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ON THE PROTEINS OF MILK WHEY

THE NITROGEN DISTRIBUTION IN WHEY, ELECTROPHORETIC CHARACTERIZATION OF PROTEINS, AND FEEDING EXPERIMENTS

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I. INTRODUCTION

1. THE WHEY PROBLEM

The so-called whey problem is well-known to every dairy expert. In the Netherlands alone the annual production of whey amounts to approximately 1 million tons, only 2 per cent of which is used for human consumption. Thus a little less than 1 million tons of whey, corresponding to 10^9 kcal of available energy are either used as animal food or completely wasted. The efficiency of domestic animals such as cows and pigs as "food producers" has been estimated to amount to about 30 per cent of the calorific value of the fodder supplied. Therefore even the use as animal food of a material which was, in the form of milk, fit for human consumption involves a loss of about 70 per cent of its total calorific value. In view of the world food shortage these losses of valuable food energy, which are paralleled in other parts of the world, need and receive increasing attention.

The limited use of whey for feeding purposes is due to the non-physiological ratio of its constituents, as shown below:

Water	93.6%
Lactose	4.8%
Crude protein	0.9%
Salt	0.7%

If it is borne in mind that of the 0.9 per cent crude protein only 0.7 per cent can be considered as real protein, the ratio protein : salt : lactose is approximately 1 : 1 : 7. Since the protein is the most valuable constituent this ratio is very unfavourable to nutritional application.

One method of concentrating the protein is its heat denaturation and separation. Thus the so-called lactalbumin, a rather well-known fodder constituent, is gained as a by-product in the milk sugar industry. However, this preparation implies serious denaturation of the protein with complete loss of many of its valuable native characteristics.

The other method might be the removal of the less valuable components (water, salt and to some extent lactose) from the whey, in order to increase the protein content. This procedure is much more difficult. Nevertheless, several attempts have been made, by ion exchange, by dialysis and by electrodialysis. From the numerous difficulties attending upon the application of any of these processes we mention: the cost of acid and alkali, necessary to regenerate the ion exchange materials and the deterioration of protein which occurs during prolonged dialysis.

In 1946 the General Technical Department of the Central National Council for Applied Scientific Research in the Netherlands (A.T.A.-T.N.O.) started an investigation into the electrodialytic desalting of whey. After some years this research appeared to open fairly promising prospects. Since 1948 another study has been undertaken into the proteins of whey in order to ascertain the possible application of desalted whey products. The results of the latter study will be presented in this report.

Two preliminary communications on this work have already been published in

The Netherlands Milk & Dairy Journal by Prof. Dr L. SEEKLES (1951) and by the author (1951).

For a short general review of the whey problem we refer to NIEMAN (1951).

2. THE MANUFACTURE OF FOOD STUFFS FROM WHEY BY ELECTRODIALYSIS

The manufacturing process includes four phases of which the electrodialysis may be considered the most essential:

a. The whey is concentrated in vacuo to 50 - 60 per cent solids.

b. A part of the lactose, crystallized during the concentration is separated by centrifuging. Thus the ratio protein/lactose can be adjusted.

c. The whey concentrate is subjected to electrodialysis. By this procedure the ratio protein/salt is regulated.

d. The remaining concentrated liquid is preserved by roller-, spray- or freeze drying.

a needs no further explanation

b is a problem in itself as will be well-known to the manufacturers of milk sugar. However it has become more or less practicable, either before or after phase *c*. This means that the ratio protein/lactose cannot be varied extensively, but the possibilities of variation are wide enough as will appear from the analytical data to be presented later. The lactose separated can be gained as a valuable by-product.

c The principle of electrodialysis may be understood from Fig. 1. The apparatus consists of three cells, separated from each other by two membranes (e.g. cellophane). The whey circulates through the middle section, whereas the outer compartments are fed with two different rinsing liquids viz., a dilute salt solution for the anodic section and a dilute alkaline solution for the cathodic one. From the electrode reactions (see fig. 1), acid and alkali originate in the anode and cathode compartments respectively; so the salt ions of the whey (M^+ and Z^-) are removed from the middle cell, while H^+, A^+ and OH^- ions move into this compartment where the H^+ and OH^- ions combine to form water. In consequence of the value of the electrolytic mobility of H^+, which is higher than that of OH^-, the respective quantities moving inwards per sec. are not equal. Now by means of a proper addition of salt (viz. K_2SO_4 or Na_2SO_4) to the anodic rinsing liquid, the transport ratio of H^+ in the anodic membrane can be kept equal to that of OH^- in the cathodic membrane. This explains the presence of the A^+ ions in Fig. 1.

For a more detailed description of the electrodialytic process and its application to whey we refer to the paper of ATEN, WEGELIN and WIECHERS (1949), the thesis of WIECHERS (1951) and the various patents on this procedure.[1]

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[1] British Patents : 637.425
Dutch Patents : 67.874, 67.868, 67.903, 68.382
French Patents : 986.196, 983.252

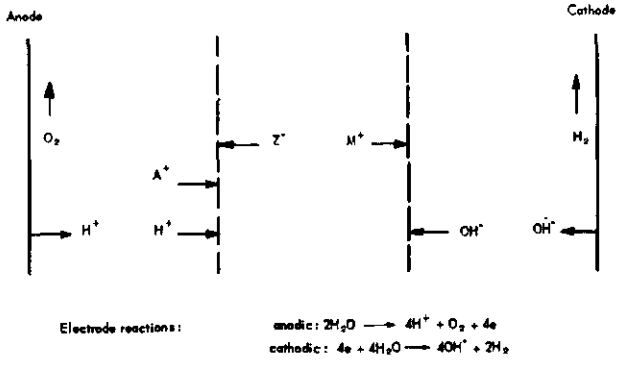

Fig.1. Scheme of the electrodialytic desalting process.

Attention may be drawn here to the facts that the process is now executed with absolute constancy of pH (mostly chosen about 7.5) and at a moderate temperature (30°C). It takes only a few hours and involves rather low costs for electrical energy.

A semi-technical installation, capable of working up a 100 litres of whey per hour has yielded completely satisfactory results and a project for a semi-technical plant with a capacity of 3600 l/h has been made.

With this method the variation of the ratio of protein to salt is almost unlimited, although the necessary energy costs rise progressively as the degree of desalting increases. By special measures the ions to be removed can be selected according to species. Generally the univalent ions (K^+, Na^+, Cl^-) can be removed for the greater part and besides Ca^{++} and/or phosphate if desirable. It is even possible to regulate the potassium - sodium ratio of the remaining salt, if this is necessary.

d The preservation of the product by drying will generally be desirable. Although every drying method can be considered, a gentle method such as spray or freeze drying is recommended because of the sensitiviness of the whey proteins to heat.

The following analytical figures of three different products will give an idea of the various possibilities of the method described:

	Desalted whey			Average composition of dried non-treated whey (%)
	sample I (%)	sample II (%)	sample III (%)	
Crude protein	14	26	35	14
Lactose	83	65	54	75
Salt	3	9	11	11

A product of mild sweet taste is obtained, when 80 per cents of the salts originally present are removed. The taste is slightly salty after removal of 50 per cents of the salts.

3. THE BEHAVIOUR OF THE PROTEIN DURING THE ELECTRODIALYTICAL DESALTING PROCESS

When liquid condensed whey containing about 45% solids is subjected to electrodialysis a partial flocculation of the protein is often observed. This phenomenon which is dependent on pH occurs only at pH values less than 7.5. The coagulum originates particularly on the cathodic membrane. This is rather surprising for the proteins are charged negatively at pH values above about 4,6, the isoelectric point. Hence the fur on the membrane cannot be due to an electrophoretic concentration effect. Neither can it be exclusively attributed to the effect of pH, for with ordinary dialysis against distilled water flocculation does not occur whatever the value of the pH may be. Nor does it occur when the dialysis is performed in the electrodialytic apparatus with the usual rinsing liquids but without current supply. So the phenomenon must be due to the combined influences of pH and electric current.

Analysis of the coagulum shows that only 16% of its solids are proteins. For the rest it consists chiefly of calcium and some phosphate, carbonate, citrate and lactose. It can be removed from the membrane by washing with 0.1 N acid or 0.1 N alkali, i.e. by the use of extreme pH values.

In view of these facts we suggest the following hypothesis for the origin of the coagulum:

In comparison with the solubility product of $Ca_3(PO_4)_2$ whey seems to be considerably supersatured with this constituent. According to recent work of SMEETS (1952), however, most of the calcium in the ultrafiltrate of milk is present as a complex calcium citrate ion $CaCi^-$, where Ci stands for $C_6H_5O_7$. This complex is in equilibrium with the trivalent citrate ion Ci^{---}:

$$Ci^{---} + Ca^{++} \rightleftarrows CaCi^-$$

As a consequence of the values of the dissociation constants of citric acid ($pK_1 = 3.0$, $pK_2 = 4.75$, $pK_3 = 5.5$) it will be clear that at alkaline pH values only the Ci^{---} ion will be present. At lower values the di- and monovalent ions will also participate in the equilibrium, but these ions are not capable of forming the above-mentioned calcium citrate complex. Hence part of the complex ions will decompose and Ca^{++} ions will be liberated. No $Ca_3(PO_4)_2$ will be precipitated as the solution is acid. When an electric current is passed through the cell the Ca^{++} ions will move towards the cathode. At the cathodic membrane, however, they will enter into an alkaline layer, which has been formed by the OH^- ions moving from the cathodic section into the middle compartment (see fig. 1). The result will be a precipitate of $Ca(OH)_2$ on the cathodic membrane. Some calcium phosphate and calcium citrate will also be precipitated, although not to such an extent as $Ca(OH)_2$, for the phosphate and citrate ions are gradually moving away from this alkaline region under the influence of the electric field. In addition, it is reasonable to expect that some protein will be entrained in the precipitate. which is a quite usual phenomenon, as will be well-known from several deproteinizing methods.

If the pH of the middle compartment is kept sufficiently high there will be nearly no Ca^{++} ions and the coagulum on the cathodic membrane will either be inhibited or it will only form at a very slow rate. On the other hand in this case there will be hardly any removal of Ca^{++} ions; for nearly all Ca^{++} ions have been converted into $CaCi^-$ ions, which move towards the anodic membrane, are

decomposed in the corresponding acid layer adjoining this membrane, whereupon Ca^{++} ions move back into the bulk of the whey. There the $CaCi^{-}$ ion is formed again and so on.

The removal of citrate will follow its normal course. So with prolonged electrodialysis the "stabilizing" influence of the citrate will decrease and a precipitate will be formed on the cathodic membrane towards the end of the desalting process. This agrees quite well with the experimental facts.

Thus in our opinion the cause of the cathodic coagulum is not to be sought in the protein but in the ionic equilibria in the ultrafiltrate.

Under conditions of extreme desalting another kind of coagulum sometimes occurs; this is rather proteinlike and will settle on both membranes, although preferentially on the anodic one. This may be due to the diminished stability of the protein itself at very low salt concentrations. As is to be expected in this case an increase of the pH will give the increase in stability needed to avoid coagulation.

4. FORMULATION OF THE PROBLEM

In view of the facts mentioned in the preceding sections, the General Technical Department T.N.O. charged the author to make an investigation into the following subjects:

a. The gathering of chemical and physico-chemical data on the proteins of whey of cows' milk and to a certain extent of human milk; this to be done by compiling data available from literature as well as by original experimental work.

b. The judging of various products, prepared from whey desalted by electrodialysis by the quality of its protein, and the study of the influence of the electrodialytic procedure on the characteristics of the protein.

c. The execution of biological and biochemical tests on the usefulness of the products mentioned for feeding purposes, especially as a fodder for poultry, cows and pigs.

The General Technical Department T.N.O. was fortunate in obtaining the co-operation of Dr L. SEEKLES, Director of the Laboratory of Veterinary Biochemistry of the State University of Utrecht, who consented to the investigation being carried out under his supervision and in his Institute. For this most valuable help the A.T.A.-T.N.O. and the author are very much indebted to him.

The subjects *a* and *b* will be treated under section II: "Chemical Researches", subject *c* under section III: "Feeding experiments".

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II. CHEMICAL RESEARCHES

1. REVIEW OF LITERATURE

a. General data on the proteins of milk whey

Whey is the yellowish green liquid that remains after the precipitation of casein from skim milk. The nitrogen content of this liquid depends on the stage of lactation and on the method of removing the casein. In the case of cows' milk the influence of the stage of lactation is largest during the first three days, and from then onwards the N content remains fairly constant. Hence it is possible to give average figures for "normal" cows' milk whey by excluding the colostrum of the first days post partum.

When the casein is clotted by means of rennet the N content amounts to 120-150 mg per 100 ml whey, whereas with acid precipitation the same samples of milk will yield whey solutions containing 100-130 mg N per 100 ml. Apparently the rennet has converted part of the casein into soluble protein. This derivative of the casein fraction is unfortunately frequently denoted in the literature as "whey protein".

Apart from this "whey protein" the further N containing substances have been differentiated into

Albumin
Globulin
Proteoses and Peptones
Non-protein nitrogen

The analytical figures obtained by various authors differ widely, especially in the case of globulin and proteoses and peptones. This is mainly due to the different methods of estimation which in turn are connected with different definitions of the above-mentioned fractions.

Since the work of SEBELIEN (1885) in the older literature (COHNHEIM, 1904; GRIMMER, 1910) albumin and globulin have been defined with the help of the following characteristics of their solubility:

Solvent	Albumin	Globulin
1. Saturated $MgSO_4$	soluble	insoluble
2. Half saturated $(NH_4)_2SO_4$	soluble	insoluble
3. Saturated $(NH_4)_2SO_4$	insoluble	insoluble
4. Water	soluble	insoluble

The classical method of separation due to OSBORNE and WAKEMANN (1918) uses the first three criteria, whereas other methods like those of SEBELIEN and SCHLOSSMANN (See ABDERHALDEN (1909)) use *Potassium alum* and *Almens reagent* (tannic acid in alcohol with some acetic acid).

Unfortunately the first three criteria do not agree with the fourth. According to the former three WICHMAN (1899) and later SJÖGREN and SVEDBERG (1930) succeeded in preparing crystallized lactalbumin from a saturated solution of

$(NH_4)_2SO_4$. PALMER (1934), however, dialyzed an amorphous precipitate of lactalbumin against frequent changes of distilled water for a long period. A clear solution was obtained from which, after prolonged dialysis, he managed to isolate a crystalline protein, nearly insoluble in salt-free water but soluble when small amounts of electrolytes were added. According to the latter criterion this product was a globulin, although it was obtained from the albumin fraction. PEDERSEN (1936) identified this protein with the β component of the ultracentrifugal diagram of milk whey; hence it has been termed β lactoglobulin. Thus it should be borne in mind that the greater part (60% or more) of the classical lactalbumin fraction consists of β lactoglobulin which is completely different from the classical globulin fraction. We will denote the latter fraction merely by "globulin".

In addition to these proteins whey contains a series of N containing substances of lower molecular weight. These can be classified as proteoses and non-proteins. The non-proteins are not coagulable by any means whereas the proteoses are coagulable by strong deproteinizing agents (e.g. trichloracetic acid) but not by heat. Some authors (e.g. KIEFERLE and GLOETZL (1931) distinguish albumoses (coagulable by saturated $ZnSO_4$) and peptones (coagulable by phosphotungstic acid). There are various other coagulation methods not mentioned here which are based on the use of other reagents at different concentrations or temperatures. The remaining non-proteins include urea, ammonia, α amino nitrogenous compounds, creatine and creatinine and xanthine derivatives (usually referred to as uric acid). In table 1 the average analytical figures of normal milk are given as obtained by SHAHANI and SOMMER (1950) and by KIEFERLE and GLOETZL (1931).

TABLE 1. Average distribution of nitrogen (mg %) in normal acid whey.

	SHAHANI and SOMMER (1950)	KIEFERLE and GLOETZL (1931)
Total nitrogen	102.1	130.9
Albumin N	37.9	} 65.6
Globulin N	24.1	
Albumoses	} 16.3	27.2
Peptones		18.7
Non-protein N	23.9	22.9
Urea	8.6	10.1
Creatine	3.9	2.2
Creatinine	0.5	1.7
α amino N	3.9	4.6
Ammonia	0.8	1.1
"Uric acid"	2.3	1.5

The higher N content of the whey samples of KIEFERLE and GLOETZL

seems to be especially due to the proteoses. However their heat treatment may have been more rigorous resulting in a shift of albumin + globulin to proteoses.

NESENI and KORPRICH (1947) studied the dependence of the amount of total protein, casein, albumin, globulin, non-protein, fat and total solids on the progress of lactation. The rapid fall of the globulin content during the first days of lactation is the most striking effect.

The amino-acid composition of various whey fractions has been the subject of many investigations. The review of DE MAN (1949) gives figures on the classical lactalbumin and some figures on the whey proteins as a whole. MC MEEKIN & POLIS' table in their review of milk proteins (1949) includes β lactoglobulin and globulin from normal milk and from colostrum. BRAND and coworkers (1945) give a fairly complete analysis of β lactoglobulin. Comparing the figures of β lactoglobulin and the classical lactalbumin there are many small but not essential differences.

The structure and properties of β lactoglobulin have received much attention. Its preparation according to PALMER (1934) has been modified by BULL & CURRIE (1946) and by CECIL & OGSTON (1949) and its molecular weight has been determined by various methods. The results are:

Authority	Method	Date	Mol. Weight
PEDERSEN	Sedimentation & Diffusion	1936	39.000
CROWFOOT & RILEY	X rays	1938	36.000
SENTI & WARNER	X rays	1948	35.000
BULL & CURRIE	Osmotic pressure	1946	35.050
CECIL & OGSTON	Sedimation & Diffusion	1949	35.400
HALWER, NUTTING & BRICE	Light scattering	1951	35.700

The structure of dry β lactoglobulin has been elucidated by electron microscopy (DAWSON, 1951) with a complementary X ray diffraction study by RILEY (1951).

The dissociation curve of β lactoglobulin at various values of the ionic strength has been studied by CANNAN, PALMER & KIBRICK (1942).

In an attempt to elucidate further the browning reaction in milk, FRAENKEL-CONRAT, COOK and FAY MORGAN (1952) examined the reaction between β lactoglobulin and lactose. It appeared that the reaction sets in at 53° C. The number of SH-groups (Native: 2 groups/mole) is reduced by heating and this reduction is independent of presence or absence of lactose.

SMITH (1946) succeeded in isolating two fractions from colostrum globulin one of which was soluble in pure water. Hence he introduced the names eu- and pseudoglobulin. Both fractions are responsible for the immunological properties of the colostrum, so the classical globulin may now also be termed immunoglobulin.

WEINSTEIN et al. (1951) have reported on the isolation and characterization of a minor protein fraction in whey, capable of producing the "Solar-activated flavour" of milk. According to their electrophoretic work this fraction is composed of at least two components or complexes.

Finally three general reviews on the proteins of whey have to be mentioned. SÖRENSEN and SÖRENSEN (1938-1941) have reviewed the literature up to 1940 in an introduction to their extensive fractionation study. Experimentally they obtained 5 fractions by cautiously salting out with $(NH_4)_2SO_4$, but some of the fractions may have been different forms of identical substances.

Little information is available in the thesis of FILLEUX (1947) whereas a condensed but fairly complete review has been published by PERCEAU (1950) quoting the literature from 1852 up to 1949.

b. Electrophoretic and ultracentrifugal data on skim milk and milk whey

The application of the above-mentioned physicochemical methods of research into the proteins of milk has opened prospects for a classification more justifiable than the one described in the previous section.

SVEDBERG (1938) made an ultracentrifugal investigation into milk which yielded nine different components characterized by the letters α - ι, indicating increasing sedimentation velocities. The components δ - ι were attributed to the casein, α and β to the lactalbumin and γ to the lactoglobulin. The differentiation of the soluble (whey) proteins into three components was due to PEDERSEN (1936). The heterogeneity of the casein fraction seems to be confirmed to some extent by electrophoretic work (e.g. WARNER (1944) and MELLANDER (1945)). These results, however, have been obtained with dialyzed casein, which is completely different from the native form. FORD & RAMSDALL (1949) have shown that native casein consists of units of highly divergent molecular weights in agreement with Svedbergs work, but in contrast to the relative homogeneity of particle types indicated by the electrophoretic work.

HEYNDRIX and DE VLEESCHAUWER (1951) published an electrophoretic study of milk and colostrum during lactation. This study confirms and extends the preceeding work of GRÖNWALL (1945) and SMITH (1946, 1946 c, 1948 a). Very clearly it shows the rise of the casein and the fall of the globulin content during the first days of lactation.

A comparative study of the proteins of milk whey and colostrum whey was recently published by SCHÄFER (1951). In addition to the differences in the electrophoretic curves he found that the immune globulins combine with iron in the iron fractionation process described, whereas the lactoglobulin remains iron free. In his opinion colostrum feeding has no advantage over the feeding of mature milk.

SLATTER and VAN WINKLE (1950) made an investigation into the influence of heat treatment, pH and ionic strength on the electrophoretic pattern of skim milk. They made the observation that the patterns of the ascending and the descending boundary were quite different.

BISERTE and MASSE (1948) made an electrophoretic study of human milk, especially of colostrum and prepartum milk.

SMITH and several coworkers (1946, 1946 a,b,c, 1947, 1948, 1948 a,b) made an extensive study of the globulins in milk and colostrum of normal and hyperimmunized cows. Hyperimmunization was performed with a mixture of antigens: Diphte-

ria toxin, vaccinia virus, and a killed culture of *Hemophilus pertussis*.

They found the serum globulin content of the calf to be closely related to the ingestion of colostrum.

As a result of electrophoretic work on blood serum HANSEN & PHILIPS (1947) reported an immediate increase in the blood serum γ globulins of calves following the ingestion of either colostrum or colostrum pseudoglobulin during the first 24 hours of life.

A comparison of the diagrams of salt-acid and saltlyophilized whey fractions was made by STANLEY, ANDREWS and WHITNAH (1950). A shift in the ratio of euglobulin to pseudoglobulin (names according to SMITH) was the only difference, which may be due to the action of HCl (pH 2!) upon the immune lactoglobulins. In a following paper the same authors (1951) compare lyophilized acid whey and lyophilized rennet whey at various pH values. At pH 5.8 rennet whey showed two fast moving components which were not present in acid whey, but at higher pH values the difference was less pronounced. This may explain the fact that DEUTSCH (1947), working at pH 8.6, did not observe this difference, at least not with cows' milk. He made electrophoretic and ultracentrifugal recordings of the whey of quite a number of animal species including man.

In the case of goat whey only, some of the fast moving components of the usual rennet whey diagram were absent in the pattern of the acid whey. Although this fact was not observed with any other species he prepared only rennet wheys in his investigation. The influence of lactation on the pattern was qualitatively the same with every species. The relative globulin content decreases hence there is some increase in the other components. In the case of cows' milk whey this change takes place in the course of few days and is very striking. With human milk the effect is much smaller and it takes a long time (90 days).

In the ultracentrifugal diagrams the same course was observed viz. an increase in the content of substances with low sedimentation constant and decrease in the content of rapid sedimentating material as lactation progressed. The effect was markedly greater with cows' whey than with human whey and the relation to lactation time was similar to that which was found by electrophoresis.

BRIGGS & HULL (1945) studied the electrophoretic pattern of β lactoglobulin during heat denaturation. The originally homogeneous protein decomposed into more components, but after prolonged heating at higher temperatures the material became homogeneous again. The conclusion was drawn that denaturation will take place in two stages of which the reaction velocities have been calculated.

LI (1946) obtained single electrophoretic diagrams of β lactoglobulin at pH 5.3 and 5.6. At pH 4.8 and 6.5, however, there were three components, which were reproducible even after recrystallizing the protein.

The heterogeneity of β lactoglobulin has been the subject of more thorough investigations. POLIS, SHMUCKLER, CUSTER and MCMEEKIN (1950) isolated an electrophoretically homogeneous crystalline compound from β lactoglobulin; heterogeneity was only observed with buffers on the acid side of the isoelectric point. The pH-mobility cürve of this new compound (β_1 lactoglobulin) showed three intersection points with the curve of normal β lactoglobulin. The effect of pH on the denaturation of β lactoglobulin has been evaluated by GROVES, HIPP & MCMEEKIN (1951). Denaturation was measured by optical rotation and by insolubility at the isoelectric point in the presence of salt.

In an immunochemical study DEUTSCH (1950) too, observed marked immunological heterogeneity of crystalline β lactoglobulin. In addition he found in

accordance with POLIS and coworkers (1950) electrophoretic heterogeneity at pH 4.2 and homogeneity at pH 6.7 and 8.6. No deviations from the normal pattern of whey could be recorded at pH 8.6, 7.7 and 6.5, but at pH 4.3 its form and the ratio of the components had changed considerably.

In general the ultracentrifugal examination of the whey proteins has yielded three components α, β and γ whereas separation by electrophoresis results in three to six components. The results of the latter method especially, depend to a large extent on the experimental conditions chosen and on the method of interpretation of the diagrams. This dependence has been a stimulus to the author to pay considerable attention to the more fundamental aspects of electrophoresis.

In this way it is hoped that the work will lead to a clearer understanding of the pecularities and anomalies of electrophoretic methods when applied to the study of milk proteins.

As a help to the reader we give in table 2 an arrangement of the various components of milk whey and some characteristic data including the classification adopted by the author. The notation α, β, γ describes increasing sedimentation constants whereas a, b, c, d, e, f, are used for the components reported by SMITH. The grouping I, II, III, IV, which has arisen from the work to be described is based on differences in ionic mobility.

The identity of the components in one column of table 2 is highly probable, although strictly proved only in the case of β lactoglobulin (β, e, d, II) and classical globulin (γ, b, a, IV).

TABLE 2. Arrangement of the protein components of whey

	"Whey-protein"	Albumin		Globulin Immuno-globulins	
		Kekwick's albumin ?	Palmer's β lactoglobulin		
Comp. Ultracentrifuge	α		β	γ	
Sed. Constant (S_{20})	1.9		2.83	7.0	
Diff. Constant ($D_{20} \times 10^7$)	10.6		7.82	3.6	
Mol. weight	17500		35400	160.000-190.000	
Comp. Electrophoresis (SMITH)	(f)	c	d,e	pseudo-globulin b	eu-globulin a
Comp. Electrophoresis (WEGELIN)	I	III	II	IV	

2. METHODS

a. Preparation of whey

The whey used in the work to be described, was prepared in the following manner:

Fresh whole milk was centrifuged three times, once in a continuous centrifuge and twice in a laboratory centrifuge operating at 3000 r.p.m. The thin layer of fat on the surface of the liquid in the centrifuge tubes was removed by suction and the bottom layer of sediment by decantation. To each litre of skim milk 1.2 ml of a saturated solution of calcium chloride was added and the casein precipitated either by means of rennet or by the addition of acid.

For rennet coagulation the milk was warmed to 35°C, 0.4 ml of a commercial rennet solution added and the liquid allowed to stand for 45 minutes. The rennet activity was estimated to be 1 : 9500 (so that one part of enzyme solution would clot 9500 parts of milk in 40 minutes at 35°C). At the end of the standing period the curd was transferred to a Buchner funnel and the whey separated.

For acid coagulation the pH of the liquid was adjusted to 4.37 by the cautious addition of concentrated citric acid solution with simultaneous stirring of the milk. The separated casein was filtered. The particular value of the pH was suggested by the work of MOYER (1940) who quotes this value for the isoelectric point of casein at an ionic strength of 0.075 which corresponds to that of a milk ultra-filtrate (cf. NÖRDBO (1939)).

After both methods of casein precipitation the resultant whey was centrifuged twice in order to obtain the clear solution which was necessary for most of the investigations to be described. When it was necessary to study concentrated or dried whey products it was essential that the reconstituted material was centrifuged at 18 000 r.p.m. in order to clarify the solution.

b. Physicochemical methods,

The examination of various solutions of whey proteins has been carried out chiefly by means of the ultracentrifuge and by electrophoresis. Both methods have been described in detail in several papers and reference books. We may refer to the excellent reviews of PICKELS (1942) and LONGSWORTH (1942) on both subjects. More recently reviews on both methods have been given by MOORE (1949) (electrophoresis) and by NICHOLS and BAILEY (1949) (ultracentrifuge) in Weissbergers *"Technique of Organic Chemistry"*. A motor direct-drive ultracentrifuge, manufactured by the *"Specialized Instruments Corporation"("Spinco")*, Belmont, California, was kindly placed at our disposal by Prof. Dr J.Th.G. OVERBEEK at the Laboratory of Physical Chemistry of the State University of Utrecht. Prof. Dr H.G.K. WESTENBRINK enabled us to make use of the electrophoresis apparatus (from the Swiss firm Strübin & Co., Basel) at the Laboratory of Physiological Chemistry of the same University. For their kind co-operation we are much indebted to them as well as to Dr H. REERINK and Dr J.A. NIEMEYER for valuable technical instruction and assistance. Both pieces of equipment were furnished with the cylindrical lens device of PHILPOT (1938) and SVENSSON (1939, 1940) for recording the patterns. The optical arrangement has been schematically drawn in Fig. 2. In the electrophoresis apparatus a wedge-

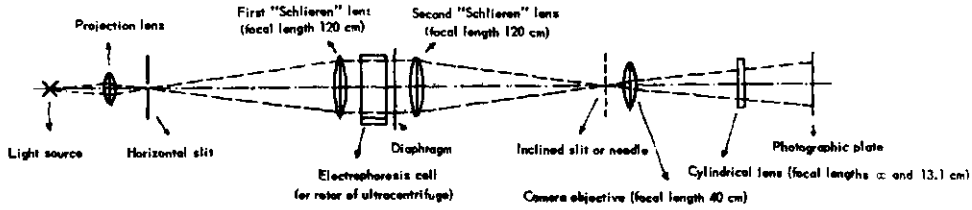


Fig. 2. Optical arrangement for examining concentration-gradients after electrophoresis or ultracentrifuging.

shaped inclined slit of adjustable breadth was used, making it possible to reduce the base line in favour of more accurate measurement of the areas under the pattern. The ultracentrifugal recordings were made with the help of a bar instead of the inclined slit. Hence the electrophoretic diagrams were light against a dark background and those of the ultracentrifuge were dark against light. The thickness of the ultracentrifugal lines could not be reduced, so that the patterns were less suitable for measurement of the respective areas.

Before introduction into the electrophoretic apparatus the whey solutions had to be concentrated and dialyzed against a suitable buffer solution. Dialysis was carried out in cellophane sausage skins at 0 °C for several days with frequent changes of the buffer solution. The concentration of the fresh whey to ± 1.5% protein, necessary for the electrophoresis experiments, was achieved by freezing the filled sausage casings in the refrigerator, followed by washing off the tiny ice crust on the outside of the casings with running tap water. By alternating this procedure with dialysis, a gradual concentration was effected in a very harmless way. When the concentration had become sufficiently great dialysis was continued till equilibrium was attained. The nitrogen content was then estimated by the Kjeldahl method after which the solution was diluted with buffer solution to exactly 1.5% protein. The nitrogen factor was taken to be 6.44, which is, according to PERLMANN & LONGSWORTH (1948), the accurate value for β lactoglobulin, the main constituent of whey protein.

The reliability of the nitrogen determinations was estimated by calculation of the standard deviations from the differences found between the duplicates. The results of 140 determinations were subdivided into several series corresponding to different materials e.g. samples of whey, condensed whey, whey powders, trichloracetic acid filtrates etc. For each series the standard deviation was calculated according to the equation

$$s^2 = \frac{\Sigma \Delta^2}{2n}$$

in which s = standard deviation, n = number of observations in the series and Δ = difference between the duplicates. This method has been recommended by the

Dutch Commission "70" for Normalisation in its Draft "V 1047" on the Reproduction of Series of Observations, published by the Chief Commission for Normalisation in the Netherlands (ed. Waltman, Delft, 1951).

It appeared that in most cases the standard deviation amounted to approximately 0.2 per cent of the mean value. In a few cases where conditions were less favourable, it was of the order of 0.6 per cent.

In the case of condensed or dried whey products this concentration procedure was of course not required. Nor was it necessary for the ultracentrifugal experiments as the optical device of this apparatus was much more sensitive and a lower protein content was therefore adequate. In general the ultracentrifugal experiments were carried out with the same dialyzed solutions (diluted to some extent with buffer solution) which were subjected to electrophoresis.

For convenience in some cases concentration was achieved by precipitation of the proteins by saturation of the whey with ammonium sulphate followed by dissolution of the precipitate in a small amount of buffer and dialysis. The influence of this precipitation has been studied in separate experiments (cf II.5.c.).

Electrophoresis was carried out at about 2.5 °C, i.e. approximately at the maximum of density of dilute salt solutions, in order to avoid convectional disturbances of the boundaries as a consequence of the Joule energy developed during the current transport. Ultracentrifugal measurements have been made at about 20 °C.

3. DISTRIBUTION OF THE N CONTAINING SUBSTANCES IN WHEY

a. The factors affecting the composition of whey.

In II.1.a. the following classification of the N-containing substances was given:

Albumin
Globulin
Proteoses and peptones
Non-protein nitrogen

Furthermore the experimental data of SHAHANI and SOMMER (1950) and of KIEFERLE and GLOETZL (1931) for normal bulked milk have been cited. However, the total composition of whey and therefore the distribution of the N-containing substances too may vary according to several factors, apart from the differences in the composition of the milk of individual cows of similar condition. The influence of the stage of lactation will be treated under *b.*, in comparison with some data on human milk. It will appear that in the case of human milk this influence is quite different from the case of cows' milk. The method of curdling the milk also influences the composition of the whey, a subject to be treated under *c.* Besides, the results of any analysis largely depend on the method of estimation. As has been mentioned in detail in II.1.a. the various fractions cannot yet be considered as completely defined chemical substances, because different methods yield different results, and even the order in which the estimations are carried out is of influence. These influences will be dealt with under *d.* By electrophoresis data are obtained on the non-dialysable nitrogen fraction only. Therefore in this chapter special attention will be given to the ratio of non-dialysable to dialysable (residual) nitrogen.

b. The influence of the stage of lactation.

During the lactation period the total nitrogen content of cows' milk decreases from a very high value in the first colostrum (approximately 3700 mg per cent) to the normal mean value (450 - 550 mg per cent). This decrease takes place in a few days. Between the 3rd and the 7th month a slight increase (about 30 mg per cent) is observed which, however, is negligible in comparison to the rapid fall in the beginning. In Fig. 3 a graph has been plotted according to the data of NESENI and KÖRPRICH (1947), in which the very first values have been

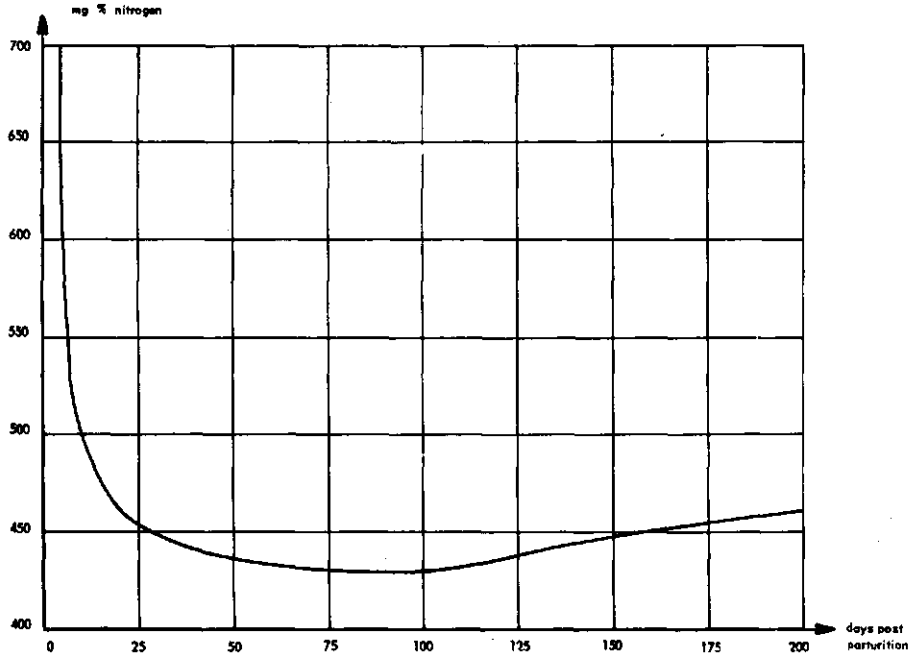


Fig. 3. Total nitrogen content of cow's milk during lactation.

left out in view of the scale. Similar curves for the total and the albumin + globulin nitrogen content of milk whey have been drawn in Fig. 4 (data of the same authors). In the case of human milk, however, the dependence of the nitrogen content on the stage of lactation is quite different. Reliable average data on human milk are still scarce, because of the difficulty of acquiring milk samples from the same subjects over a long period. Nevertheless in Fig. 5 a graph has been plotted of the total nitrogen content in human milk as a function of the stage of lactation. For the computation of this graph data have been compared of BELL (1928), MACY (1932) and CAMERER and SÖLDNER (cited by SCHLOSSMANNand SINDLER (1925)) and an intermediate curve has been drawn. Now from this graph and from the literature cited it is obvious that there is a striking difference between the curves for cows' milk and human milk. Apart from the well-known difference in average total nitrogen content of milk from

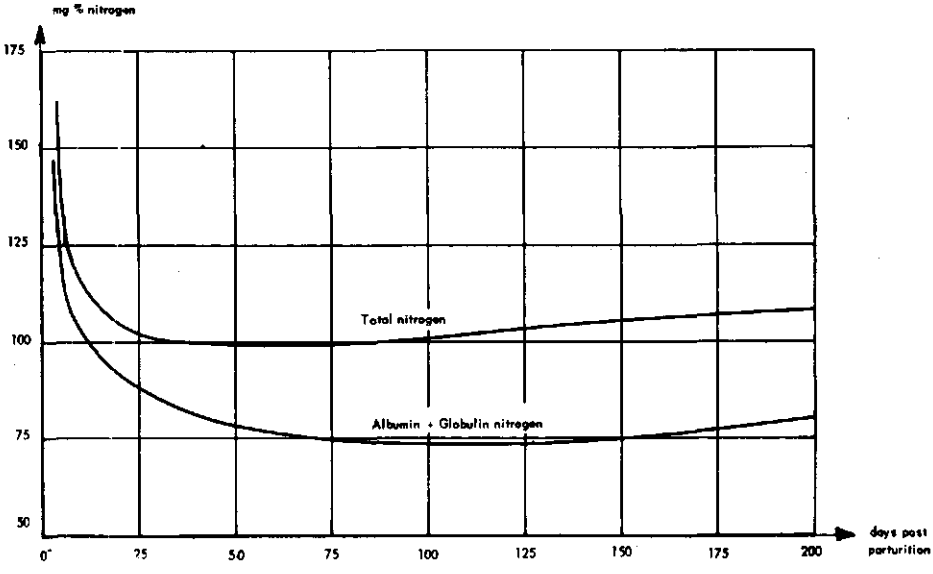


Fig. 4. Total nitrogen and albumin + globulin nitrogen content of cow's milk whey during lactation.

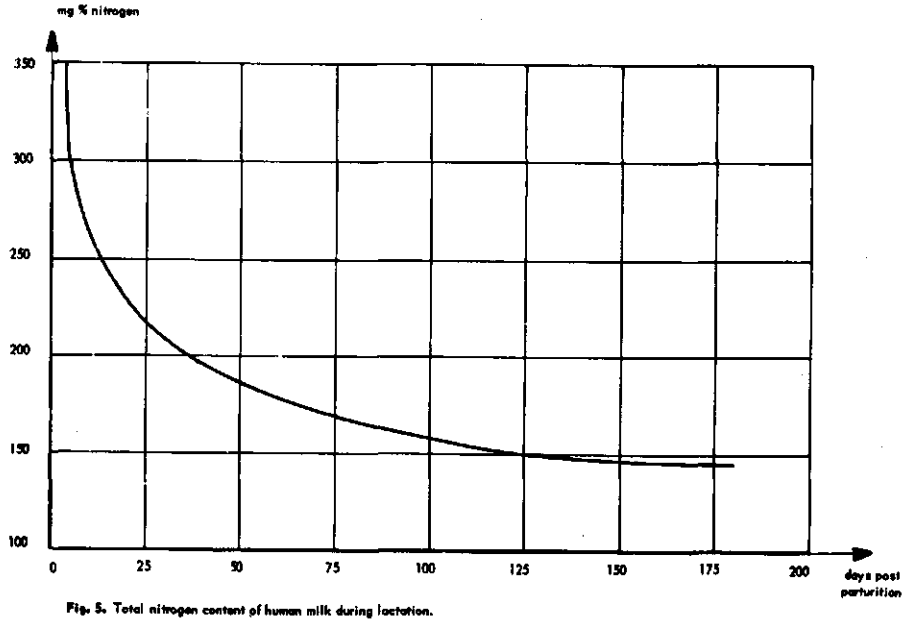


Fig. 5. Total nitrogen content of human milk during lactation.

both species the forms of the curves are different. In both cases (Fig. 3 and fig. 5) there is an enormous decrease in nitrogen content during the first few days, but from then on in the case of human milk only (Fig. 5) a gradual decrease is observed for about 180 days. Especially in comparison with the total nitrogen content this decrease is considerably greater than the changes of the nitrogen content of cows' milk during the further lactation period. Hence in contrast with cows' milk there is hardly any question of an "average normal human milk". Regarding the total whey nitrogen and the albumin nitrogen of human milk there were no sufficient data available to give a complete graph. From some figures of MACY (1932) a similarity in form with the total nitrogen curve appears probable.

c. The influence of the method of coagulation

The removal of casein from skim milk can be carried out either by rennin or by acid coagulation. We have examined this influence by estimating the nitrogen content of samples of acid and rennet whey obtained from the same milk. In addition the content of heat-coagulable nitrogen and residual nitrogen (not precipitated by 15 per cent trichloracetic acid) has been determined. All figures have been calculated as mg N per 100 g whey. One sample of milk yielded the following results:

Milk sample 1	Rennin	Acid
Total whey nitrogen	151	130
Residual nitrogen	27.7	25.2

The difference in total nitrogen is accounted for by the so-called "whey-protein" and is related to the method of coagulation, whereas the difference in residual nitrogen is not characteristic as will appear from the analysis of another sample of milk:

Milk sample 2	Rennin	Acid
Total whey nitrogen	131	116
Residual nitrogen	27.6	28.7
Heat-coagulable nitrogen	78.0	67.3

From these figures the following conclusions may be drawn:
If we identify the residual nitrogen (as estimated with 15 per cent trichloracetic) with the dialysable nitrogen (cf. *d.*), the "whey protein" content amounts to about 14-17 per cent of the total non-dialysable nitrogen in whey. This figure will be discussed again in II.6.b. in connection with the electrophoretic analysis of rennet whey.

In view of the work of HOSTETTLER and RÜEGGER (1950) the further aspects of the method of coagulation should also be of some influence. We mention the concentrations of calcium and rennet, the temperature, pH and the duration of the rennet action. HOSTETTLER and RÜEGGER (1950), coagulating solutions of pure (acid) casein by means of rennet obtained "whey protein" solutions, containing up to 25 per cent of the original casein. In a similar experiment we obtained 37 per cent. For normal rennet coagulation of milk this percentage amounts to only 5-6 per cent of the casein originally present. Although some objections can be made against an identification of milk and artificial

casein solutions with respect to the action of rennet, these figures may be used to draw attention to the accompanying reactions in the rennet coagulation.

d. The influence of the method of estimation

The treatment and experimental trial of all fractionation possibilities and methods, some of which have been mentioned in II.1.a. would be a research programme in itself. Therefore we have limited ourselves to the following classification:

Real protein nitrogen (albumin + globulin)	(R.P.N.)
Proteose nitrogen	
Non-protein nitrogen	(N.P.N.)

The R.P.N. content has been calculated as the difference between the total nitrogen content and the nitrogen remaining in solution after heat treatment. This treatment consisted in rapidly heating up to 100°C, keeping the solution in a boiling water bath for 30 minutes, followed by rapidly cooling in tap water. Previously the solution was brought to pH 4.7 by addition of some dilute HCl. According to ROWLAND (1937) in the case of whey proteins the quantity which is precipitated is largely dependent on pH and the pH - precipitation curve shows a maximum at pH 4.7. In the estimation of the N.P.N. content two essentially different methods have been compared, viz. coagulation and dialysis. Coagulation has been performed by addition of trichloracetic acid up to a final concentration of 15 per cent. This concentration has been used also by MENEFEE, OVERMAN and TRACY (1941). According to ROWLAND (1937) the amount of coagulable whey protein reaches a constant value at about 6 per cent trichloracetic acid, so a concentration of 15 per cent can be regarded as amply sufficient. After filtration the nitrogen content of the filtrate was estimated and denoted as non-protein nitrogen.

Dialysis was performed in rotating cellophane sausage skins at 0°C for about 10 days (of 24 hours) against frequent changes of the buffer solution used in the electrophoretic experiments (phosphate-citrate-KCl, pH 6.8, μ = 0.15, cf. II.5.a.). The original quantity of nitrogen inside the membranes and the quantity that was left after dialysis were determined. The difference was taken as dialysable nitrogen. In the case of whey proteins the results of both methods were in good agreement. A whey sample analysed for non-protein nitrogen (expressed in per cents of total whey nitrogen) yielded the following figures:

Non-coagulable nitrogen	23.9 per cent
Dialysable nitrogen	24.4 per cent

i.e. on coagulation, 98 per cent of the value obtainable by dialysis was found. This agreement is not at all self-evident, because it depends entirely on the material examined. It may be interesting to mention that the same techniques, applied to a sample of concentrated fish press water gave completely different values, the coagulation method yielding only 51 per cent of the figure for dialysable nitrogen. In this report of whey proteins, however, we feel justified in identifying the non-protein nitrogen as estimated by trichloracetic acid with the dialysable nitrogen.

The proteose nitrogen has been taken as total N - (R.P.N. + N.P.N.). However, the N.P.N. content, if determined in a solution after heat coagulation and filtration of the R.P.N., appears to be higher than in the original solution. The following figures (in mg N.P.N. per 100 g whey) will give an indication of the differences:

N.P.N. content	Before heat treatment	After heat treatment	Difference
Whey sample 1 (rennin)	27.7	29.9	2.2
Whey sample 2 (rennin)	27.6	31.2	3.6
Whey sample 2 (acid)	28.7	31.1	2.4

The conclusion is that during the heat treatment part of the R.P.N. or the proteoses is decomposed into low-molecular substances. In computing the nitrogen distribution in whey we shall use the figures obtained before heat treatment and thus classify this small heat-decomposable nitrogen fraction under the proteoses.

e. Analytical data

In order to compare the results obtainable by the methods described above with the figures from the literature (cf. II.1.a.) we shall give some analytical data from whey samples. The nitrogen distribution in an arbitrary sample of rennet whey is given in table 3. In table 4 the nitrogen distributions in two samples viz. rennet whey and acid whey from the same milk are compared.

TABLE 3. Nitrogen distribution in an arbitrary sample of rennet whey

Sample 1	mg per cent of the whey	per cent of total whey nitrogen
Real protein nitrogen	79.1	52.4
Proteose nitrogen	44.2	29.3
Residual nitrogen	27.7	18.3
Total nitrogen	151	100

TABLE 4. Nitrogen distribution in rennet- and acid whey from the same milk sample

Sample 2	Rennet whey		Acid whey	
	mg per cent of the whey	per cent of total whey nitrogen	mg per cent of the whey	per cent of total whey nitrogen
Real protein nitrogen	78.0	59.5	67.3	58.0
Proteose nitrogen	25.4	19.4	20.0	17.2
Residual nitrogen	27.6	21.1	28.7	24.8
Total nitrogen	131	100	116	100

In comparison with the data of SHAHANI and SOMMER (1950) and of KIEFERLE and GLOETZL (1931) (cf. table 1, II.1.a.) many differences will be observed. Comparing table 3 and the rennet section of table 4 it will be seen that the individual differences between milk samples of different cows are large. In

addition it appears from Table 4 that the "whey protein" (cf. II.3.c.) must be highly heat-coagulable (real protein!). The increase of the proteose nitrogen content as a result of the action of rennin is in itself plausible, although it is also possible that it is part of the "whey protein" which decomposes into the proteose form during the heat treatment, necessary for the - indirect - determination of the proteose nitrogen, thus reducing its own figure during the estimation.

4. THE THEORY OF ELECTROPHORESIS

a. The principle of the moving boundary

Owing to the work of TISELIUS and his school the moving boundary method has become a widespread and much appreciated means of estimating concentrations in colloid mixtures and of measuring electrolytic mobilities. It must be emphasized, however, that in several cases it is not quite clear what concentration or mobility is estimated. This is largely due to the fact that some authors do not differentiate between the microscopic and macroscopic situations in electrophoresis which are not at all identical.

A thorough study of the problems concerning the moving boundary method, which has been made at this laboratory[1] in connection with the research on the whey proteins has been published recently by DE WAEL and WEGELIN (1952) and WEGELIN and DE WAEL (1952). Nevertheless we draw the attention to some of the essentials of this study.

Several authors, e.g. SVENSSON (1946), have pointed out that any boundary in a system containing n ions may divide under the influence of electrical current transport into (n-1) boundaries. Of these (n-1) items, some are considered as "true", i.e. with the electrolytic mobility of a definite ion, whereas others are so-called "false" boundaries. Two good examples of the latter enter into nearly every Tiselius diagram viz. the stationary boundaries (δ on the ascending side, ε on the descending side). These boundaries with mobilities which are approximately zero, do not represent any ion species. In the case of milk protein these stationary boundaries are fairly large and their influence on the form of the diagram has to be considered.

A good aid to the understanding of these problems is the Kohlrausch regulating function (1897). In a system containing anionic species A, B, C, ... and the kations R, S, T ..., of concentrations $\alpha, \beta, \gamma \ldots\ldots \rho, \sigma, \tau$, and mobilities a, b, c ..... r, s, t, (concentrations and mobilities with the signs of the charges) the regulating function (Γ) has the form:

$$\Gamma = \frac{\alpha}{a} + \frac{\beta}{b} + \text{---} + \frac{\rho}{r} + \frac{\sigma}{s} + \text{---} \qquad (1)$$

According to KOHLRAUSCH (1897), (who intentionally neglected the influence of diffusion and the dependence of the transport ratios on the ionic strength) the value of Γ during electrophoresis is only dependent on the place in the

[1] Laboratory of Veterinary Biochemistry of the State University of Utrecht, The Netherlands.

electrophoresis cell where it is calculated, but independent of the time. Hence the value of Γ in the solution present at the beginning of the experiment can not be changed by electrophoresis, even when the boundaries move. This involves changes in the concentrations of every ion whenever one ion species appears or disappears as a boundary moves on.

As has been discussed in detail in the above-mentioned paper (1952, DE WAEL and WEGELIN) normal Tiselius experiments with a homogeneous protein can be suitably described as experiments with a system of three ions A, R and S, A and R representing the buffer ions and S the protein ion. Then the initial boundary will split up into two new ones, one moving (true) boundary of the protein, and one resting (false) boundary at the place of the initial one (δ or ε).

Consider first the anodic (ascending) side. The changes occurring have been drawn schematically in Fig. 6. Originally the buffer-solution 1 is placed on the protein-solution 3 which is in Donnan equilibrium with 1. Because of

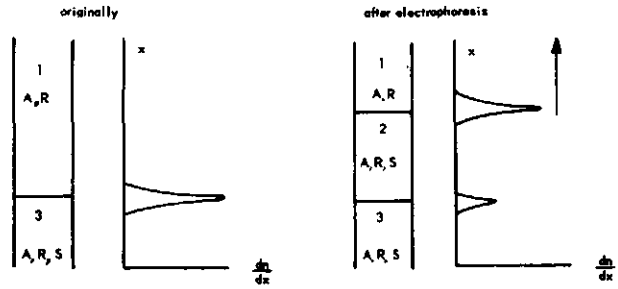


Fig. 6. Scheme of the origin of moving and resting boundaries in the electrophoretic cell and patterns belonging to them.

x = level in the electrophoretic cell

$\frac{dn}{dx}$ = refractivity gradient

the different composition of the solutions 1 and 3 (especially the absence and presence of protein) two different values of Γ are fixed all over the compartments 1 (Γ_1) and 3 (Γ_3). During electrophoresis the protein ions (S) move into the original compartment 1, thus forming a new section (2). However the value of Γ remains constant, hence

$$\Gamma_2 = \Gamma_1$$

or

$$\frac{\alpha_2}{a} + \frac{\rho_2}{r} + \frac{\sigma_2}{s} = \frac{\alpha_1}{a} + \frac{\rho_1}{r}$$

Because of the electroneutrality of the systems (i.e. $\alpha + \rho + \sigma = 0$) this can be converted into

$$\rho_1 - \rho_2 = \left(\frac{r}{a-r} \Big/ \frac{s}{a-s} \right) . \sigma_2 \qquad (2)$$

Apparently the moving boundary 1.2 involves a difference in the concentrations ρ_1 and ρ_2 on either side of this boundary, which is directly proportional to the protein-concentration σ_2.

Further consideration (cf. DE WAEL and WEGELIN, 1952) shows that the concentrations of solution 2 are strictly proportional to those of solution 3 :

$$\frac{\alpha_2}{\alpha_3} = \frac{\rho_2}{\rho_3} = \frac{\sigma_2}{\sigma_3} \qquad (3)$$

In addition it is shown that it is a quite reasonable approximation to formulate the following relation between the solutions 1 and 3 in terms of the Donnan equilibrium.

$$\alpha_1 - \rho_1 = \alpha_3 - \rho_3 \qquad (4)$$

Whereas the values of $(\alpha - \rho)$ of the solutions 1 and 3 are approximately equal, that of solution 2 will be different. This means that the salt concentration gradient at 1.2 will be compensated by an equal concentration gradient in the boundary 2.3. This also finds expression in the specific conductance, which is of great importance with the calculation of the mobilities.

On the descending side a similar effect will occur. The original boundary divides into the descending boundary and a resting (pure salt) boundary.

In Fig. 7 a schematic drawing of the specific conductance, the protein-concentration and the refractivity gradient throughout the electrophoresis cell is given.

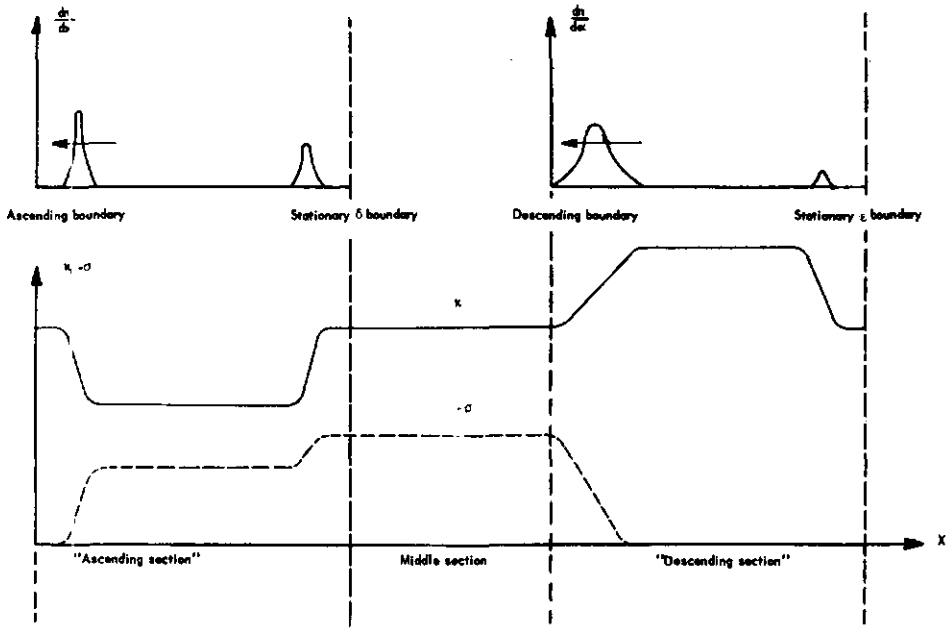


Fig. 7. Specific conductance (κ), protein concentration (-σ) and refractivity gradient $(\frac{dn}{dx})$ as function of the level in the electrophoresis cell (x).

The course of κ, recorded in fig. 7 has been predicted by LONGSWORTH and MC INNES (1940) and has been experimentally verified by LAGERCRANTZ (1945).

In view of the considerations given above and with the help of fig. 7 the following facts can be stated:

1) The total area (under all peaks) of the ascending pattern has to be equal to that of the descending pattern and is proportional only to the protein-concentration in solution 3 (fig. 6) as $(\alpha - \rho)$ has the same value in the solutions 1 and 3.

2) The δ gradient will always be larger than the ε gradient although the difference between the salt concentrations on either side of the δ boundary is smaller than the difference at the ε boundary. The specific conductances of the adjusted protein-solution under the ascending boundary and of the adjusted buffer-solution under the ε boundary may be widely different from those of the original protein and buffer-solutions. This difference depends chiefly on the ratio of protein-concentration to buffer-concentration, and in our experiments amounts to 10 - 20% of the original values.

3) In fig. 7 the descending boundary has intentionally been drawn much broader than the ascending boundary. This fact, which is experimentally well-known as sharpening and blurring of the ascending and descending boundaries respectively can be qualitively understood from the following reasoning:

The different specific conductances on either side of the boundaries involve different field strengths and hence different electrical velocities for protein ions which for some reason or another (such as diffusion!) might precede or lag behind the boundaries. Now conditions at the ascending boundary are likely to slow down ions moving ahead, whereas at the descending boundary such ions will be accelerated. Hence the ascending boundary will yield an equilibrium between the electrical sharpening effect and diffusion (the so-called steady state), whereas the descending boundary will be broadened more and more both by electrical and diffusion influences.

Now of course the question arises as to which field strength or specific conductance determines the velocity of the boundaries as a whole. HENRY and BRITTAIN (1933) have deduced that in both cases (ascending and descending) the specific conductances of the liquids just below the boundaries have to be used in the well-known equation for the velocity:

$$v = \frac{i \cdot u}{\kappa} \qquad \left\{ \begin{array}{l} i = \text{current density} \\ v = \text{velocity} \\ u = \text{mobility} \end{array} \right\} \qquad (5)$$

Up to this point we have treated the protein as being of a single nature, but when more than one protein component is present, it should be borne in mind that at each moving boundary the concentrations of all other components are changed. Analogous to the single moving boundaries from Fig. 7 which contain a counteracting salt gradient (cf. the curve of specific conductance) there will also be present superimposed protein gradients of opposite sign.

From Fig. 8 it will be obvious that in the ascending section the boundaries of the most rapid moving components will be enlarged at the expense

of the more slowly moving ones. A calculation of the errors involved is impossible as long as equivalent weight, mobility, diffusion constant and reciprocal influences of the individual components on each other are not exactly known, but even if these data are available the necessary exact treatment of a poly-ion system will be difficult. It is estimated that the error can be of the order of 15 per cents and it must therefore be emphasized that data obtained from electrophoretic experiments must never be considered to give true information concerning the quantitative composition of any protein mixtures. As a means of comparison, however, the method is most valuable.

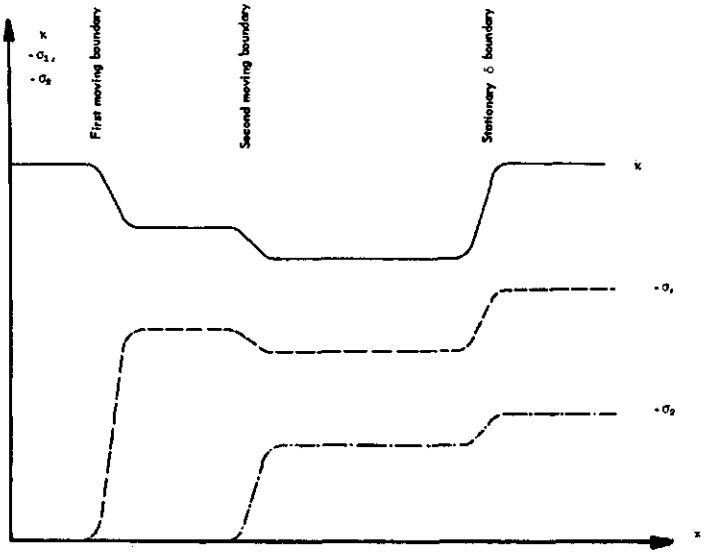


Fig. 8. Concentration of two protein components ($-\sigma_1$, $-\sigma_2$) and specific conductance (κ) as function of the level (x) in the electrophoresis cell (ascending section).

b. The analysis of electrophoretic patterns

The analysis was carried out on a great tracing of the original photograph on a sheet of drawing paper. First the total areas under both patterns (ascending and descending) were measured. This was done by cutting out and weighing, or with the help of a planimeter. Then by means of the semi-automatic method of WIEDEMANN (1947), Gaussian curves were drawn in such a way as to fit in with the peaks of the diagram, their areas covering the total area as closely as possible. This is a somewhat arbitrary operation. In some cases where reasonable separation was not possible, vertical lines were drawn according to the method of TISELIUS and KABAT (1939). Next the areas under each peak were measured. Then all areas were reduced to the value they would have had if the angle of the inclined slit had been 45° during the exposure. These values were obtained by dividing the actual values by the tangent of the angle used. The reduced total areas of ascending and descending patterns were approximately equal and the mean value was used in the further calculation. Now each indivi-

dual area was calculated, for the ascending diagram in comparison with the total area minus that under the δ peak and for the descending pattern as relative to the total area minus that under the ε peak. In several cases the separation of the various components in the descending pattern was not sufficient for analysis; the data were then taken from the ascending pattern only.

For the calculation of mobilities the values of the specific conductance under the moving boundaries had to be obtained first. As most of the diagrams contained several peaks a corresponding number of conductances had to be interpolated. The extreme values between which interpolation was to be executed were as shown in figs 6 and 7:

a. for the ascending pattern: The specific conductances of the adjusted protein solution (κ_2) and of the original buffer solution (κ_1).

b. for the descending pattern: The specific conductances of the original protein solution (κ_3) and of the adjusted buffer solution (κ'_2).

κ_1 and κ_3 were measured in separate experiments carried out in the usual way in the electrophoresis water bath at about 2.5 °C. Every time the resistance of the conductivity cell was first measured when filled with 0.1 N KCl and then with the solution concerned. After that the ratio of both values was multiplied by the specific conductance of 0.1 N KCl at 0 °C (716.10^{-5} Ω^{-1} cm^{-1}), thus yielding in addition the conductances wanted at 0 °C. When these values are used the mobilities calculated refer to 0 °C. as has been pointed out by TISELIUS (1937).

The specific conductance κ_2 was obtained by multiplying κ_3 by the dilution factor at the δ boundary (g_r). This dilution factor was found by the following reasoning (cf. Fig. 7). The area of the δ boundary (0_δ) is due to a salt gradient (contributing an area 0_{δ_s}) and a superimposed protein gradient (contributing an area 0_{δ_P}).

$$\text{Hence } 0_\delta = 0_{\delta_s} + 0_{\delta_P}$$

Both contributions are related to the separate concentration differences ΔP for the protein and $\Delta\mu$ for the salt, (in which μ stands for the ionic strength) by two constants k_1 and k_2:

$$0_{\delta_s} = k_2 . \Delta\mu \qquad\qquad 0_{\delta_P} = k_1 . \Delta P$$

As the δ boundary consists of a mere dilution with a factor g_r (defined as $g_r = \frac{\alpha_2}{\alpha_3} = \frac{\rho_2}{\rho_3} = \frac{\sigma_2}{\sigma_3}$, (cf. equation 3 in II.4.a.), we can write:

$$0_{\delta_s} = k_2 . (1 - g_r) . \mu_3 \qquad\qquad 0_{\delta_P} = k_1 . (1-g_r) . [P_3]$$

$$\text{Hence:} \qquad 0_\delta = (1-g_r) \{k_2 . \mu_3 + k_1 [P_3]\} \qquad (6)$$

From this equation g_r was calculated after k_2 had been determined in a separate experiment with a known buffer salt concentration gradient. For the product $k_1 [P_3]$, which represents the total area of the diagram, the mean value of ascending and descending pattern was available.

The specific conductance of the adjusted buffer solution (κ'_2) was found by multiplying κ_1 by the concentration factor at the ε boundary (g_d). This factor could have been derived from the area of the ε boundary (O_ε) by a similar equation as for g_r :

$$O_\varepsilon = (g_d - 1) \cdot k_2 \cdot \mu_3 \qquad (7)$$

The ε boundary, however, is much smaller than the δ boundary and therefore less suitable for accurate measurements. In addition the areas under the δ and the ε boundary are not independent of each other and their interrelation may be conveniently written in the form given by LONGSWORTH (1942) :

$$\frac{\Sigma 0 - 0_\delta}{\Sigma 0 - 0_\varepsilon} = g_r \qquad (8)$$

in which $\Sigma 0$ stands for the total diagram area. Combining this expression with the expressions for the δ and the ε boundary and remembering that $\Sigma 0 = k_1 [P_3]$, one easily obtains

$$g_d = \frac{1}{g_r} \qquad (9)$$

Thus with the help of three experimental quantities, κ_1 , κ_3 and the area under the δ peak the other extreme κ values could be calculated as follows:

$$\kappa_2 = g_r \cdot \kappa_3 \quad \text{and} \quad \kappa'_2 = \frac{\kappa_1}{g_r}$$

The specific conductance is, of course, not strictly proportional to the dilution. With the help of separate experimental data on the specific conductance of various dilutions a correction can be applied as has been described in the paper of WEGELIN and DE WAEL (1952) in the case of β-lactoglobulin. In view of the many errors possible in the case of the analysis of complex diagrams we have omitted this correction; the consequent error will not exceed 2 per cent of the total specific conductance.

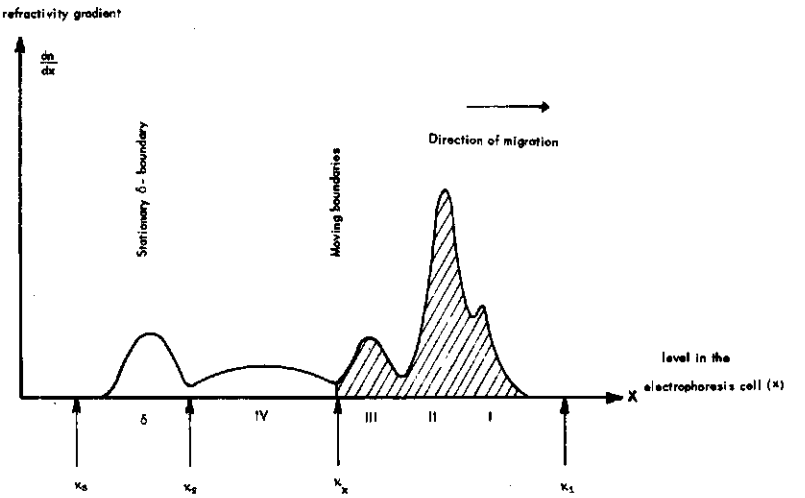


Fig. 9. Interpolation of specific conductance according to the areas concerned.

Now all through the diagram for each peak the specific conductance directly below the boundary should be known. The closest approximation to the correct value of κ at any such a place (x) has been made by interpolation from the quantities of protein above and below this level. This could be easily done by means of the areas of the diagrams as may be explained with the help of Fig. 9, which is a schematic drawing of the ascending pattern of milk whey proteins. When for example the specific conductance κ_x directly below boundary III is required, the value of κ_1 is reduced by the part of the difference $\kappa_1 - \kappa_2$ which corresponds to the ratio of the hatched area and the total area minus that under the δ peak. This involves two suppositions which are generally not quite true. First, the same specific refractive increment should be attributed to each component and secondly, the contribution of each component to the change in specific conductance of the solution should be equal.

The latter supposition implies that the value of equivalent weight (E) and transference number of the protein ion as part of an alkali proteinate (n_S) should be the same for different protein components, which will be obvious from the theoritical calculation given in the next section (II.4.c., cf. eq.7). Although this supposition is doubtless the most disputable it has to be stated that a higher value for the equivalent weight will in general be accompanied by a lower value for the mobility and vice versa. Therefore this interpolation appears to us to be the nearest possible approximation to the truth and certainly preferable to the usual neglect of any change in specific conductance.

In addition to the values of the specific conductance, the velocities of each moving boundary were calculated from the enlarged pattern relative to the stationary boundaries. The latter were used as reference points to eliminate any shift of the boundaries due to leakage. The small systematic error involved in this relative estimation has been preferred to the uncontrollable errors which are accepted by neglecting the effect.

The location of the boundaries was defined by the positions of the maxima on the pattern. From a theoretical viewpoint this is less correct than the use of the medians of the corresponding Gaussian curves. However, the positions of the maxima of the pattern could usually be located unambiguously, even in the cases where a resolution of the pattern into Gaussian curves was not or at least only arbitrarily practicable.

The current density has been calculated by dividing the actual current by the cross sectional area of the Tiselius cell. The electrophoretic current was determined as difference between the current sent through the cell during the electrophoresis experiment and the residual current measured by applying the same voltage to the apparatus while the middle section of the cell was still displaced. After the velocity (v), current density (i) and specific conductance (κ) directly below each boundary were known the mobilities (u) belonging to the boundaries could be calculated from the equation.

$$u = \frac{\kappa v}{i} \qquad \text{(cf. equation 5 in II.4.a.)}$$

c. Calculation of the equivalent weight of colloids from the concentration changes at the stationary boundaries.

With the help of equation (2), deduced in II.4.a. it is possible to calculate the equivalent weight of a colloid. This possibility has been described in full detail in a separate paper (WEGELIN and DE WAEL, 1952), so here the

subject will be treated only briefly. In equation (2) the ratios of mobilities can be denoted as transference numbers viz.

$\frac{r}{a - r} = n_R$ = transference number of the buffer anions (R)

$\frac{s}{a - s} = n_S$ = transference number of the protein ions (S)

Then equation (2) becomes:

$$\rho_1 - \rho_2 = \frac{n_R}{n_S} \cdot \sigma_2 \qquad (7)$$

From the electrophoresis experiment itself the mobility of the protein ion (s) is found, hence in combination with the mobilities of the salt ions (a,r) both transference numbers can be calculated. The value of ρ_1 (the salt concentration in the supernatant liquid) is directly available. ρ_2 and σ_2 can be calculated from the original concentrations ρ_3 and σ_3 of the protein solution, if the dilution factor at the δ boundary (g_r) is known, for $g_r = \frac{\alpha_2}{\alpha_3} = \frac{\rho_2}{\rho_3} = \frac{\sigma_2}{\sigma_3}$

Now g_r can be calculated from the area under the δ boundary with the help of equation (6) in II.4.b. Then all quantities, occurring in equation (7) are known, except that σ_2 is not available in electrochemical equivalents because the equivalent weight is not yet known. After substituting σ by $\frac{\Sigma}{E}$ in which Σ is the protein concentration in g/l and E the equivalent weight the latter is left as the only unknown quantity, which now can be calculated.

This calculation is only applicable to a system of three ions i.e. two buffer salt ions and a protein ion, a condition which can never be fulfilled. In spite of this and several other objections which could be raised, the method has appeared to be quite useful and applicable even when more than two salt ions are present. The application to experimental work on β lactoglobulin (WEGELIN and DE WAEL, (1952)) has yielded results which agreed very well with data obtained by other methods. We shall come back to this item in II.6.c. in connection with the identification of β lactoglobulin in the electrophoretic diagrams of milk whey.

Finally it should be pointed out that the size of the δ boundary of any diagram will certainly be a valuable indication as to the charge of the proteins concerned. From equation (7) it is obvious that in the case of a three ionic system the charge per unit of mass (reciprocal of equivalent weight) is directly proportional to the concentration change at the δ boundary, at least when different proteins with equal mobilities are compared. Now in the case of a protein mixture the correct relation will be far more complicated. When, however, diagrams are compared containing approximately the same components in the same ratio and with the same mobilities (as will be described further on, cf. II.6.b) a considerable difference in the size of the δ boundary can very well be ascribed to a change of one or more of the components of the mixture.

This method of estimating the equivalent weight of colloids is fairly new and may become a valuable supplement to the customary methods such as membrane potentials, direct calculation from electrophoretic velocity and

dissociation curve. As far as we know a similar method has been suggested only by SVENSSON (1946) and by CHARLWOOD (1950). For a complete description and comparison of the results, however, we refer to our papers mentioned before.

5. ELECTROPHORESIS OF NORMAL MILK WHEY PROTEINS

a. The choice of a suitable buffer solution

With regard to the buffer solution to be chosen as environment for the protein during electrophoresis three items have to be reckoned with; these are the ionic strength (μ), the pH and the chemical properties of the solution.

Regarding the ionic strength the choice is fairly limited. With the standard Tiselius apparatus the amount of electrical energy, sent into the cell should not exceed 8 Watts, to avoid thermal disturbances of the boundaries. Hence the specific conductance of the buffer solution should not be too high. In that case only a very low field strength can be applied, leading to a protracted experiment, which in its turn would needlessly complicate the diagrams by diffusion effects. On the other hand the specific conductance should not have a very low value. In this case the contribution of the protein to the specific conductance would be considerable, as the protein content has to be 1 to 2 per cent on account of the sensitiveness of the optical device. A high contribution to the specific conductance involves considerable boundary anomalies, which render the diagrams unfit for proper analysis in many respects. First the calculations given in II.4. are applicable only as far as the changes in ionic strength on either side of the δ or ε boundary do not affect the ratios of the mobilities of the ions. Secondly the accuracy of measuring the areas under the true (moving) boundaries is diminished when a considerable part of the total area is due to the false boundaries (cf. II.5.b.).

Thirdly great differences in concentrations can result in a gravitionally unstable order of solutions so that some or all boundaries are destroyed or become unreliable. A value for the specific conductance of about $700 \cdot 10^{-5}\ \Omega^{-1}\ cm^{-1}$ may be called a good compromise. This involves a value for the ionic strength of $\mu = 0.1$ à 0.2.

Regarding the pH and the chemical properties of the buffer solution we have tried to make the synthetic environment very similar to the native one in many respects. The pH was chosen to be 6.8 which is quite close to the value of milk, and at the same time sufficiently far from the iso-electric point (4.5) to ensure fairly high mobilities. As to the chemical properties the buffers of MCILVAINE (1921) are very similar to the buffering system of the ultrafiltrate of milk. To this buffer we have added KCl although we have intentionally omitted any lactose. There is much evidence that many carbohydrates can migrate electrophoretically (cf. PRAUSNITZ and REITSTÖTTER, (1931). In view of the high concentration which would be needed to give any resemblance to the milk ultrafiltrate the electrophoretic pattern of the proteins would be obscured. With whey or milk proteins an ionic strength of $\mu = 0.073$ as in milk ultrafiltrate (cf. NORDBÖ, 1939) was not desirable because of the above-mentioned reasons, hence we have taken about twice the native concentrations ($\mu = 0.15$). It is of interest to mention

that with other materials for example serum proteins, a much lower value of the ionic strength can be applied. The composition of the buffer solution used was:

	g/l	mmol/l
$Na_2HPO_4 \cdot 2H_2O$	4.47	25.08
Citric acid. H_2O	0.913	4.35
KCl	5.59	75.00

Taking into account the pK values of all ionic equilibria:

				pK
$H_2PO_4^-$	$\rightleftharpoons HPO_4^{--}$	+	H^+	6.85
H_3Ci 1)	$\rightleftharpoons H_2Ci^-$	+	H^+	3.08
H_2Ci^-	$\rightleftharpoons HCi^{--}$	+	H^+	4.75
HCi^{--}	$\rightleftharpoons Ci^{---}$	+	H^+	5.49

the concentrations and contributions of the individual ions to the total ionic strength are:

	mmol/l	$z^2/2$ (valence factor)	Contribution to ionic strength. 10^3	meq/l
Ci^{---}	4.156	4.5	18.7	12.47
HCi^{--}	0.194	2.0	0.4	0.39
HPO_4^{--}	12.12	2.0	24.2	24.24
$H_2PO_4^-$	12.96	0.5	6.5	12.96
Cl^-	75.00	0.5	37.5	75.00
Na^+	50.16	0.5	25.1	50.16
K^+	75.00	0.5	37.5	75.00
Total			149.9	250.22

Hence $\mu = 0.15$ and the total salt concentration = $\frac{250.22}{2}$ = 125.1 meq/l.

1) H_3Ci = citric acid

b. Numerical analysis of a Tiselius diagram

The result of the electrophoresis of rennet whey form normal cows' milk is shown diagrammatically in Fig. 10.

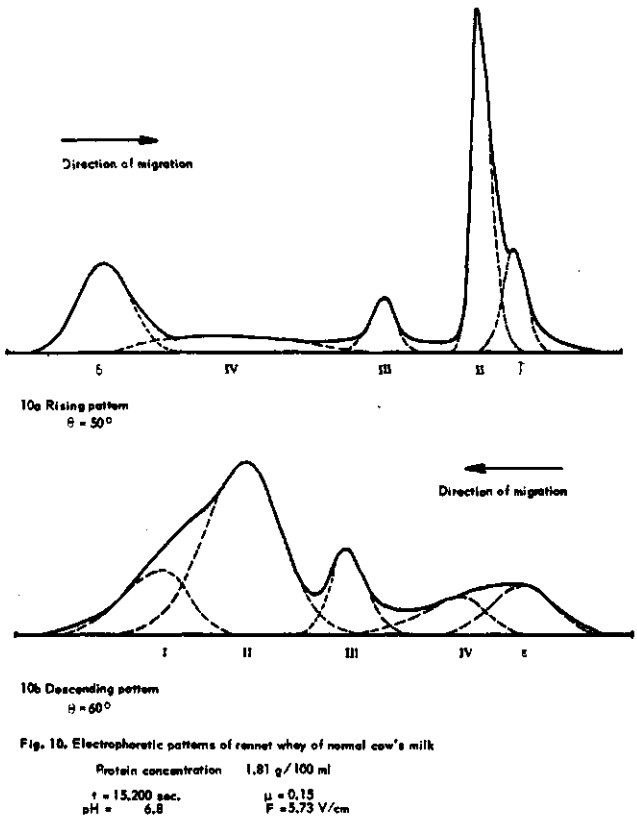


Fig. 10. Electrophoretic patterns of rennet whey of normal cow's milk

Protein concentration 1.81 g/100 ml

t = 15.200 sec. μ = 0.15
pH = 6.8 F = 5.73 V/cm

The necessary concentration had been obtained by the freezing procedure described under II.2.b. We shall now give the complete numerical analysis of these diagrams as an example of the technique described in II.4.a. For this analysis the patterns were enlarged in such a way as to make the base line 28 cm long. This line corresponded to the length of the slits (8.4 cm) directly behind the cell. The total areas as measured by cutting out and weighing amounted to $O_r = 47.15$ cm^2 and $O_d = 69.60$ cm^2 for the ascending (Fig.10a) and descending boundaries (Fig. 10b) respectively. By dividing these figures by the tangents of the angles of the inclined slit during the exposures (50° and 60° respectively) the areas were reduced to the values at 45°:

$$\frac{O_r}{\tan 50^\circ} = \frac{47.15}{1.192} = 39.56 \text{ cm}^2$$

$$\frac{O_d}{\tan 60^\circ} = \frac{69.60}{1.732} = 40.18 \text{ cm}^2$$

The mean value was calculated and denoted as $\Sigma O = 39.87 \text{ cm}^2$.

The peaks were numbered according to decreasing velocity I, II, III, IV, δ and ε respectively. The corresponding Gaussian curves were drawn (the dotted lines in Figs. 10a and 10b) and the area under each peak was measured and reduced to its value at 45°. Between the sums of these areas and the total area a small difference remained, which has been denoted as "residual area". The area under the ε peak was not measured, for from Fig. 10b it will be obvious that the separation of the component IV and the ε boundary was not sufficient. Therefore it was preferred to calculate this area from the one under the δ peak, which could be measured accurately (cf Fig. 10a). Hence we rewrite equation 6 (II.4.b.):

$$o_\delta = (1-g_r) \{k_2 \mu_3 + k_1 [P_3]\} \qquad (6)$$

Remembering that $k_1 P_3 = \Sigma O$ this equation can be transformed into

$$o_\delta = (1-g_r) \cdot \Sigma O \cdot \{1 + \frac{\mu_3}{[P_3]} (\frac{k_2}{k_1})\}$$

The values of k_2 and k_1 had been determined in two separate experiments with the identical optical arrangement and adjustment of every part of the Tiselius apparatus. The values found were:

$k_1 = 14.0 \text{ cm}^2$ per unit of the protein concentration

$k_2 = 70.4 \text{ cm}^2$ per unit of ionic strength

Hence $\frac{k_2}{k_1} = 5.03$.

From the defining equations:

The area of the protein gradient $= k_1 \cdot \Delta [P]$

The area of the salt gradient $= k_2 \cdot \Delta \mu$

it will be obvious that the ratio $\frac{k_2}{k_1}$ is only affected by the specific refractive increments of protein and buffer solution respectively, and not by the arrangement of the apparatus. Hence once this ratio has been determined it can be

applied to the data of any experiment however much the apparatus may have been altered afterwards. Substituting this value equation 6 becomes:

$$O_\delta = (1-g_r) \cdot \Sigma O \cdot \left\{ 1 + 5.03 \frac{\mu_3}{[P_3]} \right\} \qquad (6a)$$

For the experiment under discussion the following values were to be substituted:

$\Sigma O = 39.87 \text{ cm}^2$

$O_\delta = 10.13 \text{ cm}^2$

$\mu = 0.15$

$[P_3] = 1.81 \text{ g/100 ml.}$

Thus the following value for g_r, the dilution factor at the δ boundary, was obtained:

$$g_r = 0.821$$

This value was substituted in equation 8 (II.4.b.):

$$g_r = \frac{\Sigma O - O_\delta}{\Sigma O - O_\varepsilon} \qquad (8)$$

together with the values for ΣO and O_δ, which yielded

$$O_\varepsilon = 3.65 \text{ cm}^2$$

This value was then used in computing table 5, in which all the areas under the various peaks are given.

TABLE 5. Individual areas (cm^2) corresponding to the components in the diagrams of Fig. 10

Component	Ascending boundaries		Descending boundaries	
	Area	Reduced area	Area	Reduced area
I	6.12	5.13	11.25	6.50
II	17.54	14.71	34.99	20.20
III	3.42	2.87	7.70	4.45
IV	5.27	4.42	6.14	3.55
δ resp ε	12.08	10.13	-	3.65
Residual area	2.72	2.30	-	1.83
Total	47.15	39.56	69.60	40.18

Now the areas under each peak were calculated as a percentage of the value of $\Sigma O - O_\delta$ for the ascending side and of $\Sigma O - O_\varepsilon$ for the descending side. Here too the calculated value of O_ε was used. Whence:

$$\Sigma O - O_\delta = 39.87 - 10.13 = 29.74 \text{ cm}^2$$

$$\Sigma O - O_\varepsilon = 39.87 - 3.65 = 36.22 \text{ cm}^2$$

The results of this calculation are given in the next table (6):

TABLE 6. Composition of whey protein by electrophoretic analysis

Component	Ascending boundaries (%)	Descending boundaries (%)	Mean (%)
I	17.2	17.9	17.6
II	49.5	55.8	52.6
III	9.6	12.3	11.0
IV	14.9	9.8	12.4
Residual	8.8	4.2	6.4
Total	100.0	100.0	100.0

For the computation of the "Residual" figures the areas necessary to supplement the sums of all areas to $\Sigma O = 39.87$ cm^2 have been taken as residual areas.

In order to obtain the mobilities the correct individual values of the specific conductance were first calculated. All conductances are here expressed in $10^{-5}\,\Omega^{-1}\text{cm}^{-1}$. The experimental data were as follows:

Buffer solution : $\kappa_1 = 698$

Protein solution : $\kappa_3 = 670$

Substitution of these values and of $g_r = 0.821$ in equations 10a and 10b yielded:

$$\kappa_2 = g_r \cdot \kappa_3 = 550$$

$$\kappa_2' = \kappa_1 / g_r = 850$$

The differences in conductance between which interpolation had to be executed were:

$$\kappa_1 - \kappa_2 = 148$$

$$\kappa_2' - \kappa_3 = 180$$

The interpolation was performed with the help of the mean figures of table 6. As these figures do not extend to 100 per cent, we multiplied them by a factor 100/93.6, thus spreading the error of the residual area equally over all components (see table 7).

TABLE 7. Composition of whey protein, corrected for residual area

Components	% (Mean values)
I	18.8
II	56.2
III	11.7
IV	13.3
Total	100.0

The values of the conductance, to be used for the computation of the mobilities were calculated in the following way:

Ascending pattern:

Component	Specific Conductance ($10^{-5}\Omega^{-1}$ cm^{-1})
I	698 - 0.188 . 148 = 670
II	698 - 0.750 . 148 = 587
III	698 - 0.867 . 148 = 570
IV	698 - 1.000 . 148 = 550

Descending pattern:

Component	Specific Conductance ($10^{-5}\Omega^{-1}$ cm^{-1})
I	670 + 0.000 . 180 = 670
II	670 + 0.188 . 180 = 704
III	670 + 0.750 . 180 = 805
IV	670 + 0.867 . 180 = 826

The displacements of the components in relation to the δ and ε boundary were then measured; this gave the following results:

Component	Displacement (cm)	
	Ascending pattern	Descending pattern
I	18.75	17.15
II	17.30	13.40
III	12.90	8.80
IV	5.85	3.45

The equation for the calculation of the mobilities is:

$$u = \frac{\kappa v}{i} \quad \text{(cf equation 5 in II.4.a.)}$$

The velocities (v) are related to the displacements (Δ s) by

$$v = \frac{\Delta s}{\Delta t} \cdot \frac{8.4}{28}$$

in which the numerical factor is due to the ratio of the length of the base line on the drawing paper and the true proportion of the slits in the diaphragm directly behind the electrophoretic cell. The recordings were made after 15 200 sec., which value was substituted for Δ t. The measured current was 30 mA, which included a leakage current of 2.5 mA. Since the cross-sectional area of the electrophoretic cell was 0.750 cm^2, the current density (i) had the value

$$\frac{(30 - 2.5)}{0.750} \cdot 10^{-3} = 35.7 \cdot 10^{-3} \ A/cm^2$$

Substituting these values in $u = \frac{\kappa v}{i}$, we obtained

$$u = \kappa . \Delta s . \frac{8.4 \cdot 10^3}{15200 \cdot 28 \cdot 35.7} \cdot 10^{-5} =$$

$$= 5.53 \cdot \kappa \cdot \Delta s \cdot 10^{-5} \ cm^2 V^{-1} sec^{-1}$$

From this equation the mobilities were calculated with the help of the values for κ and Δs, given above. The results are shown in table 8:

TABLE 8. Mobilities of the protein components of a sample of normal rennet whey

Component	Mobility (10^{-5} $cm^2 V^{-1}$ $sec.^{-1}$)		
	Ascending pattern	Descending pattern	Mean
I	6.95	6.35	6.7
II	5.62	5.22	5.4
III	4.07	3.92	4.0
IV	1.78	1.58	1.7

To check the reliability of the figures we have calculated the mobilities of the components II and III in the same way (with the use of the same conductances) from two earlier recordings of the same experiment, viz. after 9200 and after 6000 sec. The displacements of the components I and IV could not then be measured because of insufficient separation. The results of these calculations, together with the figures for II and III from table 8 are given in table 9.

TABLE 9. Mobilities of the components II and III as estimated at different times during the experiment

Duration of current supply (sec.)	Mobility ($cm^2\ V^{-1}\ sec.^{-1}$)					
	Component II			Component III		
	Ascending	Descending	Mean	Ascending	Descending	Mean
6000	5.88	5.23	5.6	3.91	3.95	3.9
9200	5.44	5.24	5.3	4.17	3.90	4.0
15200	5.62	5.22	5.4	4.07	3.92	4.0

From this table it is obvious that the values of the mobilities remain fairly constant during the experiment although the results will be more reliable in the later stages.

For the sake of completeness the field strength in the buffer solution (F) was calculated. The leakage current was neglected. This value was not used for the computation of mobilities, but only for characterization of the conditions under which the patterns were obtained. All conductances refer to 0°C, hence this applies to the field strengths too. The calculation of the field strength (F) gave the value

$$F = \frac{i}{\kappa} = \frac{30 \cdot 10^{-3}}{0.75} \Big/ 698 \cdot 10^{-5} = 5.73 \text{ Volt/cm.}$$

A general consideration of the patterns and the numerical data obtained from them leads to the following conclusions:

Rennet whey contains at least four components. The mobilities range from approximately 1.5 to 7.0 . 10^{-5} cm^2 Volt $^{-1}$ sec. $^{-1}$. The "component" IV, characterized by the lowest mobility, shows a small but very broad boundary, which does not accurately agree with a Gaussian curve. Hence this boundary may be due to more constituents between which we are not able to make a clear distinction. The chief contribution to the diagram is given by component II (mobility 5.4 . 10^{-5} cm^2 Volt $^{-1}$ sec. $^{-1}$) which amounts to approximately 56 per cent of the total protein content. The patterns of ascending and descending diagram are highly unsymmetrical. This confirms the observations of other workers e.g. SLATTER & VAN WINKLE (1950). The phenomenon may be due to the presence of highly charged proteins with proportionally low mobilities. *According to the theory (cf II.4) it should be accompanied by great* boundary anomalies (δ and ε boundary). The latter fact was actually observed.

In contrast with the mobilities the concentrations calculated from the ascending pattern and those obtained from the descending pattern do not agree satisfactorily. This may be due in part to experimental errors, to the introduction of assumptions which do not hold completely, and to the intentional neglect of some details e.g. the influence of superimposed protein gradients. Nevertheless in our opinion these influences cannot account entirely for the differences obtained. This may therefore be an indication that our understanding of the electrophoresis of these proteins is not yet complete.

The resolution of the diagram into Gaussian curves is subject to great errors, especially with reference to the separation of the components I and II

and the measurement of the area of component IV, the latter being very flat in the ascending pattern and partly obscured by the ε-boundary in the descending one.

The influence of superimposed protein gradients has been mentioned in II.4.a. In an examination of the signs of these errors it appears that the peak, due to the most rapidly moving component will be enlarged in the ascending pattern, whereas it will be diminished in the descending one, and, in the case of the most slowly moving constituent, vice versa. As neither component I nor component IV can be estimated with great accuracy this phenomenon can only be observed with the data from component III, which are markedly influenced by extra gradients, due to the large component II. They will counteract the true gradient III on the ascending side but they will enlarge it at the descending side. Indeed it appears that for this concentration smaller values are calculated from the ascending pattern than from the descending one.

Finally the relation between the size of the δ-boundary and the charge per unit of mass has to be reviewed more accurately. It has been pointed out that the salt concentration gradients in the stationary boundaries are counterbalanced by equal gradients throughout the moving boundaries. The latter are proportional to the protein concentrations of the solutions which are moving onwards (cf II.4.c. eq.7). Hence for the ascending boundary we can write in terms of ionic strength:

$$\mu_1 - \mu_2 \propto \sigma_2$$

In practice the protein concentration is expressed in grams per liter, so that

$$\sigma_2 \propto \frac{[P_2]}{E}$$

where E = mean equivalent weight.

In addition $\mu_1 = \mu_3$, $\mu_2 = g_r \mu_3$ and $[P_2] = g_r [P_3]$.

Hence $(1 - g_r)\mu_3 \propto g_r \frac{[P_3]}{E}$

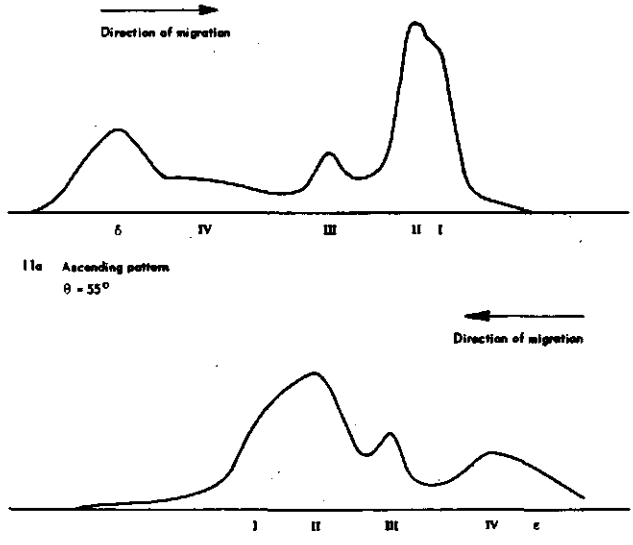
Therefore the quantity $\frac{(1-g_r)\mu_3}{g_r [P_3]}$ must be directly proportional to $\frac{1}{E}$, the mean charge per unit of mass.

For the experiment under discussion the numerical value of this quantity turns out to be:

$$\frac{1 - 0.821}{0.821} \cdot \frac{0.15}{1.81} = 0.0181$$

c. The influence of precipitation of the protein by salting out

The salting out of a protein by saturation of its solution with ammonium sulphate is a usual laboratory procedure in the preparation of proteins. In general this procedure and the resolution of the precipitate in distilled water are considered to be reversible. In addition it is the easiest way of achieving a high concentration of protein. In order to detect any possible influence of this procedure we prepared two solutions of milk whey proteins, obtained from the same sample of rennet whey. Sample 1 was concentrated by the freezing procedure described under II.2.b., sample 2 by salting out with ammonium sulphate, followed by dialysis of the precipitate against the buffer solution, discussed in II.5.a. Both solutions were dialyzed untill equilibrium with the buffer solution was attained and they were then subjected to electrophoresis on the same day viz 7 days after the coagulation of the fresh milk. The patterns obtained are shown in Fig. 11 and Fig. 12. From these drawings it is quite clear that the form of the diagrams has changed, although very slightly. In both cases the separation of the components I and II is not sufficient for a satisfactory analysis to be performed. Nevertheless gradient I appears to be somewhat larger in the case of concentration by the freezing procedure. From the numerical analysis of the patterns it appears, however, that there is hardly any difference.

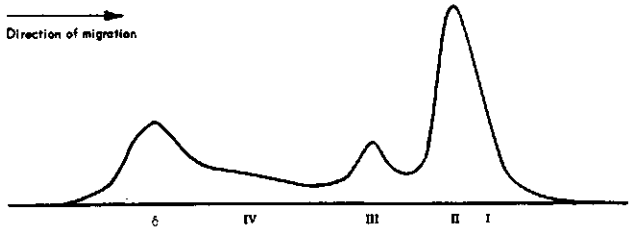


11a Ascending pattern
θ = 55°

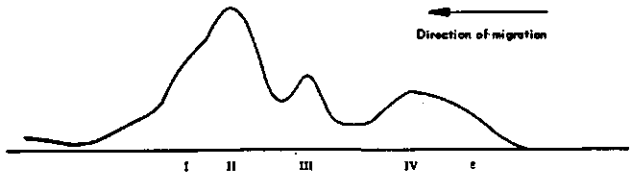
11b Descending pattern
θ = 55°

Fig. 11. Electrophoretic patterns of rennet whey proteins, concentrated by freezing out
Protein concentration 1.50 g/100 ml

t = 12.000 sec. μ = 0.15
pH = 6.8 F = 5.73 V/cm



12a Ascending pattern
θ = 55°



12b Descending pattern
θ = 55°

Fig. 12. Electrophoretic patterns of rennet whey proteins, concentrated by salting out with ammoniumsulphate.
Protein concentration 1.41 g/100 ml
t = 12.000 sec. μ = 0.15
pH = 6.8 F = 5.72 V/cm

In table 10 the concentrations and mobilities of the components are given (mean values from ascending and descending patterns). In addition the values of the dilution factor at the δ-boundary g_r and the quantity $\frac{(1 - g_r)\,\mu_3}{g_r\,[P_3]}$ have been included.

TABLE 10. Analysis of the electrophoretic patterns of whey, concentrated by the freezing procedure (fig. 11) and by salting out with ammonium sulphate Fig. 12)

Components	Mean concentration (relative %)		Mean mobility (10^{-5} cm^{2}V^{-1}sec.$^{-1}$)	
	Fig. 11	Fig. 12	Fig. 11	Fig. 12
I	} 65.8	} 64.7	6.4	6.6
II			5.4	5.6
III	14.2	16.6	3.9	4.0
IV	20.0	18.7	1.6	1.6
Dilution factor at the δ-boundary			0.850	0.843
Index of mean charge $\frac{(1 - g_r)\,\mu_3}{g_r\,[P_3]}$			0.0176	0.0186

Neither the concentration nor the mobilities show differences of any importance, although there is no complete identity of the concentrations. The mobilities can be regarded as unaffected. The values of g_r and $\frac{(1 - g_r)\,\mu_3}{g_r\,[\,\nu_3\,]}$ show a small difference. As the latter are obtained from the areas under the δ-boundaries which can be estimated with fairly good accuracy the difference should not be disregarded. It implies that after salting out the mean specific charge of the proteins has somewhat increased. In view of the work to be described under II.7.c-d. on the heat denaturation of the whey proteins it is probable that this increase of charge is a symptom of incipient denaturation.

The conclusion is that although the electrophoretic patterns of whey proteins are undeniably influenced by the process of salting out with ammonium sulphate, this influence is only of minor importance.

This conclusion does only apply to ammonium sulphate. In the case of other reagents a similar test should be carried out before the question whether the protein studied is wholly or partly an artefact can be answered.

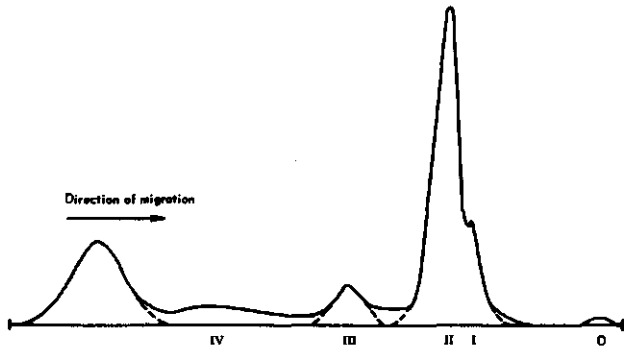


Fig. 13a Ascending pattern
$\theta = 55°$

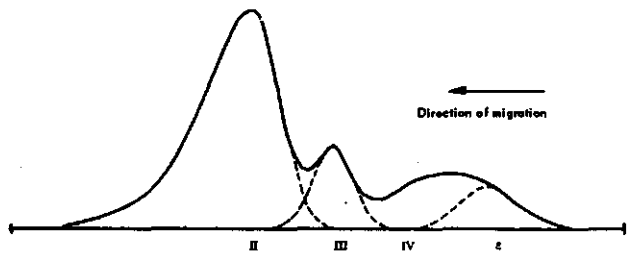


Fig. 13b Descending pattern
$\theta = 65°$

Fig. 13. Electrophoretic patterns of rennet whey proteins, concentrated by freezing out.
Protein concentration 1,50 g/100 ml
t = 14000 sec μ = 0.15
pH = 6.8 F = 5,74 V/cm

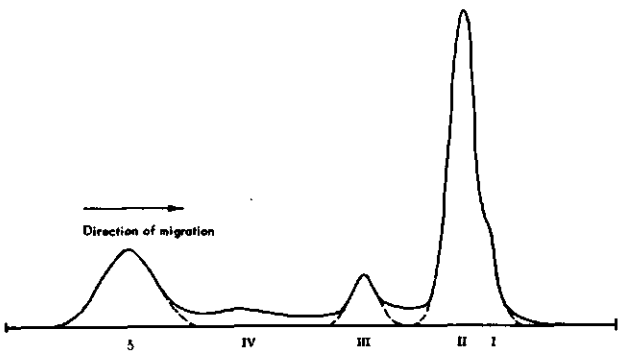


Fig. 14a Ascending pattern
$\theta = 55°$

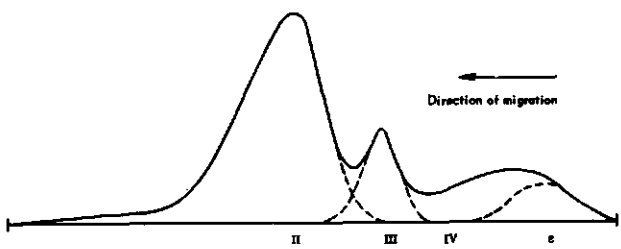


Fig. 14b Descending pattern
$\vartheta = 65°$

Fig. 14. Electrophoretic patterns of rennet whey proteins, concentrated by salting out with phosphate mixture (3,5 M, pH = 6,5)
Protein concentration 1,50 g/100 ml
$\tau = 14000$ sec $\mu = 0,15$
pH = 6.8 F = 5,74 V/cm

Among the other salts or salt mixtures we have also studied the equimolecular mixture of mono and di-potassium phosphate (3.5 M, pH 6.5), which has been extensively used by DERRIEN (1947) in his fractionation work by salting out.

By analogy with the experiment involving ammonium sulphate electrophoretic patterns were recorded of two samples of the same whey. The first sample was concentrated by the freezing procedure, the second by salting out with the phosphate mixture. The patterns have been drawn in Fig. 13 (freezing procedure) and Fig. 14 (salting out). It appears that the diagrams are almost identical, except that the velocity of the components in Fig. 13a appears somewhat higher than that in Fig. 14a. The velocities in the descending patterns are approximately equal. In addition in Fig. 13a, a small amount of a very rapidly moving component (denoted as O) is observed, which is absent in Fig. 14a.

The analysis of the patterns confirms these preliminary statements. The only differences are to be found in the ascending patterns and hence only these are summarized in table 11.

TABLE 11. Analysis of the electrophoretic patterns of whey, concentrated by the freezing procedure (Fig. 13) and by salting out with phosphate mixture (3.5 M, pH 6.5) (Fig.14)

Component	Concentration (relative %)		Mobility (10^{-5} cm^2 V^{-1} sec.$^{-1}$)	
	Fig. 13	Fig. 14	Fig. 13	Fig. 14
I	} 69.6	} 70.8	-	-
II			5.4	5.1
III	9.7	10.5	3.8	3.5
IV	14.4	13.5	-	-
Dilution factor at the δ boundary			0.842	0.840
Index of mean charge $\frac{1 - g_r}{g_r} \frac{\mu_3}{[P_3]}$			0.0188	0.0190

The only difference which is confirmed in some degree by the descending patterns, is the difference in globulin content (component IV). In the ascending patterns it amounts to 14.4 - 13.5 = 0.9 per cent as against 13.9 - 13.4 = 0.5 per cent in the descending patterns. Here the concentration of component IV has been computed by subtraction of the area corresponding with the ε boundary from the area ascribed to component IV and the ε gradient together. The areas under the ε peaks were calculated from the areas under the δ peaks of the ascending patterns according to the method described in II.5.b. The resultant ε areas have been bounded by dotted lines in the figs. 13b and 14b. In view of this rather complicated procedure the difference of 0.5 per cent is negligible. The difference in the mobilities of the components II and III is not great but it may be of some importance, even though it is not found in the case of the descending patterns. The dilution factor at the δ boundary and hence the index of mean charge has remained unaffected within the limits of error.

Therefore as a result of the experiments described in this section, it can be stated that salting out with ammonium sulphate or with phosphates does not produce any important change although slight effects are observed.

d. The number of components after prolonged electrophoresis

With every method of analysis of a protein mixture the question arises how many different constituents can be detected. The electrophoretic experiments described up to this point have yielded four components, but in our less extensive ultracentrifugal investigations - to be described under II.11. - the presence of only three components could be proved.

According to DERRIEN (1947) blood serum should contain 20-30 different constituents, which can only be detected by cautiously and gradually salting out, a method especially developed by him. When the slope of the salting out curve is plotted graphically against the protein concentration in solution, a large number of small peaks is found. In a preliminary experiment the application of this method to the proteins of milk whey also yielded a great

many peaks. Although it is questionable whether these peaks are related to native components, the observation has been a stimulus to us to examine if still more components could be detected by electrophoresis.

When the electrophoresis of rennet whey is carried out for approximately 5000 seconds under the circumstances described in previous sections only three components can be detected. After 10.000 seconds one new constituent (I) begins to separate from the most rapidly moving component (apparently I + II) of the three. After 15.000 seconds when the pattern has extended over the complete length of the cell, only 4 components are visible still. Is there a chance that after prolonged electrophoresis any new separation will occur? In answer to this question we have carried out one experiment without bringing the initial boundaries into the optical field before starting. Twenty thousand seconds' exposure to the electric field was necessary before the most rapidly moving component reached the further end of the viewing slit. In order not to let the elongated components be obscured in the base line an extraordinarily high protein concentration was used viz. 2.29 g/100 ml. The latter resulted in a very large δ -boundary. In Fig. 15 the ascending pattern of this experiment has been drawn. The descending pattern which is of inferior resolution and thus less suited to the purpose has not been recorded. The dotted left hand side of the δ -boundary was brought into the optical field at the end of the experiment by the cautious injection of some liquid in the cathodic vessel of the apparatus. It will be obvious that no characteristic new component has separated, at least not from the main complex (I + II). Only the elongated form of component IV suggests that it has a complex nature.

The analysis of this diagram yielded the figures recorded in table 12.

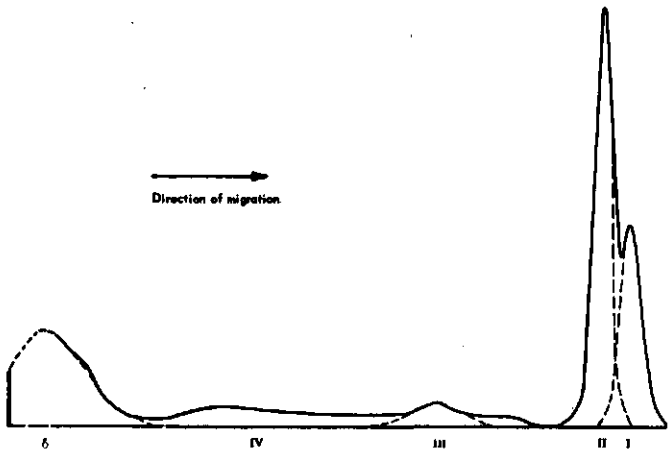


Fig. 15. Electrophoretic pattern of rennet whey after prolonged electrophoresis (ascending boundaries)

Protein concentration 2.29 g/100 ml

t = 20.100 sec. μ = 0.15 θ = 65°

pH = 6.8 F = 5.76 V/cm

TABLE 12. Electrophoretic analysis of rennet whey proteins after prolonged electrophoresis (calculated from the ascending pattern)

Component	Concentration (relative %)	Mobility 10^{-5} cm^{2}V^{-1}sec.$^{-1}$)
I	28.7	6.8
II	46.1	5.8
III	11.7	3.9
IV	13.5	undefined
Dilution factor at the boundary (g_r)		0.770
Index of mean charge $\frac{(1-g_r).\mu_3}{g_r \quad [P_3]}$		0.0196

The concentrations are rather different from the values obtained in the previous experiments (cf II.5.b. table 7). However, the whey had been prepared from another sample of milk, the composition of which depends on several other factors. The mobilities are not exactly equal to the other values found, but the differences are not great. The values obtained depend to some extent upon the duration of the experiment chiefly because of the progressive separation of the mixture (cf II.5.b. table 9). The dilution at the δ-boundary is very high (23%); this is due to the high protein concentration (2.29%). The "mean charge" value is somewhat higher than the values obtained in the previous experiments, but this difference may be completely due to the differences in composition of this sample compared with the other samples of whey. In any event the order of magnitude of the two values is the same.

The conclusion of the experiment is that even after prolonged electrophoresis no new constituents can be detected. This does not necessarily exclude the possibility of their existence.

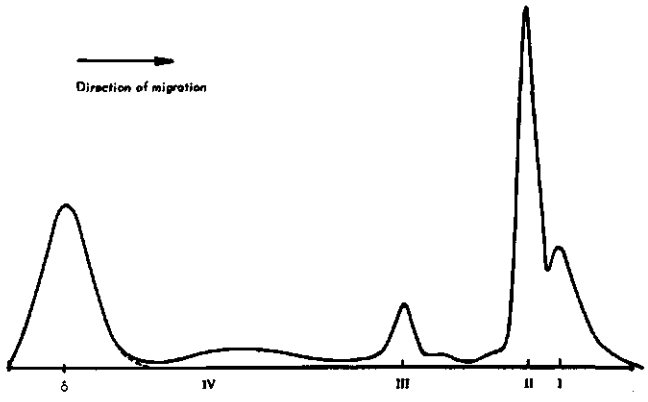
6. IDENTIFICATION OF THE COMPONENTS

a. The stationary boundaries

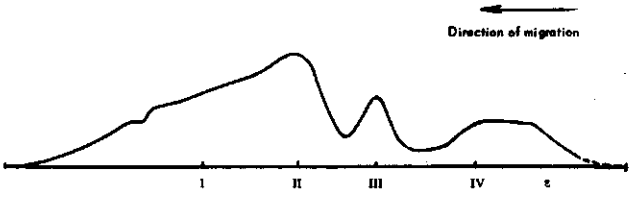
According to the theory in section II.4. stationary boundaries can be identified by means of the following criteria:

1. They should not move or at least only very slowly.
2. They should be reduced by increasing the ratio of the ionic strength of the buffer to the protein concentration.
3. They should be reduced, if desired even to zero, by suitable dilution of the dialyzed protein solution with water.

The first condition was fulfilled by the peaks denoted as δ and ε-boundaries in all the electrophoretic diagrams treated in this essay. However, there was a possibility of these nearly stationary boundaries being protein gradients characterized by extremely low mobilities. Therefore they were also tested by their behaviour towards the effects in 2 and 3 above. The diagrams of four experiments were compared. These latter were carried out under different



16a Ascending pattern



16b Descending pattern

Fig. 16. Electrophoretic patterns of rennet whey of normal cow's milk.
Protein concentration 2.50 gr/100 ml
Ionic strength μ = 0.10

t = 13.400 sec. F = 6.11 V/cm
pH = 6.8 θ = 45°

conditions of ionic strength, protein concentration and previous dilution of the protein solution. The patterns obtained in these experiments have been drawn in Figs 16, 17, 18 and 19. Form each of these patterns several characteristic quantities were calculated viz.

1. the area under the δ -boundary expressed in per cents of the total area under the ascending pattern (% δ).
2. the dilution factor (g_r) as calculated from the area under this supposed δ -boundary.
3. the function $\frac{1 - g_r}{g_r} \frac{\mu_3}{[P_3]}$, introduced in II.5.b. to give information as to mean the charge of the protein mixture.

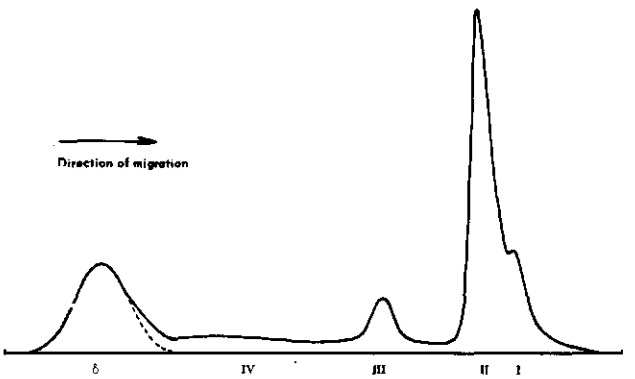
For the experiments where a previous dilution with water had been applied the factor g_r was multiplied by the factor of previous dilution, which yielded the total dilution factor (g_r total), and this factor was used in the calculation of the function

$$\frac{(1-g_r)}{g_r} \cdot \frac{\mu_3}{[P_3]}$$

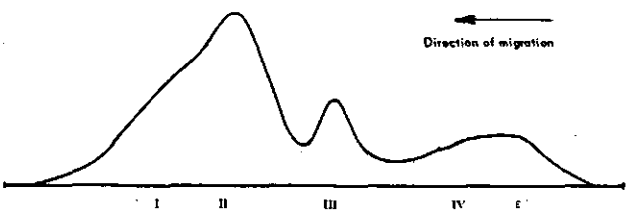
In table 13 the necessary experimental and calculated data have been recorded for the four experiments together.

TABLE 13. Experimental and calculated data in connection with the patterns recorded in Figs. 16, 17, 18 and 19 on the identification of the stationary boundaries

Fig. No.	Protein conc. $[P_3]$ (g/100 ml)	Ionic strength	% δ	g_r	Factor of previous dilution	g_r total	$\frac{(1-g_r)}{g_r} \cdot \frac{\mu_3}{[P_3]}$
16	2.50	0.10	35.4	0.70	1.000	0.70	0.017
17	1.81	0.15	25.6	0.82	1.000	0.82	0.018
18	1.81	0.15	15.1	0.90	0.900	0.81	0.019
19	1.86	0.15	2.4	0.99	0.806	0.80	0.020



17 a Ascending pattern
θ = 50°



17 b Descending pattern
θ = 60°

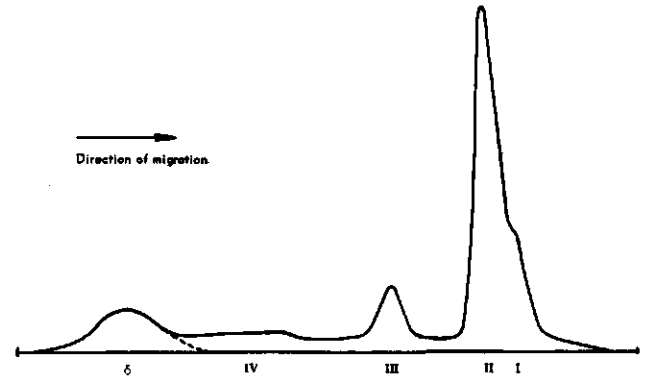
Fig. 17. Electrophoretic patterns of rennet whey of normal cow's milk.
Protein concentration 1.81 gr/100 ml
ionic strength μ = 0.15

t = 15200 F = 5.73 V/cm
pH = 6.8

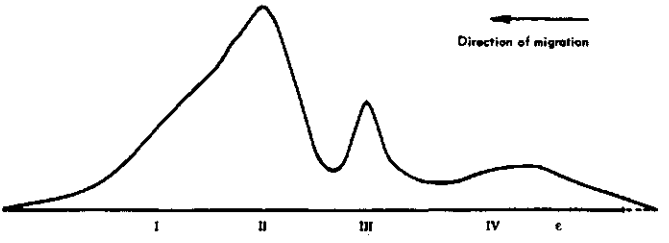
The figures inserted in the cases where previous dilution had been applied Figs. 18 and 19) denote the original values of protein concentration and ionic strengnt before dilution. In these cases the calculation of g_r was complicated by a difference in salt concentration between the buffer solution (1) and the diluted protein solution (3). Here equation 6a (II.5.b.) was provided with an additional correction factor in the denominator by which the influence of the dilution gradient was discounted. The corrected form of the equation was:

$$O_\delta = (1-g_r) \cdot \Sigma O \cdot \frac{\{1 + 5.03 \frac{\mu_3}{[P_3]}\}}{\{1 + 5.03 \frac{(\mu_3' - \mu_1)}{[P_3]'}\}}$$

Here μ_3 and $[P_3]$ stand for the original values of ionic strength and protein concentration and μ_3' and $[P_3]'$ for their real values after the dilution preceding the experiment. This equation has been derived by analogy with the



18 a Ascending pattern
θ = 50°



18 b Descending pattern
θ = 65°

Fig. 18. Electrophoretic patterns of rennet whey of normal cow's milk.
Original protein concentration 1.81 gr/100 ml
Original ionic strength $\mu = 0.15$
Dilution before electrophoresis to 90% of the original values
t = 14300 sec. F = 5.73 V/cm

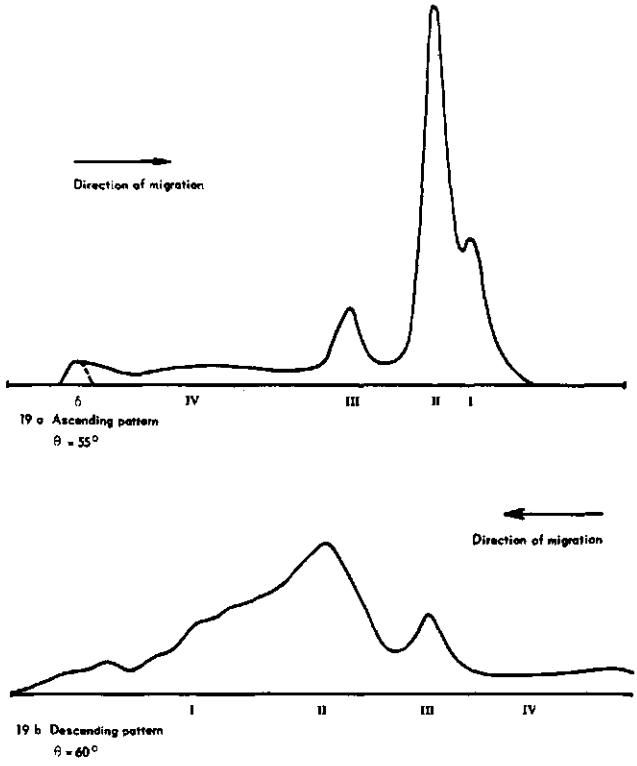


Fig. 19. Electrophoretic patterns of rennet whey of normal cow's milk.
Original protein concentration 1.86 gr/100 ml
Original ionic strength 0.15
Dilution before electrophoresis to 80.6% of the original values.
t = 14000 sec. F = 5.69 V/cm
pH = 6.8

original equations 6 (II.4.b.) and 6a (II.5.b.). Its derivation is not given here as it does not include any new arguments. Regarding Figs. 16, 17, 18 and 19 and table 13 the following facts can be stated.

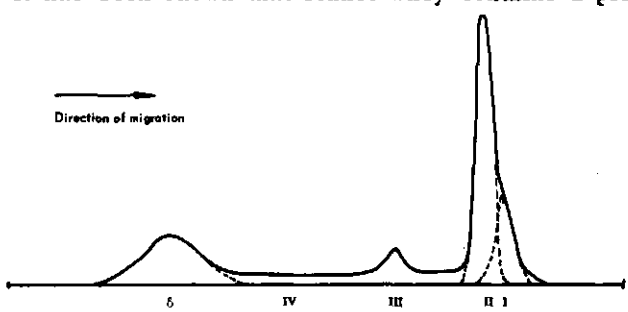
The reduction of the ratio of protein concentration to ionic strength (cf Fig. 16 and Fig. 17) leads to a considerable decrease of the relative area occupied by the δ-boundary. A similar further decrease is obtained by dilution of the protein solution to 90 per cent of its original value (Fig. 17 and Fig. 18). From the latter patterns (obtained from samples of the same whey) the dilution necessary to reduce the δ-boundary to approximately zero was calculated. This dilution (to 80.6 per cent) was used in the experiment which yielded the patterns of Fig. 19 where the δ-boundary has disappeared almost completely. The value of the function

$$\frac{1-g_r}{g_r} \quad \frac{\mu_3}{[P_3]}$$

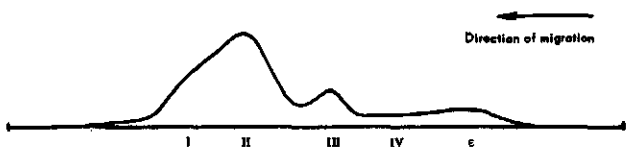
however, remains fairly constant, which means that the mean charge of the protein mixture has not been affected. In the case of the patterns of figs. 17 and 18 the values ought to be identical; this is correct within the limits of experimental error. The small difference from the two other experiments be due to the different origin of the whey samples. Hence it is quite clear that the peak denoted as the δ-boundary is in fact stationary. Concerning the ε-boundary it will be obvious from Figs. 16b to 19b that the most slowly moving peak is reduced at the same rate as the δ-boundary but not completely to zero. Apparently the separation of the ε-boundary and the slow component IV is very unsatisfactory. For this reason we prefer the calculation of the ε area from the experimental δ area to the use of any experimental ε curve in the descending patterns.

b. The so-called "Whey protein"

In II.3.c. it has been shown that rennet whey contains a protein fraction



20 a Ascending pattern



20 b Descending pattern

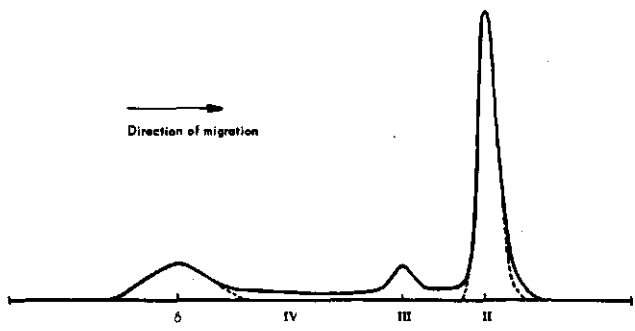
Fig. 20. Electrophoretic patterns of rennet whey of normal cow's milk (cf. Fig. 21 acid whey from same milk sample)

Protein concentration 1,50 gr/100 ml

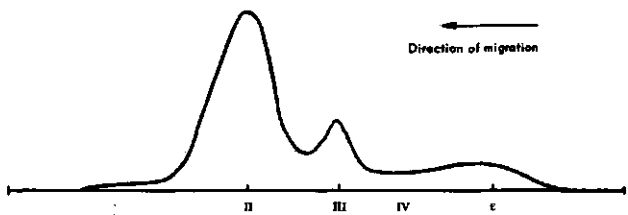
Ionic strength $\mu = 0.15$

$t = 12000$ sec. $F = 5.76$ V/cm

pH = 6.8 $\theta = 55°$



21 a Ascending pattern
θ = 55°



21 b Descending pattern
θ = 65°

Fig. 21. Electrophoretic patterns of acid whey of normal cow's milk (cf. Fig. 20 rennet whey from same milk sample).

Protein concentration 1.45 gr/100 ml
Ionic strength $\mu = 0.15$

t = 12000 sec. F = 6.16 V/cm
pH = 6.8

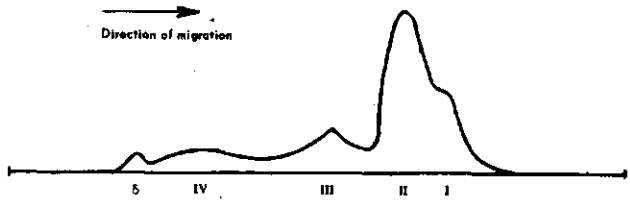
due to the action of rennin on casein. From the values of the nitrogen content of comparable samples of rennet and acid whey it appeared that this fraction amounted to 14-17 per cent of the total non-dialysable nitrogen present in rennet whey. By means of electrophoretic examination it should be possible to decide which of the components of rennet whey can be identified as "whey protein".

For this purpose two samples of whey were prepared from the same milk, one by means of rennin and the other by addition of HCl. Both samples were concentrated by precipitation with ammonium sulphate and afterwards dialyzed against the buffer solution. The patterns obtained are drawn in Figs. 20 (rennet whey) and 21 (acid whey). It appears that in the case of acid coagulation of the casein, component I has disappeared. In addition the δ -boundary is somewhat smaller in Fig. 21 than in Fig. 20.

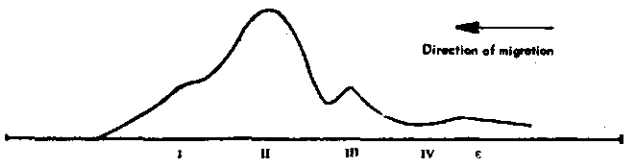
Numerical analysis of these patterns yields the figures of table 14.

TABLE 14. Electrophoretic analysis of rennet and acid whey from the same milk sample

Concentrations (relative %)				Mobilities (10^{-5} cm^2 V^{-1} sec^{-1})	
Component	Rennin	Acid	Rennin*	Rennin	Acid
I	20.6	-	-	6.9	-
II	55.1	73.6	69.4	5.4	5.4
III	13.2	14.2	16.6	3.7	3.7
IV	11.1	12.2	14.0	undefined	undefined
Dilution at the δ-boundary				0.837	0.858
Index of mean charge $\frac{1-g_r}{g_r}\frac{\mu_3}{[P_3]}$				0.0195	0.0171



22 a Ascending pattern
θ = 55°



22 b Descending pattern
θ = 65°

Fig. 22. Electrophoretic patterns of rennet whey of normal cow's milk (cf. Fig. 23 acid whey from same milk sample).

Elimination of boundary anomalies by previous dilution to 88.5% of the original concentrations.

Original protein concentration 1.30 gr/100 ml

Original ionic strength μ = 0.15

t = 12000 sec. F = 5.72 V/cm

pH = 6.8

Under the heading Rennin* the values of the concentrations II, III and IV in rennet whey have been converted into the values that would have been found in the case of the complete absence of Component I, by multiplication of the former values by a factor 100/79.4.
There is some difference from the values actually found for acid whey. As the concentration value of Component I is highly subject to the error due to counteracting protein gradients, it cannot be expected that the above mentioned conversion will lead to a satisfactory agreement between the two sets of figures. In addition, the magnitude of the counteracting protein gradients within the boundaries II, III and IV will also have changed.

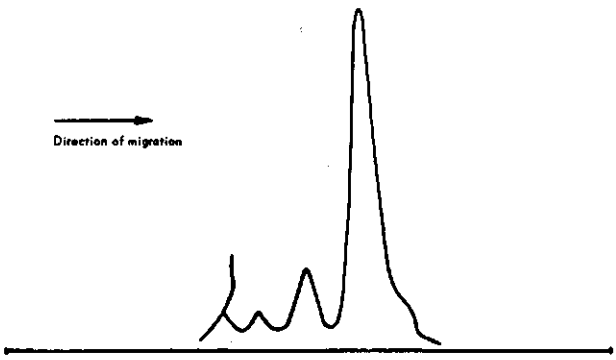
The mobilities of Components II and III are equal. Those of the component IV have not been calculated as in both patterns these boundaries could not be located unambiguously. In any event it appears that the character of the three remaining components is the same.

The figures for the "mean charge" indicate a lower value for acid whey than for rennet whey. However, this conclusion is disputable although the difference in dilution at the δ-boundary is quite real. As has been emphasized in II.4.c. and II.5.b. a difference in size of the δ-boundaries is only significant if neither the mobilities nor the relative concentration values in either experiment shows a difference of importance. The latter condition is not fulfilled in the experiments under discussion.

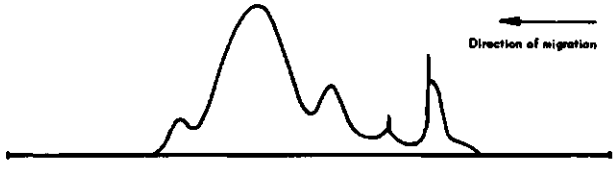
Concerning the dilution at the δ-boundary two similar experiments deserve to be mentioned. Figs. 22 and 23 represent the patterns of rennet whey and acid whey proteins concentrated by the freezing procedure, from the same milk and recorded under the same circumstances. In these experiments the protein solution was previously diluted to 88.5 per cent of the original concentrations Now this dilution was almost sufficient to eliminate the boundary anomalies in the case of rennet whey under the given conditions. In the case of acid whey, however, this dilution appeared to be inadmissable. During electrophoresis solutions were developed which were gravitationally unstable with respect to the diluted solution present in the lower parts of the cell; hence gravitational disturbances of the boundaries occurred, thus resulting in the distorted patterns of fig. 23.

In view of the experiments described in this section we feel justified in identifying Component I in the patterns of rennet whey as the so-called "whey protein". In the sample under discussion the electrophoretic analysis yields a concentration of approximately 20 per cent. In general values of 15-20 per cent are found. This agrees sufficiently with the values, mentioned in II.3.c., viz. 14-17 per cent, obtained by direct estimation of the nitrogen contents of samples of rennet whey and acid whey.

Finally we wish to draw attention to the fact that DEUTSCH (1947), working at pH 8.6 did not observe any difference in the patterns of rennet whey and acid whey, prepared from cow's milk. In the case of goat's milk, however, he recorded similar differences to those described in this section, viz. the disappearance of the most rapidly moving component and a decrease in size of the ε-boundary after acid coagulation of the casein. Apparently there is some similarity in the action of rennet on the caseins of milk of different animal species.



23a Ascending pattern
$\theta = 55°$



23 b Descending pattern
$\theta = 60°$

Fig. 23. Electrophoretic patterns of acid whey of normal cow's milk (cf. Fig. 22 rennet whey from same milk sample).

Distorted patterns because of previous dilution to 88.5% of the original concentrations.

Original protein concentration 1.30 gr/100 ml . t = 12000 sec. F = 5.72 V/cm
Original ionic strength $\mu = 0.15$ pH = 6.8

c. *β lactoglobulin*

According to PALMER (1934) 60 per cent or more of the classical lactalbumin fraction should consist of β lactoglobulin. As the classical lactalbumin fraction forms the greater part of the proteins in whey, β lactoglobulin would have to correspond with one of the major peaks of the electrophoretic patterns of whey. In the notation adopted in this essay this would have to be component II (cf e.g. Fig. 10, II.5.b.). The concentration of this constituent amounts to approximately 50 per cent of the proteins in rennet whey and its mobility averages $5.4 \times 10^{-5} cm^2 V^{-1} sec^{-1}$. Following the instructions given by PALMER (1934), BULL and CURRIE (1946) and CECIL and OGSTON (1949) we have prepared a solution of β lactoglobulin from an arbitary sample of normal cow's milk. As only a solution was needed we made no special attempts to obtain it in the crystalline form. In addition some small modifications of the methods applied by the above-mentioned authors were introduced. The complete method of preparation has been described in detail in the paper of

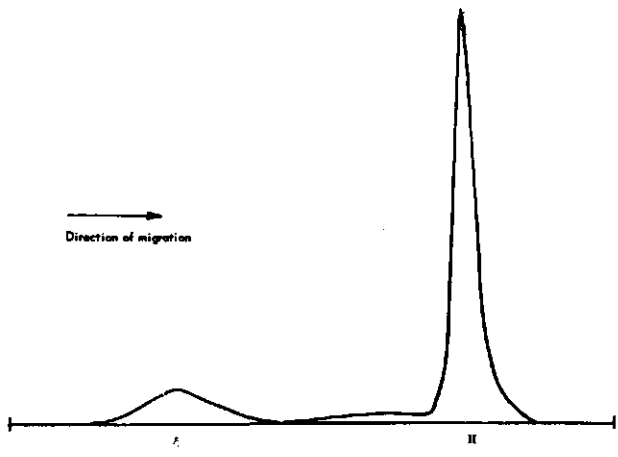


Fig. 24a Ascending pattern

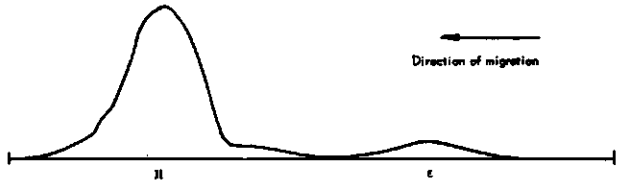


Fig. 24b Descending pattern

Fig. 24. Electrophoretic patterns of β-lactoglobulin
Protein concentration 1.50 g/100 ml
Ionic strength = 0.15
t = 14000 sec. F = 5.62 V/cm
pH = 6.8 θ = 45°

WEGELIN and DE WAEL (1952) on the determination of the charge of β lactoglobulin from which only the essentials will be quoted here.

The fraction which precipitates from skim milk between 55 per cent and 80 per cent saturation with ammonium sulphate was separated. After dissolution and dialysis the fractionation was repeated and the precipitate dialyzed first against distilled water and afterwards against the electrophoretic buffer solution (pH 6.8, μ 0.15). The material appeared to be homogeneous on electrophoretic as well as on ultracentrifugal examination (cf II.11.c.). The sedimentation constant (S^0_{20} = 2.71 Svedberg units) agreed reasonably well with the values obtained for β lactoglobulin by CECIL and OGSTON (1949) viz. 2.81 Svedberg units.

The dissociation curve of the protein in the solution was also determined (pH 5-7), according to the method described by CANNAN, PALMER and KIBRICK (1942); the results agreed almost completely with the curve obtained by these authors.

The patterns, obtained by electrophoresis have been drawn in Fig. 24.

The mobilities calculated from either pattern were $4.89 \times 10^{-5} cm^2 V^{-1} sec^{-1}$.

In addition the equivalent weight of β lactoglobulin was calculated. This was done with the help of equation (7) according to the principle treated in II.4.c. The value was found to be 2710 (at pH 6.8 and μ 0.15).

The equivalent weight at pH 6.8 and μ 0.15 was also calculated from the dissociation curve. To this purpose part of the solution of β lactoglobulin was dialyzed against 0.15 M KCl at pH 5.18. Samples from this dialyzed solution were mixed with increasing volumes of a solution 0.13 M in KCl and 0.02 M in NaOH and the pH was determined. The amount of alkali necessary to reach a given pH was converted into equivalents alkali per 40 000 g β lactoglobulin. This quantity h was introduced for the sake of comparison with the data of CANNAN, PALMER and KIBRICK (1942). It appears that the curves are almost identical. At pH 6.8 the values of h are 12.4 and 12.7 eq./mol respectively. (cf. Fig. 25)

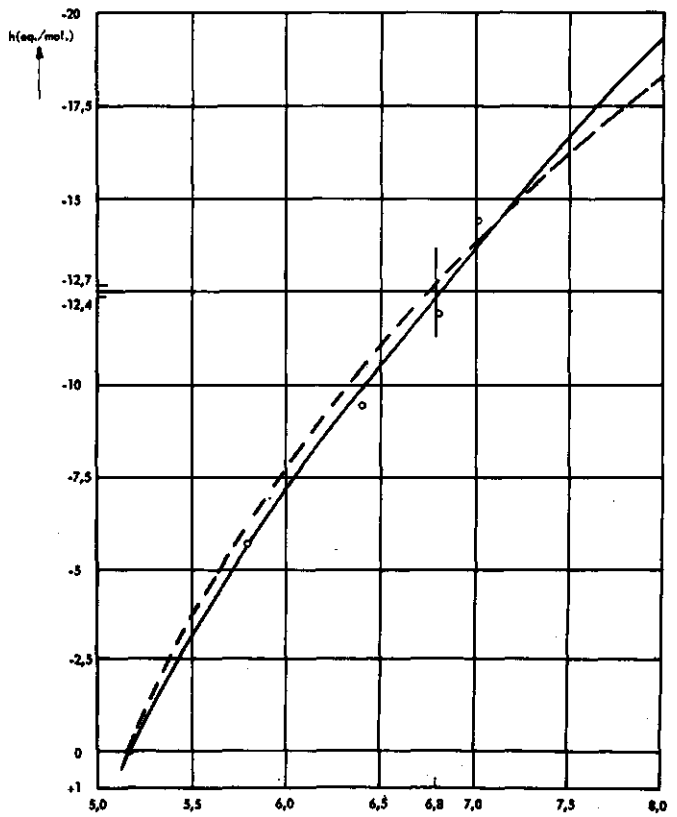


Fig. 25 Dissociation curve of β lactoglobulin.
Protein concentration 1.08 per cent
Ionic strength (KCl) μ = 0.15
– – – – Cannan, Palmer and Kibrick (1942)
——— Own observations

This corresponds to an equivalent weight of 3225 or 3140, if no dissociation of other groups takes place. With regard to the various possibilities of error these results may be considered to agree reasonably well with the value obtained by electrophoresis (2710). Hence the conclusion can be drawn that the charge of the proteins is almost exclusively due to the acid and alkaline groups.

When a comparison is made with the patterns of whey proteins as a whole Fig. 10, table 8, II.5.b.), it is obvious that the mobilities of β lactoglobulin and component II of the total pattern are not equal (4.9 and 5.4 $\times$ 10^{-5} $cm^2V^{-1}sec^{-1}$ respectively). However, the difference in mobility between β lactoglobulin and the adjacent component III is considerably greater. In addition, the concentration of this constituent (12 per cent) is not sufficient to account for the high value of the β lactoglobulin content in whey mentioned in the first paragraph of this section. Hence peak number II in the electrophoretic patterns must be ascribed to β lactoglobulin. The difference in mobility may be due to the other surrounding components, and to possible small alterations of the protein during its preparation. No values of the mobility under exactly the same circumstances were available in the literature. The values recorded by various authors under different circumstances are very divergent as is shown in table 15.

TABLE 15. Mobility of β lactoglobulin as quoted by various authors

Author	Material	Buffer	pH	Ionic strength	Protein concentration (%)	Temperature (°C)	Mobility (10^{-5} cm^2 V^{-1} sec^{-1})	Particulars
BRIGGS and HULL (1945)	"pure" β lactoglobulin	phosphate	6.9	0.1	?	4	- 6.4	homogeneous
LI (1946)	"pure" β lactoglobulin	acetate	4.8	0.1	1.5	1.5	+ 2.3 + 1.9 + 1.2	heterogeneous
LI (1946)	"pure" β lactoglobulin	acetate	5.3	0.1	1.5	1.5	- 1.4	homogeneous
LI (1946)	"pure" β lactoglobulin	acetate	5.6	0.1	1.5	1.5	- 2.5	homogeneous
LI (1946)	"pure" β lactoglobulin	acetate	6.5	0.1	1.5	1.5	- 5.6 - 5.2 - 4.5	heterogeneous
SMITH (1946)	colostrum fraction 0.5-0.9 sat.amm. sulph.	veronal	8.6	0.1	?	1	- 4.3	heterogeneous
SMITH (1946 c)	whey	veronal	8.6	0.1	0.51	1	- 5.6	heterogeneous
SMITH (1946 c)	whey	veronal	8.6	0.1	1.23	1	- 5.1	heterogeneous
SMITH (1946) c	whey	veronal	8.6	0.1	1.85	1	- 5.0	heterogeneous
POLIS et al. (1950)	"pure" β lactoglobulin	veronal	8.4	0.1	1.0	0	- 5.1	homogeneous
POLIS et al. (1950)	"pure" β_1 lactoglobulin	veronal	8.4	0.1	1.0	0	- 5.6	homogeneous
POLIS et al.	"pure" β lactoglobulin	acetate	4.8	0.1	1.0	0	+ 1.9 + 3.0	heterogeneous
POLIS et al. (1950)	"pure" β_1 lactoglobulin	acetate	4.8	0.1	1.0	0	+ 1.8	homogeneous
WEGELIN	"pure" β lactoglobulin	phosphate-citrate	6.8	0.15	1.5	0	- 4.9	homogeneous
WEGELIN	whey	phosphate-citrate	6.8	0.15	1.5	0	- 5.4	heterogeneous

From this table it will be clear that the values obtained for the mobility of pure β lactoglobulin and that of component II in the pattern of whey are plausible. In addition it shows that accurate comparison of electrophoretic mobilities obtained by various authors is hardly possible, as the results depend on so many different circumstances. In any event, the identification of component II as β lactoglobulin appears to us to be completely justified.

d. The further components

Apart from the boundaries dealt with in the preceding sections (II.6.a., b. and c.), there are still two constituents left viz. those denoted as III and IV.

Sufficient data concerning component IV are available in literature. According to SMITH (1946 c) the content of components with low mobilities amounts to approximately 10 per cent of the total whey proteins. He records two constituents (mobilities - 1.7 and - 2.5. $10^{-5}cm^2V^{-1}sec^{-1}$ at pH 8.6) each of which contributed 5 per cent to the total protein content. By repeated precipitation with ammonium sulphate he succeeded in isolating two crystalline fractions from the classical globulin fraction of whey. One of these fractions, which was soluble in water and was termed euglobulin, corresponded with the component with mobility - 1.7 . $10^{-5}cm^2V^{-1}sec^{-1}$. For the isoelectric points he gives the values 5.6 and 6.05 respectively.

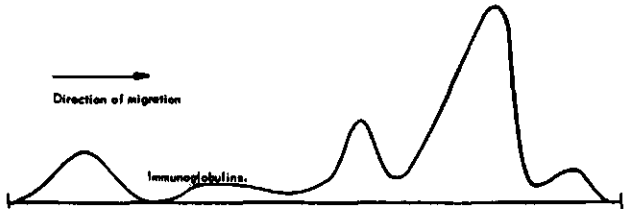


Fig. 26. Electrophoretic pattern of acid whey proteins (normal bovine milk, descending boundaries) According to Smith (1946 c).
Protein concentration 1.85 per cent.
Diethyl barbiturate buffer μ = 0.10.
t = 13000 sec.
pH = 8.6

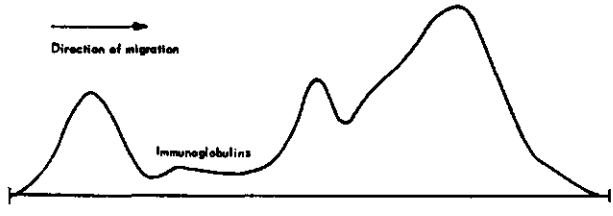


Fig. 27. Electrophoretic pattern of rennet whey proteins (pooled samples of bovine milk, descending boundaries)
According to Deutsch (1947)
Barbiturate-citrate buffer μ = 0.088
pH = 8.6
F = 8.5 V/cm

In another paper SMITH (1946) reports on the proteins of colostrum whey of hyperimmunized cows. Here the content of both constituents together appeared to be considerably higher viz. 32 per cent. After several immunological tests he concluded that these globulins were responsible for all the immunological properties of the whey and denoted them as immunoglobulins.

DEUTSCH (1947) records electrophoretic data on the composition of whey during the complete lactation period. For the content of the slowly moving component (mobility - 2.2 . 10^{-5} $cm^2V^{-1}sec^{-1}$) he reports 70 per cent (of the total protein) in the very first colostrum, and 10 per cent at the end of the lactation period. Approximately the same figures were recorded for a rapidly settling constituent in the ultracentrifuge (S^0_{20} = 6 Svedberg units).

The patterns obtained by SMITH (1946 c) and by DEUTSCH (1947) for normal cow's milk have been drawn in Figs. 26 and 27. By comparison with the patterns recorded in our experiments e.g. Fig. 10 (II.5.b.) it is obvious that component IV corresponds to the globulins dealt with by these authors. We have not subdivided this "peak" into more components, although in view of its attenuated form it is quite probable that it is heterogeneous.

In addition it is of interest to quote some more results of DEUTSCH. He reports only small differences in the globulin content of human milk whey (at least in the content of components with very low mobilities) during lactation, viz. a decrease from 34 per cent in colostrum to 26 per cent after three months. Apparently ingestion of globulin is far more essential for the new-born calf than for the human infant. DEUTSCH (1947) suggests the interesting explanation that this may be connected with the difference in thickness of the placenta. The placental structure of humans is only one cell thick. In animals like the pig, goat and cow the placental membranes contain three or more cell layers. Now it is probable that the thin layer of the human placenta allows the immunoglobulins to pass from the maternal blood to the young in utero, which will, however, be impossible in the case of the cow. Hence cow's milk is the indispensable source of antibody proteins for the new-born calf, which may explain the markedly high level of immunoglobulins in bovine colostrum.

As far as we know, component III from our patterns with mobility 4.0 . 10^{-5} $cm^2V^{-1}sec^{-1}$ has not yet been isolated. When in the preparation of β lactoglobulin from whey, fractionation with ammonium sulphate is not repeated, the electrophoretic analysis of the solution still shows the presence of this component (mobility 4,0 . 10^{-5} $cm^2V^{-1}sec^{-1}$), whereas the constituents I and IV are absent. The patterns of such a solution have been drawn in Fig. 28. The ratio of the concentration of β lactoglobulin to that of component III as calculated from these diagrams, is approximately 7; whereas in general for normal rennet whey patterns this ratio is approximately 4. After a second precipitation by 80 per cent saturation with ammonium sulphate component III disappears completely (cf Fig. 24, II.6.c.). It is obvious that component III has remained in solution even at 80 per cent saturation. Hence we conclude that this component is a highly soluble albumin. From experiments which will be described later (cf II. 7.d., e. and II. 9.b.), it appeared that, under the influence of heat, this component is relatively stable in comparison with the other constituents. Its coagulation takes place at somewhat higher temperatures and it is not denatured during the usual condensing and drying

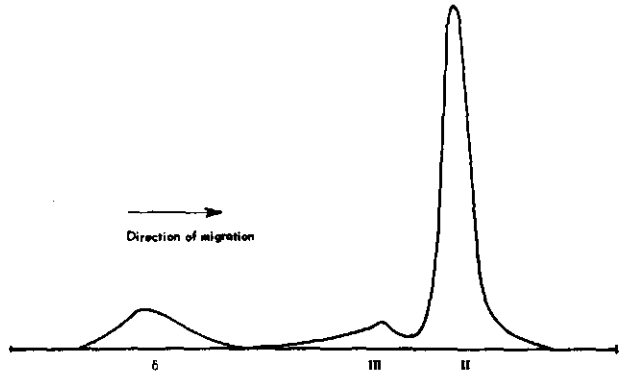


Fig. 28a Ascending pattern

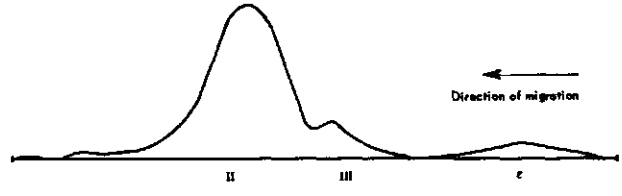


Fig. 28b Descending pattern

Fig. 28. Electrophoretic patterns of the whey fraction, precipitating between 55 and 80 per cent saturation with ammonium sulphate. (Fractionation not repeated)

μ = 0.15 F = 5.66 V/cm
t = 14000 sec $\angle$ = 45°
pH = 6.8

processes. It is far more stable than the immunoglobulins and even more stable than β lactoglobulin. PEDERSEN (1936) has attributed the α component of his ultracentrifugal whey pattern (sedimentation constant $S^{0} = 1.9$ Svedberg units) to an albumin, isolated by KEKWICK. Unfortunately the work of the latter author has not been published, so that it is impossible to find out whether this preparation might correspond to our component III.

e. *Comparison with the diagrams of whole milk and skim milk*

The whey proteins other than component I (the rennet "whey protein") should also be present in the electrophoretic patterns of the milk proteins as a whole. Hence it was of interest to compare the diagrams of milk and whey and identify some peaks of the milk patterns as whey proteins, the remaining peaks being due to casein. In order to obtain clear solutions of milk proteins it was necessary to remove the fat by centrifuging. In addition the casein which in the native condition is present in the form of very large macromolecules (cf FORD and RAMSDALL, 1949) had to be brought in true solution.

This can be achieved by dialysis against a phosphate buffer solution as has already been observed by SVEDBERG (1938). It had to be considered whether the removal of the fat should take place before or after dialysis. In the former case, during the separation of the fat, part of the casein would be removed because of the large size of the native casein particles. This would alter the electrophoretic pattern if the components of dialyzed casein did not equally originate from native casein particles of different size. If dialysis should precede the removal of the fat such changes could not occur.

We decided to follow both methods and compare the results.

The following calculation could be made before the experiments: In view of the results of the experiments treated up to this point the average composition of rennet whey could be assumed to be:

Component	Concentration (%)	Mobility $10^{-5}cm^2V^{-1}sec^{-1}$
I	18	6.6
II	54	5.4
III	13	4.0
IV	15	1.6

The average nitrogen ditribution in milk is

total nitrogen	500 mg per cent
whey nitrogen	130 mg per cent
residual nitrogen	30 mg per cent

After subtraction of the residual nitrogen the ratio of the nitrogen contents, corresponding with total milk proteins and total whey proteins can be calculated. This yields:

milk protein : whey protein = 470 : 100 = 4.7 : 1

Now division of the average concentrations of the components II, III and IV in whey by 4.7 will yield the concentrations to be expected for the same components in the electrophoretic analysis of milk proteins. Component I is discarded as it should not be present in milk. This calculation yields the data of table 16.

TABLE 16. Calculated concentrations of whey constituents in milk proteins

Component	Concentration (%)	Mobility $10^{-5}cm^2V^{-1}sec^{-1}$
II	11.5	5.4
III	3.0	4.0
IV	3.4	1.6

From these figures it will be clear that the components III and IV will be hardly detectable in the electrophoretic patterns of milk. Only component II (β lactoglobulin) will give a reasonable contribution to the total pattern.

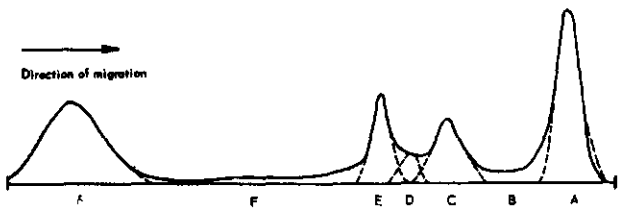


Fig. 29a Ascending pattern

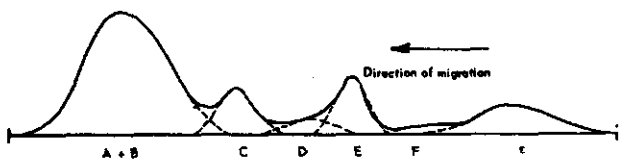


Fig. 29b Descending pattern

Fig. 29. Electrophoretic patterns of the proteins of whole milk.

Protein concentration 1.53 g/100 ml.
$\mu = 0.15$ F = 5.76 V/cm
$\tau = 15000$ sec. $\alpha = 65^{\circ}$
pH = 6.8

The electrophoretic patterns obtained with milk proteins are given in Figs. 29 and 30. Dialysis was performed against the citrate-phosphate buffer solution used in the previous experiments. This yielded satisfactory solution of the casein. Fig. 29 ("whole milk") refers to an experiment in which dialysis preceded the fat removal and Fig. 30 ("skim milk") was obtained from an experiment with the same milk but with reversed order of these manipulations.

The patterns show many peaks which have been denoted A, B, C, D etc. according to decreasing values of the mobility. Many parts of the diagram areas could not be covered by Gaussian curves. For the sake of completeness we have not eliminated these residual areas by a proportionate spreading over the more distinct peaks so that the areas under them would total 100 per cent. On the contrary we have treated every area as a separate component, which will be useful for a more accurate comparison of the patterns. The mobilities could only be calculated in the case of the more distinct peaks and the values found are given in table 17.

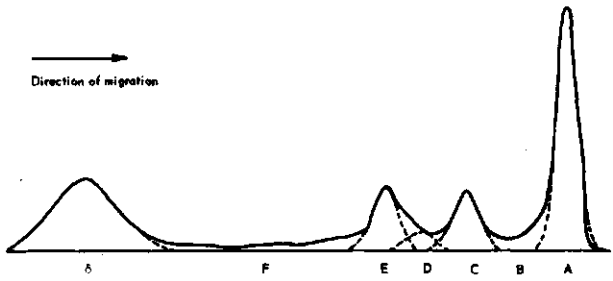


Fig. 30a Ascending pattern

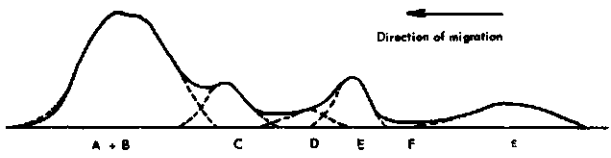


Fig. 30b Descending pattern

Fig. 30. Electrophoretic patterns of the proteins of skim milk.
Protein concentration 1.50 g/100 ml,
μ = 0.15
t = 15000 sec.
pH = 6.8
F = 5.76 V/cm
α = 65°

TABLE 17. Mobilities of the main components of milk proteins (mean values from ascending and descending patterns

Component	Mobility (10^{-5} cm^{2}V^{-1}sec^{-1})	
	Whole milk (fig. 29)	Skim milk (fig. 30)
A	7.0	6.9
C	5.5	5.2
D	4.4	4.5
E	3.7	3.7

As was to be expected there is hardly any difference between these data. In comparison with the mobilities included in table 16 it appears that the components C and II may be identical, whereas the components III and E differ somewhat, although not to a great extent. In table 18 we give a comparison of the concentrations, obtained by analysis of the patterns of figs.

29 and 30, with the calculated concentrations of table 16. In spite of the small difference mentioned above, we have put the components III and E on the same level and in addition components IV and F, the latter being completely undefined.

TABLE 18. Comparison of the mean concentrations of the components of milk proteins (from skim milk and from whole milk) with the calculated concentrations of whey constituents

Milk component	Concentration (%)			Whey component
	Whole milk (fig. 29)	Skim milk (fig. 30)	Whey proteins (calculated)	
A	54.2	52.4	-	
B	8.9	8.4	-	
C	13.6	14.3	11.5	II
D	4.8	5.7	-	
E	12.1	13.6	3.0	III
F	6.4	5.6	3.4	IV

It is improbable that the small differences between the figures for whole milk and skim milk are significant. The influence of centrifuging before dialysis seems negligible. This should not lead to the conclusion that the protein sediment after centrifuging has exactly the same composition as the casein remaining in solution. The quantity that settles during the centrifuging process is too small in relation to the total protein content to exert a strong influence. Hence it is possible that the effect is not detected in these experiments.

The following conclusions can be drawn concerning the whey proteins: The identity of the constituents II and C is highly probable. It may be possible that peak C contains a very small casein contribution, but for the rest it is certainly due to β lactoglobulin (component II). The difference in the concentrations obtained from milk and whey patterns can be attributed as well to a different value of counteracting protein gradients in these two very different cases of electrophoretic analysis.

Component III can be classified under D or E as far as the values of the mobilities are concerned, for

$$\begin{aligned} \text{D} \quad & 4.4 \,.\, 10^{-5}\,\text{cm}^2\text{V}^{-1}\text{sec}^{-1} \\ \text{III} \quad & 4.0 \,.\, 10^{-5}\,\text{cm}^2\text{V}^{-1}\text{sec}^{-1} \\ \text{E} \quad & 3.7 \,.\, 10^{-5}\,\text{cm}^2\text{V}^{-1}\text{sec}^{-1} \end{aligned}$$

In view of the large concentration of constituent E we are inclined to assume that it is obscured rather more by this large peak than by constituent D which only amounts to approximately 5 per cent.

Finally it is obvious that the immunoglobulins (IV) have to be classified under the residual area F. Whether they are only responsible for this area is a question which cannot be answered definitely from these single experiments.

7. THE BEHAVIOUR OF WHEY PROTEINS UNDER THE INFLUENCE OF HEAT TREATMENT

a. The temperature of visible heat denaturation

In anticipitation of more thorough physicochemical experiments it was of interest to know at what temperature the first visible coagulation of whey proteins occurs. This temperature depends largely on the circumstances under which it is determined. Arranged according to decreasing importance these circumstances are:

1. pH
2. Ionic strength
3. The character of the salts and other substances present.

Some data on the influence of the pH will be given here, whereas for the latter two items the normal conditions have been chosen, i.e. the experiments were carried out with native whey.

Three samples of clear centrifuged whey were adjusted to pH 4.5, 6.0 and 8.0. The last mentioned sample had to be filtered again from precipitated calcium phosphate. The test tubes containing the samples were placed in a water bath at 60 °C and the temperature was raised to 90 °C over a period of 35 minutes, with thorough stirring of the water. Any visible change in the appearance of the solutions was noted (table 19).

TABLE 19. Occurrence of turbidity (t) and coagulation (c) during the heating of whey

Temperature (°C)	pH of the sample		
	4.5	6.0	8.0
68			
69	t		
70			
71			
72	c		t
73		t	
74		c	
75			
—			
—			
90			c

As was to be expected the sample at pH 4.5 was the most sensitive one. Nevertheless it is possible that with other values of the pH some change in the structure of the protein will occur at approximately 69 °C which does not result in a visible decrease of the solubility. Strong evidence of such changes will be given in II.7.d.

b. The soluble nitrogen content as a function of the temperature of heat treatment

From the observations treated in the previous section (II.7.a.) it is clear that there is an interval of temperature in which the initial turbidity changes into complete coagulation. It is to be expected that the complete process of coagulation will extend over a wide range of temperature. In order to obtain more information on the behaviour of the proteins within this interval, the following experiment was made.

Rennet whey prepared from centrifuged fresh milk, was divided into eight portions of 150 ml. Each portion was rapidly heated on the free flame to a definite temperature with simultaneous vigorous stirring to avoid overheating. The flask was then kept in a water bath at the fixed temperature for half an hour after which it was rapidly cooled to room temperature by running tap water. The precipitate was filtered off and the nitrogen content of the filtrate estimated by the Kjeldahl method. The nitrogen contents of the solutions obtained by this procedure are given in table 20.

TABLE 20. Nitrogen content of whey filtrates (whey samples kept for half an hour at the temperatures recorded)

Temperature (°C)	Nitrogen content (mg per 100 g)	Nitrogen content (percent of original value)
18	123	100
60	122	99
65	117	95
70	103	84
75	86	70
80	70	57
85	58	47
100	54	44

The nitrogen content of the solution heated to 100°C is due to residual nitrogen plus proteoses as has been described in II.3.d.

In Fig. 31 a graph has been plotted from the data of table 20. This graph gives a good insight into the course of the denaturation. It appears that no marked effect can be observed at 69°C (the lowest temperature of initial turbidity). The slope of the curve shows its maximum between 70° and 75°C. This is the same region where clearly visible coagulation at pH 6.0 was noted (cf. II.7.a.). Apparently the test tube trial yields approximately this point of maximum slope of the thermal coagulation curve.

From 65° to 80°C the slope is approximately constant. This involves a long horizontal part in a plot of the first derivative of the line of Fig. 31. Such a form is not to be expected when only one pure material is present. This points to the presence of various constituents which differ as to their resistance to heat treatment. In the next sections (II.7.d., e.) it will appear evident that there are considerable differences between the various whey proteins with regard to their resistance to heat treatment.

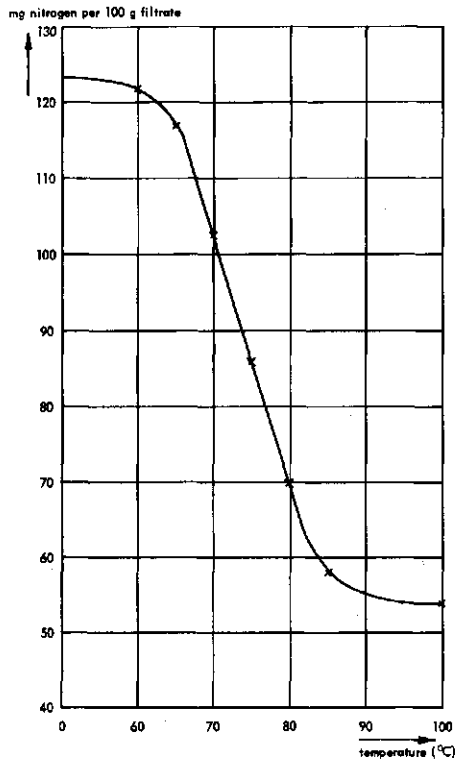


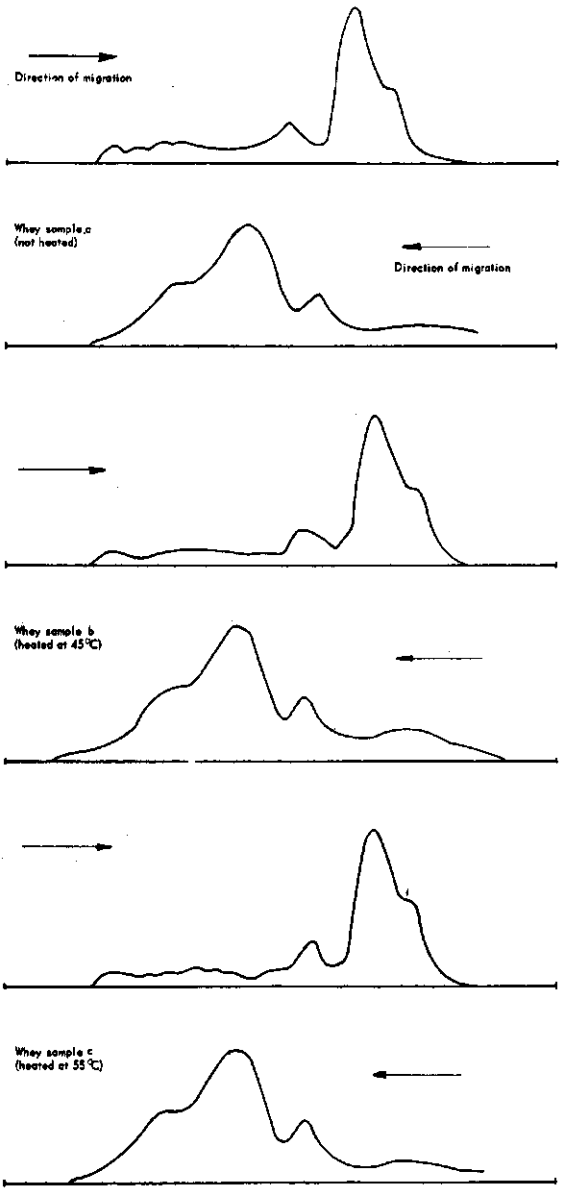
Fig. 31. Nitrogen content of whey filtrates.
(Whey samples kept for half an hour at different temperatures)

At present it can only be stated that denaturation takes place between 60° and 90°C, chiefly between 65° and 85°C with a maximum from 70 - 75°C.

c. The electrophoretic patterns of whey proteins after heat treatment up to 55°C

In this and the following section (II.7.d.), the influence of heat treatment on the electrophoretic pattern of whey proteins will be treated. In the light of the coagulation curve recorded in the previous section (Fig. 31, II.7.b.), we could expect no or negligible influence of temperatures under 60°C and great influence of temperatures from 60 - 90°C. In this section the lower temperatures are reviewed.

The electrophoretic patterns of three samples (a, b and c) of the same whey have been drawn in Fig. 32. Sample a was not heated before electrophoresis and samples b and c were kept for half an hour at 45°C and 55°C, respectively. Electrophoresis was carried out under exactly the same circumstances as far as possible. In all three experiments a previous dilution with buffer solution was applied which will explain the almost negligible small δ boundaries (cf. II.6.a.).



Ascending patterns $\theta = 55°$

Descending patterns $\theta = 65°$

Fig. 32. Electrophoretic patterns of whey samples kept at different temperatures.

Original protein concentration 1.30 g/100 ml.
Original ionic strength $\mu = 0.15$
Previous dilution of protein solution to 88.5 per cent of its concentration.

$t = 12000$ sec. $F = 5.71$ V/cm.
pH = 6.8

The patterns are morphologically similar. For the sake of comparison the successive patterns have been drawn accurately above each other with the positions of the δ and ε boundaries as common origins. Analysis of the patterns yielded approximately the same values for the concentrations of components I, II, III and IV. The expanded component IV in the ascending pattern shows some irregularities which however are not significant because of the thickness of the curve on the photograph and the uncertainty of drawing accurately the center of this broad line. The mobilities of the components show a slight shift in the patterns a, b, and c as can be seen from table 21.

TABLE 21. Mobilities of whey components after heat treatment at moderate temperatures

Whey sample	a	b	c
Temperature of heat treatment (°C)	-	45	55
Component	Mobility (10^{-5} cm^2 V^{-1} sec^{-1})		
I	5.6	5.9	6.0
II	4.4	4.7	4.9
III	3.2	3.5	3.7

Unfortunately in these preliminary experiments the current due to leakage has not been measured, and hence the total current had to be used in computing the mobilities. This accounts for the difference with the mean values calculated from other experiments with normal whey viz. 6.6, 5.4 and 4.0 10^{-5} cm^2 V^{-1} sec^{-1}. Therefore the possibility may not be excluded of the observed small differences being due to accidental differences in the value of the leakage current. Anticipating the results of the more comprehensive experiments within the critical interval of temperature (cf. II.7.b.) that will be treated in the next section we feel justified to conclude that the changes, occurring up to 55°C are only negligible.

d. The electrophoretic patterns of whey proteins after heat treatment from 65°C up to 100°C

To study the influence of heat treatment at temperatures at which serious denaturation takes place six samples of one portion of rennet whey were taken and kept at different but fixed temperatures for half an hour. After filtration of the precipitates they were subjected to electrophoresis at pH 6.8 and μ 0.15. It was the intention to carry out the experiments under exactly the same circumstances at a protein concentration of 1.50 g/100 ml. However, some differences occurred as to the total protein concentration, due to insufficient degree of concentration within the time available. In Fig. 33 (a, b, c, d, e, f) the patterns have been drawn with records of the corresponding temperatures of heat treatment and other particulars.

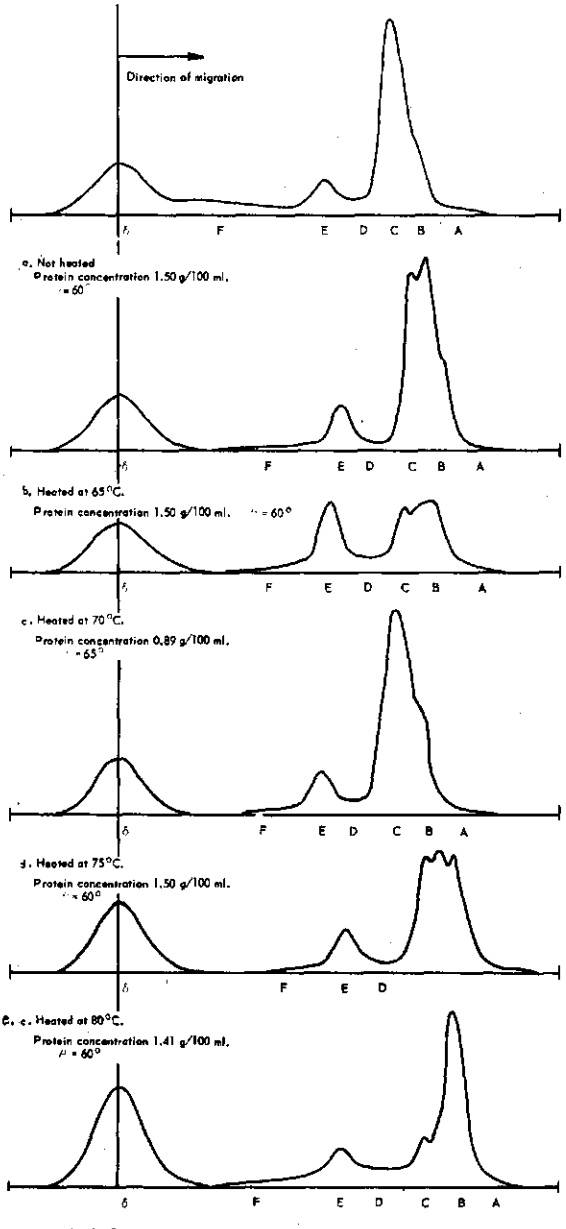


Fig. 33. Electrophoretic patterns of samples of the same whey, after heat treatment at different temperatures.

Ascending patterns
μ = 0.15 F = 5.77 V/cm
t = 12500 sec.
pH = 6.8

As the differences that could be observed were far more pronounced in the ascending patterns than in the descending ones, only the ascending patterns have been recorded. The components, as well as the residual areas between them, were denoted by the symbols A, B, C, D, E, F according to decreasing mobility. For the sake of accurate comparison the residual areas were not discarded and hence many times these symbols refer to areas which do not resemble Gaussian curves. In addition it should be kept in mind that the symbols A, B, C, D, E, and F do not necessarily represent the same materials in the six patterns. Whether they do or do not will be decided in the later discussion. Only in the case of the first pattern (sample a, not heated) can it be stated that B, C, E and F refer to the components I (rennet "whey protein"), II (β lactoglobulin), III (KEKWICKS albumin?) and IV (immunoglobulin) respectively, which components have been amply discussed in previous sections.

The mobilities will be first reviewed. They can only be calculated for the components B, C and E which show fairly distinct peaks and the values are given in table 22.

TABLE 22. Mobilities of the components, denoted as B, C and E in the patterns of whey subjected to heat treatment (cf. Fig. 33)

Whey sample	a	b	c	d	e	f
Temperature of heat treatment (°C)	-	65	70	75	80	100
Component	Mobility (10^{-5} $cm^2 V^{-1} sec^{-1}$)					
B	6.2	6.4	6.6	6.5	-	6.7
C	5.2	5.6	5.7	5.3	-	5.8
E	3.8	4.1	3.9	3.8	4.0	3.9

In the case of pattern e no values for the mobilities of B and C have been recorded as the curve shows three peaks instead of two which cannot even approximately be separated.

From the data of this table it can be seen that the mobilities of component E remain fairly constant. Those of components B and C show increasing values in the range a, b, c, d, e, f, which, however, in some place are interrupted by a lower value. Although the total increase is not great it does not point identity of these components in the successive patterns. For component E such identity seems probable at least as to mobility.

Regarding the patterns of Fig. 33 it is striking that component F (IV) has almost disappeared after heating to only 65 °C. Apparently the immunoglobulins are fairly unstable under heat treatment. At the same time component II (β lactoglobulin, C) has divided into two constituents, which makes it difficult to find back the original component II in Figs. 33 b, c, d, e, f. Component III (E) increases, possibly due to the precipitation of the other ones; at higher temperatures it decreases again.

In table 23 we give a review of the concentrations of the components A, B, C, D, E and F, keeping in mind that thus far they still may represent different materials in the different patterns.

TABLE 23. Concentrations of the successive components of whey proteins after increasing degree of heat treatment

Whey sample	a	b	c	d	e	f
Temperature of heat treatment (°C)	-	65	70	75	80	100
Component	Concentration (% of total protein)					
A	2.9	1.5	3.8	3.6	-	3.5
B	19.0	38.9	30.4	24.0	-	49.7
C	49.2	37.9	23.9	55.3	-	15.6
D	2.2	3.4	7.0	2.5	-	7.8
E	12.0	13.9	28.3	12.5	13.4	15.6
F	14.7	4.4	6.6	2.1	3.9	7.8
B + C	68.2	76.8	54.3	79.3	-	65.3

Many concentrations from sample e have been left out for the same reasons as in the case of the computation of the mobilities (table 22). The separation of the areas, due to the components B and C was in some cases very arbitrary. For that reason the sum of these areas has also been recorded. All data have been taken from the ascending patterns only (Fig. 33). As long as it is not known whether the components from A up to and including F are the same in the successive samples these data are not very suited for discussion. The following calculation will give some information on this question. If it is assumed that the components are all the same in all samples it is possible to calculate the content of these components per 100 g whey filtrate with the help of the data on the total nitrogen content of the filtrates (similar to those recorded in II.7.b.) and the residual nitrogen contents. After subtraction of the residual nitrogen from the total nitrogen the content of non-dialysable nitrogen is obtained (cf. II.3.d.). By multiplication by the percentages of the components the non-dialysable nitrogen can be divided into the contents due to each component separately. The total non-dialysable nitrogen contents, used for this calculation were (in mg per 100 g filtrate) 119, 86, 72, 55, 39 and 23 for the respective samples a, b, c, d, and e. With the use of these figures table 24 has been computed.

TABLE 24. Concentrations of the successive components of whey protein after increasing degree of heat treatment, expressed in mg nitrogen per 100 g whey filtrate

Whey sample	a	b	c	d	e	f
Temperature of heat treatment (°C)	-	65	70	75	80	100
Component	Concentration (mg nitrogen/100 g filtrate)					
A	3.5	1.3	2.7	2.0	-	0.8
B	22.6	33.5	21.9	13.2	-	11.4
C	58.5	32.5	17.2	30.4	-	3.6
D	2.6	2.9	5.0	1.4	-	1.8
E	14.3	12.0	20.4	6.9	5.2	3.6
F	17.5	3.8	4.8	1.2	1.5	1.8
B + C	81.1	66.0	39.1	43.6	-	15.0

From these data it appears that none of the components shows a concentration, continuously decreasing with the rising of the temperature. Now it seems impossible for the content of any component to increase with progressive heat treatment, unless the possibility of conversion of one native component into another is assumed. Such an assumption is non-physiological, whereas we may expect that any chemical conversion between the native components will take place under physiological circumstances only. Hence we conclude that during heat treatment the components are not only gradually precipitated but in addition the constituents remaining in solution change in character. In other words, as the native components break down, new ones emerge. The mobilities observed for the new constituents do not differ seriously from the original ones. There is, however, not the least indication that for example, the new component E should originate from the old constituent E. Therefore it may be possible that it is only accidental that the total "mobility pattern" remains approximately the same.

Reviewing table 24 and Fig. 33 we can make the following statements:

The concentration of component I (B, "whey protein") increases already at 65 °C, whereas component II (C, β lactoglobulin) decreases at 70 °C to approximately one quarter of its original value; afterwards it increases again. More reliable are the data on I + II (B + C) whose concentration at 70 °C falls to approximately 50 per cent of the original value, rises a little at 75 °C, and then gradually decreases to approximately 20 per cent at 100 °C. Up to 70 °C this points to a breakdown of the native components, at higher temperatures the further course is obscured by the appearance of other constituents. Component III (E) shows its first but marked increase at 70 °C followed by a pronounced decrease at 75 °C and from then onwards a gradual decrease to 100 °C. In view of the constancy of the mobility of this component a slow but continuous denaturation of the native component can be assumed; this is temporarily obscured by an intermediate product of denaturation of some component.

Component IV (F) has vanished for the greater part at 65 °C.

After arrangement according to resistance to heat treatment we can say that component III seems to be the most stable, followed by β lactoglobulin and the "whey protein", whereas the immunoglobulins are very unstable.

It should be noted that fig. 33 f apparently represents the proteoses, for in this case the real proteins have been precipitated, whereas the non-protein nitrogen has been removed by the dialysis preceding electrophoresis. Comparison of the patterns a and f yields the conclusion that none of the peaks of the whey pattern can be exclusively attributed to the proteoses. It is even questionable whether the proteoses as such are present in whey. Our experiments leave both possibilities open. If they are present their contribution to the patterns becomes of importance only after the precipitation of the principal amount of the real proteins. If not, they may be the result of the thermal breakdown of the proteins. ROWLAND (1937) records equally high amounts of nitrogen in whey filtrates after coagulation by heat treatment and after coagulation with trichloracetic acid at a concentration of 4.4 per cent. At this concentration he observed a remarkable increase in the slope of the coagulation curve of whey protein with increasing concentrations of trichloracetic acid. Because of these facts he assumes that the material precipitating from 4.4 up to 10 per cent trichloracetic acid corresponds to the proteoses. This might be an indication of the proteoses being already present in the original whey. Nevertheless the possibility cannot be excluded that the breakdown under the influence of trichloracetic acid (up to 4.4 per cent) might have the same course as the thermal denaturation. Hence we do not feel justified in recommending either of these suppositions.

An interesting feature of the process of heat denaturation is the size of the δ boundary. From Fig. 33 it can be seen that the δ boundary of the proteose patterns (f) is considerably greater than that of the native whey pattern (a). As the total protein concentrations and the angles of the inclined slit during the exposures have not been the same in all intermediate cases we have calculated the values of the "index of mean charge" $\frac{1-g_r}{g_r} \cdot \frac{\mu_3}{[P_3]}$ for all temperatures. These values have been tabulated in table 25.

TABLE 25. Index of mean charge of whey proteins during heat denaturation

Temperature (°C)	$\frac{1-g_r}{g_r} \cdot \frac{\mu_3}{[P_3]}$
-	0.0203
65	0.0209
70	0.0357
75	0.0174
80	0.0304
100	0.0364

Although there is no sense in a comparison of the values of this function except in the case of patterns which show the same concentrations for components of equal mobility, it can be applied very roughly to the patterns of

fig. 33. In these patterns the condition mentioned above is not fulfilled but on the other hand there is much similarity between them. The data of table 25 prove that heat denaturation is accompanied by pronounced alterations of the charge of the proteins. Up to 65 °C the charge has not yet changed appreciably, but at higher temperatures a great increase is observed, reaching its maximum in the proteose pattern. For a spherical polyvalent ion the relation between the radius r and the mobility u can be assumed to be (approximately)

$$u = f\frac{n}{r}$$

where f = a proportionality "constant" and n = the number of charge units per molecule.
The value of f is subject to numerous secondary influences. These are neglected as only similar materials are compared.
As $n = \frac{M}{E}$ (M = molecular weight, E = equivalent weight) we can write

$$\frac{1}{E} = \frac{n}{M} = \frac{1}{f} \cdot u \cdot \frac{r}{M}$$

or $$\frac{1}{E\,u} = \infty \frac{r}{M} = \infty \frac{1}{r^2}$$

Now the index of mean charge is proportional to $\frac{1}{n_S E_S}$ (transference number and equivalent weight of the S ions) (cf. II.4.c. eq. 7 and II.5.b.). Replacing the transference number by the mobility (which quantities are indeed almost proportional in the case of proteins) we see that the index of mean charge is approximately proportional to

$$\frac{1}{r^2}$$

This means that the higher value shown by this function in the case of the proteoses points to a lower average value of the radii of the particles i.e. lower molecular weights. This is quite in agreement with the usual classification of the proteoses between the proteins and the normal organic or inorganic ions. The exceptional value of the function calculated for sample c (heated at 70 °C) needs not point to the same cause but it may also be due to the exceptional distribution of the concentrations of this pattern, which is essentially different from the other ones.

Finally the conclusion can be drawn that the electrophoretic pattern proves to be a fairly sensitive criterion of the effect of heat treatment. In view of the changes recorded in this section, it is not surprising that many condensed or dried whey products show considerably modified electrophoretic patterns as will be shown in the next sections (II.7.e., II.9.c.).

e. The influence of condensing whey in an industrial vacuum pan

When the electrophoretic patterns of the proteins in various samples of spray-dried desalted whey are compared with the patterns of fresh whey

proteins, considerable differences are observed. This subject will be treated in detail in II.9.b. and c. By examining the successive stages of processing of these materials, we have tried to decide to which treatment such changes might be due. In this connection we considered it necessary to add a study of the influence of an industrial condensing process to this chapter on the influence of heat treatment.

For this purpose samples were taken from fresh industrial rennet whey which was condensed immediately afterwards in an industrial vacuum pan. From this pan samples were taken after 2, 3 and 4½ hours' condensing at reduced pressure at 49°C, the final concentration being approximately ten times its original value i.e. about 65 per cent dry matter. The experiment has one drawback. Owing to our special request for accurate recordings of the temperature the condensation has doubtless received more than usual attention. Hence it has to be realised that the samples which have been studied are not fully representative of commercially prepared condensed whey.

The samples were stored in the frozen state at approximately - 20°C for some days. They were then transported to our laboratory where they arrived still in the frozen state and were examined at once.

After the usual dialysis the electrophoretic pattern of the proteins was recorded under exactly the same circumstances for each sample. In the case of the first sample (taken before the starting of the condensing process) it was necessary to concentrate the solution by the freezing procedure described in II.2.b. In addition the content of residual nitrogen in each sample was estimated with the help of 15 per cent trichloracetic acid and expressed as a percentage of the total nitrogen content.

The results of the estimations have been given in table 26.

TABLE 26. Data on whey samples taken during condensation in an industrial vacuum pan

Sample	Heating time (h)	Ratio of original volume to actual volume	Residual nitrogen (in % of total nitrogen)	Corresponding electrophoretic patterns (Fig. 34)
1	-	1 : 1	23.2	a
2	2	3 : 1	24.4	-
3	3	4 : 1	24.9	b
4	4½	10 : 1	26.7	c

It can be observed that the content of residual nitrogen rises, especially during the period in which the final high concentration is reached. The difference between samples 2 and 3 may not be significant for it is within the limits of error of the estimations which are based on two separate determinations of the nitrogen content. Nevertheless the gradual increase in the content of residual nitrogen during the process is evident. In Fig. 34 the electrophoretic patterns of the samples 1, 3 and 4 have been drawn. The descending patterns have not been shown as they were less suited to the detection of small differences. The pattern of sample 2 was roughly similar to those of the other samples. Owing to an accident the photograph of this pattern has not been exposed and hence it cannot be reproduced together with the other ones.

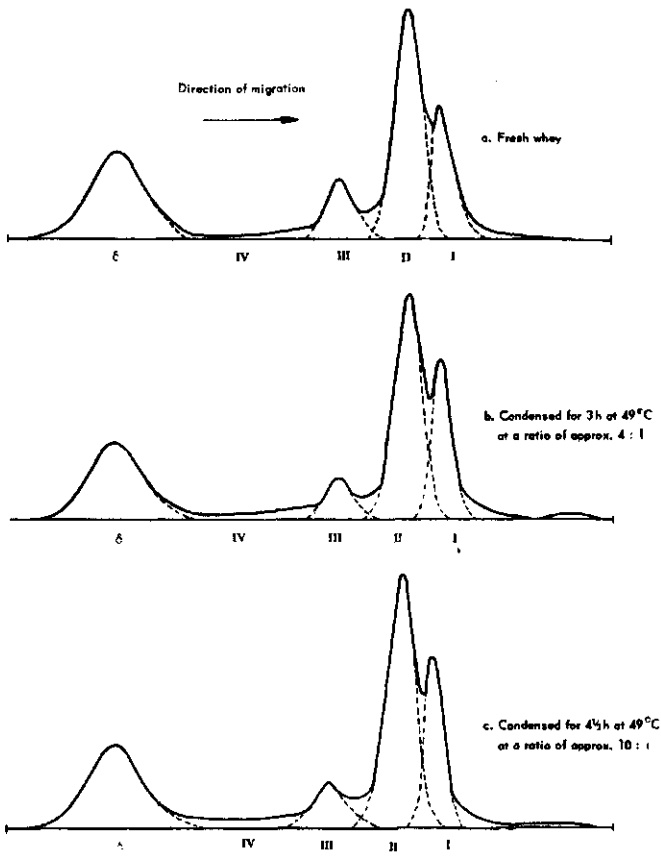


Fig. 34. Electrophoretic patterns of whey proteins at different stages of the condensing process.
Ascending side

Protein concentration	1.45 g / 100 ml
μ = 0.15	F = 5.76 V / cm
t = 12000 sec.	θ = 65°

A consideration of the patterns of Fig. 34 leads to the following statements:

1. The fresh whey shows an abnormally low content of immunoglobulins (component IV).
2. The patterns of condensed whey show no important differences with the pattern of the original whey.
3. Only component III appears to be somewhat greater in pattern a. than in the patterns b. and c.

These statements are confirmed by the complete analysis of the patterns. This can be seen with the help of the tables 27 and 28.

TABLE 27. Mobilities of the components of whey proteins subjected to condensing in an industrial vacuum pan (cf (Fig34)

Whey sample	Mobility ($10^{-5}cm^2V^{-1}sec^{-1}$)		
	a	b	c
Component			
I	6.6	6.5	6.6
II	5.3	5.2	5.2
III	3.9	3.9	3.9

TABLE 28. Concentrations of the components of whey proteins, subjected to condensing in an industrial vacuum pan (cf fig. 34)

Whey sample	Concentration (% of total whey protein)		
	a	b	c
Component			
I	25.5	27.8	24.6
II	52.9	55.1	57.1
III	15.1	9.8	11.0
IV	6.5	7.3	7.3
Index of mean charge	0.023	0.021	0.021

The mobilities have not changed. The mobility of component IV, which is almost absent, has not been included as it could not be located unambiguously. The concentrations show a decrease of component III and an increase in component IV and also in the sum of the components I and II after three hours' condensing (b). After that they remain constant within the limits of error of the analysis. The individual variations of the componentsI and II are due to the more or less arbitraty choice of the Gaussian curves, used for their analysis.

For comparative purposes the average data mentioned in II.6.e. on samples of fresh whey prepared at the laboratory may be recalled:

Component	I	II	III	IV
Mobility ($10^{-5}cm^2V^{-1}sec^{-1}$)	6.6	5.4	4.0	1.7
Concentration (% of total protein)	18	54	13	15

It now appears that the mobilities in the samples under discussion are quite normal. The concentrations are, however, rather different. The content of "whey protein" (I) is markedly higher and that of immunoglobulin (IV) is markedly lower. Apparently these differences are due to the cheese manufacturing procedure of the factory concerned. In view of the behaviour of whey proteins under the influence of heat treatment at approximately 65°C

(cf II.7.d., tables 23 and 24) it is probable that these differences point to a preheating of the milk before cheese making. If we assume that heating of the milk exerts a similar influence on the composition of the whey proteins as direct heat treatment of the whey, this can explain the high value of component I and the low value of component IV. In the case of component III the suggestion can be made that it has been enlarged somewhat in pattern a. because of this previous heat treatment. If this enlargement is really due to a superimposed product of denaturation (cf II.7.d.) it is quite plausible that during the condensing process this new product is coagulated, which renders the original component III at an apparently lower level after condensing than before. The values of the index of mean charge point in the same direction. The original value is somewhat high, which is due to the previous heat treatment of the milk. During condensing (several hours at 49 °C) the denatured products coagulate, whereas at this temperature no denaturation of native components occurs. Hence the value of the function is reduced to the original figure, where it remains constant during the last stage of condensing. The difference observed in the calculation of this function is not great, hence we need not attach too much importance to these figures. They are in any event not inconsistent with the conclusions drawn above.

After inquiry at the cheese factory concerned it appeared that the milk in fact had been pasteurized at approximately 65 °C. Hence it can be stated that the electrophoretic pattern of whey can give valuable information on previous heat treatment of the cheese milk.

In conclusion we can say that the industrial method of condensing milk whey in a vacuum pan at 49 °C for several hours does not exert a detrimental influence on the proteins. The effect of pasteurizing the milk is far greater. If however the temperature of the pan is raised, considerable changes have to be expected.

8. THE INFLUENCE OF THE ELECTRODIALYTIC DESALTING PROCESS

a. Experimental data

The influence of the electrodialytic desalting process has been studied in some special experiments.

At the semi-technical installation for electrodialysis of whey, mentioned in I.2., samples were taken from the solution entering the apparatus and from the resultant desalted liquid. In addition two modifications of the electrodialytic procedure were compared viz. working with high and with low circulation velocity of the liquid to be desalted. The ratio of the velocities was approximately 5 : 1. This implies more or less vigorous mixing of the liquid in the middle compartment of the apparatus. The desalting was carried out at pH 7.5 - 8.0 and at temperatures not exceeding 32 °C (average 23 °C). The salt content was decreased to approximately 25 per cent of its original value.

The material to be desalted consisted of condensed whey from which a great part of the crystallized lactose had been removed. The nitrogen content was approximately 1100 mg/100g which implies that the whey had been condensed in the ratio of approximately 10 : 1. The samples which were examined will be denoted as follows:

sample a : before desalting
sample b : after desalting with low circulation velocity
sample c : after desalting with high circulation velocity

The samples were frozen during the night and transported the next day to our laboratory where they arrived still partially frozen.

After defrosting the samples were centrifuged and the sediment discarded. The clear solutions were dialyzed against frequent changes of the usual electrophoretic buffer solution (pH 6.8, μ 0.15, cf II.5.a.). In each case, the amount of solution placed in the cellophane skin was weighed and its nitrogen content estimated. After dialysis for several days, the same estimations were made so that the amounts of dialysable nitrogen could be calculated. Expressed as the percentage of total nitrogen the results were:

sample a : 33.5%
sample b : 22.4%
sample c : 18.9%

The samples were subjected to electrophoresis under the usual conditions (protein concentration 1.50 g/100 ml, pH 6.8, μ = 0.15) and the patterns obtained have been drawn in Fig. 35. For the sake of comparison the three patterns have been drawn in the same figure. The stationary boundaries have been used as common starting points for the ascending and the descending patterns.

Apart from the well-known components I, II, III and IV a small rapidly moving component was present, as can be seen from Fig. 35. We have denoted this component as O.

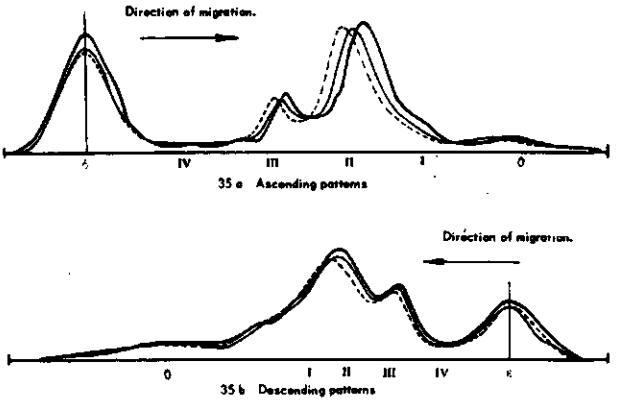


Fig. 35. Electrophoretic patterns of whey proteins before and after the electrodialytical desalting process.

Protein concentrations 1.50 g/100 ml.
t = 12000 sec. μ = 0.15
pH = 6.8 F = 5.76 V/cm

Sample a, before desalting
Sample b, after desalting with low circulation velocity
Sample c, after desalting with high circulation velocity

Accurate analysis of the patterns into all their components was impossible owing to insufficient separation. In table 29, however, we are giving all the data that could be calculated with reasonable accuracy.

TABLE 29. Analysis of the patterns of Fig. 35 (whey subjected to electrodialysis)

Mean values of ascending and descending patterns

	Sample		
	a	b	c
Concentration component O (%)	10.9	11.3	11.2
Concentration component I + II (%)	63.6	62.5	64.8
Concentration component III + IV (%)	25.5	26.2	24.0
Mobility component O	9.0	8.7	8.9
Mobility component II	4.7	4.6	4.6
Mobility component III	3.4	3.4	3.4
Index of mean charge $\frac{1 - g_r}{g_r} \frac{\mu_g}{[P_g]}$	0.033	0.030	0.031

The concentrations have been expressed in per cent total non-dialysable protein, the mobilities in $10^{-5} cm^2 V^{-1} sec^{-1}$. The index of mean charge has been calculated as described in II.5.b.

b. Discussion and conclusions

The data on the contents of non-dialysable nitrogen yield interesting conclusions. First it appears that during electrodialysis approximately one third of the dialysable nitrogen disappears. It is self evident that the electrodialysis is accompanied by normal dialysis due to diffusion, and the figures mentioned confirm this logical supposition. Now in addition it appears that the loss of dialysable nitrogen also depends on the circulation velocity, this loss being greater than with low velocities. The difference is not great (22.4 and 18.9 per cent respectively) but it proves the stirring effect of circulation at high speed. Hence it has to be kept in mind that although stirring in the middle compartment is desirable in view of variations of the pH in the layers adjoining the membranes, it will involve greater loss of dissolved substances, including some nitrogen compounds.

Reviewing the mobilities in table 29, it appears that no appreciable change can be observed. This means that the components are qualitatively almost unaffected by the desalting procedure. Having regard to Fig. 35 it seems remarkable that the mobilities of component II can be the same, as the distances to the reference point (δ or ε boundary) are obviously not equal. This is due to the fact that the differences have opposite signs in the ascending and descending patterns and hence disappear when the mean value is calculated. It is partly due to differences in the size of the δ boundaries, corresponding to differences in the conductances which are used in the computation of the mobilities.

The variations in the concentrations are within the limits of experimental error. Hence in this respect we can conclude that no influence of the desalting

procedure can be observed. However, the differences in the size of the δ boundaries are expressed in the index of mean charge which shows a small but not wholly negligible decrease after electrodialysis. The values for the two modifications of the electrodialysis can be regarded as identical.

For the discussion of this effect we have to refer again to II.4.d., the influence of heat treatment. After comparison with the figures reported for the index of mean charge in table 25, II.4.d., it can be stated that a value of 0.033 is abnormally high. Although we do not know the previous history of this condensed whey in detail it is certain that the milk has been pasteurized. In addition it is probable that the condensing of the whey was carried out less carefully than in the case of the samples treated in II.7.d. In any event the patterns point to some denaturing influence before electrodialysis.

After electrodialysis the value of the index decreased to 0.030. It is plausible to assume that during electrodialysis the small amount of denatured material is removed (probably precipitated) which is expressed by a reverse tendency of the index of mean charge. In view of the form of the patterns, it is less plausible to assume a continued denaturation, as this would involve further alterations of the patterns.

Summarizing the results we can describe the influence of the electrodialysis as follows:

The desalting procedure involves a loss of approximately one third of the residual nitrogen. The other nitrogen containing substances (proteins, proteoses) are only slightly affected, and possibly the coagulation of previously affected material is favoured.

9. ELECTROPHORETIC PATTERNS OF THE PROTEINS IN VARIOUS KINDS OF SPRAY-DRIED DESALTED WHEY AND NORMAL WHEY, TO BE APPLIED IN THE FEEDING EXPERIMENTS

a. Description of the various products

In the feeding experiments six different whey powders have been tested for their value as feeding stuffs. All of them had been previously desalted by electrodialysis except powder VI which had been prepared from normal whey. For the rest the preparation had been different in many respects. The degree of desalting was not the same; an important part of the lactose had been removed by centrifuging from some materials, from others by filtration and in one case no lactose removal had been carried out at all. Hence it will be understood that the chemical composition of the materials was widely divergent.

In order to be able to correlate any effect in the feeding trials with every possible peculiarity in the composition of the whey powders, a fairly extensive analysis was made of each of these materials. In addition these data could be used to find out whether the desalting procedure or the accompanying treatments exerted a special influence on any constituent of the powders, other than the proteins.

Estimations were made of the contents of moisture, nitrogen, residual nitrogen, ash and fat. The residue up to 100 per cent was denoted as carbo-

hydrate. From the mineral compounds the contents of CaO, MgO and $Na_2O + K_2O$ and those of P_2O_5 and Cl were determined. In addition the alkalinity of the ash was determined. To check the results the contents of all oxides have been added after which a correction was made for chloride. This yielded a calculated minimal value for the ash content as no carbonates had been included. Next the total alkalinity of the ash was interpreted as due to CO_2. After addition of this contribution a calculated maximal value for the ash content was obtained. The small contribution of other mineral constituents e.g. SO_3 has been discarded. The results were in good agreement with the total ash contents that were obtained experimentally.

The pH of each powder was determined in a solution containing 140 mg N/100 ml i.e. at approximately the original dilution. Furthermore estimations were made of the contents of thiamin, riboflavin, pyridoxin, nicotinic acid and pantothenic acid and of the important indispensable amino acids, methionine and lysine. The analyses of vitamins and amino acids were carried out at The Central Institute for Nutritional Research T.N.O., Utrecht. We are much indebted to Dr M. VAN EEKELEN and Dr C.G.J.M. ENGEL of this Institute for their kind co-operation.

All these analytical data have been arranged in table 30. In addition to the composition of the various whey powders, data from the literature are given in the last column. They have been taken chiefly from STAEL and KREDIET'S "Jaarboek voor de Zuivelbereiding" (1951) and from HUNZIKER'S "Condensed Milk and Milk Powder" (1946).

TABLE 30. Composition of whey powders used in the feeding experiments

Constituent	Unit	Whey powders						According to literature
		I	II	III	IV	V	VI	
Moisture	per cent	4.6	3.25	4.5	5.35	4.5	3.9	6.8
Total nitrogen	per cent	2.12	3.79	4.05	4.56	4.91	2.01	1.89
Residual nitrogen	per cent	0.598	1.050	1.146	1.537	1.537	0.706	0.473
Fat	per cent	1.2	0.4	1.1	0.3	0.1	0.8	1.1
Carbohydrate	per cent	78	65	60.5	54	56	75	71
CaO	per cent	0.97	0.43	0.94	0.39	0.35	1.11	0.74
MgO	per cent	0.17	0.22	0.38	0.04	0.16	0.20	0.25
$Na_2O + K_2O$	per cent	0.75	3.9	4.9	7.0	5.1	3.9	3.8
Cl	per cent	0.07	0.29	0.15	0.91	0.31	1.74	1.59
P_2O_5	per cent	0.60	1.97	1.15	1.31	0.82	1.44	1.90
Ash calculated (lower limit)	per cent	2.5	6.5	7.3	8.5	6.4	7.3	7.3
Ash (experimental)	per cent	2.9	7.7	8.55	11.7	8.7	8.0	9.2
Ash calculated (higher limit)	per cent	2.9	8.2	9.35	11.5	8.9	7.9	
Alkalinity of the ash	m.aeq./g ash	7.2	10.2	10.7	11.3	13.5	3.5	
pH		7.3	5.7	8.4	7.0	6.75	6.9	
Thiamin	mg/100 g	0.28	1.17	0.69	0.7	0.66	0.38	0.75
Riboflavin	mg/100 g	2.7	2.6	3.5	2.5	1.8	2.3	3.0
Pyridoxin	mg/100 g	0.4	1.15	0.7	0.8	1.05	0.3	0.97
Nicotinic acid	mg/100 g	2.2	3.7	3.3	2.2	3.3	1.2	1.7
Pantothenic acid	mg/100 g	3.5	8.5	7.6	15.1	16	4.7	5.4
Vitamin B_{12}	µ g/100 g	0.8	3.0	3.6	12.6	6.0	1.3	0.9
Methionine	per cent	0.24	0.40	0.53	0.60	0.64	0.21	0.31
Lysine	per cent	0.99	1.92	2.48	2.57	2.76	1.06	0.94

Although this table yields sufficient information as to the composition of the whey powders it is not especially suitable for quantitative comparison of the various powders. Such comparison is more effective when the contents are expressed in parts per 16 g nitrogen. Since no appreciable nitrogen loss occurs during electrodialysis or centrifuging such data are approximately representative of composition relative to a fixed amount of original whey. In addition the contents of amino acids are directly comparable with data from the literature. In this way the data of table 30 have been converted into those of table 31.

The discussion of these figures will be given in II.9.c. after the recording of the electrophoretic data.

TABLE 31. Composition of whey powders expressed in parts per 16 g nitrogen

Constituent	Unit	Whey powders						According to literature
		I	II	III	IV	V	VI	
Total nitrogen	grams	16	16	16	16	16	16	16.
Residual nitrogen	grams	4.51	4.51	4.53	5.39	5.01	5.62	4.0
Fat	grams	9.1	1.7	4.3	1.0	0.3	6.3	9.3
Carbohydrates	grams	588	273	239	190	181	588	603
CaO	grams	7.3	1.8	3.7	1.4	1.1	8.8	6.3
MgO	grams	1.3	0.9	1.5	0.1	0.5	1.6	2.1
$Na_2O + K_2O$	grams	5.7	16.4	19.4	24.6	16.6	31.0	32.2
Cl	grams	0.5	1.3	0.6	3.2	1.0	13.8	13.5
P_2O_5	grams	4.5	8.3	4.5	4.6	2.7	11.4	16.2
Ash (experimental)	grams	21	34	34	41	28	63	78
Thiamin	mg	2.1	4.9	2.7	2.5	2.15	3.0	6.3
Riboflavin	mg	20.4	11.0	13.8	8.8	5.8	18.2	25.4
Pyridoxin	mg	3.0	4.0	2.8	2.8	3.4	2.4	8.2
Nicotinic acid	mg	16.8	15.6	13.0	7.7	10.7	9.5	14.4
Pantothenic acid	mg	26.4	36.7	30.0	53.0	52.5	37.3	45.8
Vitamin B_{12}	μg	6.0	12.2	14.2	44.2	19.5	10.3	7.6
Methionine	grams	1.8	1.7	2.1	2.1	2.1	1.7	2.6
Lysine	grams	7.5	8.1	9.8	9.0	9.0	8.4	8.0

b. Electrophoretic data

The powders were dissolved in buffer solution (pH 6.8, μ = 0.15) to a concentration of approximately 600 mg per cent nitrogen. In the case of powders I and VI the amount of lactose was too high to permit complete solution. Here the rest of the lactose was removed by centrifuging. From the nitrogen content of the solutions it appeared that the proteins had been almost completely dissolved. The other samples were also centrifuged in order to remove impurities. All the samples were then supercentrifuged for approximately 5 minutes at a speed of 14.000 r.p.m. The solutions were cleared considerably by this procedure. Nevertheless according to nitrogen determinations before and after centrifuging the loss of nitrogen was only approximately 1 per cent.

After dialysis against the usual buffer solution (pH 6.8, μ =0.15), electrophoresis was carried out under the usual circumstances. The results of electrophoresis are shown diagrammatically in Fig. 36 to 41. The patterns are rather different from those of normal rennet whey (cf Fig. 10, II.5.a.) and the separation of the components is unsatisfactory, especially in the case of the descending patterns; only the ascending patterns were therefore analyzed. The areas ascribed to the various components have been bounded by the dotted lines in Figs. 36a to 41a. The relative concentrations, the mobilities and the values of the index of mean charge were calculated. The results are recorded in tables 32 and 33, together with the average data, reported in II.6.e. for normal rennet whey.

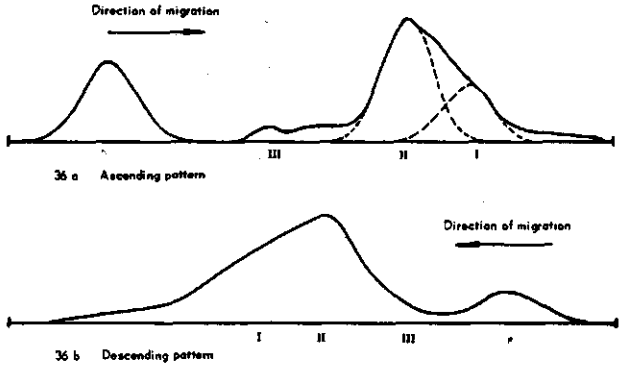
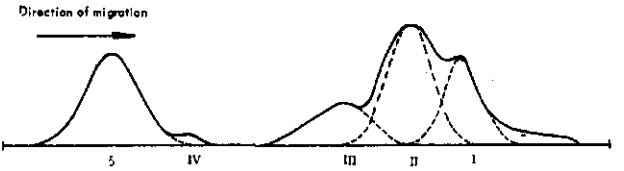
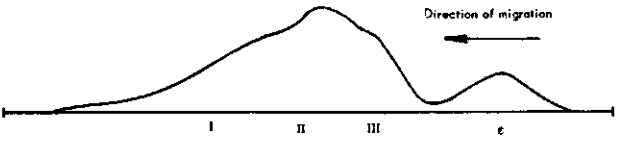


Fig. 36. Electrophoretic patterns of whey powder I.

Protein concentration 1.50 g/100 ml
μ = 0.15 F = 5.75 V/cm
t = 12000 sec. ρ = 60°
pH = 6.8



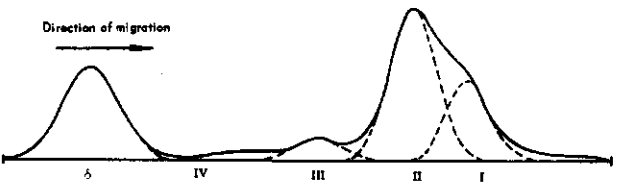
37 a Ascending pattern



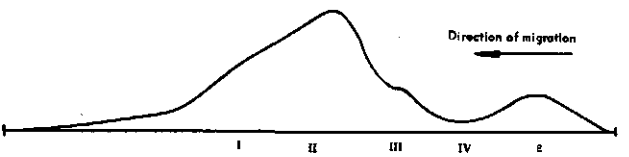
37 b Descending pattern

Fig. 37. Electrophoretic patterns of whey powder II.

Protein concentration	1.50 mg/100 ml
μ = 0.15	F = 5.75 V/cm
t = 12000 sec.	θ = 60°
pH = 6.8	



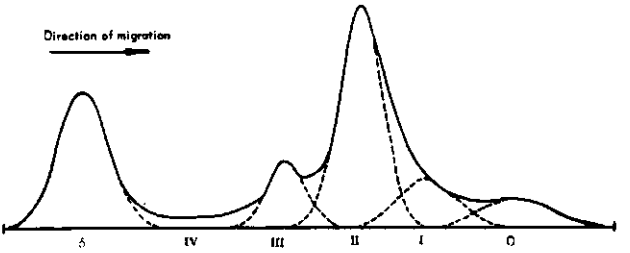
38 a Ascending pattern



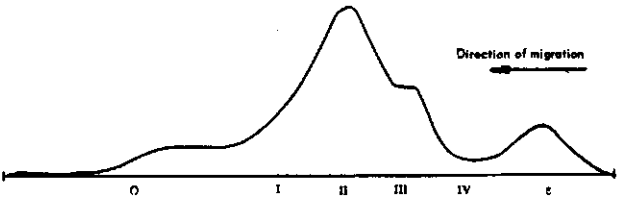
38 b Descending pattern

Fig. 38. Electrophoretic patterns of whey powder III.

Protein concentration	1.50 mg/100 ml
μ = 0.15	F = 5.77 V/cm
t = 12000 sec.	θ = 65°
pH = 6.8	



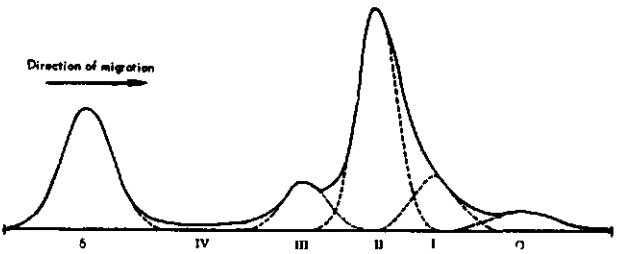
39 a Ascending pattern



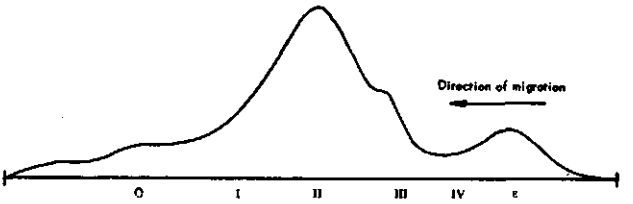
39 b Descending pattern

Fig. 39. Electrophoretic patterns of whey powder IV.

Protein concentration 1.50 g/100 ml
$\mu = 0.15$ F = 5.78 V/cm
t = 12000 sec. $\theta = 65°$
pH = 6.8



40 a Ascending pattern



40 b Descending pattern

Fig. 40. Electrophoretic patterns of whey powder V.

Protein concentration 1.50 g/100 ml
$\mu = 0.15$ F = 5.78 V/cm
t = 12000 sec. $\theta = 65°$
pH = 6.8

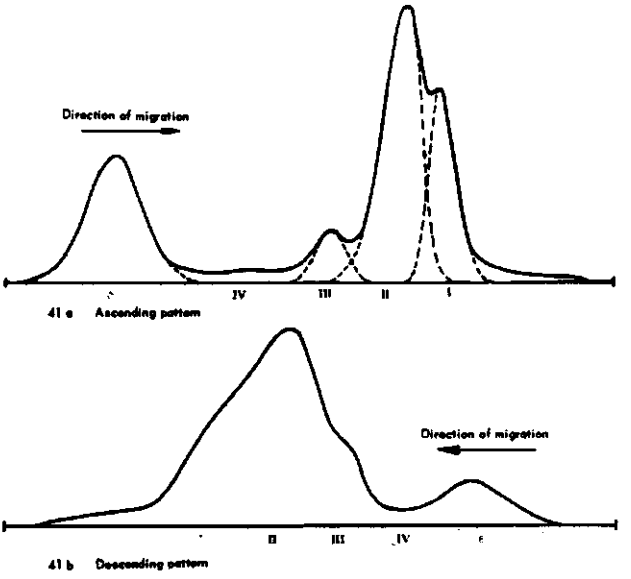


Fig. 41. Electrophoretic patterns of whey powder VI.
Protein concentration 1.50 g/100 ml
μ = 0.15 F = 5.78 V/cm
t = 12000 sec. θ = 65°
pH = 6.8

TABLE 32. Relative concentrations (%) of the protein components in the various whey powders, and in normal rennet whey

Component	Whey powders						Normal whey
	I	II	III	IV	V	VI	
0	5.9	5.8	4.0	11.3	8.8	4.4	-
I	25.2	26.4	26.8	16.1	16.3	27.0	18
II	58.5	43.8	54.1	52.1	55.2	53.7	54
III	10.4	22.0	9.9	13.7	15.1	8.2	13
IV	-	2.0	5.2	6.8	4.6	6.7	15

TABLE 33. Mobilities of the protein components in the various whey powders and in normal rennet whey ($10^{-5}cm^2V^{-1}sec^{-1}$)

Component	Whey powders						Normal whey
	I	II	III	IV	V	VI	
0	-	-	-	8.7	9.0	-	-
I	7.3	7.0	6.9	6.7	7.0	6.3	6.6
II	5.3	5.4	5.2	4.8	5.1	5.0	5.4
III	3.6	3.9	3.6	3.4	3.6	3.6	4.0
IV	-	-	-	-	-	-	1.7
Index of mean charge	0.0187	0.0191	0.0187	0.0175	0.0183	0.0177	0.0185

c. *Discussion and conclusions*

Reviewing table 31 we can state several interesting correlations.

The degree of desalting is expressed by the various ash contents. Similar variations can be observed in the contents of Cl and the alkali oxides. The contents of P_2O_5 do not exactly follow the same course whereas the contents of MgO and CaO do not show any correlation with the ash contents. This can be due to the fact that the elements Ca, Mg and P are partly present in non-ionized forms and on the other hand, to the interference of other abstracting processes such as centrifuging.

The amounts of residual nitrogen show some correlation with the ash contents. The effect is in agreement with the loss of residual nitrogen during electrodialysis, stated in II.8.a. and b. All contents of residual nitrogen are rather high compared with the average value in literature. The origin of the whey and the storage of the condensed whey will be of importance here and the deviations of the general correlation might certainly be due to such differences in previous history of the whey.

Among the minor constituents the distribution of vitamin B_{12} is very remarkable; in the powders I to V a fairly good correlation with the ash contents can be observed. The degree of electrodialytic desalting is expressed by the value of the ash contents. Highly desalted products show low vitamin B_{12} contents and vice versa. This points to the electrical removal of this vitamin. In fact some electrolytic properties of vitamin B_{12} have been described in literature. According to FANTES et al. (1949) it has an electrolytic mobility of $1.7 \cdot 10^{-5}cm^2V^{-1}sec^{-1}$ at pH 7.0 in silica gel. This might explain the correlation. However, the values for whey powder VI (normal whey powder) and those found in literature for dried whey do not confirm this supposition at all. For the occurrence of higher values in the desalted whey two possibilities can be considered. Either the B_{12} content of whey is subject to very large seasonal variations or the vitamin is formed as a result of some fermentation during the storage of the condensed whey from which the desalted products were manufactured. Then, in the case of whey powder VI, there could be no question of fermentation, as it was prepared directly from fresh whey. The assumption of seasonal variations is less probable because of the relatively high B_{12} content of all powders. This would imply that all of them would have been prepared from whey obtained in approximately the

same season, which was actually not the case. Hence we are inclined to the fermentation hypothesis. This suggests that the correlation of the other values with the ash content will not hold accurately. A different degree of fermentation of the condensed whey will cause interference with the assumed relation.

The degree of removal of undissolved substances is expressed best by the contents of carbohydrates (lactose). The course of these values is paralleled by the contents of CaO and MgO and to some extent also by those of P_2O_5. This means that a considerable part of the Ca and Mg in condensed whey is present in the form of fine precipitates which are centrifuged or filtered off, along with the lactose crystals. The P_2O_5 value in whey powder I is low, although in this case no centrifuging or filtration had been applied. This is due to the prolonged desalting. Apparently the removal of phosphate ions had been accomplished electrically whereas removal of Ca and Mg ions can be achieved only by precipitation.

The correlation of riboflavin with carbohydrate removal is high. This confirms the work of LEVITON (1943, 1944) on the concentration of riboflavin by selective adsorption by cristalline lactose.

Concerning the other constituents it can be stated that the values are not specially influenced by any stage of the manufacturing process with the exception of some loss of vitamins (thiamin, pyridoxin). This loss does not seem to be due to the procedure, as the values for normal whey powder (VI) are approximately the same and also lower than those calculated from data in the literature.

After considering the electrophoretic patterns (cf figs. 36 to 41) it appears that the deviation from the normal whey pattern (cf fig. 10, II.5.b.) is considerable. The least deviation in form can be observed in the patterns of normal whey powder (VI, fig. 41) which show a resemblance to the patterns of factory whey, treated in II.7.e. (cf fig. 34).

The mobilities, summarized in table 33, agree reasonably with the values for the components of fresh whey proteins. Hence there is no doubt as to the identity of the components I, II and III. Component IV is practically absent in many patterns. From table 32 it appears that the highest quantity observed (whey powders IV and VI) is still less than 50 per cent of the value in normal whey. The powders IV and V appear normal with respect to the quantities of components I, II and III. However component I has spread over an area much wider than usual and in addition a rapidly moving component (0) is present in quantities which are not negligible.

In comparison with fig. 33 and table 23 (II.7.d.), the greater part of the deviations can be ascribed to the influence of heat treatment. The high content of component I (whey powders I, II, III and IV) is obviously a first symptom of heat denaturation. The high value of component III in whey powder II (fig. 37a) and the widened form of the corresponding area is similar to the deformation in fig. 33c (heat treatment at 70 °C) although not to such an extent. Stress should be laid upon the values of the index of mean charge (table 33). It appears that whey powder II shows the highest value (0.0191) which means that this powder is the most denaturated, although the variations are not great. The virtual absence of component IV points to heat treatment at 65 °C or higher. Finally the relatively high values of the residual nitrogen contents might have been caused in part by previous heat treatment, as will

be obvious from the experiments described in II.3.d. Summarizing we can say that the various whey powders show several characteristics of initial denaturation. These may be due chiefly to heat treatment, especially of the cheese milk and perhaps to a smaller extent to the spray drying of the whey. There is no indication that the electrodialytic desalting procedure involves the changes observed in the electrophoretic patterns of the products which have been examined (cf II.8.).

10. HUMAN MILK WHEY

a. Fresh human milk

It is well-known that the composition of human milk is essentially different from that of cows' milk. Although it is difficult to define an "Average human milk" because of the dependence of composition of the stage of lactation (cf II.3.b.), several differences are very marked during the greater part of the lactation period.

With regard to proteins the ratio casein : whey protein in cows' milk usually has a value of approximately 4.5. For the same ratio in human milk a value of approximately 1.0 is more generally found.

In view of the many attempts to develop artificial infant foods having approximately the same composition as human milk, it is of interest to determine whether bovine whey proteins are similar to human whey proteins. We have therefore recorded the electrophoretic patterns of whey prepared from a sample of fresh human milk. The milk sample was obtained from the Department of Gynaecology of the State University of Utrecht by the kind co-operation of its Director Professor W.P. PLATE, M.D. The milk was centrifuged to remove the fat and coagulation of the casein was carried out by the combined action of calcium ions, acid and rennin. For this purpose 2 ml of a saturated solution of $CaCl_2$ were added per 1000 ml of centrifuged milk; next the pH was reduced to 4.37 by addition of citric acid and finally 1.2 ml of rennet solution (cf II.2.a.) were added. Precipitation took place in approximately one hour at 37°C. The precipitate filtered slowly, but a clear filtrate was obtained. The nitrogen contents of the milk and the filtrate were 253 and 158 mg per 100 g respectively. Dialysis against the citrate-phosphate-KCl buffer (μ = 0.15, pH = 6.8) was alternated with concentration by the freezing procedure (cf II.2.a.). Dialysis was continued until equilibrium was attained. The final protein concentration was only 1.04 g/100 ml.

An interesting feature of the human casein should also be mentioned here. Another portion of the centrifuged milk was dialysed with the additional intention of recording the patterns of human milk as a whole. In contrast with cows' milk, this milk sample did not become translucent during dialysis (cf II.6.e.). Hence it could not be used for electrophoresis. Apparently the degree of dispersion of human casein is not altered by dialysis. This points to a different construction of the particles of casein in human and bovine milk.

Electrophoresis was carried out under the usual conditions and the patterns have been drawn in Fig. 42. The ascending and descending diagrams are very dissimilar. The ascending pattern shows four main constituents, the descending one three, apart from the stationary boundaries.

In table 43 the results of analysis of the patterns are summarized, together with average data on bovine whey.

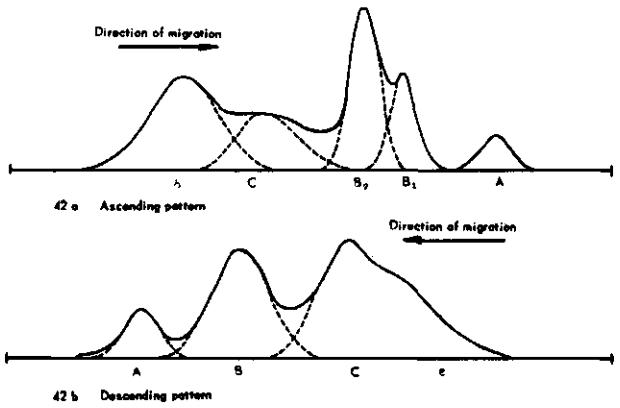


Fig. 42. Electrophoretic patterns of whey prepared from fresh human milk.

Protein concentration 1.04 g / 100 ml
μ = 0.15
t = 14000 sec.
pH = 6.8
F = 5.76 V / cm
θ = 65°

TABLE 34. Results of the electrophoretic analysis of fresh human milk whey together with average data on bovine whey

Human milk whey sample					Average bovine whey		
Component	Mobility (10^{-5} cm^2 V^{-1} sec^{-1})		Concentration (%)		Component	Mobility (10^{-5} cm^2 V^{-1} sec^{-1})	Concentration (%)
	Ascending	Descending	Ascending	Descending			
A	-	-	-	-	I	6.6	18
B_1	6.0	5.4	9.8	11.2	II	5.4	54
B_2	4.0	-	20	-	-	-	-
$B_1 + B_2$	2.9	-	38	-	-	-	-
C	-	3.6	58	42	III	4.0	13
	1.2	1.6	32	47	IV	1.7	15

Index of mean charge:	Human whey	Bovine whey
	0.037	0.0185

The following statements can be made in the light of table 34.

Human milk whey patterns do not contain a constituent corresponding with the rennet "whey protein" (component I) in bovine rennet whey. This fact will be related to differences between human and bovine casein (cf II. 1.a.).

Component A of the human whey pattern shows approximately the same mobility as β lactoglobulin in the patterns of bovine whey. The concentration is completely different viz. only approximately 10 per cent as against 54 per cent in bovine whey protein.

The main contribution to the patterns is given by the more slowly moving constituents B and C. The distribution of these components is, however, very different in ascending and descending patterns. In the ascending pattern two distinct peaks B_1 and B_2 are observed. Such differentiation is impossible in the descending pattern and even the ratio B : C is completely different from the ratio $(B_1 + B_2)$: C in the ascending one. We are quite aware that the resolution of the patterns in Gaussian curves recorded in Fig. 42 is very unsatisfactory but we have not been able to give a better one, except by the introduction of a great many components of rather arbitrary mobilities and concentrations. As yet we can only conclude that the separation is not sufficient for a satisfactory analysis. Nevertheless it is certain that the greater part of the human milk whey proteins consists of constituents different from the proteins of bovine whey as identified in II.6.

The values of the index of mean charge show a very marked difference. Whether this is due to the different composition or to the different charge of the individual constituents cannot be decided; it is probably due to the action of both influences together.

It is outside the scope of this work to make an extensive study of various samples of human milk whey, but it is suggested here that the index of mean charge may become a most valuable criterion to distinguish human from bovine whey.

The high value of the index of mean charge is connected with the large δ boundary. Great boundary anomalies are consistent with pronounced dissimilarity of ascending and descending patterns as recorded in Fig. 42.

b. Whey from lyophilized pooled human milk.

By the courtesy of the Human Milk Bank of the Netherlands Red Cross Society we were enabled to extend our investigations to a more standardized product viz. pooled human milk, transformed into a stable powder by means of a lyophilic drying process. A complete description of this process has been given by Mr G.G.A. MASTENBROEK (1951) who kindly placed some samples at our disposal. It should be mentioned that the treatment at the Human Milk Bank includes pasteurization for half an hour at 67°C. In view of our work on the sensitiveness of bovine whey proteins to heat treatment this feature of the previous history should not be disregarded.

The powder was dissolved in water to the concentration of normal human milk (approximately 12 per cent dry matter). After one day's storage in the refrigerator the cream was separated by centrifuging and the casein was removed by the combined action of rennin and acid; a clear solution was obtained. Dialysis and concentration by the freezing procedure were alternated in the usual way. Finally electrophoresis was performed at pH 6.8, $\mu = 0.15$ and a protein concentration 1.06 g/100 ml.

Although the powder could be easily suspended in water, a small amount of nitrogen containing substances was not in fact dissolved. After centrifuging approximately 96 per cent of the nitrogen present in the milk powder was found in the fat free solution. In addition a rather small part of nitrogen remained in the whey viz. approximately 58 per cent of the total nitrogen in the milk powder. The value of the total nitrogen content was quite plausible viz. 1635 mg/100 g. According to the values found in literature (cf BEACH et

al., 1941) approximately 70 per cent of the total nitrogen ought to have remained in the whey. We are inclined to ascribe the low value found, to partial denaturation of the non-casein fraction during pasteurization. The denatured whey proteins coagulate together with the casein during the latter's removal. In the case of cow's milk this phenomenon is well-known to the cheese manufacturer. At high milk pasteurization temperatures high yields of cheese are obtained and vice versa.

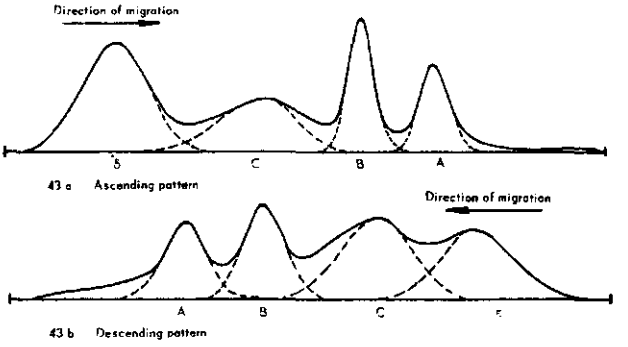


Fig. 43. Electrophoretic patterns of whey prepared from lyophilized human milk powder.

Protein concentration 1.06 g/100 ml
μ = 0.15
t = 14000 sec.
pH = 6.8
F = 5.74 V/cm
$\theta = 60^0$

The results of electrophoresis of the soluble whey proteins are shown diagrammatically in Fig. 43. The differences from the patterns of cow's milk (cf Fig. 10, II.5.b.) and from those of the fresh human milk sample (cf Fig. 42, II.10.a.) are remarkable. The results of analysis of the patterns are given in table 35, together with average data on bovine whey.

TABLE 35. Results of the electrophoretic analysis of whey, prepared from lyophilized human milk, together with average data on bovine whey

Whey prepared from lyophilized pooled human milk					Average bovine whey		
Component	Mobility (10^{-5} cm^{2}V^{-1} sec^{-1})		Concentration (%)		Component	Mobility (10^{-5} cm^{2}V^{-1} sec^{-1})	Concentration
	Ascending	Descending	Ascending	Descending			
-	-	-	-	-	I	6.6	18
A	5.6	5.5	21	20	II	5.4	54
B	4.0	4.3	29	25	III	4.0	13
C	2.1	2.2	42	48	IV	1.7	15

	Human whey	Bovine whey
Index of mean charge	0.041	0.0185

The statements made in II.5.a. will now be reviewed successively by comparing tables 34 and 35 and Figures 42 and 43.

1. Neither kind of human whey contains the so-called rennet "whey protein".
2. In the sample under discussion component A, characterized by a mobility similar to that of β lactoglobulin (II) shows approximately half the concentration of β lactoglobulin in bovine whey.
3. Component B appears as a single boundary. No diffentiation into B_1 and B_2 can be made. Its mobility is similar to that of component III in bovine whey but its concentration is approximately twice as high. This value is much lower than the values for $B_1 + B_2$ in table 34.
4. The concentration of component C is of the same order as in fresh human milk, but its mobility is different.
5. The value of the index of mean charge is slightly higher than in the case of fresh human milk. In bovine milk whey this would point to heat denaturation.

Reviewing the facts and data given in this section we conclude that obvious differences are found between the patterns of bovine and human whey and also between those of fresh human milk whey and whey obtained from lyophilized human milk.

In connection with the latter differences the influence of the stage of lactation should be mentioned. In view of the different relation between nitrogen content and stage of lactation for human and bovine milk (cf II.3.b.) it might be assumed that the milk obtained directly from the Department of Gynaecology would be collected during another stage than the powder from the Human Milk Bank. The latter milk is pooled from a large number of donors. Therefore it can be considered as representative of "Average human milk". The former sample was obtained from a smaller number of subjects, but nevertheless it was pooled. It is, however, probable that on average this milk was obtained in an earlier stage of lactation. According to fig. 5 (II.3.b.) its content of soluble nitrogen (253 mg per cent) corresponds to a date of approximately 12 days post partum. This might partly explain the differences found between both kinds of milk.

Nevertheless in our opinion the major cause has to be sought in pasteurization. The low nitrogen content in the whey clearly points to heat denaturation. In addition the amount of insoluble whey proteins is small enough (4 per cent) to consider the remaining soluble fraction which is the only one to be connected with the electrophoretic pattern, representative for the non-casein fraction of the milk.

This comparison of the three kinds of whey proteins leads to the following conclusion.

From a chemical point of view not only bovine whey proteins but even pasteurized human whey are markedly different from the native protein mixture of human whey. This does not necessarily involve a depreciation of either or both kinds of proteins from the point of view of their biological value.

11. ULTRACENTRIFUGAL RECORDINGS

a. The principle of the ultracentrifuge

For a clear comprehension of the results of the experiments to be treated under b, c and d we give a short description of the principle of the ultra-

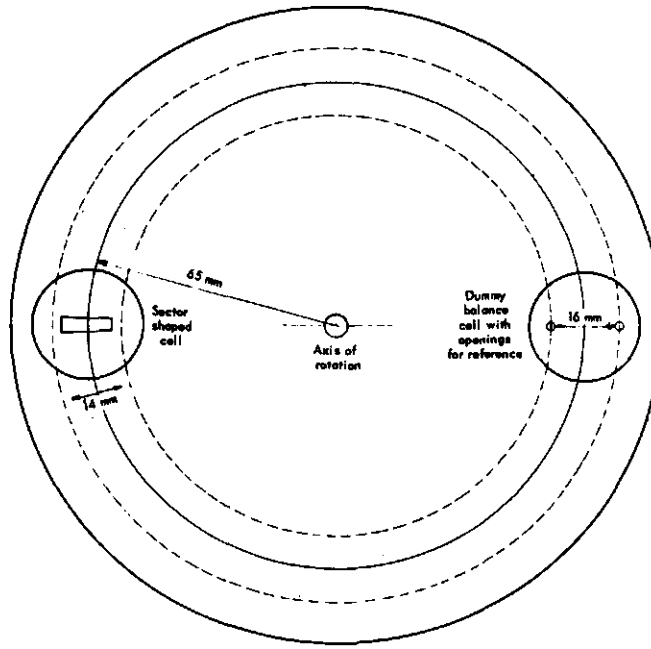


Fig. 44. Schematic drawing of rotor of ultracentrifuge

centrifuge and some special data on the apparatus used in the experiments. For a complete treatment we refer to the reviews in literature e.g. those of PICKELS (1942) and GUTFREUND (1950).

Ultracentrifugal analysis of a protein mixture is based on separation of the components according to their sedimentation velocities and a large centrifugal force has to be applied in order to obtain reasonable velocities. The centrifugal field strength is proportional to $\omega^2 r$, where ω is the angular velocity and r is the distance from the axis of rotation. The choice of suitable values for ω and r seems rather arbitrary but it is limited by various experimental factors. The experience of SVEDBERG and coworkers has lead to more or less standardized dimensions for a practicable type of ultracentrifuge.

In the case of the apparatus at our disposal the radial distance r was 6.5

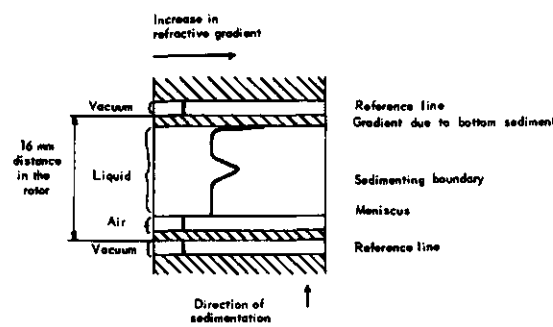


Fig. 45. Schematic drawing of ultracentrifugal pattern

cm and the angular velocity applied was approximately 6000 radians per sec.

A schematic drawing of the rotor of the ultracentrifuge used for the experiments is given in Fig. 44. Light rays pass perpendicularly to the plane of the drawing and are transmitted only by the sector shaped cell and the two openings for reference in the dummy balance cell. The further optical arrangement is similar to that of the electrophoretic equipment (cf II.2.b. Fig. 2.) except for the substitution of the inclined open slit by an opaque strip. It is to be expected that the patterns obtained by this device will resemble the one drawn schematically in Fig. 45. The distance between the reference lines corresponds to the distance of 16 mm between the openings in the dummy cell. Owing to the inclined opaque strip and the cylindrical lense device a black line on a light field is obtained. The lines within the reference areas indicate the position of zero deviation of light rays passing through vacuum viz. the openings in the dummy cell, because the rotor is spun in high vacuum. The cell is made airtight so that the space above the meniscus remains filled with air. Light rays passing through this air layer will also show approximately zero deviation. Owing to the centrifugal force the liquid in the cell will be compressed; this involves a density gradient. The density of the liquid layer increases almost proportionally to the distance from the axis of rotation because the centrifugal force is proportional to this distance. This involves a constant density gradient throughout the liquid layer which is expressed by the straight vertical parts of the pattern at a position different from that of zero deviation. Owing to diffusion the separation of protein from the solution results in the well-known Gaussian curve. The sedimenting protein accumulates at the bottom of the cell and causes a final increase in the refractivity gradient. The final value is reached gradually because of diffusion.

The rotor was spun at a velocity of 59.780 r.p.m., thus giving a field of centrifugal force of approximately 260.000 x gravity. The experiments were carried out at room temperature. Before and after the experiments, which lasted 1-2 hours, the temperature of the rotor was recorded. Usually an increase of 1-2 °C was observed.

Most of the experiments were carried out at pH 6.8 and $\mu = 0.15$ with samples of the dialyzed solutions that were used for electrophoresis, except that a somewhat lower protein concentration was used, usually 0.75 per cent.

At first sight dialysis does not seem necessary in these experiments, because there is no need for a supernatant liquid as in the electrophoretic investigations. This possibility of direct examination of native protein solutions would be an advantage over electrophoresis. It appeared, however, that the centrifugal force was high enough to cause even the dissolved lactose to separate. Owing to the high concentration of lactose the contribution of this gradient partly obscured the real protein gradients as will be seen in the next section (II.11.b.). Hence it was necessary to work with dialyzed solutions.

b. Native milk whey and dialyzed milk whey

In Fig. 46 the ultracentrifugal pattern of milk whey is shown, after one hour's centrifuging at 59.780 r.p.m. The solution was a sample of the same solution used for the electrophoretic experiment described in II.5.d. Three components can be observed in this pattern; these have been denoted α, β

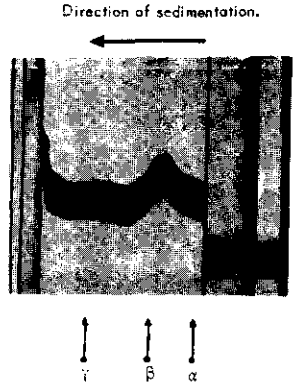


Fig. 46 Ultracentrifugal pattern of milk whey proteins after one hour's centrifuging at 59780 r.p.m.

Protein concentration 0.75 g/100 ml.
pH = 6.8 μ = 0.15

and γ corresponding to increasing sedimentation velocities. It appears that the chief constituent is the β component. This component was identified with β lactoglobulin by PEDERSEN (1936). The sedimentation constants of the three components have also been reported by PEDERSEN (1936) viz. 1.9, 3.12 and 7.2 Svedberg units for the α, β and γ components respectively. CECIL and OGSTON (1949) report S°_{20} = 2.83 Svedberg units for β lactoglobulin.

It will be clear that patterns like Fig. 46 are not very suitable for detecting small contributions of minor constituents. Even the α component is only expressed by an asymmetry on the curve of β lactoglobulin. As to the identity of the α and γ components it is assumed that the γ component of high sedimentation constant and high molecular weight (cf SVEDBERG (1938) and PEDERSEN (1936), corresponds with the classical globulin fraction (immunoglobulin, component IV in the electrophoretic patterns). The α component has been ascribed by PEDERSEN (1936) to the albumin isolated by KEKWICK (unpublished). After two hours' centrifuging the separation of the α and β components was still insufficient as will be seen hereafter.

The first question to be solved by means of the ultracentrifuge was whether the dialysis, preceding electrophoretic experiments might alter the properties of the protein mixture. To answer this question ultracentrifugal patterns were recorded of samples of native milk whey and samples of the same whey dialyzed against the buffer solution of electrophoresis (pH 6.8, μ = 0.15, cf II.5.a.). In Fig. 47 b and c the patterns are shown; they were obtained with two samples of milk whey, one after one hour's and the other after two hours' centrifuging. The differences between the patterns are so great that the possibility of influences other than dialysis has to be considered. The only notable difference in composition of native and dialyzed solutions is the presence of a large amount of lactose (approximately 4.8 per cent) in the former. Hence we decided to record in addition the patterns of a lactose solution of the same concentration. For the sake of accurate comparison we dissolved the lactose in the same buffer solution (pH 6.8, μ = 0.15), which yields a kind of synthetic milk ultrafiltrate. The patterns obtained with this solution are presented in Fig. 47a. It appears that the lactose concentration does not remain constant throughout the cell during centrifuging. On the contrary, the concentration falls from an increased value at the bottom via the original concentration in the middle of the cell down to a reduced

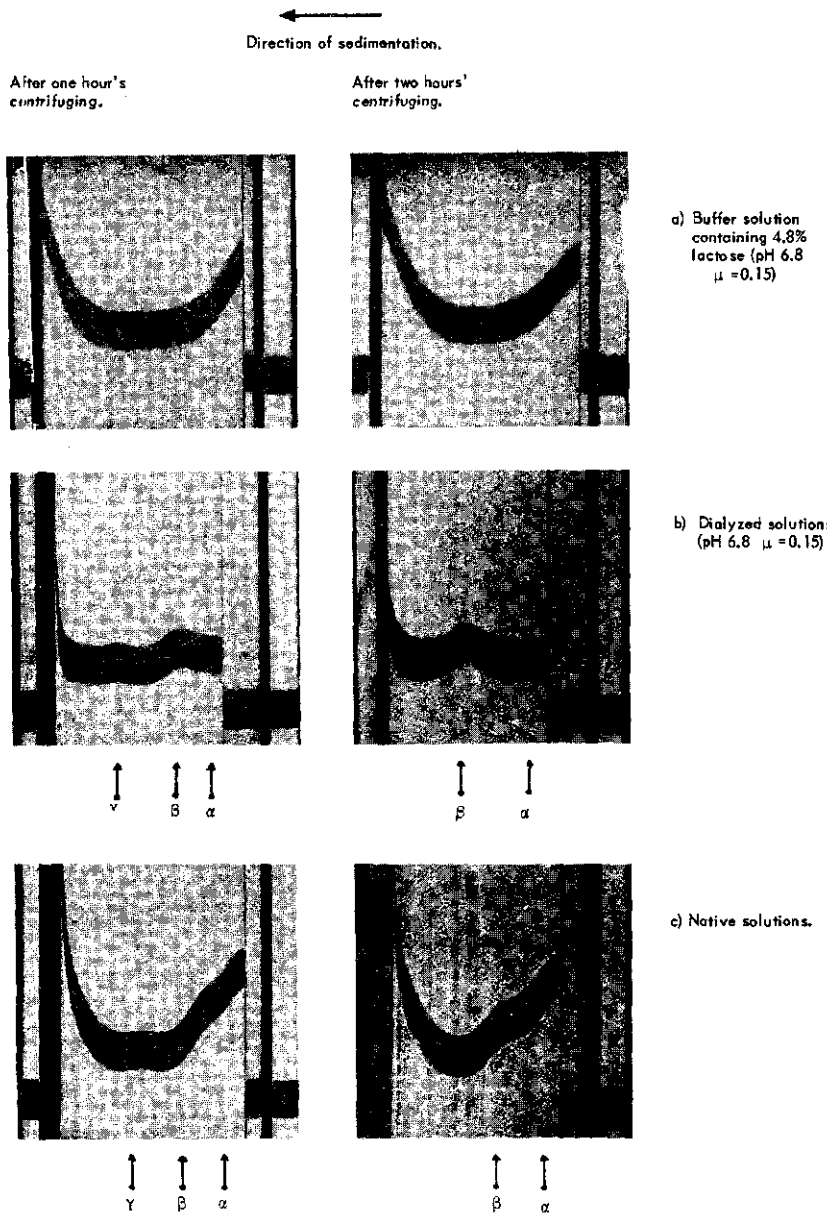


Fig. 47. Ultracentrifugal patterns of native and dialyzed whey and of synthetic milk ultrafiltrate.

value near the meniscus. The lactose patterns give an excellent explanation of the differences between the patterns of figs. 47b and 47c. The latter are approximately the sums of the lactose patterns and those of the dialyzed solutions. After two hours' centrifuging the patterns are less additive as can be seen from the shift in the places indicated as α and β boundaries. Apparently the sedimentation of the proteins is influenced by the presence of lactose. In order to explain such influences we might assume the formation of a weakly bound complex of proteins and lactose by analogy with the so-called Maillard reaction during heat denaturation.

It will be obvious that the native environment of the whey proteins i.e. the complete milk ultrafiltrate is not suitable for ultracentrifugal studies. However, the differences observed between the patterns of native and dialyzed solutions need not point to an essential alternation of the proteins, as they are chiefly due to the sedimentation of lactose.

c. β *lactoglobulin*

In section II.6.c. the main component of the whey proteins has been identified as β lactoglobulin. This was determined by the characterization

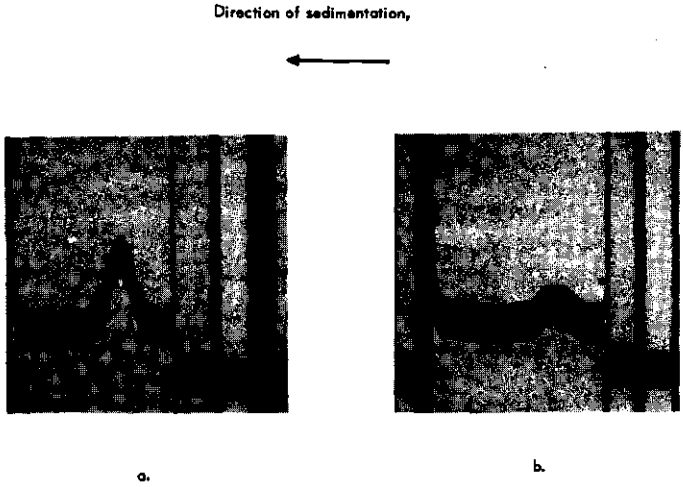


Fig. 48. Ultracentrifugal patterns of β lactoglobulin.
a. Protein concentration 1.0 g/100 ml. pH =6.8, μ =0.15, citrate-phosphate KCl. (After 64 minutes' centrifuging)
b. Protein concentration 0.5 g/100 ml. pH =4.8, μ =0.18, acetate. (After 80 minutes' centrifuging)

of the peaks of the electrophoretic pattern by their mobilities and comparison with the mobility of a pure β lactoglobulin preparation. To check the identity of this preparation with the materials described in literature, we have also recorded its ultracentrifugal pattern. Recordings were made in the usual buffer solution (pH 6.8, μ = 0.15) at several protein concentrations. One experiment was also made in acetate buffer (pH 4.8, μ = 0.18) near the iso-electric point. The material appeared to be completely homogeneous in all the experiments. Two patterns out of the series are shown in Fig. 48. The velocity of the boundary was measured accurately and the sedimentation constant S^{0}_{20} was calculated. S^{0}_{20} represents the velocity the material would have in a hypothetical medium having the density and viscosity of water at 20°C and is expressed in Svedberg units (10^{-13} sec). The values obtained are given in table 36.

TABLE 36. Sedimentation constant (S^{0}_{20}) of β lactoglobulin as calculated from the sedimentation velocities observed in five experiments

Experiment	Protein concentration (%)	pH	μ	S^{0}_{20} (Svedberg units)
1	1.0	6.8	0.15	2.59
2	0.3	6.8	0.15	2.81
3	0.1	6.8	0.15	2.68
4	0.54	6.8	0.15	2.72
5	0.5	4.8	0.18	2.71

It appears that the results at pH 6.8 differ only slightly from that obtained in the approximately iso-electric condition (pH 4.8). CECIL and OGSTON (1949) report S^{0}_{20} = 2.81 Svedberg units at a protein concentration of 1 g/100 ml. At zero protein concentration they find S^{0}_{20} = 2.83 Svedberg units. As has been mentioned by CECIL and OGSTON (1949), differences like those recorded here may easily originate from different methods of temperature reading etc. It is not our intention, however, to give new accurate values of the sedimentation constant, but only to ascertain whether the material under discussion was really homogeneous β lactoglobulin. In view of the results (fig. 48 and table 36) this is apperently the case. Hence the method of preparation of β lactoglobulin described in II.6.c. may be recommended here for all those purposes where only a solution is needed.

d. The whey powders used in the feeding experiments

A comprehensive description of the whey powders used in the feeding experiments has been given in II.9. Several differences in composition were stated and even the electrophoretic patterns of the proteins were not identical (Figs. 36 - 41). For the sake of completeness we have also recorded the ultracentrifugal patterns of the proteins of these whey powders. They were

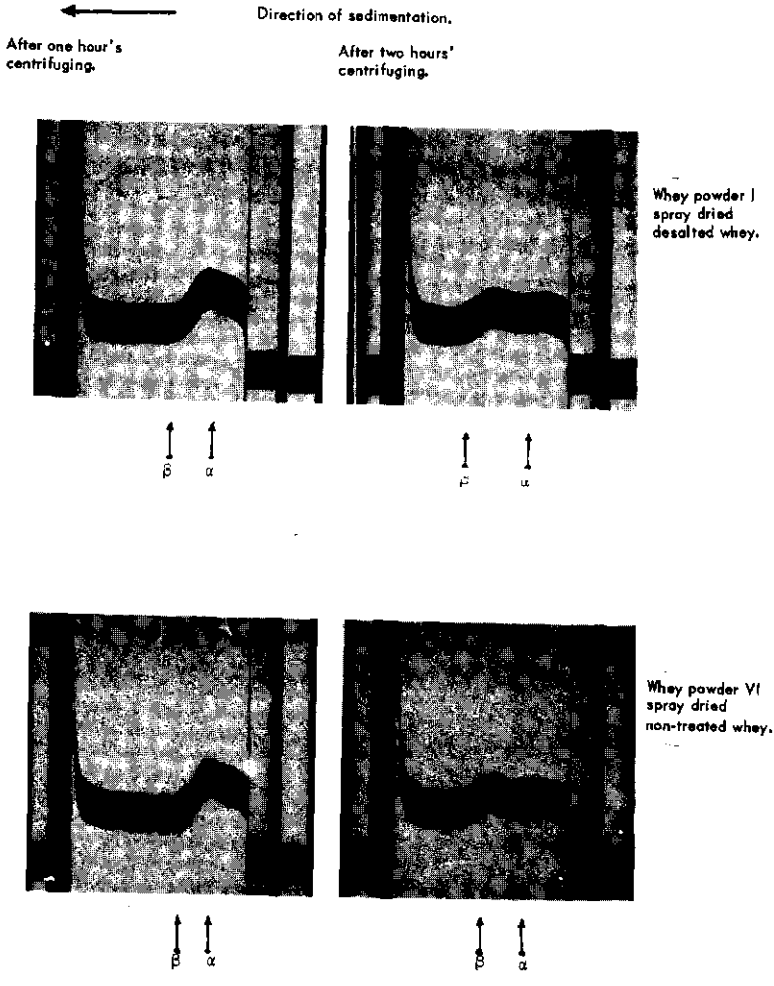


Fig. 49. Ultracentrifugal patterns of spray dried desalted and non-treated whey (Whey powders I and VI).

Protein concentration 0,75 g/100 ml.

almost completely identical, but somewhat different from the pattern of fresh whey. Therefore we will not reproduce all the patterns obtained. In Fig. 49 the patterns of the whey powders I and VI are given after one hour's and two hours' centrifuging. These powders are the most divergent from the point

of view of electrodialytic desalting. Powder I contains only 21 g ash per 16 g nitrogen whereas powder VI which has been prepared from non-treated whey, contains 63 g ash per 16 g nitrogen (cf table 31, II.9.a.). Even these patterns are almost identical. This confirms the results of the electrophoretic experiments described in II.8., where the influence of the electrodialytic desalting procedure on the electrophoretic pattern appeared to be negligibly small.

In comparison with the patterns of fresh whey Fig. 47b) some differences can be stated. The γ component which is clearly visible in the pattern of fresh whey after one hour's centrifuging, is absent in the patterns of the whey powders. This is in agreement with the results of electrophoresis where it appeared that component IV (immunoglobulin) was either absent or markedly reduced in comparison with fresh whey proteins (cf table 32, II.9.b.). Furthermore the α component appears to be slightly greater in the case of the whey powders than in fresh whey. With regard to table 32 (II.9.b.) it can be suggested that the component corresponds to the rapidly moving components of electrophoresis (O, I). According to SMITH (1946 c), the α component should be connected with Kekwicks lactalbumin. Hence it is probable that the α constituent also corresponds with component III. Whether this is true or not can only be decided by preparative work.

The conclusion of this examination of the powders prepared from processed whey is that they are completely equivalent to powders from non-treated whey with regard to the character of their proteins. There are on the other hand differences compared with the proteins of fresh whey prepared from raw milk. These differences can be ascribed to the pasteurizing of the cheese milk and to heat treatment during incautious condensing of the whey.

12. SUMMARY

The chemical researches described in this section concern the nitrogen distribution in whey and the composition of the proteins. The latter was studied especially by electrophoresis. In addition the influence of several treatments viz. heat treatment, salting out, electrodialysis and condensing on this composition have been examined. Furthermore the detailed analyses of several whey products, to be applied in the feeding experiments were included; this yielded information on the total influence of the manufacturing process of these powders. A comparison with human milk was also made. Finally some supplementary ultracentrifugal studies were discussed.

The nitrogen distribution in whey was reviewed. Apart from data in the literature experimental analytical data were given on the composition of samples of acid and rennet whey prepared from the same milk. From the figures obtained the following values, expressed in mg nitrogen per 100 g whey are quoted:

	Rennet whey	Acid whey
(1) Real protein nitrogen	78.0	67.3
(2) Proteose nitrogen	25.4	20.0
(3) Residual nitrogen	27.6	28.7
Total nitrogen	131.0	116.0

The definitions adopted for these fraction were:

1. Precipitated by 30 minutes' heating of the whey at 100°C and at pH 4.7
2. Precipitated by 15 per cent trichloracetic acid, but not by heat treatment.
3. Not precipitated by 15 per cent trichloracetic acid.

The sum of real protein nitrogen and proteose nitrogen appeared to be approximately equal to the content of non-dialysable nitrogen. The examination by means of electrophoresis or in the ultracentrifuge was always preceded by dialysis. Hence the patterns obtained by these methods do not include the residual nitrogen.

Electrophoretic analysis yields information on the relative concentrations of the various components and the values of the electrolytic mobilities of these constituents. The concentrations are expressed as percentages of the total "protein" examined(i.e. real protein nitrogen + proteose nitrogen). The mobilities serve as a means of characterization of the components and are expressed in $cm^2V^{-1}sec^{-1}$. The average results of electrophoretic analysis of fresh rennet whey, expressed in these units were:

Component	Concentration	Mobility
I "Whey protein"	18	$6.6 \cdot 10^{-5}$
II β Lactoglobulin	54	$5.4 \cdot 10^{-5}$
III Component	13	$4.0 \cdot 10^{-5}$
IV Immunoglobulins	15	$1.7 \cdot 10^{-5}$

The components mentioned above were identified as follows:

Component I represents the so-called "Whey protein". This is a constituent which has originated by the action of rennin on casein during its coagulation. It gives rise to the difference between the total nitrogen contents of rennet and acid whey as it is not present in acid whey.

Component II is identified as β lactoglobulin. It constitutes the greater part of the classical lactalbumin fraction (defined as the protein precipitating between half and full saturation with ammonium sulphate). According to PALMER (1934), it has some characteristics of a globulin although it is completely different from the classical globulin fraction of the milk. It is the main constituent of whey proteins.

Component III which is present only in rather small amounts is a highly soluble albumin, which has probably not yet been isolated. It may however correspond with a lactalbumin isolated by KEKWICK (unpublished).

Component IV represents the classical globulin fraction of the milk (defined as the fraction precipitated by saturation with $MgSO_4$). From this fraction two proteins have been isolated by SMITH (1946 c), which have been termed eu- and pseudoglobulin. These fractions are connected with the immunological properties of milk; hence the name "immunoglobulins" has been adopted. The fraction denoted as component IV is heterogeneous, but a justified analysis into its constituent parts was impossible because of insufficient separation.

Ultracentrifugal analysis of whey proteins yields three components, α, β and γ, characterized by the sedimentation constants 1.9, 2.83 and 7.0 Svedberg units respectively. Their relation to the components detected by electrophoresis is probably as follows:

$$\alpha = I + III$$

$$\beta = II$$

$$\gamma = IV$$

The first relation has not been proved. The second relation was proved by PEDERSEN (1936) who suggested the name β lactoglobulin for this constituent in accordance with the notation α, β, γ. The third relation is certainly true and is proved i.a. by the work of SMITH and co-workers (1946 up to 1948), and the results of the work described in this thesis.

The electrophoretic and ultracentrifugal studies were carried out in a special buffer solution (pH 6.8, $\mu = 0.15$) which contained sodium and potassium phosphates and citrates and potassium chloride in such a ratio as to resemble milk ultrafiltrate.

In view of the large boundary anomalies observed in the electrophoretic

patterns of whey, the theory of electrophoresis was thoroughly studied. With the "Regulating Function" of KOHLRAUSCH (1897) as the starting point the influence of the boundary anomalies on the migration velocities of the protein boundaries was discounted. In addition the relation between the size of the so-called δ boundary and the equivalent weight of the protein under investigation was studied. For the case of protein mixtures a corresponding quantity, the "Index of mean charge" was introduced. The calculation of the equivalent weight was applied to a homogeneous preparation of β lactoglobulin at pH 6.8 and $\mu = 0.15$; the value found was 2710. By means of the dissociation curve of the same solution a value of 3225 was obtained, in agreement with values reported in the literature.

The electrophoretic patterns of milk whey were compared with those of milk and some components of milk were identified as whey proteins.

The influence of salting out of whey proteins with ammonium sulphate or with an equimolecular mixture of mono and di-potassium phosphate was studied and it appeared to be small. The patterns obtained after re-solution and dialysis were only slightly different from those of fresh whey proteins.

The influence of heat treatment was studied extensively. The lowest temperature at which visible turbidity of whey occurred was 69 °C at a pH of 4.5. The precipitation of protein at increasing temperatures was examined by estimation of the nitrogen contents of the filtrates. Precipitation occurred chiefly between 65 and 85 °C with a maximum between 70 and 75 °C. After heat treatment at 65 °C the classical globulin fraction has already disappeared from the electrophoretic pattern. Heat treatment up to 55 °C does not cause any change in the patterns. When arranged according to decreasing resistance to heat treatment the following order of the components is found: III >II >I >IV. The value of the index of mean charge is highly dependent on the temperature of heat treatment.

The influence of condensing whey in an industrial vacuum pan was also examined. The changes observed were small and pointed to a further coagulation of products, initially denatured during pasteurization of the cheese milk. In this case condensing has been carried out very cautiously at temperatures not exceeding 49 °C. Condensing at higher temperatures was not studied but may cause considerable alteration of the proteins.

Electrodialytic desalting of whey in the semitechnical plant developed by the General Technical Department "T.N.O." is accompanied by two effects. First, approximately one third of the content of residual nitrogen is removed by diffusion, this amount being greater when thorough stirring (high circulation velocity) is applied to the liquid to be desalted. Secondly, very slight changes in the electrophoretic patterns are observed and possibly coagulation of previously affected materials has taken place.

The examination of whey powders obtained from various kinds of desalted and normal whey, yielded supplementary information as to the accompanying effects of the electrodialytic procedure. The removal of certain minor constituents (vitamins B_2 and B_{12}) appeared to be dependent on the method. Removal of lactose is accompanied by a decrease of the vitamin B_2 content, whereas the possiblity of electrolytic removal of vitamin B_{12} is suggested. Several characteristics of incipient denaturation of the proteins were observed which, however, were chiefly due to previous heat treatment of the cheese milk.

Bovine whey proteins appeared to be essentially different from human whey proteins in many respects from a chemical point of view. Examination of whey

prepared from lyophilized pooled human milk yielded differences from fresh human milk; these were ascribed to previous pasteurization.

Ultracentrifugal studies of native milk whey and dialyzed milk whey yielded large differences which were ascribed to the presence of lactose. The possibility of weakly bound complexes between proteins and lactose in native whey was suggested. No differences could be observed between the patterns of whey powders prepared either from normal technical milk whey or from desalted whey. Differences with the patterns of fresh rennet whey prepared from raw milk were consistent with similar differences between the electrophoretic patterns. They were ascribed to the pasteurization of the cheese milk.

13. REFERENCES

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III. FEEDING EXPERIMENTS

1. INTRODUCTION

As has been mentioned in the general introduction, the subjects to be treated in this thesis include the execution of biological and biochemical tests on the usefulness of products manufactured from desalted whey for feeding purposes, especially as a fodder for poultry, cows and pigs (cf I.4.c.).

Feeding experiments were carried out to this end, with the whey powders described in II.9. Some preliminary tests were carried out with rats, followed by more extensive trials with chicks, calves and pigs. The kind co-operation of several specialized Institutes should be mentioned here. The rats were supplied by the Central Institute T.N.O. for the Breeding of Experimental Animals, where the experiments were carried out. We are indebted to Prof. Dr G.M. VAN DER PLANK and Dr W.K. HIRSCHFELD (Chairman and Director of the Institute) not only for this co-operation but also for the execution of a trial with chicks at the Zootechnical Institute of the State University of Utrecht, and for valuable advice in the experiment with calves. In the case of this experiment, three sets of twin calves were placed at our disposal by the kind co-operation of the Unit for Research on Animal Husbandry T.N.O. We are much indebted to Prof. P. HOEKSTRA D.V.M., Mr C. VAN DER GIESSEN and Miss A. HOETINK, Director, Secretary and Co-worker respectively of the Unit for their valuable contribution to this research. The experiment with pigs was executed at the Institute for Modern Live-Stock Feeding "De Schothorst" at Hoogland (near Amersfoort). Its Director Dr J. GRASHUIS consented to the trial being carried out at his Institute. We are indebted to him and also to his co-workers Dr S.T. HOFSTRA, Mr J.G. DE HEUS and Mr B. BLOM for valuable help and assistance.

Statistical analyses of the experiments were made by the Statistics Department T.N.O. We are indebted to Mr Th.J.D. ERLEE and Dr E.F. DRION for their valuable co-operation.

In the next four sections the experiments with rats, chicks, calves and pigs will be treated successively.

2. FEEDING EXPERIMENTS WITH RATS

Before the experiments with chicks, calves and pigs some laboratory experiments were carried out with rats. These experiments were necessary to yield information as to the quantities of different whey powders that could be fed without detrimental effects.

Three experiments were performed, which will be denoted as A, B and C. Experiment A was a preliminary trial with widely divergent quantities of whey powder. Experiment B was a repetition of A, within the limits obtained as a result of the first feeding trial. Experiment C was carried out with another whey powder of higher protein content.

The animals were albino rats, obtained by breeding in of a strain from the Wistar Institute (London). The strain is now denoted Z.I. (Zootechnical Institute).

Experiment A

Five groups, each of five male rats (Z.I. strain) were placed on different diets

in which approximately 0, 5, 10, 20 and 50 per cent of the protein was supplied in the form of whey powder I. The composition of this powder has been given in tables 30 and 31 (II.9.a.). It is a typical example of a dried desalted whey, without any removal of lactose. The only remarkable difference from normal whey powder is the low ash content (2.9 per cent as against 8-9 per cent in normal whey powder).

The diets were calculated in such a way that the values of total protein, lysine, methionine, minerals and the starch value were approximately the same for the five groups. In table 37 the composition of the diets is represented. In table 38 the analyses of the mixed feeds as to the constituents mentioned above are given, including the contents of lactose.

TABLE 37. Composition of diets, supplied in the feeding trials A and B on rats

	Control group (C)	Test groups			
		1	2	3	4
Ground corn	24	19	10	-	-
Ground oats	18	18	18	18	-
Linseed meal	3	3	3	3	1
Soybean oil meal	6	8.5	8	6	1
Corn gluten feed	8	8	8	4	1
Wheat bran	5	5	6	5	1
Grass meal	6	6	6	6	-
Skim milk powder	6	-	-	-	-
Fish meal	14	14	14	14	14
Yeast	3	3	3	3	2
"Vital" (substitute for yeast)	5	5	5	5	-
Minerals	2	2	2	2	-
Vitamins A and D	0.3	0.3	0.3	0.3	0.3
Whey powder I (desalted)	-	8.5	17	34	80
Total	100	100	100	100	100

TABLE 38. Analyses of diets, supplied in the feeding trials A and B on rats

	Control group (C)	Test groups			
		1	2	3	4
Whey protein in % of total protein	-	5	10	20	47
Whey powder I in % of total diet	-	8.5	17	34	80
Total crude protein (%)	22.5	22.4	22.8	22.5	20.4
Lysine (%)	1.35	1.37	1.43	1.51	0.87
Methionine (%)	0.58	0.54	0.54	0.52	0.28
Starch value	68	68	68	70	86
Lactose (%)	3.0	6.6	13.3	26.5	62.4
Minerals (%)	8.5	8.4	8.5	8.9	5.7

Diet 4 contained an extreme amount of whey powder. Therefore it was not possible to maintain the constant values of the analysis. The high content of lactose resulted in an increased starch value, whereas the content of minerals was considerably lower than that of the other diets, in consequence of the low ash content of the desalted whey.

The animals were fed ad libitum as groups and the increase in weight was measured at the end of two weeks. The average increase per animal per week was calculated. The values found are given in table 39.

TABLE 39. Growth of rats in feeding trial A

Groups	C	1	2	3	4
Percentage of protein substitution in the diet (%)	0	5	10	20	47
Average increase in weight per animal (g/week)	18.2	19.2	14.5	11.5	-

All the animals of group 4 died within one week, showing symptoms of severe diarrhoea. This was probably due to the excessive amount of lactose in the diet. The growth of the other groups shows a slight maximum at 5 per cent protein substitution. At higher percentages, lower growth values were found. The data of this preliminary experiment have not been analyzed statistically. Therefore they will not be discussed in detail.

Experiment B

In this trial the same diets as used in experiment A were tested. The experiment lasted 60 days and was performed with five groups, each composed of two male and two female rats (Z.I. strain). For the composition and analyses of the diets we refer to tables 37 and 38. In addition, every animal received 0.1 mg thiamin per day (per os) in order to prevent a possible thiamin deficiency as a result of intensified carbohydrate metabolism. Nevertheless the animals of group 4 showed diarrhoea and died within one week. The increases in weight are recorded in table 40.

TABLE 40. Growth of rats in feeding trial B

Groups	C		1		2		3		4	
Percentages of protein substitution in the diet (%)	0		5		10		20		47	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Individual growth of rats in 60 days (g)	170 179	135 160	196 220	119 76	183 183	150 125	132 111	110 123	- -	- -
Total growth (g)	349	295	416	195	366	275	243	233	-	-
Mean growth per rat (g)	161		153		160		119		-	
Mean growth per week (g)	18.7		17.8		18.7		13.9		-	

Average growth of all rats in 60 days: 148.25 g.

These results are in agreement with those recorded in table 39, as far as the groups C, 3 and 4 are concerned. Group 2 shows better growth than in the first experiment, whereas at 5 per cent protein substitution in group 1, no maximum is found.

An analysis of variance was applied to the individual growth values. The total growth of each animal (G) was assumed to be additively composed of five components viz.:

1. The mean level of growth (M)
2. The influence of sex (S)
3. The influence of diet (D)
4. An interaction I_{SD} representing the deviation of additivity of the S and D effects
5. The residual variance ε.

This scheme can be represented by the equation

$$G = M + S + D + I_{SD} + \varepsilon$$

Whether any influence is significant or not depends on the value of the probability of the null hypothesis being correct. In this and in the next sections this probability will be denoted by the symbol P.

It appeared that not only the influences of sex and diet were significant ($P < 1$ per cent), but also the interaction. If, however, the same calculation is made, group 1 being left out, the interaction is no longer significant ($P = 16$ per cent). The first result is probably due to the second female rat of group 1 which showed an abnormally low growth (76 g as against the mean value 148.25 g). Although it cannot be proved, it is probable that the influence of this extreme value is too great in view of the small number of animals per group. According to the second calculation, the differences between groups C, 2 and 3, due to feeds were higly significant ($P = 0.5$ per cent), which shows that 34 per cent of whey powder I in the feed is inadmissible whereas 17 per cent can be applied without detrimental effect.

The faeces of each group of animals were collected daily during several weeks, and were analyzed for their content of reducing substances ("sugars"). The analyses were carried out according to the ferricyanide method of HAGEDORN and JENSEN (1923), which was preceded by deproteinization with cadmium hydroxide according to FUJITA-IWATAKE (1931). The reducing power was calculated as glucose. In addition the content of dry matter was estimated so that the amounts of "glucose" could be expressed as per cents of total solids. Furthermore the same estimations were made in the protein free filtrates after inversion of complex carbohydrates to monosaccharides by one hour's heating at 100°C after addition of hydrochloric acid up to a final concentration of 3.2 per cent.

The results of the estimations are given in table 41.

[1] TABLE 41. Total reducing power of rat's faeces, calculated as glucose as a percentage of the total solids

	"Glucose" before inversion					"Glucose" after inversion				
Group	C	1	2	3	4	C	1	2	3	4
Date										
11/7	1.03	1.29	1.44	2.54	3.09	1.22	1.70	1.30	3.70	3.96
12/7	-	1.66	2.36	-	4.9	-	2.50	2.58	-	7.7
13/7	1.59	2.42	2.03	2.27	5.2	1.73	3.86	2.73	3.87	8.4
14/7	1.46	1.75	1.34	1.30	8.1	2.04	2.80	1.37	2.09	9.9
15/7	1.52	1.46	1.83	1.70	5.4	1.78	2.10	2.46	2.26	9.1
16/7	1.19	0.99	-	1.61	7.6	1.29	1.88	-	2.33	9.8
17/7	-	1.58	1.12	1.22	14.8	-	2.88	1.31	1.77	20.0
18/7	1.21	1.30	1.37	1.03		1.62	2.04	1.69	1.15	
19/7	0.77	1.57	1.70	2.07		0.89	2.70	1.99	2.43	
20/7	-	1.44	1.16	2.12		-	2.17	1.09	2.69	
21/7	0.85	1.28	1.15	1.90		1.27	1.47	1.52	2.47	
22/7	0.89	0.85	0.99	0.92		1.40	1.29	1.45	1.37	
24/7	0.75	0.85	1.06	1.78		0.98	1.30	1.51	2.00	
25/7	0.99	0.91	1.55	1.02		1.05	1.32	2.00	1.25	
26/7	0.49	1.54	1.77	1.94		0.55	2.57	2.47	2.49	
27/7	1.37	1.34	1.31	1.46		1.66	2.30	1.90	1.98	
28/7	1.01	1.55	1.83	1.44		1.39	2.19	2.48	1.95	
29/7	0.35	1.00	1.37	1.29		0.40	0.94	1.63	1.95	
30/7	-	1.04	1.17	0.82		-	1.30	1.36	1.12	
4/9	0.47	0.73	1.02	0.62		0.44	1.20	1.65	1.00	
5/9	0.57	0.74	0.22	0.43		0.62	0.87	0,19	0.60	
6/9	0.64	1.02	0.64	0.73		1.03	0.97	1.18	1.00	
7/9	0.52	0.50	0.65	1.05		0.74	0.52	0.83	1.33	
8/9	0.17	0.33	0.53	0.42		0.12	0.38	0.50	0.52	
Mean	0.88	1.18	1.25	1.36		1.10	1.71	1.62	1.86	

[1] The mean values have been computed only from the observations at dates where analyses of the faeces of all groups except 4 were available.

It appears that the faeces of group 4 show an abnormally high sugar content. In general the values of the groups 3, 2 and 1 are higher than those of the control group. In addition the values decrease in the course of the experiment. The ratios of the values before and after inversion were also calculated but are not represented here. There was no significant difference in these values either between the groups or between successive periods of the experiment.

The data of the analyses were examined statistically by means of the method of m arrangements, as described by KENDALL (1948). The differences of the groups 3, 2 and 1 as compared to the control group were highly significant in both cases, before and after inversion ($P = 0.1$ per cent). In view of the relatively small difference, between the "glucose" contents before inversion of group 1 and group C, in this case a special test was applied viz. that of HEMELRIJK (1950). According to this test the difference between the groups 1 and C was still very significant ($P = 0.2$ per cent), whereas the corresponding difference between the groups 2 and 3 was not significant ($P = 10$ per cent).

Examination of the ratios of the values before and after inversion yielded no significant difference between the groups (probability of occurrence of the arrangement values found: 26 per cen).

From the results of this experiment the following conclusions can be drawn.

Feeding of 80 per cent of whey powder I is detrimental. Several authors have reported that rations containing 62 - 100 per cent lactose cause early death in rats (cf FISCHER and SUTTON, 1949). The ration of group 4 contained 62.4 per cent. It can be stated that the diarrhoea observed is caused by the lactose and is not prevented by the other constituents of this whey powder. Neither is it prevented by oral administration of thiamin. A ration containing 34 per cent whey powder I (26.5 per cent lactose) does not cause death but results in retardation of growth, probably in consequence of a slight diarrhoea. This confirms the observation of RIGGS and BEATY (1947) who report the lowest lactose level

causing diarrhoea in weanling rats (Sprague-Dawley strain) to be 20 per cent. Rations containing 17 per cent whey powder or less do not cause any retardation of growth but still involve an increased "sugar" content in the faeces. Metabolic breakdown of lactose to monosaccharides is accomplished in all cases at the same rate, for no difference was observed in the ratio of reducing power of the faeces between the groups, before and after inversion.

Experiment C

In view of the difficulties encountered in the previous experiments because of the high levels of lactose in the rations, another experiment was carried out with a whey powder containing a more favourable ratio of protein to lactose. The powder used was whey powder II which has been described in section II.9.

TABLE 42. Composition of diets, supplied in feeding trial C on rats

	Control group (C)	Test groups		
		1	2	3
Ground corn	24	20.7	17.3	14
Ground oats	17	14.7	12.3	10
Soy bean oil meal	6	5.3	4.7	4
Corn gluten feed	8	7.7	7.3	7
Wheat bran	5	4.3	3.7	3
Fish meal	16	14	12	10
Peanut oil meal	5	4.3	3.7	3
Linseed meal	3	3	3	3
Grass meal	6	6	6	6
Yeast	3	3	3	3
"Vital" (substitute for yeast)	5	5	5	5
Minerals	2	2	2	2
Vitamins A and D	0.3	0.3	0.3	0.3
Whey powder II (desalted)	-	10	20	30
Total	100	100	100	100

TABLE 43. Analyses of diets, supplied in feeding trial C on rats

	Control group (C)	Test groups		
		1	2	3
Whey protein as % of total protein	-	10	20	30
Whey powder II as % of total diet	-	10	20	30
Total crude protein (%)	24.0	24.0	24.0	24.0
Lysine (%)	1.40	1.45	1.50	1.55
Methionine (%)	0.57	0.56	0.54	0.52
Starch value	68	69	70	71
Lactose (%)	-	6.5	13	19.5
Minerals)(%)	8.8	8.9	9.0	9.1

The degree of desalting is somewhat lower than in whey powder I but its protein content is approximately twice as high because of the removal of lactose (cf tables 30 and 31, II.9.a.). With the help of this powder, diets were composed in which 10, 20 and 30 per cent of the total protein was supplied as whey protein. Nevertheless even with 30 per cent protein substitution, the lactose content of the diet did not exceed the 20 per cent level of RIGGS and BEATY (1947).

The compositions and analyses of the feeds are represented in tables 42 and 43, together with the same data on the ration of the control group.

From these tables it is obvious that this whey powder is more suited to the composition of well-balanced diets.

The experiment was carried out for five weeks with four groups each composed of 4 male and 6 female white rats (Z.I. strain). The feed was supplied ad libitum to each group.

Weight recordings were made each week and the faeces were collected each two days and analyzed for reducing power as described in experiment B. After five weeks, the average increase in weight per animal per week of each group was calculated. Five animals, distributed over the groups C, 1 and 3, had died and were left out. In addition 7 other animals were intentionally left out of the calculation although the choice within each group and sex was made at random. This was done to make the experiment orthogonal and more accessible to the application of an analysis of variance. In this way equal numbers of males and females per group were left. The growth values are given in table 44.

TABLE 44. Growth of rats in feeding trial C

Groups	C	1	2	3
Percentage of protein substitution in the diet (%)	0	10	20	30
Average increase in weight per animal (g/week)	15.8	16.6	15.2	14.4

The analysis of variance of the individual growth values of the selected 28 animals was carried out in the same way as described under experiment B. There appeared to be no interaction of the influences of sex and diet. The influence of sex was very significant ($P = 0.01$ per cent) whereas the influence of the diets was not significant ($P = 78$ per cent).

In addition the course of growth was analyzed. In fig. 50 the average weights of the animals of each group have been plotted. The curves are fairly similar. More exact information was obtained by application of the method of m arrangements (KENDALL, 1948) to the individual weekly increases of weight. Here only the dead rats were left out as the application of this method does not require orthogonality. A first arrangement of the data of the 35 animals together yielded a probability value of only 0.8 per cent. This value, however, may be due to the influence of sex, which is already very marked in the case of total

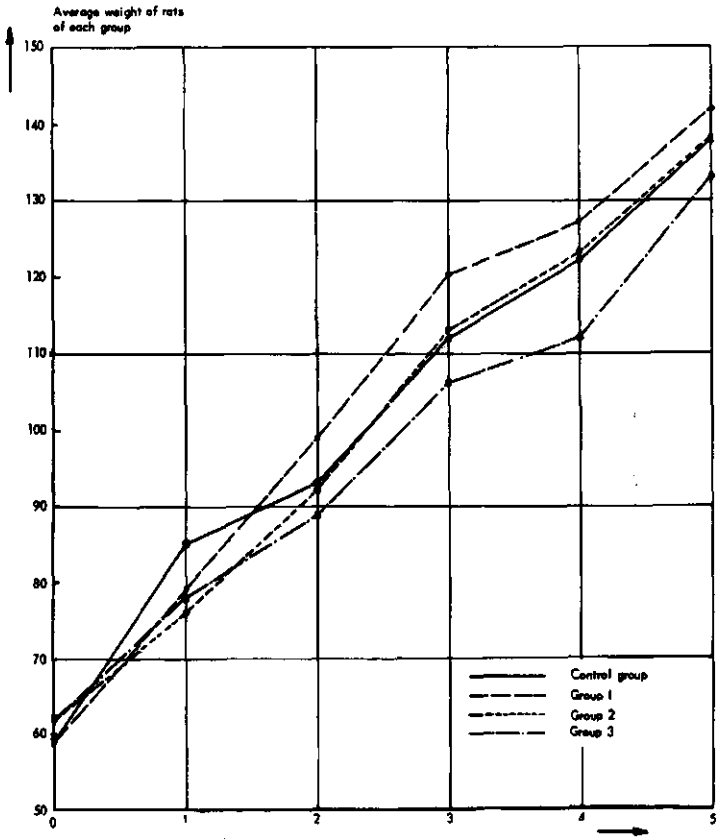

Fig. 50. Growth of rats in feeding trial C

growth. When separate arrangements were made for the males and the females the probabilities of occurring were 45 and 85 per cent respectively. This proves that the differences between the groups are not significant either in total growth or in the rate of growth.

The analyses of the faeces for reducing power yielded a general decrease in the course of the experiment, and a constancy of the ratio before and after inversion. No significant differences between the groups could be observed either in the "glucose" contents before or after inversion or in the ratios of the two values. This was found by application of the method of m arrangements to the data from the analyses. In table 45, the average data per group are given, together with the probability of finding a similar arrangement of the individual differences by chance.

TABLE 45. Mean reducing power of faeces of rats in feeding trial C per group, calculated as glucose and expressed as percentage of the total solids (averages of 16 observations in the course of 5 weeks)

	Groups				Probability of occurring by chance
	C	1	2	3	
Glucose before inversion	0.79	0.70	0.73	0.75	95%
Glucose after inversion	0.90	0.85	0.89	0.86	98%
Ratio after/before	1.13	1.19	1.22	1.22	40%

Hence there is no significant difference in reducing power of the faeces of the four groups.

Reviewing the experiments with rats, we can state that the feeding of whey powder is limited by the lactose level of the diet. At levels over 20 per cent retardation of growth and diarrhoea are observed. Substitution of 30 per cent of the protein in the feed by whey protein does not give rise to any difficulty if the lactose content remains below 20 per cent of the diet. It is remarkable that in experiment B, an increase of the reducing power of the faeces was observed with diets containing 8.5 and 17 per cent lactose respectively, whereas in experiment C such an increase was not observed, even with 19 per cent lactose in the diet.

3. FEEDING EXPERIMENTS WITH CHICKS

The rate of growth of chicks, fed on a diet containing whey protein (W) was compared with that of chicks on two control diets, one containing animal proteins (A) and one containing plant proteins (P).

The source of whey protein was whey powder III, described in II.9. From the analysis of this powder (tables 30 and 31, II.9.a.) it appears that the degree of desalting is the same as in whey powder II, which was used in feeding trial C with rats; the content of lactose however is somewhat lower. Therefore a substitution of 35 per cent of the protein by whey protein was carried out. This involved a lactose content of 18 per cent of the diet, which was still below the 20 per cent level observed in the experiments with rats. On the other hand it also implied that all animal protein in the diet A could be amply replaced by whey protein without the 20 per cent lactose level being exceeded. In this way the possible influence of minor constituents such as vitamin B_{12} and/or the so-called "whey factor" would be expressed in the results of the experiment. The control diets A and P would show the influence of presence or absence of the Animal Protein Factor (A.P.F.) as diet A contained 10 per cent fish meal.

The compositions and analyses of the diets supplied to the three groups are given in the tables 46 and 47.

TABLE 46. Composition of diets, supplied in the feeding trial with chicks

	W	A	P
Ground corn	15.0	32.0	35.0
Ground oats	7.5	17.5	12.5
Soybean oil meal	10.0	17.0	27.0
Mixed bran	18.0	13.0	10.0
Extracted sunflower seed	5.0	-	-
Peanut oil meal	4.0	-	5.0
Fish meal	-	10.0	-
Yeast	3.0	3.0	3.0
"Vital" (substitute for yeast)	5.0	5.0	5.0
Minerals	2.0	2.0	2.0
Vitamins A and D	0.4	0.4	0.4
Whey powder III	30.0	-	-
Total	100	100	100

W =Diet containing whey powder
A =Diet containing animal protein
P =Diet containing plant proteins

TABLE 47. Analyses of diets, supplied in the feeding trial with chicks

	W	A	P
Whey powder as % of total protein	35	-	-
Whey powder III as % of total diet	30	-	-
Total crude protein (%)	21.5	21.3	22.1
Lysine (%)	1.43	1.27	1.10
Methionine (%)	0.48	0.49	0.44
Starch value	69	68	71
Lactose (%)	18.2	-	-
Minerals (%)	7.7	7.3	5.7

It is worth mentioning that the starch value and the mineral content of diet W remained on a normal level in spite of the high content of whey powder. The lysine contents are far above the norm according to ALMQUIST (1948) viz. 0.9 per cent, whereas the methionine contents are exactly up to the norm (0.45 - 0.50 per cent).

The experiment was carried out with three groups, each composed of 100 White Leghorn newly hatched chicks (♂ ♂). The animals were housed in electrically heated cages with raised screen floors, each cage containing 25 birds. The chicks were weighed at one day old and distributed over the groups at random. After that weights were recorded at weekly intervals. In the course of the experiment some chicks died and two of them turned out to be females

and were removed. After four weeks the chicks were transported into three normal chicken-runs. Unfortunately they became infected with coccidiosis, which became evident two weeks after moving; this caused an increased mortality in the last weeks of the experiment. The numbers of dead birds in both periods of the experiment are recorded in table 48.

TABLE 48. Number of chicks that died in the course of the experiment

	Groups		
	W	A	P
First 4 weeks (before coccidiosis)	11	4	2
Last 5 weeks (after coccidiosis)	11	3	18
Total	22	7	20

The mortality in the group which was fed animal protein is lower than in the W and P groups. At the end of the experiment 249 chicks were left, viz. 77 in the whey group, 80 in the vegetable protein group and 92 in the animal protein group. Growth data will be reported on these birds only. During the first four weeks, faeces of each cage of 25 birds were collected separately at weekly intervals and analyzed for reducing power before and after inversion. The method of analysis was the same as reported in section III.2. Feed and water were supplied ad libitum. The general appearance of the chicks of group A (animal protein was the best, although almost the same as that of the animals of group P (plant protein). The appearance of the birds of group W (whey protein) was somewhat poorer. The group consumed much more water than the other groups and the birds were less clean. Obvious diarrhoea was not observed.

The average growth of the chicks of the three groups was slightly divergent. When the average weights of the surviving birds were plotted against the time, there appeared to be no change in the slopes and the relative positions of the curves after the fourth week. Apparently the interference due to the outbreak of coccidiosis is not very important. Therefore the results of the experiment are represented completely. In any event these curves can be considered to be completely reliable with respect to the first four weeks of life. The small differences in growth become more pronounced when the average weights per animal of each group are expressed as a percentage of the average weights of one of the groups at corresponding dates. In this way the graph represented in Fig. 51 has been plotted, with the weights of group A as 100 per cent level. Hence it has to be realized that fluctuations of the growth of the control group will will result in simultaneously occurring peaks or valleys of the curves of the other groups. The corresponding average weights of the birds of each group are given in table 49.

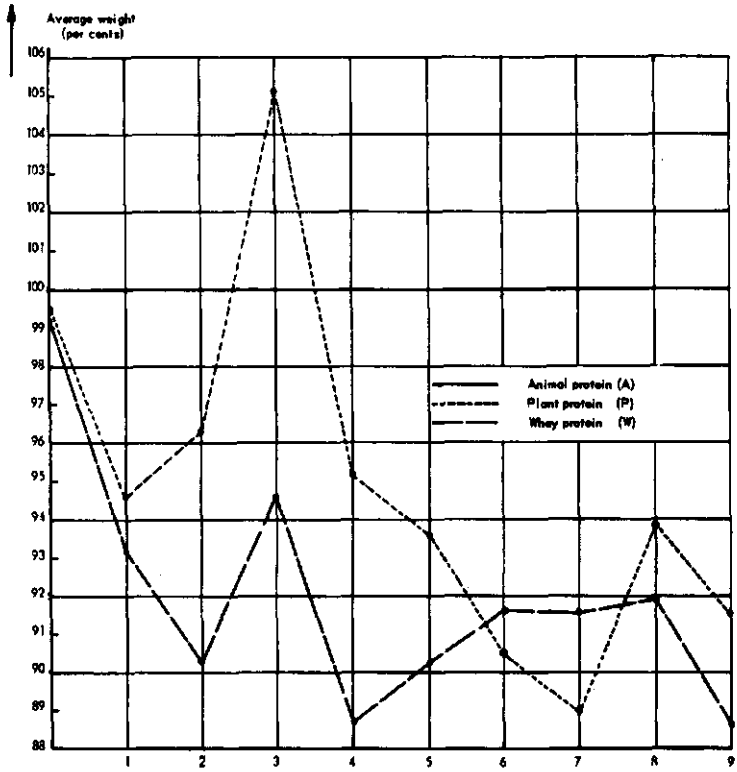

Fig. 51. Average weights of chicks at successive dates, expressed in percents of weights of control group, fed animal protein (A)

TABLE 49. Average weights (g) of chicks which were fed animal (A), plant (P) or whey (W) protein, during 9 successive weeks

Weeks	Group		
	W	A	P
0	38.9	39.3	39.1
1	72.4	77.7	73.6
2	125	137	132
3	186	196	206
4	272	306	291
5	371	411	385
6	470	513	464
7	581	635	575
8	664	722	678
9	764	862	789

Statistical analysis of the data was carried out with the help of the method of m arrangements. No significant differences could be proved when the method was applied to the average weekly increases in weight of the birds of four cages within one group. The probability values found for the groups W, A and P

were 24, 46 and 79 per cent respectively. Hence the arrangements found can be ascribed to chance, which means that the chicks formed a homogeneous batch of material. The average weekly increases in weight of the birds of the three groups were then calculated. A probability value of 2.4 per cent was obtained by the method of m arrangements. Hence the data show an obvious significance. The mean values of the average weekly increases in weight during the experiment were:

W 80.5 g/week
A 91.4 g/week
P 83.3 g/week

This significance was chiefly due to the higher growth values of the A group. With regard to Fig. 51 the same conclusion could be logically expected.

The weekly analyses of the faeces during the first four weeks of the experiment yielded the average results represented in table 50.

TABLE 50. Average reducing power of chicks' faeces before and after inversion, calculated as glucose and expressed as percentages of the total solids

	Group		
	W	A	P
"Glucose" before inversion (%)	2.47	0.74	0.81
"Glucose" after inversion (%)	5.52	2.60	2.42
Ratio after/before	2.31	3.58	3.09

By application of the method of m arrangements to the weekly data, there appeared to be a significant difference between the reducing power before inversion (P = 0.4 per cent) and that after inversion (P = 0.25 per cent). The arrangement of the values of the ratio "after/before" appeared to be almost significant (P = 7 per cent). The significance is due to the higher values of the reducing power of group W (whey protein). There is certainly no significant difference between the groups A and P. The higher reducing power of the faeces of the whey group was to be expected. It is remarkable, however, that the values of the ratio in the case of the whey group, is almost significantly lower than those of the P and A groups, whereas in the experiments with rats, no differences between these values were observed.

The following explanation is suggested. By inversion of lactose the reducing power increases to approximately twice its original value. In the case of rats' faeces, the value of the ratio was lower than 2 for the control diet. Hence the presence of lactose might cause the total value of the ratio to increase; this was not observed, so that no presence of lactose could be assumed. In the case of chicks' faeces, the normal value of the same ratio appears to be higher than 2 (3.58-3.09 in our experiment). Hence in this case the presence of monosaccharides and even that of lactose might cause the value to decrease. No significant time variation of the values was observed.

The presence of an increased amount of sugars, including lactose, in the faeces of the birds which were fed the whey powder diet, may be connected with the relatively small intestinal tract of chicks. Apparently the feed and especially the lactose is not completely digested in the short time available. This may explain the retardation of growth.

According to MONSON et al. (1950) the rate of growth of chicks fed on diets containing dextrine, glucose, sucrose or lactose, was most retarded in the case

of lactose. Attention should be drawn to their observation that diets with a carbohydrate consisting of 50 per cent dextrin and 50 per cent lactose did not involve the difficulties attendant upon the feeding of lactose, such as diarrhoea, short excretion time and low rate of growth.

According to FRENS and UBBELS (1950) who also experimented with chicks, the role of 8 per cent dried whey in the diets was not clear. They observed better growth when the whey powder was substituted by vegetable materials (corn meal and wheat bran) and some chalk, although the level reached by a control group, which was fed animal protein, was not attained.

The general practical experience in the Netherlands with such percentages of whey powder in mixed feeds for chicks is favourable.

The adequacy of whey protein as compared to vegetable proteins is completely confirmed in our experiment. Nevertheless in comparison with diets containing animal protein, the results are less favourable.

The experiment leads to the conclusion that the diet W containing whey powder cannot be recommended as poultry feed. This may be due to the influence of the lactose in the diet. Hence several possibilities can be suggested for an improvement of the results. We mention the feeding of smaller amounts of whey powder, of whey powders containing less lactose, or a feed composition in which the detrimental effects of lactose are suppressed by the presence of other substances such as dextrin.

4. FEEDING EXPERIMENT WITH TWIN CALVES

All over the world attention is given to the possibility of rearing calves with limited amounts of whole and skim milk or without it. We refer for example to the experiments of CONVERSE (1949), to the review on the nutrition of calves given by SAVAGE and MC CAY (1942) and to the recent Dutch experiments of DAMMERS, DIJKSTRA and FRENS (1951). The feeding of dry calf starters is studied and also the substitution of skim milk by milk whey or condensed whey. In that respect we refer to the experiments of DE MAN (1949, 1950).

The experiment to be described in this section gives a comparison between two calf starters, one of which contained dried desalted whey.

The calf starter to be tested contained 15 per cent of whey powder IV, described in II.9. Owing to centrifuging and desalting, the whey from which the powder was prepared showed lactose and ash contents reduced to approximately 30 and 60 per cent of their original values respectively. The main differences between the composition of the two calf starters were the substitution of 5 per cent blood meal and 2.5 per cent fish meal in the control diet by 15 per cent of whey powder in the test diet. The blood and fish meal mentioned above formed the only source of animal protein in the diet. According to the experiment of RUSOFF and HAG (1951) vitamin B_{12} is not essential to the growth of calves weaned at an early age, so that this substitution did not imply a great risk. In order to obtain reasonably equal values for the most important chemical constituents, this substitution in the test diet was accompanied by an extra amount of 5 per cent soybean oil meal and 4.5 per cent linseed meal and a decrease of the amount of ground oats by 6 per cent, ground barley by 6 per cent and ground corn by 5 per cent. Both mashes contained 10 per cent of ordinary whey powder. The analyses of the resulting mixtures are given in table 51.

TABLE 51. *Analyses of calf starter meals, used in the feeding experiment on twin calves*

	Test group	Control group
Whey powder IV (% of total protein)	15	-
Whey protein (% of total protein)	29.5	6.7
Crude protein (%)	18.5	17.4
Digestible protein (%)	15.7	14.0
Lysine (%)	1.14	1.08
Methionine (%)	0.33	0.33
Minerals (%)	7.7	6.7
Starch value	70	69
Crude fibre (%)	5.6	6.8
Lactose (%)	15.6	7.5
Crude fat (%)	2.4	3.0

It will be clear that the addition of 15 per cent whey powder IV constituted a marked contribution of whey protein to the total protein, whereas the lactose content of the diet increased only to twice the value of the control feed. Although cow's milk contains lactose as the only source of carbohydrate for the young calf the percentage of lactose in the diet should not reach too high a level. ROJAS et al. (1948) report that doubling the lactose content of whole milk or

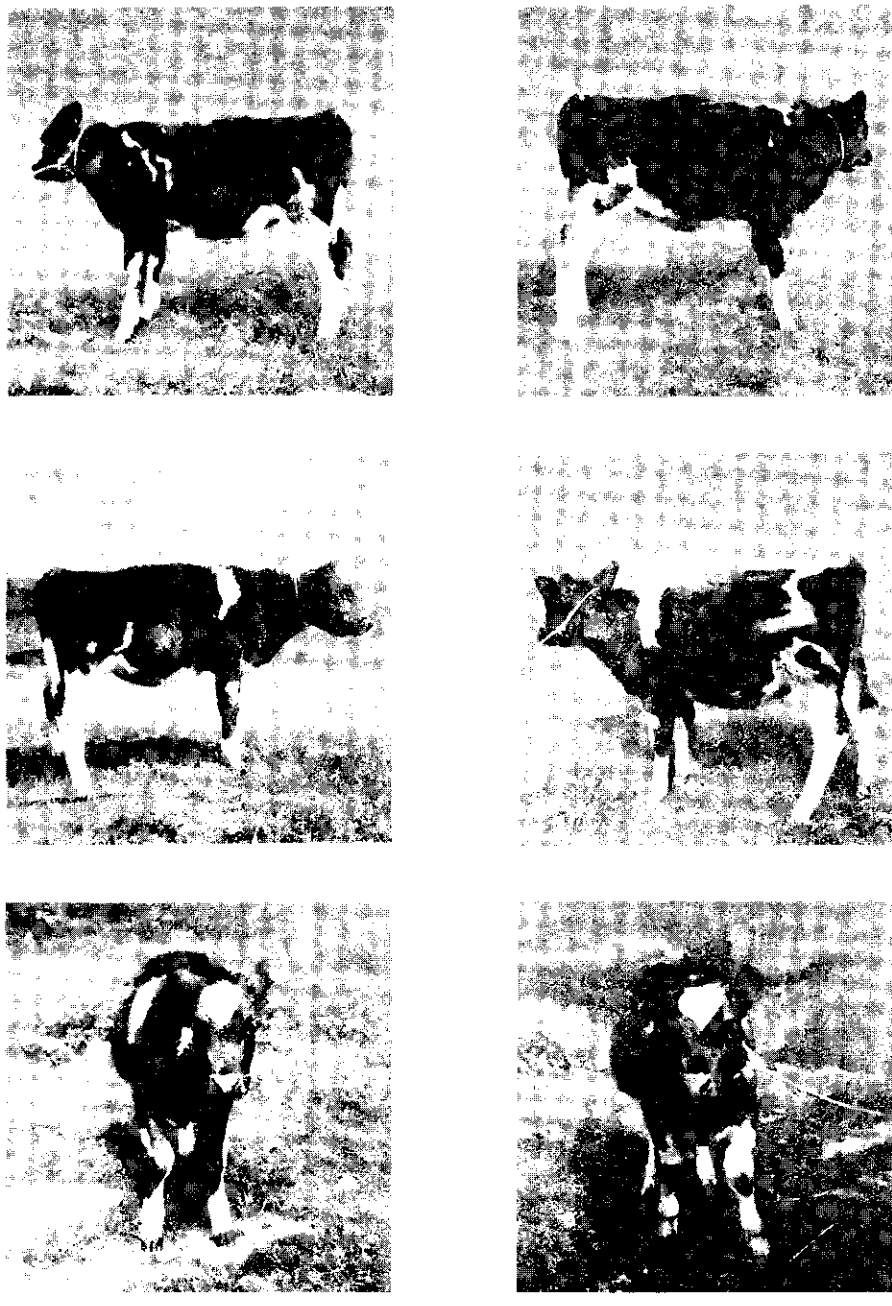

Fig. 52. Twin calves of the red and white "Meuse, Rhine and Ysel breed" Left: Test calf (A)
Pair A α Right: Control calf (α)
Age: 19 weeks

skim milk fed to calves results in an increase of the excretion of galactose in the faeces and in diarrhoea. Therefore the lactose contents of the calf starters should not be too high. There are several small differences between the protein and mineral contents of the two diets, although they were designed to yield equal values for these constituents.

The experiment was carried out on three sets of monozygotic twin calves, which will be denoted A α, B β and C γ respectively, the Roman capitals referring to the animals of the test group and the Greek symbols to the control calves. The pairs B β and C γ were black and white Friesian calves, whereas the pair A α was of the red and white "Meuse, Rhine and Ysel Breed". Six photographs of the calves A and α are shown in Fig. 52. The resemblance of the calves can be clearly seen from these pictures, although there are certainly some minor differences. The twins were examined by several experts and found to be materially identical. On the first day of the experiment (July 31st) the ages were 13, 22 and 23 weeks for the A α, B β and C γ calves respectively. The calves (A, B and C) were kept in one cow shed and the animals α, β and γ in another. They were fed in groups. Water was supplied ad libitum. The supply of mash and roughage is tabulated in the feeding scheme given in table 52.

TABLE 52. Feeding scheme of mash and roughage (Rations are given in kg per animal per day)

Weeks of the experiment	Calf starter meal	Hay	Fodder beets
0 - 7	2	2.4	-
7 - 12	2	4.8	-
12 - 13½	2½	4.8	-
13½ - 18½	3	4.0	5

Although it was decided that the supply of hay should be ad libitum, during the first period an insufficient amount was provided. This shortcoming in the care of the animals was corrected after seven weeks. The intake of hay increased immediately to about twice the previous amount. The consumption of hay was lower in the last weeks, due to the feeding of fodder beets and an increased amount of calf starter meal.

The growth curves of the calves are shown in Fig. 53. During the first 10 days the calves gradually became accustomed to the new fodder. In this period and especially in the next week remarkable increases in weight were observed. After that growth was very slight, probably as a result of the shortage of hay. After seven weeks the curves again become steeper. The utilisation of energy and protein of the calf's ration is influenced among other things by vitamin D, as has been pointed out by COLOVOS et al. (1951). To be sure that the diminished growth from the second up to the tenth week was not due to a vitamin deficiency, one of the sets (A α) received an extra amount of *"Dohyfral oil"* (vitamins A and D) during the latter part of the experiment. It appeared, however, that the growth of the other sets improved to the same extent following the increased supply of hay. The appearance of the animals was quite good although they were somewhat pot-bellied, a condition which seems to be unavoidable with calf starter feeding. It will be obvious from Fig. 53 that the diets do

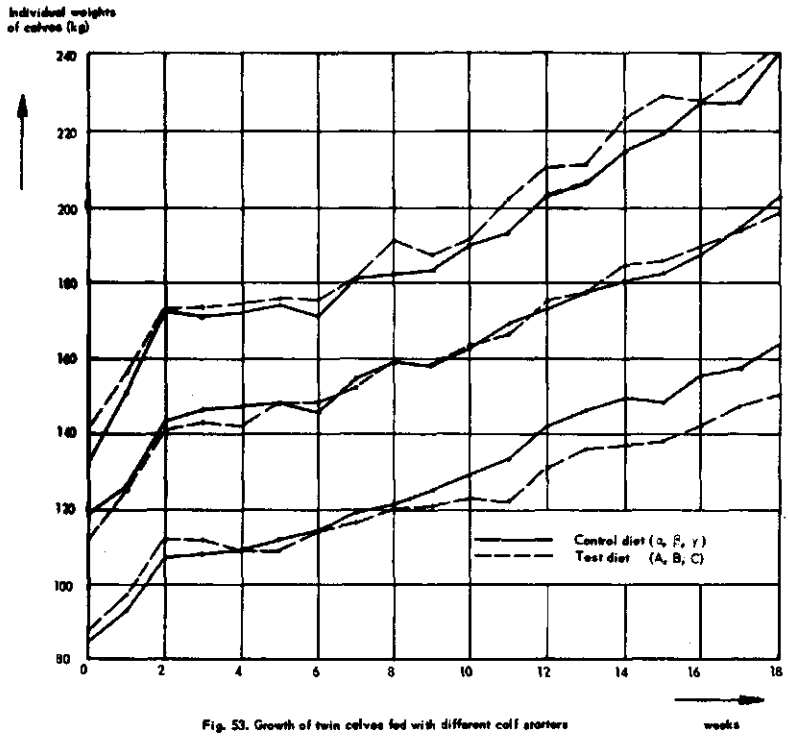

Fig. 53. Growth of twin calves fed with different calf starters

not differ as to their influence on the growth. Application of the method of m arrangements to the individual increases in weight of the six calves proved the probability of chance effects to be high (32 per cent) which means that the observed differences are not significant. The greatest difference between the total increases in weight during the experiment was observed in the case of the A α pair viz. 16 kg. By applying Student's t test to the differences between the weekly increases in weight of the A α calves it appeared that this value was not significant (probability of occurence by chance was 15 per cent).

This is caused by the large fluctuations of the weekly increases in weight of the calves around the mean values of each pair at each day. This may be shown by calculation of the mean standard deviations of the weekly increases in weight of each pair. The equation used was:

$$s^2 = \frac{\sum_i \frac{\sum_i (x_{ij} - \bar{x}_i)^2}{k - 1}}{n}$$

in which x_{ij} represents the increase in weight of calf j during the week i, $\bar{x}_i$ the mean increase in weight of the two calves in this week, k the number of calves (2) and n the number of weeks (18).

The values found for the three pairs were:

A α 1.85 kg

B β 2.41 kg

C γ 3.65 kg

The same quantity was calculated for the results of one group of another experiment performed at the Zootechnical Institute. The data concerned were those from a control group of three calves, which was fed the same diet as our control diet, at approximately the same age. In this case the standard deviation was found to have the value 2.74 kg. Apparently the fluctuations between the twins of one set are of the same order of magnitude as those within the set of three arbitrary calves. From a theoretical point of view this might be ascribed to the difference between the diets. In practice this is highly improbable as no special fluctuations in appetite or digestion were observed. Hence with respect to the weekly increases in weight we have to conclude that the twin calves did not behave more homogeneously than an arbitrary set of comparable calves.

The average total increase in weight during the experiment was 83.0 kg for the calves of the test group and 89.7 kg for those of the control group. The amounts of feed used in this period by each calf were:

Calf starter meal	300 kg
Hay	473 kg
Fodder beets	175 kg

Hence the consumption of calf starter meal per kg of growth was:

Test group 3.61 kg/kg

Control group 3.34 kg/kg

Of the consumption of crude protein per kg of growth, only a very rough estimation can be made as the amounts of hay were not weighed accurately. The crude protein content of the hay was estimated in seven samples taken at different intervals. The values found ranged between 6 and 8 per cent with an average of 7.1 per cent. The protein content of the beets was taken to be 1.3 per cent. With the use of these data the following values for the crude protein consumption per kg of growth were obtained:

Test group 1.10 kg/kg

Control group 0.98 kg/kg

These values are fairly high compared with the usual values (0.6 - 0.8 kg/kg). This is doubtless due to the diminished growth during the week of insufficient hay supply. The differences between test and control calves are chiefly due to the corresponding differences in total growth. As the latter differences are not significant (at least, it cannot be proved that they are) the same has to be said for the differences in food utilisation.

In conclusion we can say that no significant influence of the diets on growth rate has been proved. This means that approximately 30 per cent of the protein in the calf starter can be supplied in the form of whey protein in the absence of any other source of animal protein. In view of this result the possibility may be suggested of saving fodder constituents with high protein content (blood meal, fish meal, tankage, soybean oil meal) by the use of whey protein concentrates, obtained by removal of minerals and lactose from condensed whey.

5. FEEDING EXPERIMENT WITH PIGS

Feeding of skim milk and milk whey is common practice in the fattening of pigs. It is even possible to replace the greater part of skim milk by milk whey, as has been reported by KRISTENSEN (1951). In the absence of milk the supply of 10 per cent of the dry fodder in the form of fish meal or tankage seems to be necessary as a source of animal protein. GODDEN (1949) reports that this value may be lowered to 7 per cent, together with a lowering of the total content of digestible crude protein. According to DE MAN (1950) 50 per cent of the animal protein in the normal rations can be substituted by whey protein in the form of condensed whey. Feeding of greater amounts of condensed whey is unprofitable. In our opinion this might be due to the unbalanced composition of the whey rather than to the feeding of whey proteins as such. This question could be examined with the help of whey products with increased protein contents. For this purpose the experiment, described hereafter, was designed.

Six groups, each containing seven pigs, were fed on six different diets. The control diet (group I) contained 10 per cent fish meal in the first period and 10 per cent tankage in the last period of the experiment, which was necessary with a view to the quality of the meat of the pigs. For the groups II and III diets were designed in which 60 and 75 per cent of the animal protein was supplied in the form of dried normal whey viz. whey powder VI. This product has been described extensively in II.9. The groups IV, V and VI received diets in which 60, 75 and 100 per cent of the animal protein was substituted by whey protein in the form of whey powder V (see II.9.). This was a whey powder of high protein content, as approximately one third of the salts and the lactose of the original whey had been removed by electrodialytic desalting and centrifuging respectively. The complete analysis of these powders can be found in tables 30 and 31 in section II.9. Two sets of similar diets were calculated, one based on the control diet with fish meal and one on the control diet with tankage. The compositions and analyses of the diets are given in tables 53, 54, 55 and 56.

TABLE 53. Compositions of diets supplied to the young pigs (first period)

	Groups					
	I	II	III	IV	V	VI
Ground corn	24	10	7.5	24	23.6	24
Ground barley	50	35.7	30.8	43.5	41	39.1
Grass meal	5	5	5	5	5	5
Wheat bran	10	10	10	10	10	10
Fish meal	10	4	2.5	4	2.5	-
Whey powder VI	-	29.7	37.1	-	-	-
Whey powder V	-	-	-	11.2	15.2	18.6
Sunflower seed without hulls (extracted)	-	5.6	7.1	1.3	1.7	2.3
Minerals	1.0	-	-	1.0	1.0	1.5
Total	100	100	100	100	100	100

TABLE 54. Compositions of diets supplied to the older pigs (second period)

	Groups					
	I	II	III	IV	V	VI
Ground corn	20	9	6.5	20	20	20
Ground barley	22.5	22.5	20	16.5	18.4	20.2
Ground rye grain	23.5	20	17.1	24	22.2	19.3
Cassava meal	12.5	-	-	8.5	7	4.7
Grass meal	5	5	5	5	5	5
Coconut oil meal	5	5	5	5	5	5
Tankage	10	4	2.5	4	2.5	-
Whey powder VI	-	30.7	38.4	-	-	-
Whey powder V	-	-	-	11.5	14.4	19.3
Sunflower seed without hulls (extracted)	-	2.8	4.5	4	4	5
Minerals	1.5	1.0	1.0	1.5	1.5	1.5
Total	100	100	100	100	100	100

TABLE 55. Analyses of diets supplied to the young pigs (first period)

	Groups					
	I	II	III	IV	V	VI
% substitution of animal protein by whey protein	-	60	75	60	75	100
Whey powder V (%)	-	-	-	11.2	15.2	18.6
Whey powder VI (%)	-	29.7	37.1	-	-	-
Whey protein (% of total protein)	-	25.7	31.9	23.4	32.2	39.4
Crude protein (%)	15.8	14.5	14.6	14.7	14.5	14.5
Digestible crude protein (%)	14.4	13.8	13.8	13.8	14.0	13.7
Lysine (%)	0.85	0.81	0.80	0.84	0.86	0.83
Methionine (%)	0.29	0.32	0.31	0.29	0.29	0.29
Crude fibre (%)	6.3	5.6	5.7	5.4	5.3	5.1
Crude fat (%)	3.2	2.4	2.2	3.1	3.0	2.8
Starch value	69	70	71	69	69	69
Lactose (%)	-	22.2	27.8	6.3	8.5	10.4
Minerals (%)	5.6	6.0	6.2	5.3	5.4	5.2
Sodium chloride (%)	0.48	1.12	1.22	0.48	0.52	0.59
Calcium (%)	1.12	0.71	0.65	0.73	0.70	0.62
Phosphorus (%)	0.72	0.76	0.72	0.67	0.60	0.56

TABLE 56. Analyses of the diets supplied to the older pigs (second period)

	Groups					
	I	II	III	IV	V	VI
% substitution of animal protein by whey protein	-	60	75	60	75	100
Whey powder V (%)	-	-	-	11.5	14.4	19.3
Whey powder VI (%)	-	30.7	38.4	-	-	-
Whey protein (% of total protein)	-	28.6	35.7	25.2	32.3	43.9
Crude protein (%)	14.3	13.5	13.5	14.0	13.7	13.5
Digestible crude protein (%)	12.7	12.6	13.1	12.7	12.6	12.7
Lysine (%)	0.70	0.74	0.76	0.82	0.80	0.83
Methionine (%)	0.23	0.25	0.27	0.26	0.25	0.26
Crude fibre (%)	4.3	4.2	4.2	4.3	4.0	4.0
Crude fat (%)	3.3	2.6	2.4	2.8	2.6	2.6
Starch value	71	72	73	71	71	71
Lactose (%)	-	23.1	28.8	6.4	8.1	10.8
Minerals (%)	4.9	6.0	5.9	4.5	4.8	5.0
Sodium chloride (%)	1.00	1.36	1.65	0.72	0.67	0.64
Calcium (%)	0.80	0.73	0.70	0.60	0.64	0.58
Phosphorus (%)	0.51	0.58	0.58	0.49	0.50	0.48

The "minerals" were a standard mixture, generally used at "De Schothorst" and composed of chalk, bone meal, NaCl, KI, $FeSO_4$, $CuSO_4$ and $MnSO_4$. This mixture is described by GRASHUIS and VAN VLIET (1950) in their manual on the feeding of pig and swine. In the cases where the mineral mixture was left out the minor elements were supplied to the diets separately. In addition the rations were provided with a sufficient amount of Dohyfral oil (vitamins A and D).

In tables 55 and 56 the special possibilities of whey products like whey powder V as compared with normal whey powder are clearly expressed. Considerable substitution of animal protein by whey protein is easily accomplished by a relatively small amount of whey powder V, and does not involve high lactose or ash contents. In the case of normal whey powder VI the values of the ash contents cannot be kept on the same level and the contents of lactose become considerable.

The lysine and methionine contents of the various diets are almost equal. They are somewhat lower than the values suggested by SHELTON et al. (1951, 1951 a) for the requirements of weanling pigs. They report 1.0 per cent lysine and 0.3 per cent methionine (in the presence of 0.3 per cent cystine) to be the minimum values. The control diets I are recommended by the Institute for Modern Live-stock Feeding "De Schothorst" and have found general application. As fas as we know it has never appeared that these diets were deficient in any respect.

The animals were obtained from seven litters, each composed of six pigs. They were distributed in groups in such a way that each group contained one animal of each litter. In addition the sexes were evenly distributed so that the groups contained either three males and four females or three females and four males. On the day of arrival at the Institute they were approximately 10 weeks old and their weights averaged 21 kg. For two weeks the control diet I (tables 53 and 55) was fed to all the animals. After that they were weighed again and the groups separated. Apart from the considerations mentioned above the groups were designed in such a way as to make the total weights of each group

almost equal, not only at the first but also at the second weighing (146 and 180 kg respectively). From then onwards the six different diets described in tables 53 and 55 were fed (first period). After twelve weeks, when the weights of the pigs averaged approximately 65 kg, the diets described in tables 54 and 56 were fed until slaughter weight (90 kg) was reached (second period). Most of the animals reached this weight four weeks later i.e. after 16 weeks of feeding. Weight recordings were made every two weeks. No animals died, nor did any special case of disease occur. The average weights of the pigs of each group have been plotted in Fig. 54.

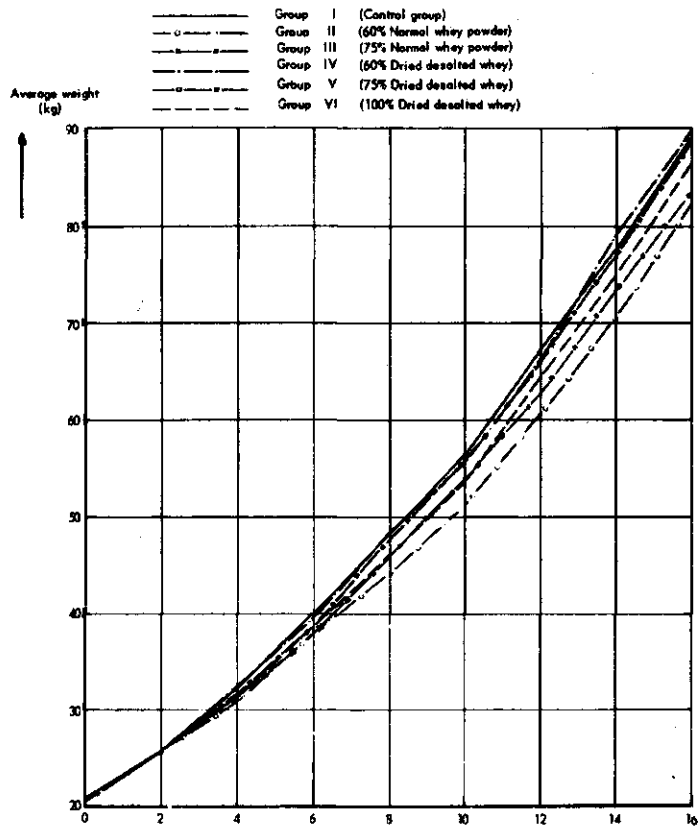

Fig. 54. Average weights of pigs of each group

It will be clear that the differences between the groups are small. Therefore in fig. 55 the same data are plotted, expressed as percentages of the values of the control group at each date.

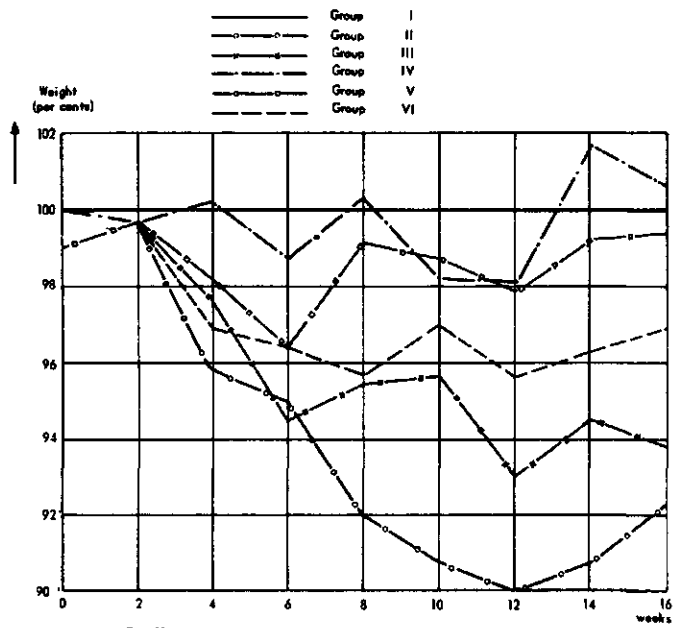

Fig. 55. Average weights of pigs of each group, expressed as percentages of the weights of the control group

The differences are now more pronounced. Apparently Groups II and III (normal whey powder) lag behind, while Group IV (60% substitution by desalted whey powder) appears to grow as much as the control group, and the pigs of Group V (75% substitution) only a little less. Group VI (100% substitution) held an intermediate position. The retardation of Groups V and VI is due chiefly to lower growth during the first four weeks. From then onwards the growth parallels that of the control group.

When the method of m arrangements was applied to the weekly increases in weight of the six groups, the results were not significant, although a rather low probability value was found (9 per cent). This was an indication that some significant influence might be present although it was not yet clearly detected.

Therefore the following grouping was examined:

A	Group I	(Control group)
B	Groups II + III	(Normal whey powder, 60 and 75%)
C	Groups IV + V	(Desalted whey powder, 60 and 75%)
D	Group VI	(Desalted whey powder, 100%)

When the increases in weight of these four groups were examined with the help of the method of m arrangements the probability value amounted to only 0.6 per cent. This was clearly due to group B (normal whey powder) which on an average showed the lowest increases in weight. No significant differences between the other groups could be proved. Hence we can conclude that retardation of growth of pigs to which any form of whey is fed is due to the accompanying constituents of the whey, but not to the protein. The whey proteins themselves are the equal of any other source of animal protein.

The data on feed consumption and utilisation per group are given in table 57.

TABLE 57. Feed consumption and utilisation per group of pigs

	Groups					
	I	II	III	IV	V	VI
Kilogrammes consumed:						
Mash I (First period)	944	903	903	944	944	944
Mash II (Second period)	790	935	788	790	790	790
Total	1734	1838	1791	1734	1734	1734
Total growth (kg)	464.5	447	429	473.5	465	441
kg mash/kg growth	3.73	4.11	4.17	3.66	3.73	3.93
kg crude protein /kg growth	0.564	0.575	0.555	0.527	0.527	0.527
Average growth per animal per day (kg)	0.64	0.58	0.59	0.65	0.64	0.61

Although the differences are not great it is clear that the Groups II and III are slightly inferior to the other groups. The differences between Groups II and III or between Groups I, IV, V and VI are very small and often have opposite signs with respect to different criteria so that we cannot consider them to be significant.

Finally all the animals were transported to the abattoir and after one day's fasting they were weighed and slaughtered. The Dutch Commission of Superintendance of Testing Stations has kindly judged the slaughtered animals and we herewith express our thanks to the Commission for this contribution. The results of their judgements were averaged per group and the averages are summarized in table 58.

TABLE 58. Average results of judgment of the slaughtered animals

	Groups					
	I	II	III	IV	V	VI
Final living weight (kg)	86.3	86.3	83.0	89.1	88.6	85.0
Slaughter weight (kg)	67.0	66.7	62.4	67.7	67.0	64.3
Slaughter loss (%)	22	23	25	24	24	24
Number of animals in Class I	6	6	6	5	7	7
Number of animals in Class II	1	1	0	1	0	0
Number of animals in Class III	0	0	1	1	0	0
Trunk length (cm)	79	79	77	79	79	79
Thickness of shoulder bacon (cm)	4.6	4.5	4.3	4.6	4.4	4.4
Thickness of dorsal bacon (cm)	2.5	2.5	2.4	2.6	2.5	2.6
Thickness of lumbar bacon (cm)	3.5	3.5	3.4	3.5	3.5	3.5
Judgment in points: *)						
Length	8	8	6	8	7	8
Firmness of bacon	7	8	7	7	8	7
Fleshedness	6	6	6	6	6	6
Shoulder	5	5	5	6	5	5
Thickness and distribution of dorsal bacon	6	6	7	6	7	6
Form and thickness of belly bacon	6	6	6	7	6	7
Form and thickness of hams	6	6	6	7	6	7
Fineness of skin	7	7	6	7	6	6
Heaviness of skeleton	7	7	8	7	7	7
Type	6	6	5	6	6	6

From this table it is obvious that no important differences were found in any respect. The different rates of growth are not reflected in the quality of the meat of the animals.

The results of this experiment lead to the following conclusion and summary. Whey, from which the water, two thirds of the salts and two thirds of the lactose are removed, forms an excellent replacement for fish meal or tankage in the fattening of pigs. Substitution of 60 or 75 per cent of the animal protein by this form of whey protein yielded exactly the same results as are obtained from a good standard diet. Substitution of all the animal protein produced a very small retardation of growth, which, however, could not be proved significant. Similar results could not be obtained with normal whey powder fed at levels of 65 and 75 per cent. Apparently the substitution may not exceed the limit of 50 per cent. This difference between normal and purified whey is only connected with growth for the slaughter quality of the meat of the pigs is the same in all cases. The retardation of growth by feeding high quantities of normal whey powder is not due to the whey proteins but it has to be ascribed to the accompanying salts and/or lactose. The whey proteins themselves are an excellent form of animal protein.

*) Scale: a = 10, ab = 8, b^+ = 7, b = 6, b^- = 5, bc = 4, c = 2.

6. SUMMARY

A description is given of several feeding experiments with various kinds of dried desalted and centrifuged whey. The intention was to test the products mentioned with regard to their usefulness for feeding purposes, especially for poultry, cows and pigs. In addition it was possible to compare whey proteins with other proteins.

In three preliminary trials with rats it appeared that the feeding of whey powder to rats is limited by the lactose level of the diet. At levels over 20 per cent retardation of growth and diarrhoea were observed. This was not prevented by an increased dosage of thiamin. A level of 62 per cent lactose caused early death. In the experiments with lactose levels under 20 per cent no retardation of growth was observed, whatever amounts of whey protein were provided.

With the whey powders used for the experiments on rats 30 per cent of the total protein in the diet could be substituted by whey protein without exceeding the 20 per cent lactose level. Addition of dried whey to the test diets was made chiefly at the expense of the amounts of corn, oats, fish meal, soybean oil meal, peanut oil meal and bran in the control diet. Hence these trials prove that whey protein is equivalent to the mixture of proteins in the fodder constituents mentioned above. In several cases an increased "sugar" content in the faeces was observed, as estimated by means of their reducing power. Metabolic breakdown of lactose to monosaccharides was accomplished in all cases at the same rate, as no differences between the groups were observed in the ratio of reducing power of the faeces before and after inversion.

In an experiment with chicks a diet in which 35 per cent of the protein was replaced by whey protein was compared with a diet containing vegetable proteins only and a diet which included animal protein (fish meal). The replacement was made chiefly at the expense of the amounts of corn, oats and soybean oil meal in both control diets and the addition of the fish meal to the animal protein diet. The experiment lasted nine weeks. After 4 weeks the chicks became accidentally infected by coccidiosis which makes the results of the next five weeks less reliable, although the relative position of the growth curves remained the same. The group to which a diet containing animal protein was fed showed the best results as to growth, general appearance and resistance to coccidiosis. No significant differences were observed between the groups to which diets containing whey protein or vegetable proteins were fed. The reducing power of the faeces of the whey group appeared to be higher than that in the other groups. The ratio of the reducing power before and after inversion was also different from that found with the other groups. This was ascribed to insufficient digestion of lactose, resulting in increased amounts of sugars in the faeces. It is suggested that the less favourable results obtained with the whey protein diet should not be considered to be due to the protein but to the lactose content of the diet, which was still fairly high (18 per cent).

Another experiment was carried out with three sets of twin calves aged 13 - 23 weeks. This trial lasted 4 months. The test calves received 30 per cent of the total protein in the diets in the form of whey protein. This was accomplished chiefly at the expense of the animal protein (fish and blood meal) and of the amounts of oats, barley and corn in the control diet. The fodder was supplied in the form of calf starter mash. In addition the calves received hay

and fodder beets. No significant differences were observed either in growth or in general appearance of the calves. The consumption and utilisation of feed were rather unfavourable. This was ascribed to insufficient supply of roughage during part of the experiment.

In a feeding trial on pigs six different diets were prepared. The control diet (I) contained 10 per cent fish meal during the first period and 10 per cent tankage during the last weeks before the animals were slaughtered. This was the only source of animal protein. In two other diets (II and III) 60 and 75 per cent of the animal protein was replaced by whey protein which was supplied in the form of dried normal whey. The three remaining groups (IV, V, VI) received amounts of whey protein, corresponding to 60, 75 and 100 per cent respectively of the animal protein in the diets. This whey protein was supplied in the form of dried desalted centrifuged whey. The growth of the pigs of Groups II and III was less than that of the other groups. The experiment was carried on until slaughter weight was reached. The result of judgements of the slaughtered animals was that no influence of the different diets upon the quality of the meat could be stated. The animals of all groups gained approximately the same marks. As to growth and feed consumption, however, the pigs to which normal whey powder was fed were inferior to those of the other groups. This backwardness is obviously caused by the accompanying constituents of normal whey viz. lactose and/or the salts. Hence it was concluded that the whey proteins themselves are an excellent form of animal protein.

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SUMMARY

This communication forms part of an extensive research programme for new methods of utilizing milk whey and improving its properties. This programme is sponsored by the General Technical Department of the Central National Council for Applied Scientific Research in the Netherlands (T.N.O.). In this communication a special study of the proteins of milk whey has been made. The study was carried out at the Laboratory of Veterinary Biochemistry of the State University of Utrecht under supervision of Dr L. Seekles, Professor of Veterinary Biochemistry at the University.

In Part I (Introduction) the so-called whey problem is reviewed first. Then the working method, developed by the General Technical Department T.N.O. is treated, a method which consists of removing the salts by electrodialysis and the lactose by centrifugal force. Some peculiarities observed during this process are mentioned and a formulation of the questions, to be treated in this communication is given.

In Part II (Chemical Researches) the whey proteins are reviewed as a chemical problem. Attention is given chiefly to examination of the proteins by means of electrophoresis. A review of the literature is given. The nitrogen distribution in whey is treated and the way it depends on stage of lactation, on the method of precipitating the casein and other influences is described. The theory of electrophoresis is studied and a special method of interpretation of the patterns is developed. A great many electrophoretic patterns of several kinds of fresh and processed whey are recorded. The individual protein constituents are identified and reviewed. The influences of heat treatment and of electrodialytic desalting are discussed. A comparison of bovine milk whey and human milk whey (fresh and lyophilized) is given. Finally ultracentrifugal patterns of many of the materials studied by means of electrophoresis are recorded and discussed.

In Part III (Feeding experiments) a description is given of various feeding trials with the whey powders described in part II. After three preliminary trials with rats, experiments were carried out on chicks, calves and pigs. It appeared that these powders were fairly suitable for use in calf starters and fodders for pigs and it has been concluded that it will be possible to compose diets applicable to poultry breeding. Feeding of whey or whey powder has certain drawbacks, which are not connected with the whey proteins which appeared to be of high biological value.

More detailed summaries of the chemical researches and the feeding trials are given at the end of Part II and Part III respectively.

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