than on the whole protein.

CHAPTER VI

Studies to determine the mechanism of action of PDA with histidine

Nakai digested PDA-alkylated -casein with the enzyme pronase and ran the mixture on T.L.C. plates in order to identify the modified histidine residue. It was postulated that PDA might react with histidine in -casein in a similar way as does N-bromoacetyl-l-arginine methyl ester in that carboxymethyl histidine derivatives could be formed. This was supported by an increase in absorbance at 280 nm of -casein after alkylation. However, due to two bromines in PDA the hydrolysis products could be different and more complicated. The loss of one histidine residue after alkylation of -casein with PDA could not be totally accounted for by the 3-carboxymethyl histidine peak on the amino acid analyser during analysis of the acid-hydrolyzed PDA--casein. A difficulty in identifying carboxymethyl histidine derivatives is that several derivatives overlap with several of the amino acids present in -casein. The attempts to purify the modified amino acid by pronase digestion followed by thin layer chromatography were not successful. Therefore, to simplify the experiments, Dr. Nakai reacted PDA with histidine and compared the derivatives with CM-histidine derivatives prepared according to the method of Moore, Crestfield and Stein (33). Several experiments which Nakai performed are as follows:

1. Preliminary Experiments

Four hundred and fifty milligrams of l-histidine hydrochloride monohydrate (Mann Research Laboratory) dissolved in 90 ml of 0.1 M phosphate, pH 7.5, were alkylated with 1.35 g of PDA dissolved in 110 ml of methanol and freeze-dried after 16 hours in the dark. Thirty millilitres of water and 15 ml 0.2 M sodium citrate buffer, pH 3.25, were added and the pH was adjusted to 1.6 with HCl. The residue of the extraction was

dissolved in 15 ml of methanol. The aqueous extract was then chromatographed on a column 4 x 32 cm of Dowex 50 x 8, 100-200 mesh washed with 0.2N NaOH and equilibrated with citrate buffer, pH 3.25. The effluent was analyzed in 0.1 ml amounts by a ninhydrin method. The fractions were concentrated by rotary evaporation and the concentrates transferred to test tubes were cooled at 4°C. The supernatant after crystallization of buffer salts was used for identification. N-Acetyl-l-histidine (Calbiochem) 450 mg in 65 ml of the phosphate buffer, was also used instead of histidine to avoid alkylation of α-amino groups. The derivatives produced were hydrolyzed to remove the acetyl group by boiling for 6 hours under reflux after adding 155 ml of 8.5N HCl. The HCl was removed by rotary evaporation.

Nakai obtained five fractions by chromatography of both the PDA-histidine and the PDA-N-acetyl-l-histidine reaction mixtures, two of which appeared to be the same derivative from both reactions.

The respective T.L.C. Rf values of the Dowex 50 peaks were 0.25, 0.49, and 0.62 for peak (I), peaks (II and IV) and peaks (III and V). These were all higher than three derivatives of carboxymethyl-histidine.

As the derivatives, especially those derived from histidine, produced an odour similar to that of acetophenone after warming in the presence of strong bases, it was reasoned that they may contain the phenylketone group. Since it was known that the PDA decomposes and produces acetophenone by warming with bases, Nakai tested for the presence of this group by a modification of the method of Tikhonova (33).

Acetophenone and PDA yielded a yellow colour reaction while acetone produced a pink colour. Fractions (II) and (III) of PDA derivatives from histidine indicated a distinct yellow colour. Fractions (IV) of the derivatives from acetyl-histidine reacted positively but with a decreased

intensity. The reaction of fractions (I) and (V) of the derivatives was very faint. Carboxymethyl-histidine did not react.

It was reasoned from these results that the derivatives are possibly addition products and that production of acetophenone in the presence of bases may be a sign of release of a phenylketone group attached to histidine and may partially explain why the stabilizing ability of the PDA-alkylated whole -casein at pH 6.6 was restored by the action of base (32).

The methanol extract from the preparation of PDA-alkylated histidine produced a faint spot of histidine by T.L.C.

2. Isolation of PDA-Alkylated Histidine

The reaction of PDA on histidine is so mild that the yield without further purification was approximately 0.2 to 0.3%. Raising the reaction pH above 7.5 increased the reaction rate but at the same time decomposed the products to form carboxymethyl-histidines which were confirmed by T.L.C. Using Dowex 1 and gelatin to remove bromine from the reaction products increased the yield without further purification to 2%. Raising the temperature of the reaction beyond 25 °C was not effective at all.

Ion exchange resins were utilized for desalting without success because of restriction of the operating pH range. Use of the mild condition of Dowex 2 equilibrated with pyridine-acetate buffer, pH 6.0, and elution with 1N acetic acid resulted in conversion of the derivatives to carboxymethyl histidines. The recrystallization of the derivatives from ethanol hydrolyzed them as well.

Thus, in the final experiments Nakai used a chromatography on Dowex 50 x 8 to separate the five fractions, followed by cellulose chromatography to desalt each fraction.

3. Final Experiments

Ten grams (64.4 mM) of l-histidine free base were dissolved in 1.4 litres of water at 25°C in which 21 g of gelatin was dissolved by warming to 70°C. Thirty grams of PDA (93.2 nM) dissolved in 2.3l of methanol were added slowly to the histidine solution, followed by 420 g of wet Dowex 1 x 4, OH-form. The pH of the solution was 7.2 to 7.3. The solution was stored in the dark for 5 days while the pH was maintained at 7.2 to 7.3 with 2N NaOH. The solution was filtered on Whatman No. 1, washed with N acetic acid, flash-evaporated to 45 ml. Nine hundred ml of absolute methanol were added, and the solution was centrifuged at 8,000 x g for 30 minutes at -15°C; this precipitated most of the gelatin. The supernatant was evaporated to 60 ml and desalted on a Sephadex G-50 column, 3.6 x 60 cm, equilibrated with 0.1N acetic acid. Effluents eluted with 0.1N acetic acid were analyzed by the ninhydrin reaction for 10 ul aliquots from each test tube containing 20 ml fractions. The ninhydrin-positive fractions were evaporated to 60 ml, centrifuged at 3,600 x g for 20 minutes and evaporated further to dryness. Approximately 20 ml of water was added and the solution redried. The residues were dissolved in 25 ml of water and the pH adjusted to 2.2 with 6N NaOH. The solution was centrifuged again at 3,600 x g for 20 minutes and chromatographed on a Dowex 50 x 8, 200-400 mesh column, 2 x 90 cm, with a 0.2 M sodium acetate buffer at pH 3.25. The effluent of 10 ml fractions was analyzed by the ninhydrin method for 100 ul from alternate test tubes. Usually three derivatives were separated between tubes 120 and 220. The combined fractions for each derivative were evaporated to approximately 20 ml, cooled to 4°C overnight to crystallize most of the buffer salts, centrifuged, the supernatant adjusted to pH 3.0 with HCl and freeze-dried. The residues were dissolved in

20 ml of the solvent, isopropanol-formic acid-water 20:1:5, and chromatographed with the same solvent on a cellulose column, 2 x 90 cm, in which 100 g of Cellex-N-1 (BioRed Laboratories, Richmond, California) were packed with the same solvent. Aliquots of 150 ul from alternate 5 ml fractions were analyzed by the ninhydrin method. The ninhydrin-positive fractions were combined, 50 to 100 ul of which was used for amino acid analysis, evaporated after adding 2 drops of HCl and finally freeze-dried. The highest yield obtained was Fraction (II), 70 mg, which had a salt content of approximately 90%.

It was found that the Sephadex G-50 treatment described above did not desalt the derivatives and in fact resulted in a large decrease in total yield.

The purpose of this chapter is to report further work done in an attempt to elucidate the mechanism of reaction between PDA and histidine. The objectives were as follows:

(a) to test the possibility of purifying the three derivatives by a one step procedure using cellex chromatography;

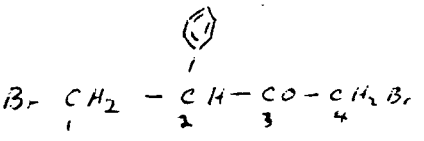
(b) to test several basic factors of the reaction for the purpose of increasing the yield;

(c) to test the possibility of changes in the products caused by treatment of proteins for amino acid analysis; and

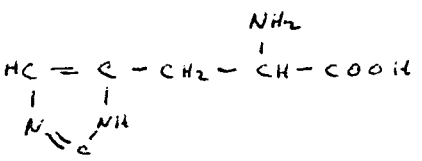
(d) to test whether each product was a histidine derivative.

THEORY

Based on the work done by Crestfield et al on the preparation of carboxymethyl-histidine derivative (33), it is predicted that the following products (A,B & C) could result from a combination between PDA (I) and histidine (II).

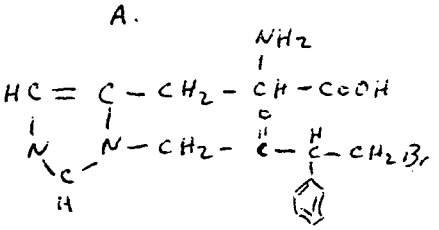


(I)

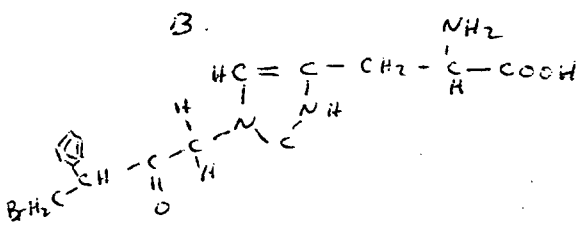


(II)

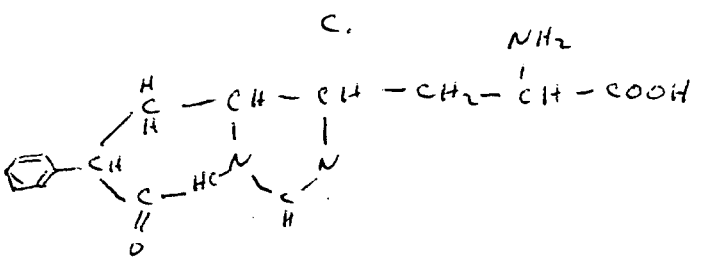
A.



B.



C.



It is felt that the 4-bromine is the most reactive of PDA because of its proximity to the carbonyl group. Nakai has proven that the α-amino group of histidine is not a main participant and also that treatment with the reaction products of PDA and histidine with base gives rise to CM-histidine.

If the reaction between PDA and histidine acts in the proposed way then the ratios of the three (A), (B) and (C) should be close to 1:1:2 in line with the work of Crestfield et al on carboxymethyl histidine derivatives.

One problem in predicting the reaction is that PDA is an optically active compound and no attempt has been made to resolve it (4).

Another difficulty with this compound is that acetoins are readily hydrolyzed in water and this could account for the very low yields obtained by Nakai. Other solvents such as formamide should be attempted as suggested by Dr. Bose (4). Also the concentration of reactants may play a role in the type and ratio of products.

The replacement of the hydrogen atoms on histidine and the removal of bromines from PDA should result in a decrease in pH through the formation of HBr. Thus there is a possibility that this could be used to monitor the effect of changing the conditions of the reaction.

Several changes in the reaction were carried out following preliminary attempts to purify the products by chromatography on cellex-N-1 monionic cellulose.

Experimental Plan

Series one

The first series of experiments were designed to test the possibility of eliminating the Dowex 50 x 8 step by carrying out an immediate fractionation of a reaction mixture after removal of the gelatin and concentration of the mixture by flash evaporation. Also attempted was the purification of fractions by both thin layer and paper chromatography.

Series two

Several basic factors of the reaction were studied, including varying the concentration of reactants, changing the solvent system, and running the reaction in the presence of aluminium amalgum and refluxing in an attempt to

more rapidly push the reaction to completion.

Series three

The reaction mixture was treated in boiling 6N HCl in the same way as are proteins in preparation for amino acid analysis. This was done to see if this treatment caused changes in the products which would explain the difficulty of not being able to account for the modification product during amino acid analysis of whole PDA-κ-casein.

Also paper chromatographs of the reaction mixture were stained with Pauley's reagent for histidine.

MATERIALS

PDA was prepared by Dr. Nakai according to the method of Ruggli (44).

l-Histidine hydrochloride free base was obtained from Nutritional Biochemicals Corporation.

Cellulose-N-1 nonionic precoated plastic plates were purchased from Sigma Chemical Company.

Whatman No. 3 filter paper was used for paper chromatography.

Formamide was obtained from Nutritional Biochemical Company.

Aluminium amalgum was obtained from Fischer Scientific Company and treated as described in Chapter VI.

Hydrochloric acid was glass distilled, of which the first and the last 10% of the distillate was discarded.

METHODS

Series one

Preliminary experiments designed to fractionate the products by cellex-N-1 chromatography were carried out by reacting PDA with histidine following Nakai's method up to and including the point of removing the gelatin. The

gelatin-free concentrate was then flash evaporated to dryness and the residue dissolved in the solvent isopropanol-formic acid-water (20:5:1) and chromatographed, using this solvent, on a short cellex column (15 x 0.9 cm). The reactions were carried out using small amounts of reactants for these preliminary experiments. One hundred milligrams of l-histidine was dissolved in 14.0 ml of water in which 0.21 g of gelatin had been dissolved by heating to 70°C. To this was added 300 mg of PDA dissolved in 23 ml of absolute methanol. Finally 0.42 gr of wet Dowex 1 x 4 (OH-form) was added and the pH of the mixture was raised to 7.2 with 2N NaOH. The reaction was allowed to run in the dark for 24 hours. When the reactions were run longer than 24 hours the pH was readjusted to 7.2 daily.

Purification of fractions by paper chromatography was carried out by placing a strip of concentrated reaction mixture dissolved in the solvent mentioned above along the fold at the top of an 8 inch wide strip of Whatman No. 3 filter paper and allowing the chromatographs to run for 16-18 hours in descending fashion in the same solvent. After the runs were completed a ½ inch strip was cut off the right side of the paper and stained. This was then aligned with the remainder of the paper and the spots marked with pencil. The strips containing spots were then cut from the paper and eluted with the same solvent by placing them between glass slides in petri dishes and cutting the bottom ends of the strips to a point and placing this in the mouth of small beakers. When it was found that the solvent would not move up the glass sides and that the spots were water-soluble, water was used to elute the spots. The liquid in the beakers was then freeze-dried and the residue stored under vacuum in a desiccator.

Series two

Several basic factors involved in the reaction were studied in an attempt to increase the yield. It was first of all suggested by Dr. Bose

that PDA would possibly be hydrolyzed in water and therefore the concentration of the latter should be kept to a minimum.

The molar ratio of reactants used by Nakai was a 1.80 molar excess of PDA to histidine. Thus ratios of 0.1 to 30 were set up using total reaction volumes of 5 or 10 ml. Again the pH and the T.L.C. patterns were measured after reaction in the dark for 24 hours.

The reaction was also carried out in the presence of formamide instead of water. This material was flash evaporated until no more liquid could be removed and then run on T.L.C.

One other attempt taken to increase the yield was to reflux 100 mg of histidine dissolved in 1.4 ml of water with 300 mg of PDA dissolved in 23 ml of absolute methanol in the presence of aluminium amalgum prepared as described earlier. One ml aliquots were withdrawn at intervals over a three hour period, centrifuged and 10 µl spotted on cellulose plates for thin layer chromatography.

Series three

Ten milligrams of the freeze-dried water-soluble products of the reaction mixture was dissolved in 5.0 ml of 6N HCl and boiled under vacuum for 24 hours. During the first experiment in this series the residue of solvent-soluble material was treated in this way. The material which was not soluble in the HCl after boiling was removed by centrifugation. The clear solution of HCl was then evaporated to dryness with 4 washes of distilled water and the residue taken up in 1.0 ml of water. Ten microlitres of sample was applied to T.L.C. plates and run in Jones' solvent for five hours followed by staining with ninhydrin spray.

The directions for the preparation and use of Pauley's reagent are as follows:

Solution A - dissolve 1 gram of sulphanilic acid in 100 ml of N HCl

Solution B - prepare just before using a 0.7% solution of $NaNO_3$

Solution C - prepare a 10% aqueous solution of $NaHCO_3$

Carefully mix solutions A and B in a ratio of 1:1. Spray the paper with this mixture and dry the paper in a stream of air. Spray again lightly with solution C. It is important to spray the paper lightly, otherwise large red spots appear. Care must be taken in the staining of precoated thin layer plates because of the possibility of liquid running down the plates.

RESULTS AND DISCUSSION

Series one

Fractionation of Reaction Products of PDA-Histidine by Cellex Chromatography

Reactions involving 100 mg of histidine and 300 mg of PDA in a total volume of 37 ml were carried out for 24 hours; the gelatin removed; the reaction mixture was dried down; and the residue was dissolved in 5.0 ml of solvent. Various amounts were applied to a 15.0 x 0.9 cm cellex column previously equilibrated with the same solvent (isopropanol-formic acid-water, 20:1:5). After eluting the mixture with this solvent 1.0 ml fractions were collected and 250 ul aliquots of every second fraction analyzed by the ninhydrin reaction (33). Figure 23 shows the results of a successful fractionation of the mixture for the whole reaction mixture in 0.5 ml of solvent. The chromatographic fractionation of larger volumes of sample was unsuccessful. It will be noted from Figure 23 that all of the peaks contained traces of CM-histidine-like material. Fraction C_I of two runs was combined, dried down and taken up in 1.0 ml of solvent and rechromatographed with the results shown in Figure 24. These experiments showed that it is possible to obtain the three main products of the reaction mixture by cellex chromatography. There was, however, a trace of CM-histidine-like substance in all fractions.

Figure 17. Fractionation on cellex of 0.5 ml of reaction mixture of PDA and histidine.

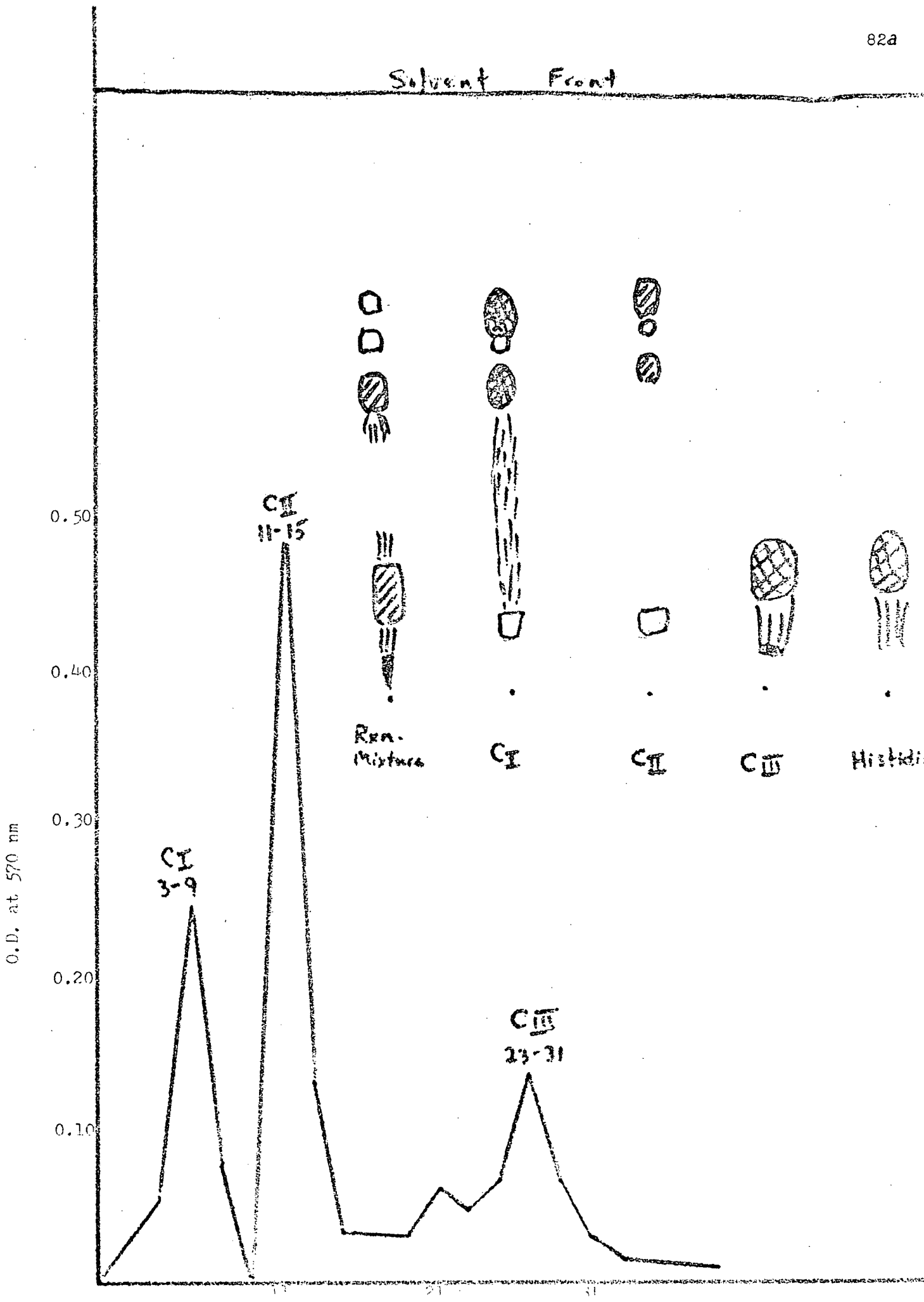
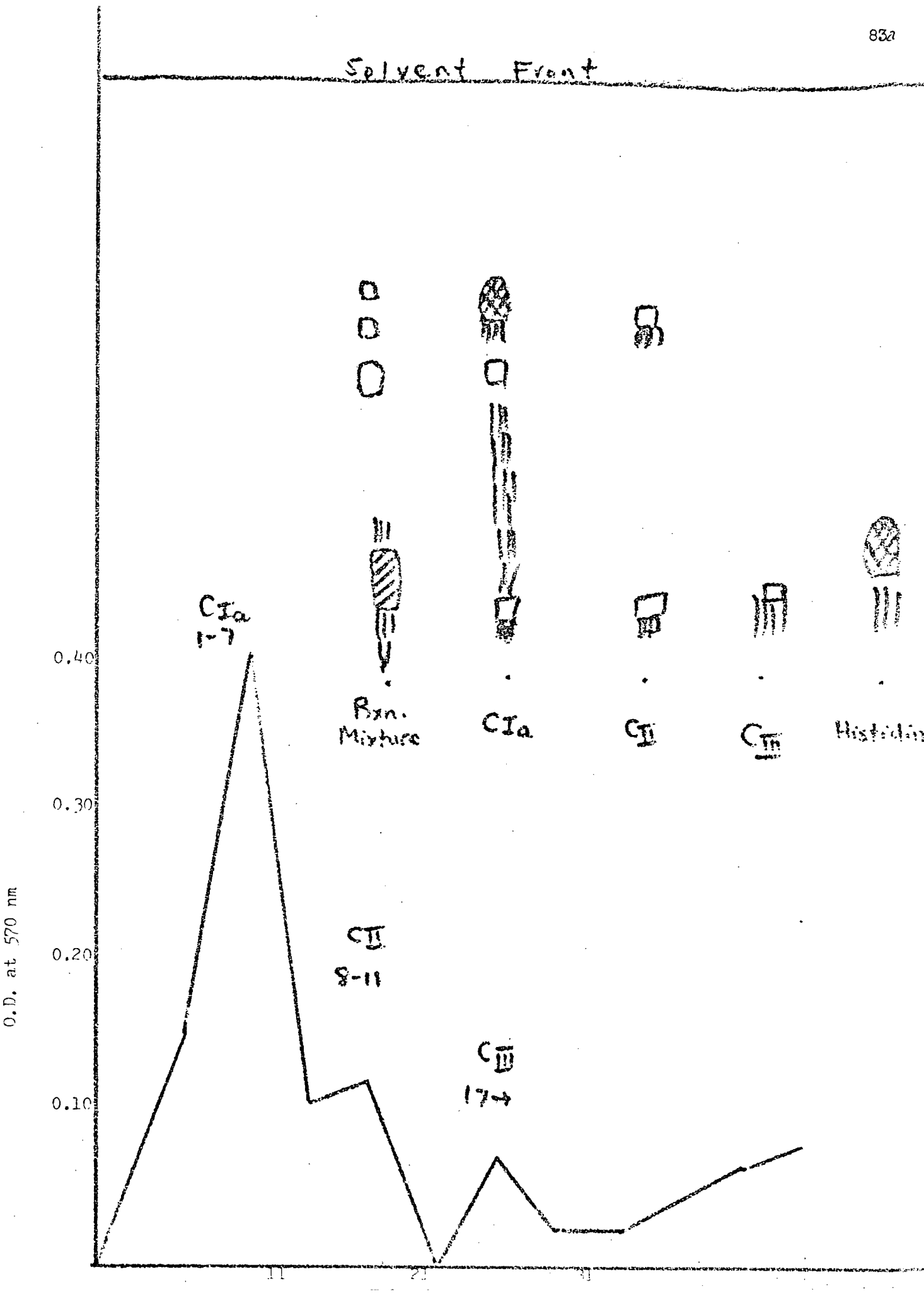

Solvent Front
0.50
0.40
0.30
0.20
0.10
O.D. at 570 nm
CI
3-9
CII
11-15
CIII
23-31
Rxn. Mixture
CI
CII
CIII
Histidi

Figure 18. Rechromatography on cellex of several preparations of Fraction C_I shown in Figure 17.


Solvent Front
CIa
1-7
CII
8-11
CIII
17→
0.40
0.30
0.20
0.10
O.D. at 570 nm
Rxn. Mixture
CIa
CII
CIII
Histidin

It was then decided to scale up the experiment by using a large cellex column (60 x 2.4 cm). Also, since it was apparent that whenever the reaction mixture was dissolved in solvent and then chromatographed, every fraction contained CM-histidine-like material, the freeze-dried reaction mixture was extracted with water and the insoluble material discarded. Figure 25 shows the fractionation of 1.5 ml of solvent containing 80 mg of water-soluble material on a large cellex column. Fraction I contained the Rf 0.62 pink spot and a small amount of the Rf 0.59 material. Fraction II contained the Rf 0.50 and 0.48 spots, and Fraction III contained mainly the 0.48 spots. Fractions IV-VII all contained histidine. All of the first three spots contained CM-histidine-like material.

It was found that washing the cellex with approximately 100 ml of formic acid followed by several washings in solvent was an effective method of equilibration.

Figure 26A shows the fractionation of 80 mg of water-soluble components dissolved in 3.0 ml of solvent and chromatographed on a 60 x 2.4 cm cellex column which had been equilibrated by washing with 300 ml of solvent. Fraction I (Tubes 31-42) contained a large amount of Rf 0.62 spot and CM-histidine. Fraction II (Tubes 43-48) contained a mixture of Rf 0.49 and 0.47 spots and traces of CM-histidine. Fraction III (Tubes 49-57) contained a mixture of all fractions except histidine. Fraction IV contained only histidine and a trace amount of Rf 0.47 spot. The tubes spanning Fraction I were analyzed with the results shown in Figure 26B. The last three tubes contained a mixture of 0.62 spot and more CM-histidine than the first three tubes. The fractions rich in Rf 0.62 spot were combined. Figure 27 shows a rechromatography on the small cellex column of 1 ml of solution of this material gathered from several cellex runs as well as several paper chromatographic runs. Fraction I and several individual tubes of the second fraction

Figure 19. Fractionation of 1.5 ml of solvent containing 80 mg of reaction mixture of PDA and histidine on a large cellex column.

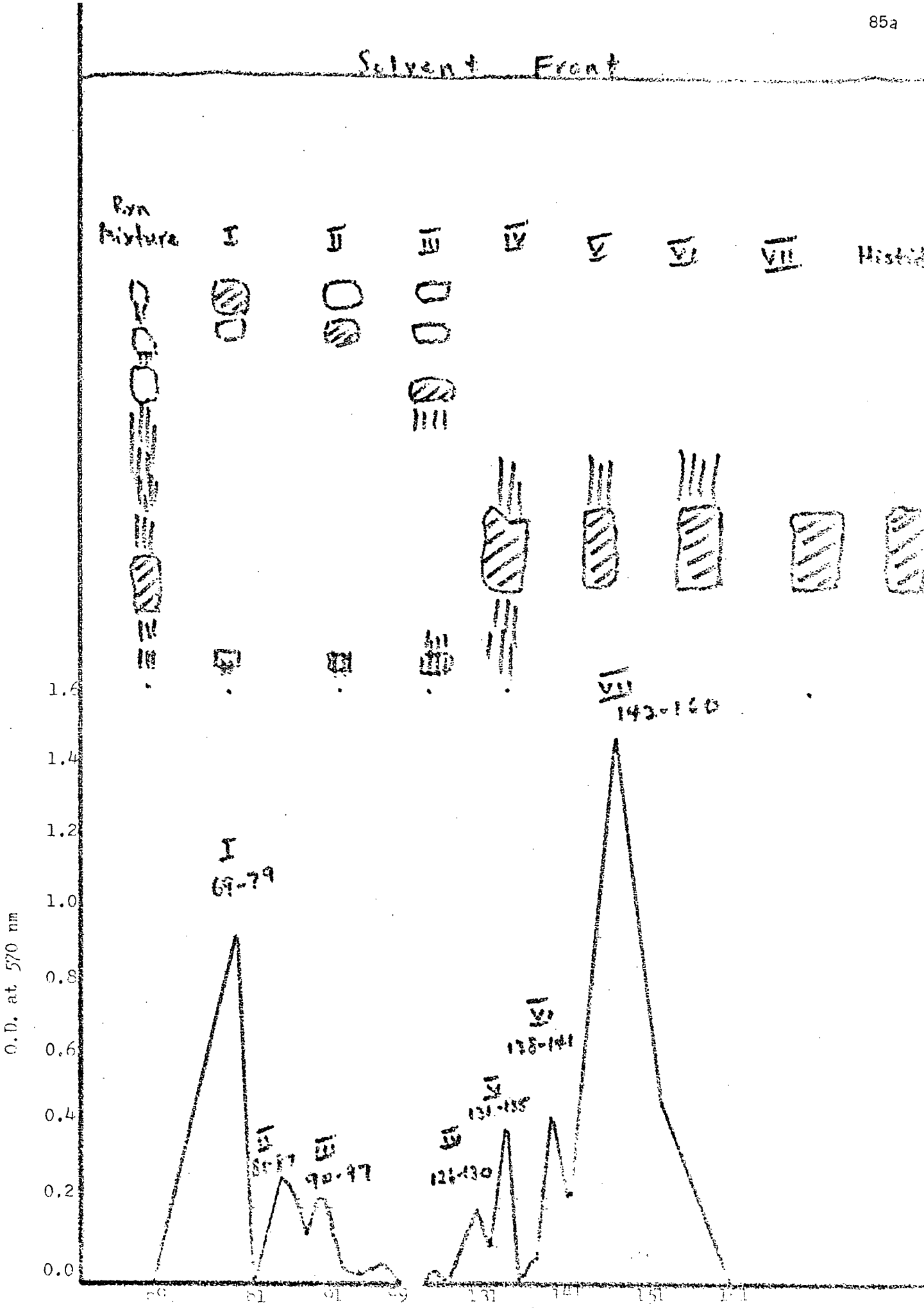

Solvent Front
Rvn Mixture
I
II
III
IV
V
VI
VII
Histid
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
O.D. at 570 nm
I
61-79
III
90-97
IV
124-130
V
131-135
VI
136-141
VII
143-160

Figure 20A. Fractionation of 3.0 ml of solvent containing 80 mg of reaction mixture and histidine on a large Cellex column.

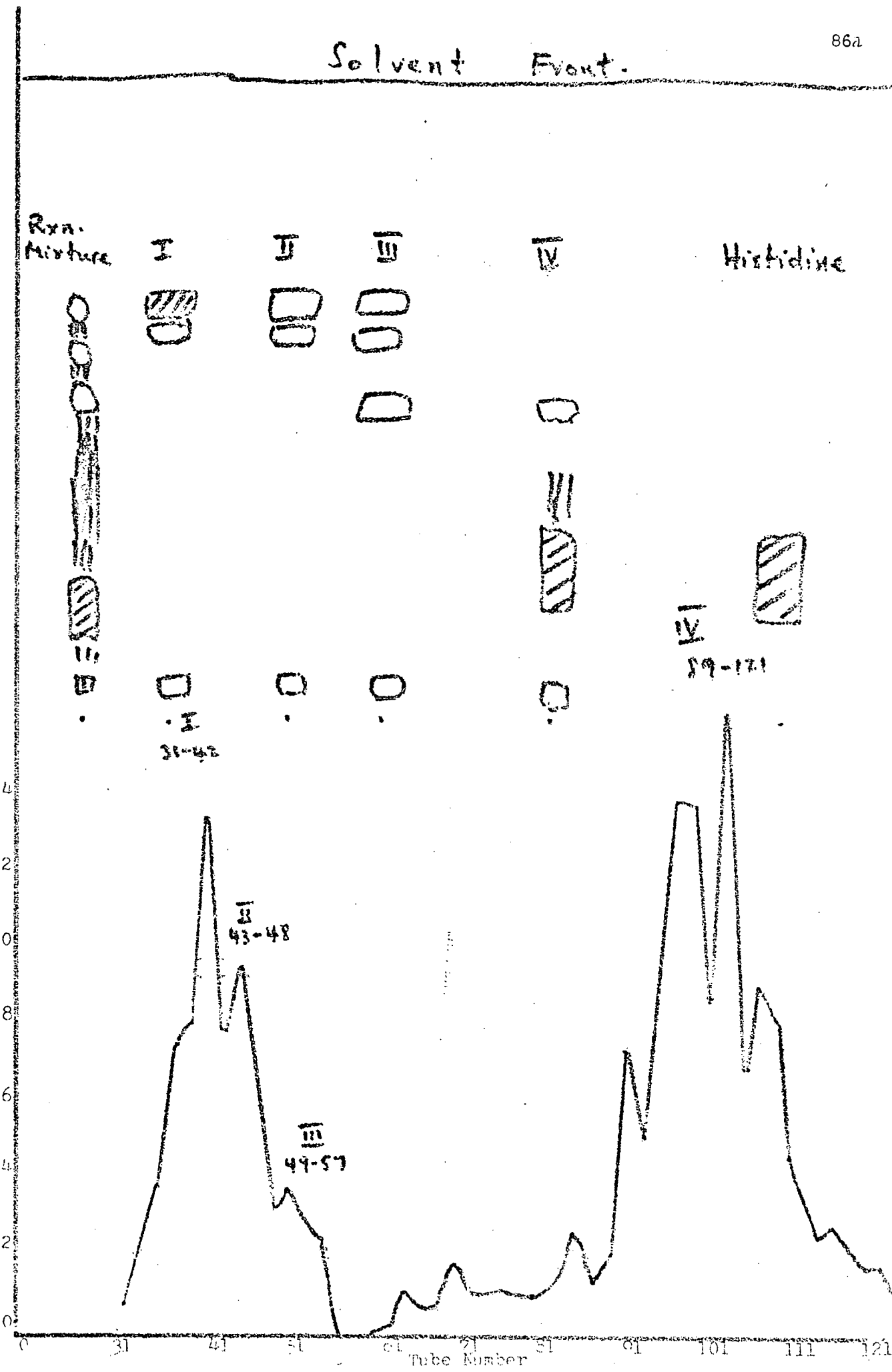
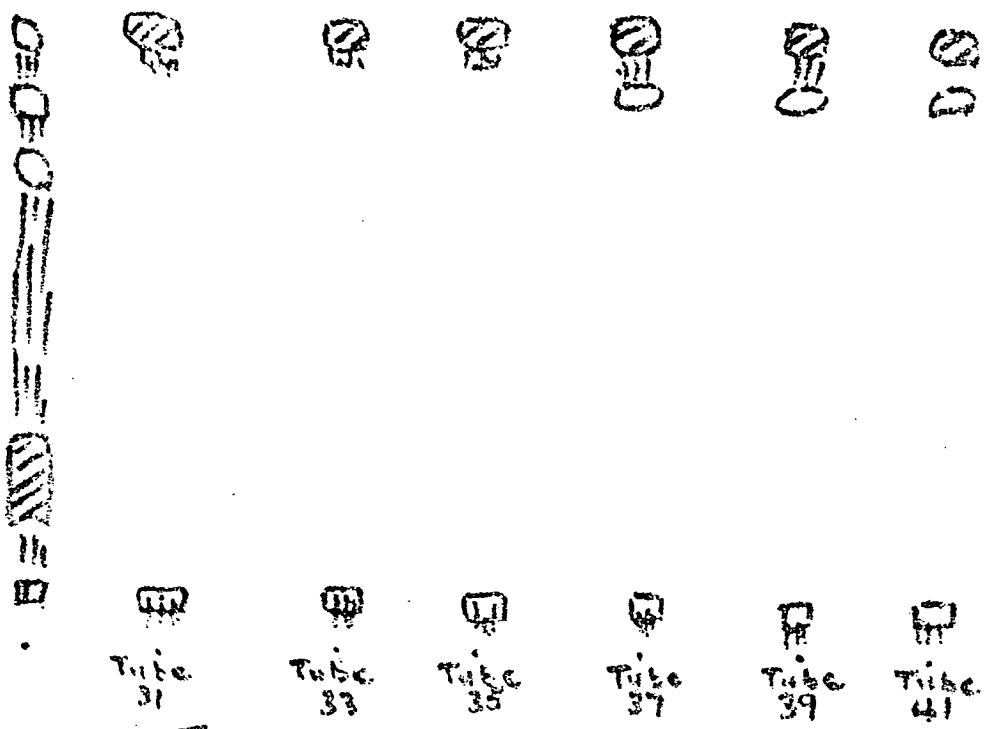

Solvent Front.
Rxn. Mixture
I
II
III
IV
Histidine
I
31-42
II
43-48
III
49-57
IV
59-121
O.D. at 570 nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
31
41
51
61
71
81
91
101
111
121
Tube Number

Figure 20B. Thin layer chromatography of the tubes spanning Fraction I of Figure 20A.

Solvent Front



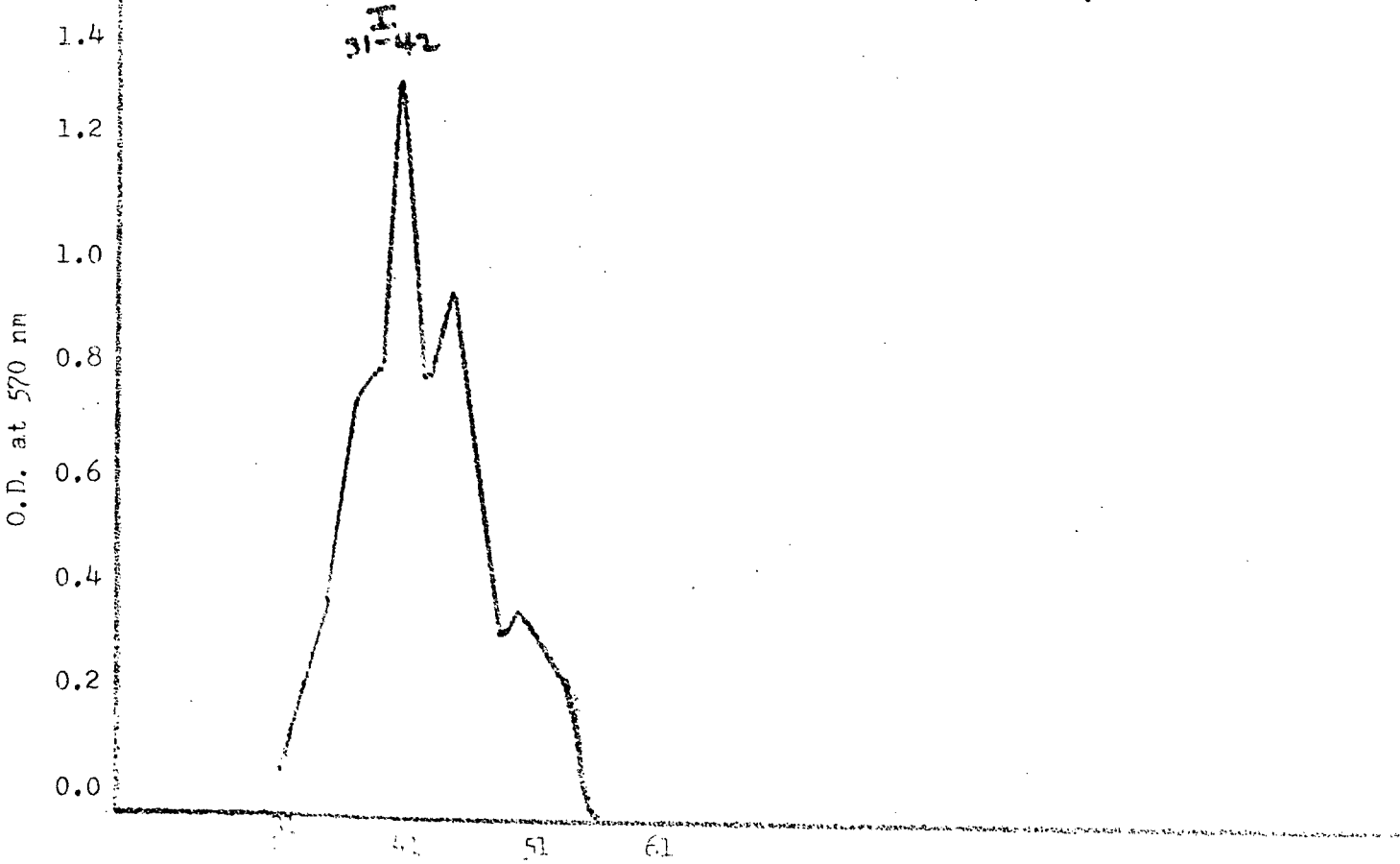
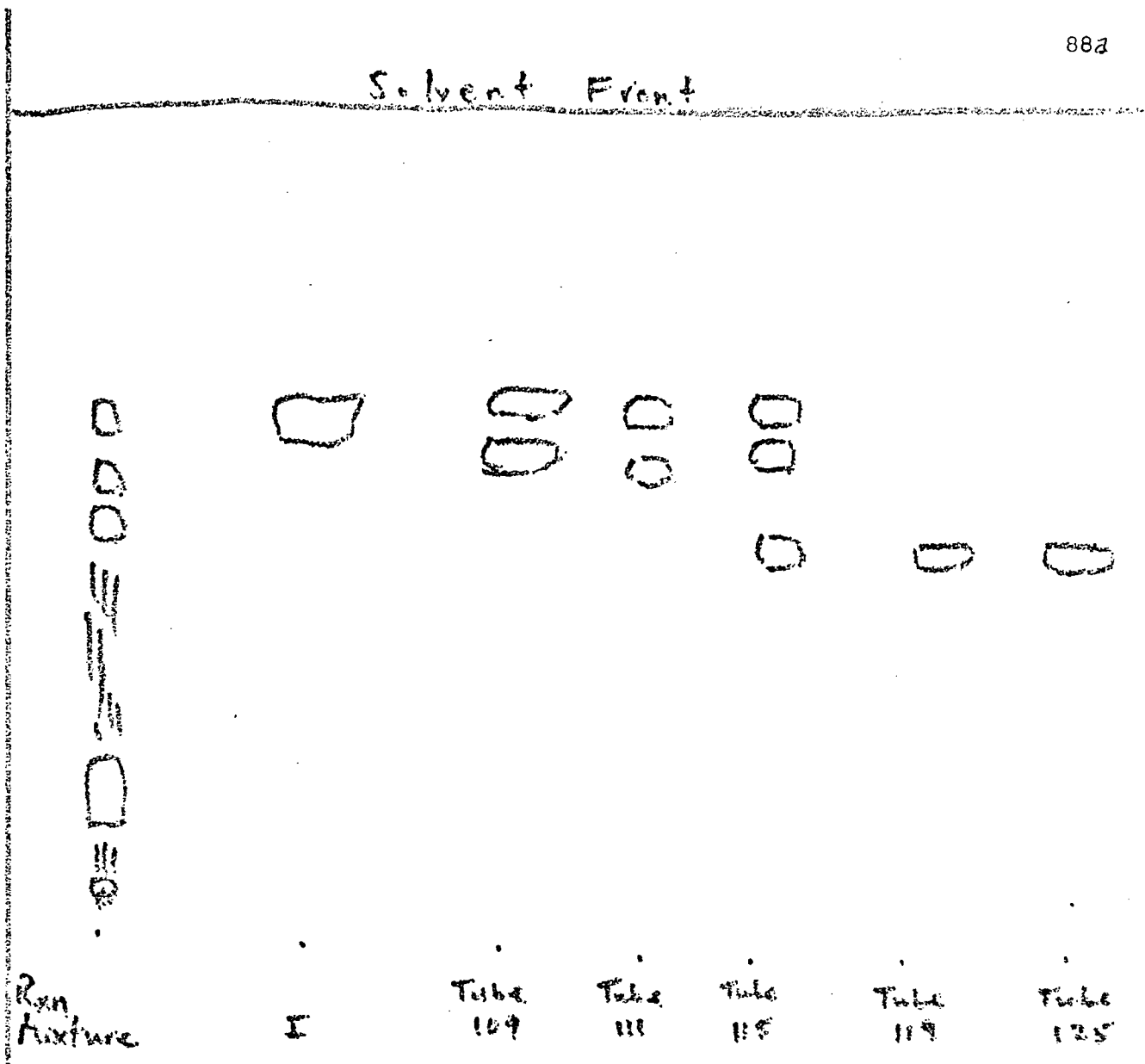
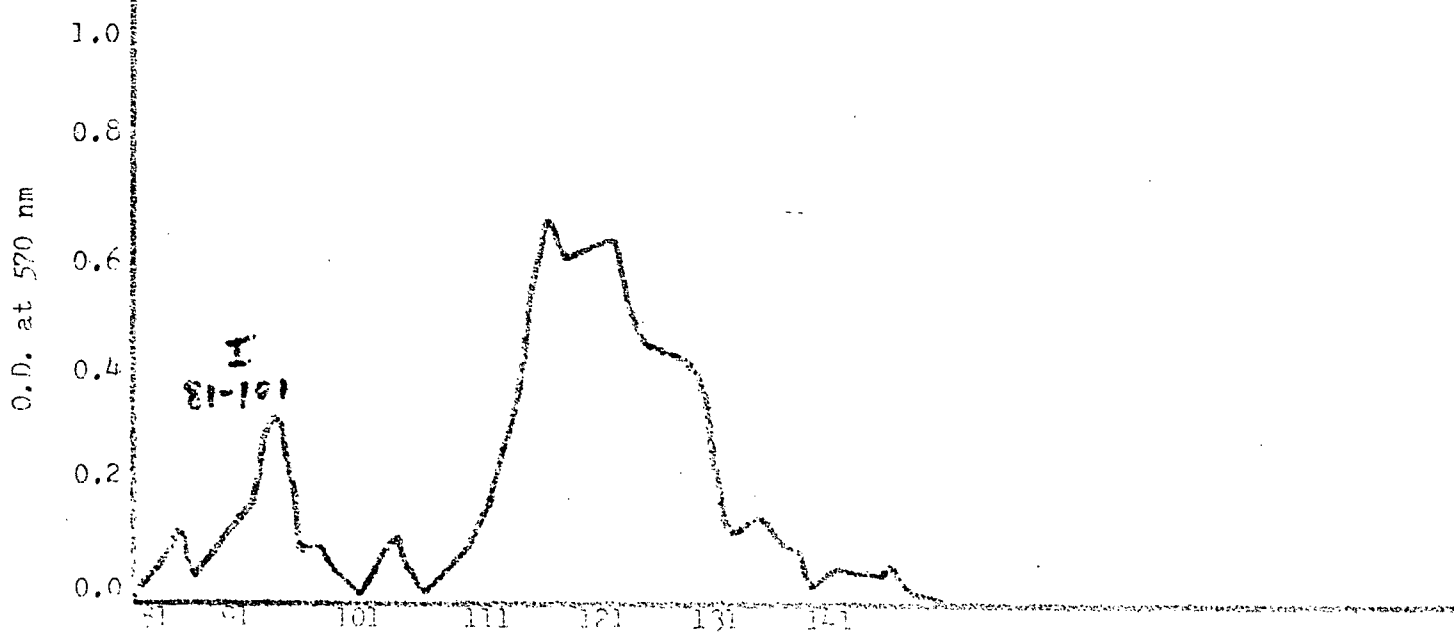


Figure 21. Rechromatography of 1 ml of solvent containing material rich in Rf 0.62 substance.


Solvent Front
Origin
Run Mixture
I
Tube 109
Tube 111
Tube 115
Tube 119
Tube 125


O.D. at 570 nm
1.0
0.8
0.6
0.4
0.2
0.0
I
81-101
91
101
111
121
131
141

were tested. Fraction I contained mainly the Rf 0.62 spot, tubes 109 and 111 a mixture of the Rf 0.62 and 0.59, and tube 115 all three. Tubes 119 and 125 contained only the Rf 0.48 spot.

By repeating this type of procedure the total amount of Rf 0.62, 0.58, and 0.48 material purified was 16 mg, 10 mg., and 5.0 mg. The actual weight of dry material was much larger but the salt content of each fraction was approximately 90% and thus there was not enough material for a proper chemical analysis. One serious difficulty which was found to happen with all three fractions was that when they were stored in water they broke down to the unfractionated reaction mixture.

These results show that it is possible to fractionate the reaction mixture of PDA-histidine on cellex columns using the standard Jones solvent for amino acid analysis by thin layer chromatography. The low yields, however, did not permit the second phase of this work being done, that is, attempting to desalt on a cellex column using formic acid-water (1:5) as the solvent. Also these studies emphasized the instability of the reaction products in water.

Series two

A. The Effect of varying the Concentration of Reactants

Table VI shows the effect on pH of the reaction mixture after 24 hours and 72 hours of varying the concentration of reactants. One great difficulty in carrying this experiment longer than 24 hours was that the methanol evaporated and had to be replenished daily. It is clear from Table VI, however, that increasing the molecular ratio of PDA to histidine led to a larger decrease in pH after a reaction time of 24 hours. Raising the ratio beyond 10 did not seem to result in further decreases in pH after a 24 hour reaction time, nor did it result in a further pH decrease when the reaction was allowed to go for 72 hours. It was then decided to set up

Table VI. The effect of concentration of reactants on the pH of the reaction mixture (PDA + histidine) after 24 and 72 hours.

Molar Ratio PDA: Histidine	pH of Reaction Mixture 24 hour	72 hour
1:2	6.22	6.22
1:5	5.99	5.99
1:10	5.20	5.20
1:20	5.20	5.15
1:30	5.20	5.15

a small scale reaction mixture using a tenfold excess of PDA and to freeze-dry, extract, and fractionate this mixture. Figure 22 shows the results of this experiment and seems to indicate that more reaction products were present than was the case with the reactions involving 1.8 molar excess of PDA. It also appears that the Rf 0.62 material constitutes more of the product. Figure 23 is a T.L.C. of 10 µl of reaction mixtures set up using various molar ratios of PDA to histidine. The solvent was allowed to run off the plate in order to obtain maximum separation. No obvious difference was detected by thin layer chromatography.

B. The effect of carrying out the reaction in the presence of Formamide

The same concentration of reactants as used by Nakai was used for this experiment, and water was replaced by formamide. The reaction was allowed to go for 24 hours, freeze-dried and extracted with water.

It was found that the solution turned from clear to a dark brown at the end of 2 hours reaction. The mixture also could not be freeze-dried to complete dryness. Attempts to extract with water resulted in no precipitation of unreacted PDA whatsoever. Nakai found only a slight increase in yield and recommended that no further attempts be made with this reaction.

C. The effect of refluxing PDA and Histidine in the presence of Aluminium Amalgum

Figure 24 shows the T.L.C. of 10 µl of the reaction mixture set up by refluxing for 6 hours at 65°C 2 grams of PDA dissolved in 23 ml of absolute methanol with 300 mg of histidine dissolved in 3.0 ml of water at 65°C. It seemed that this reaction did not give the same products as did the reaction at room temperature and for this reason no further work was done in this area.

Figure 22. Fractionation on cellex of PDA and histidine reaction mixture containing a 10 molar excess of PDA.

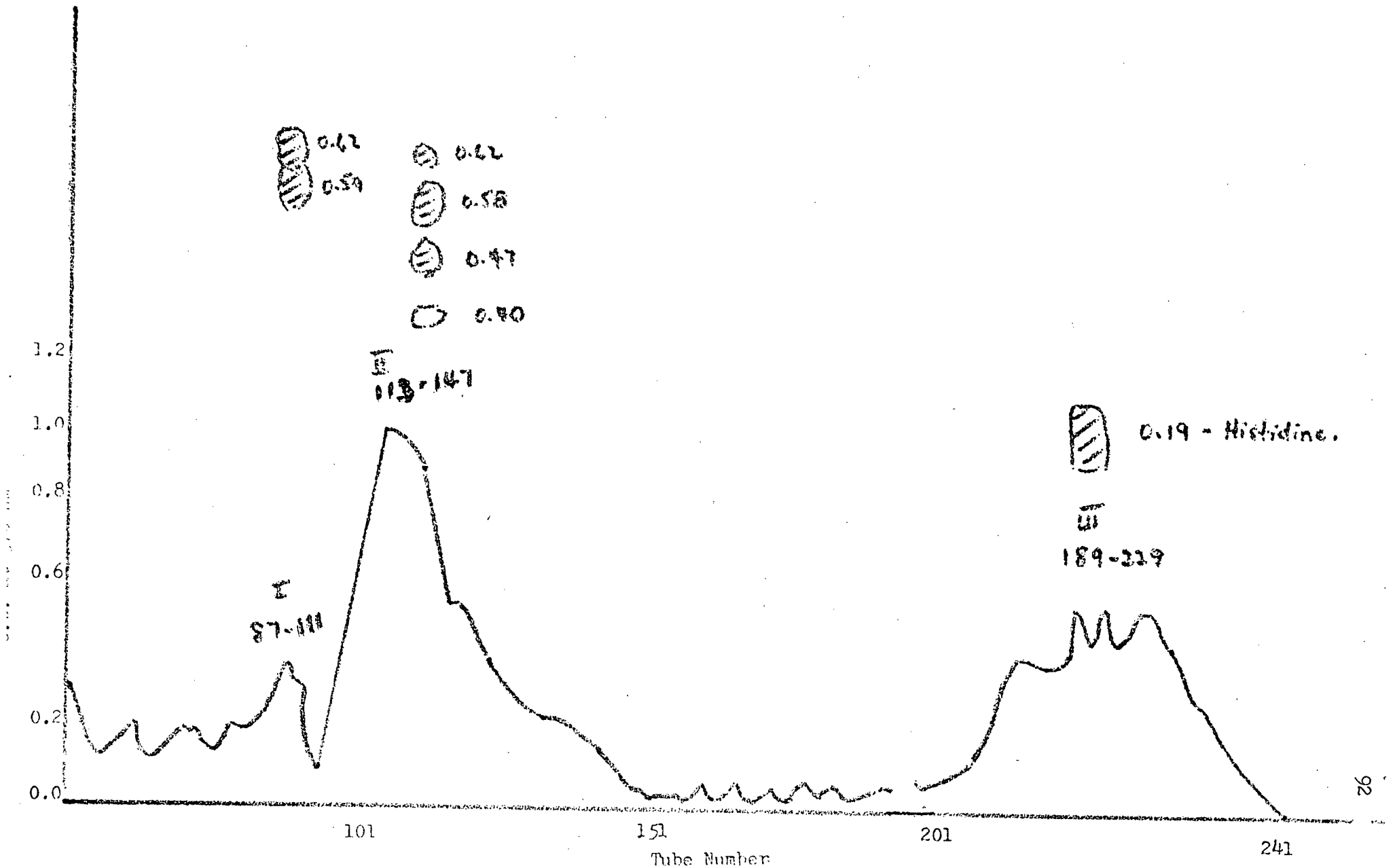


Figure 23. Thin Layer chromatograms of reaction mixtures of PDA and histidine containing various concentrations of reactives.

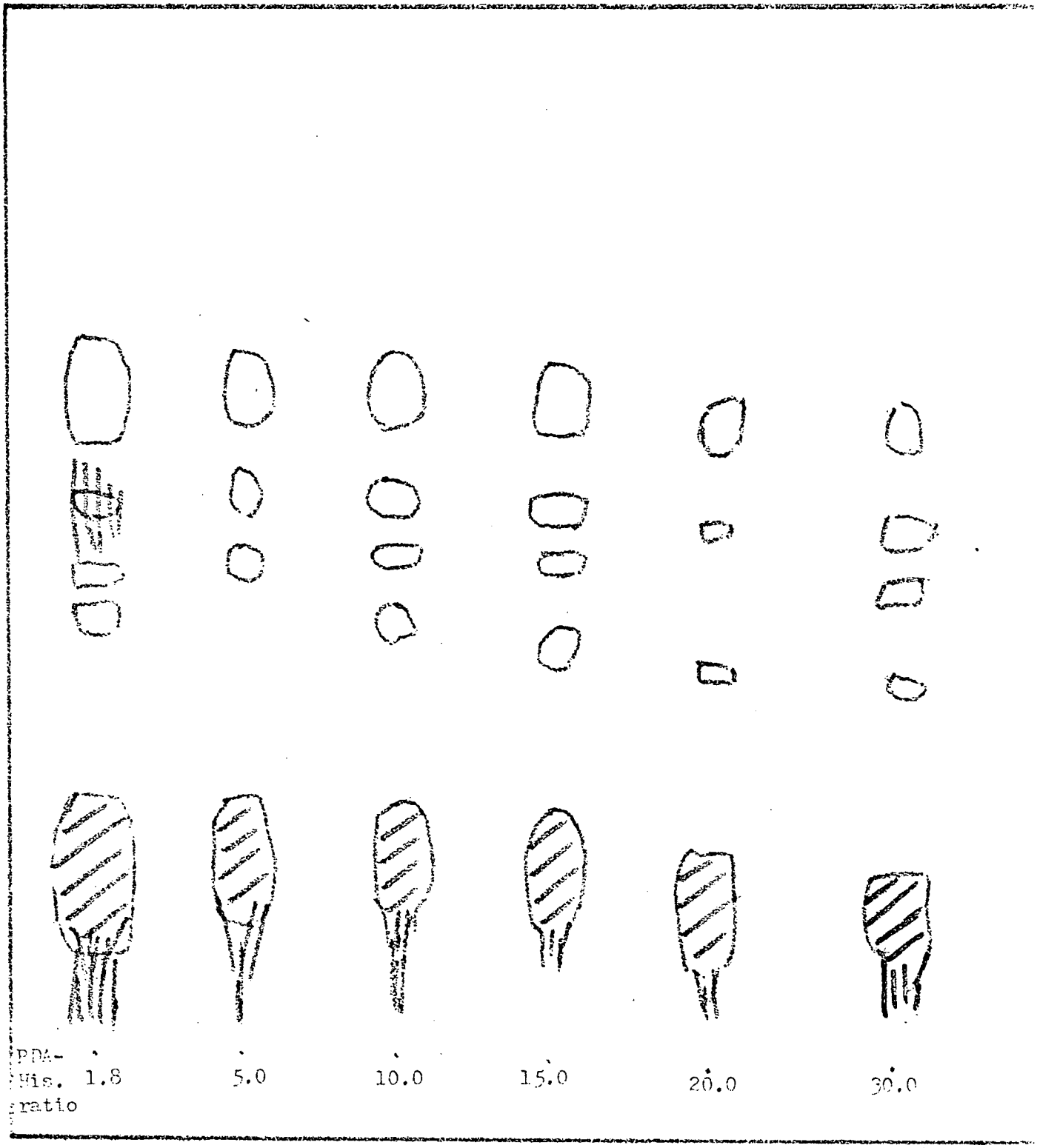
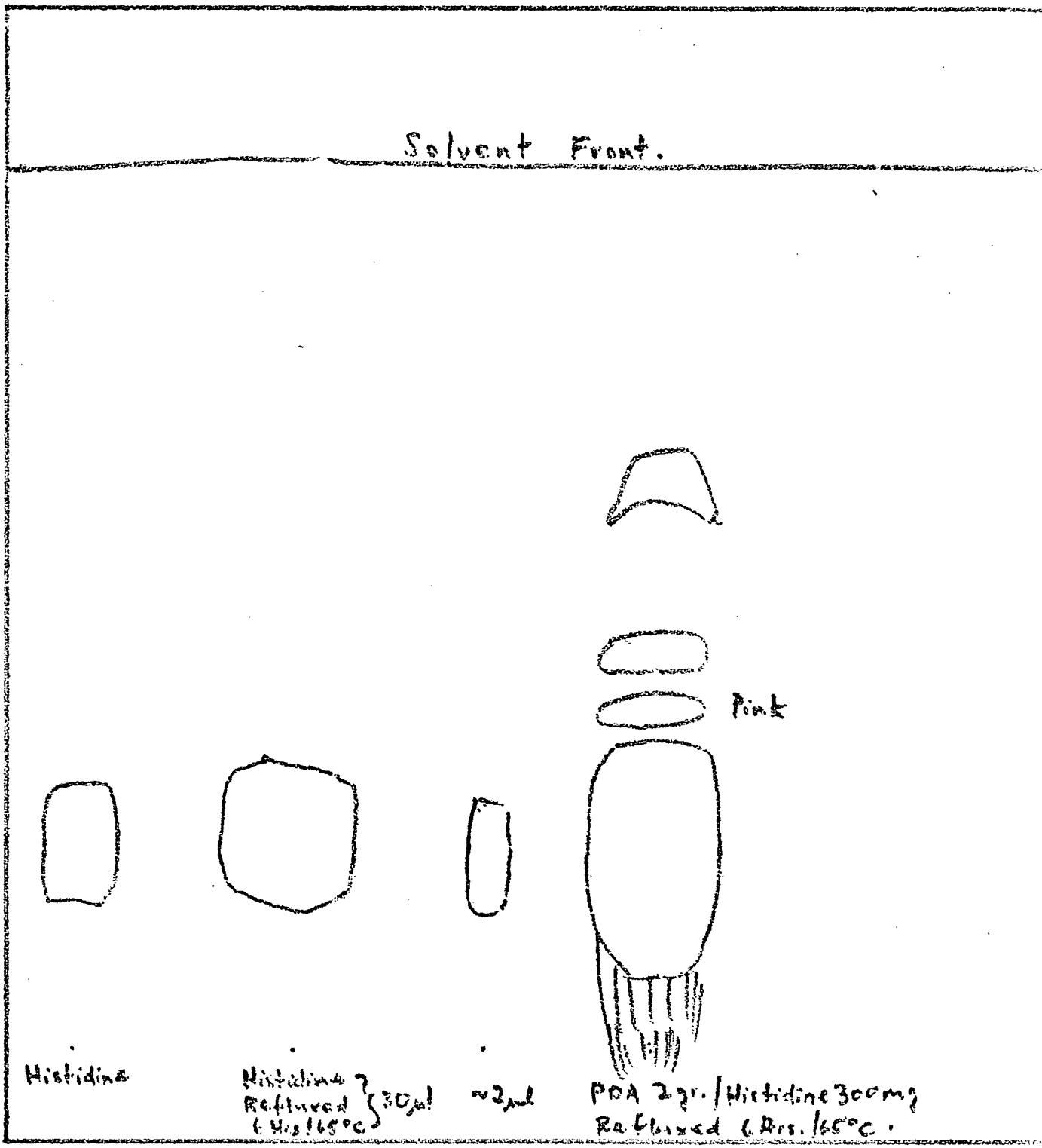


Figure 24. Thin Layer Chromatography of reaction mixtures of PDA and histidine refluxed in the presence of Aluminium Amalgum.



Series three

A. The effect of refluxing the Reaction Mixture in 6N HCl for 1 hour

Figure 25 shows the results of T.L.C. of 10 µl of the reaction mixture refluxed in 6N HCl in the same way as is done in the preparation of proteins for amino acid analysis. No difference was apparent indicating that no change takes place in PDA-histidine during hydrolysis of the modified protein.

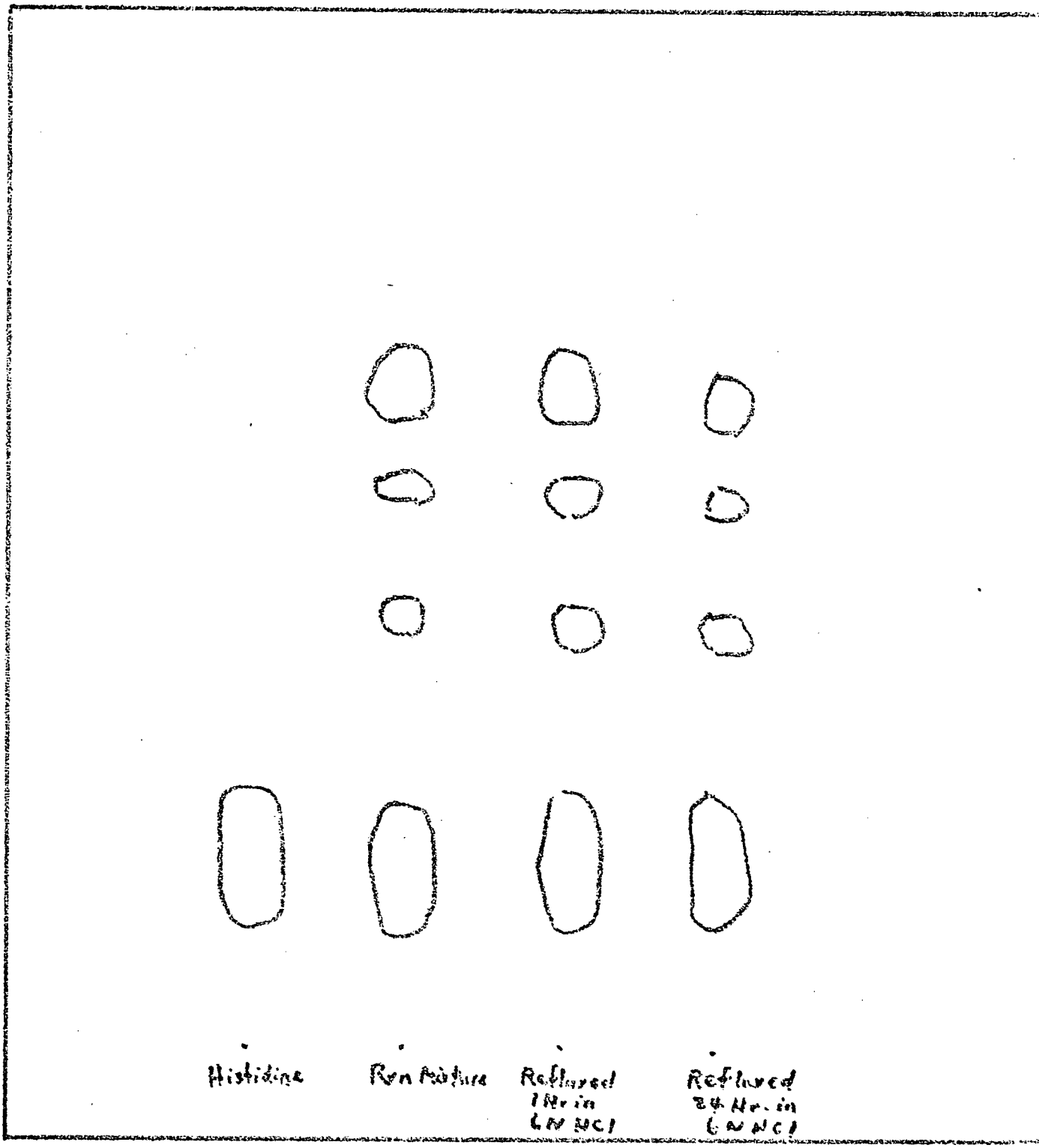
B. The reaction of spots of the Reaction Mixture to Pauley's Reagent

All were cherry red, which indicates that all spots contained the imidazole ring. This indicates that the reaction of PDA with histidine results in addition products and one method which might be used to give a preliminary indication of the chemical composition is to compare the absorbance at 220 nm. One difficulty in doing this is the low yields of products.

SUMMARY

Cellex chromatography in Jones' solvent can be used to separate the products of the reaction between PDA and histidine. Paper chromatography is also an effective method of purifying the individual fractions. Using a 10 molar excess of PDA seems to increase the amount of product having an Rf of 0.62 on thin layer chromatography plates. Refluxing PDA and histidine at 65°C in the presence of aluminium amalgum for 6 hours did not lead to an increase in yield. Carrying out the reaction in the presence of formamide resulted in only a slight increase in yield and no advantage since the formamide could not be easily removed. The products were not damaged by the normal method of preparing proteins for amino acid analysis. All of the products were Pauley reagent-positive, which indicates that histidine addition products were formed.

Figure 25. Thin Layer chromatography of reaction mixtures of PDA and histidine refluxed in 6N HCl for 1 and 24 hours.



CHAPTER VII

SUMMARY AND CONCLUSIONS

During preliminary studies in this thesis observations were noted in detail regarding the practical aspects of large scale production of Zittle's κ-casein. Temperature was found to be a critical factor throughout the procedure. Skimming was more efficient when cool milk was warmed to 45°C; the yield of casein was higher when the temperature of the milk was raised to 30°C; the mixture of the α_s - and β-casein precipitate and κ-casein supernate had to be held at room temperature during centrifugation because the α_s-casein was soluble at 4°C. During alcohol fractionation stirring had to be minimal, otherwise aggregation occurred. Electrophoretic analyses at various steps in the procedure showed that fractionations were successfully carried out even though the amounts of material used were at least ten times that in Zittle's original work.

The lowest yield of κ-casein obtained in these studies was 6.0% and the highest was 7.5%. The amino acid composition of two preparations of the κ-casein resembled that published in a recent review by McKenzie (23). Electrophoretic analysis showed that the κ-casein was heterogeneous. The subfractions could not be seen and studies were then carried out to improve the electrophoresis technique.

Four modifications which gave promising results were described in detail. They involved increasing the voltage through the gel, equilibrating the system by carrying out preliminary runs without samples, increasing the concentration of dissociating agents in the gel by equilibration in 0.0875M Tris-glycine buffer pH 9.1 containing 8M urea and 0.001M 2-mercaptoethanol, and decreasing the ionic strength of the system. The subfractions were clearly seen when methods incorporating these principles were applied and

were purified by DEAE cellulose chromatography during which several suggestions of Mercier (28) had to be closely followed.

The steps in Mercier's method which proved to be the most important included sifting the cellulose and using only that having a mesh size of 100-200 u, recrystallyzing the urea, and using low molarity NaOH and HCl for regenerating the cellulose. Subfractions 1 and 2 were purified and used, along with κ-casein, in reactions with PDA and PMA.

It was found that these reactions did not support the hypothesis that histidine plays an important role in the stabilizing ability of κ-casein because a derivative of PDA-κ-casein was prepared in low ionic strength buffer which had 0.6 residue of histidine less than untreated κ-casein but had the same stabilizing ability as untreated κ-casein. Moreover, PMA-KA1 and PMA-KA2, which had 1.05 histidine and 1.60 lysine residues less than the corresponding untreated proteins had the same stabilizing ability as KA1 and KA2. These results support the theory that the mechanism by which PDA causes κ-casein to aggregate is cross-linking between histidine residues on separate κ-casein molecules. Studies using the blocking group, sodium sulphenyl sulfonate, indicated that the role of disulfide bonds in the process of aggregation of PDA-κ-casein is remote.

An interesting phenomenon which was not investigated further was that a preparation of κ-casein whose state of aggregation was higher than that used in the preparation of the low ionic strength PDA-κ-casein, precipitated when it was reacted for 24 hours with PDA in the low ionic strength buffer. This indicates that the degree of cross-linking could be dependent upon the state of aggregation of the protein and the ionic strength of the reaction. Also, it may be conjectured that if PDA acts more rapidly with κ-casein when the ionic strength is low, its selectivity might also decrease.

Removing 1 bromine from PDA to make PMA seemed to result in a change of selectivity from histidine to lysine in that 1.6 out of 9.6 lysine residues per molecular weight 20,000 in KA1 and KA2 were modified by PMA whereas only 0.2 lysine residue was modified by PDA in these proteins. It might be possible to use PMA to modify lysine in other proteins. Incidentally, these studies involving the modification of lysine did not support those of Talbot and Waugh (50) who reported that decreasing 1.0 lysine residue in κ-casein led to a decrease in stabilizing ability.

One other approach taken to explain the mechanism of action of PDA was to react it with the amino acid histidine. The reaction was very slow and low yields were obtained. Three main products having Rf values of 0.62, 0.58, and 0.40 on thin layer plates were purified by paper and cellex chromatography using Jones' solvent (33) for the chromatographic separation of amino acids. The derivatives were histidine addition products as judged by their positive reaction to Pauley's reagent. Because of the low yields this approach has not led to an explanation of the mechanism of action of PDA. However, the products break down to carboxymethyl histidine-like products when reacted in base and this may help to explain the restoration of stabilizing ability obtained when Nakai (32) treated PDA-κ-casein in 0.1N NaOH.

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