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Studies in Physico-Chemical Properties of Caseins
Part I : *p*H-Titration Curves

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The *p*H-titration curves of caseins isolated from milk by different methods, using NaOH, $Ba(OH)_2$, NH_4OH and HCl as the titrants, have been obtained. The caseins isolated by the rennet and ultracentrifuge methods, being the original calciumcaseinate-phosphate complexes, have higher initial *p*H values and consume less alkalies and more acids than those isolated at their isoelectric points by the addition of acids (acid caseins). The amount of ammonia taken up by casein is more than that of other alkalies, probably due to hydrogen bonding with some of the functional groups. The acid caseins behave as dibasic acids like the constituent amino acids. The heats of ionisation at different *p*H values have been determined by using van't Hoff equation and necessary information regarding *p*H range in which various acidic groups ionise has been obtained. This permits the determination of the various ionisable groups in caseins.

A good deal of data on *p*H-titration curves of proteins of different kinds with acids and bases have been reported in recent years [1–4]. These curves have been useful in determining acid- and alkali-binding capacities of proteins as well as in estimating the number of various ionisable groups. Similar work on caseins has not been reported in as much details. Moreover, the effect of the method used for the isolation of casein from milk of different species on the amount of acidity or alkalinity and the number of ionisable groups has not received adequate attention. The present work was, therefore, undertaken.

EXPERIMENTAL

Materials and Methods:

One sample each from cow milk and buffalo milk was prepared by each one of the five methods described below. Thus 10 samples of casein in all were obtained.

(i) *Rennet method*: One litre milk was heated to 35-37° and enough of rennet (E. Merck 1 : 100,000) was added to cause coagulation in about half-an-hour. The curd was broken into small lumps by stirring mechanically after which the whey was separated by filtration and the mass was washed in a blender first with water and then with acetone to remove free water and finally with ether to remove acetone. It was finally dried over sulphuric acid in a vacuum desiccator.

(ii) *Ultracentrifuge method*: Sixty ml skim milk, taken in 6 tubes, were whirled in an ultra-centrifuge @30,000 r.p.m. for a period of 30 mins. The residue was washed first with

1. Esther Breslow and Frank R. N. Gurd, *J. Biol. Chem.*, 1962, **237**, 71.
2. A. Wishnia, I Weber and R. C. Warner, *J. Amer. Chem. Soc.*, 1961, **83**, 2071.
3. N. J. Hipp, M. L. Groves and T. L. McMeekin, *J. Amer. Chem. Soc.*, 1952, **74**, 4822.
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water and then with acetone and ether as in the above method and finally dried over sulphuric acid.

(iii) *Hammarsten method* : One litre skim milk diluted with 4 litres water was stirred mechanically in a glass trough while dilute acetic acid was added slowly through a dropping funnel till casein precipitated out at its isoelectric *p*H value of 4.6 The precipitate was dissolved in ammonia and reprecipitated on the addition of dilute acetic acid. The mass was washed and dried as above.

(iv) *Zoller method*: The procedure was essentially the same as in the Hammarsten method except that an aqueous solution containing 5 per cent hydrochloric and 5 per cent citric acid was used as the precipitant. The casein was dissolved in excess of dilute HCl to bring down its *p*H value to 3 and was precipitated on raising the *p*H value to 4.6 on the addition of ammonia.

(v) *Van Slyke and Bosworth method*: The procedure initially was the same as in the Hammarsten method. The casein was then dissolved in ammonia and sufficient ammonium oxalate was added to precipitate calcium. Casein was reprecipitated from the solution on the addition of dilute hydrochloric acid to bring down the *p*H value to about 4.6.

pH-titration curves: 0.25 g portions of caseins were taken in 50 ml pyrex bottles and increasing amounts of 0.1 *N* NaOH, $Ba(OH)_2$ or NH_4OH were added, making the volume to 25 ml in every case by the addition of 0.1 *N* NaCl, $BaCl_2$ or NH_4Cl so that the concentration of cation was 0.1 *N* in every case. 0.25 g portions were also mixed with increasing amounts of 0.1*N* HCl and the volume was made to 25 ml in every case by the addition of 0.1 *N* NaCl. The suspensions or solutions were allowed to stand at 30° ±(0.1°) for 48 hr. with occasional shaking after which *p*H values were determined by using glass electrode in conjunction with calomel electrode. The reversibility of the complete titration curve (plotted on the addition of acid on one side and on the addition of alkali on the other) was checked by starting with casein solution in HCl at *p*H 2.5 and adding increasing amounts of NaOH.

RESULTS AND DISSCUSSION

The caseins prepared by the rennet and ultracentrifuge methods were, more or less, in their natural states and, being calcium-caseinate-phosphate complexes, their initial *p*H values (table II) were much higher than those of the rest of the caseins precipitated at about their isoelectric points on the addition of acids.

The titration curves of the various samples of cow's caseins with different bases are plotted in figs. 1-5. The curves for buffalo's caseins were similar and have not been given for reasons of space.

It is seen that the characteristic shape of the curve with each base is maintained in every sample. The points of equivalence are quite sharp and are seen to lie between *p*H 8.1 and 9.0 with NaOH and $Ba(OH)_2$ and between *p*H 7.7 and 8.3 with ammonia. The amounts of the various alkalies neutralised at the points of equivalence are given in table I. It is seen, in the first instance, that these values are relatively low in the case of

rennet and ultracentrifuge caseins and appreciably higher in the rest of the caseins. It is also seen that while the amounts of sodium hydroxide and barium hydroxide neutralised by a given sample are fairly close to one another, those of ammonium hydroxide are appreciably higher. It appears that apart from neutralisation ammonia interacts with casein in a different manner and probably involves in hydrogen bonding. This aspect needs further investigation. It is well known that casein undergoes swelling much more in ammonia than in any other alkali.

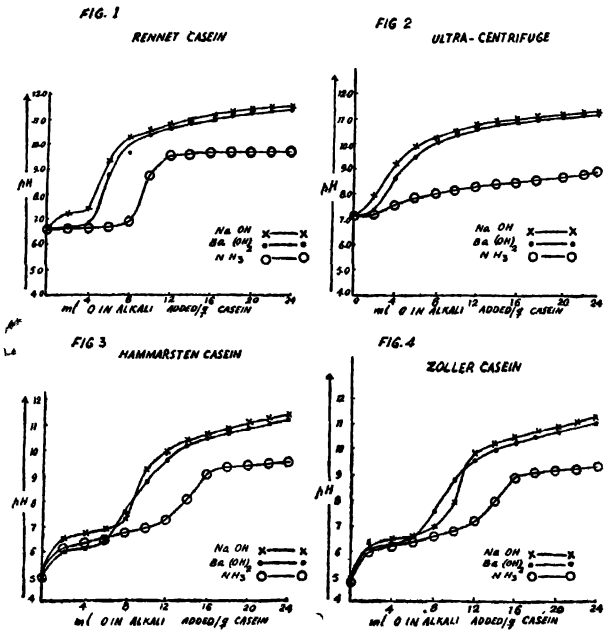


Fig. 1-4 *p*H titration curves of different samples of cow's casein with different alkalies.

It is also interesting to note that the values for cow's caseins are invariably higher than those for buffalo's caseins.

The titration curves on the addition of NaOH, on the one hand, and on the addition of HCl on the other, starting in each case from the respective isoionic point, are plotted in fig 6 for the various samples of cow's caseins. The curves for the buffalo's caseins had similar features. The caseins prepared by the rennent and ultracentrifuge methods are seen to stand out in a separate category from the rest.

The points of equivalence with HCl, though less sharp than those with NaOH, can neverthelsss be located. The acid values, corresponding to these points, are included in table I. The rennet and ultra centrifuge caseins show higher values than the rest. The values for cow's caseins are again slightly higher than those for buffalo's caseins.

The back-titration curves starting with acid solutions at the lowest *p*H values and adding increasing amounts of NaOH were found to be almost identical with those plotted in fig. 6.

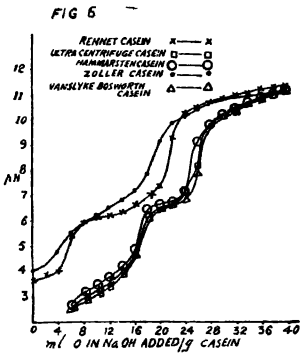


Fig. 6. Titration curve of cow's casein both in acidic and alkaline range.

Since in the case of the three acid caseins (Hammarsten, Zoller and Van Slyke caseins) there are two distinct points of inflection, one in the acid and the other in the alkaline range and the titre values corresponding to these points of equivalence are quite close to one another (table I), it appears that these caseins, like the constituent amino acids, behave

TABLE I

Base and acid binding capacities of caseins as obtained from titration curves

Method of preparation	Base binding capacity (m. eq./100 g.)						Acid binding capacity (m.eq./100 g.)	
	Cows' casein			Buffalo's casein			Cows' casein	Buffalos' casein
	NaOH	$Ba(OH)_2$	NH_3	NaOH	$Ba(OH)_2$	NH_3	HCl	
Rennet	66	68	84	52	62	68	124	116
Ultra-centrifuge	32	31	100	30	30	88	172	170
Hammarsten	96	96	136	90	86	100	100	100
Zoller	108	104	144	100	98	132	104	98
Van Slyke-Bosworth	100	100	106	90	96	96	102	100

as dibasic acids; the two acidic groups being 'carboxyl' and 'amino' groups. The quotation marks indicate that these terms include other allied groups as well. The pK_1 and pK_2 values of these acid groups, corresponding to pH values at half neutralisations, are given in table II.

TABLE II

pk_1 and pk_2 values of acid caseins obtained from their titration curves

Method of preparation	Cows' casein				Buffalos casein			
	Initial pH	pK_1	pK_2	pHiso	Initial pH	pK_1	pK_2	pHiso
Hammarsten	5.0	3.50	6.70	5.10	4.8	3.30	6.75	5.03
Zoller	4.7	2.85	6.55	4.70	4.8	2.60	6.85	4.73
Van Slyke-Bosworth	4.8	2.80	6.65	4.73	5.1	2.80	7.10	4.95

The accuracy of these values was checked by calculating isoionic pH values from the equation

$$p\mathrm{H}_{iso} = 1/2\ pK_1 + 1/2\ pK_2,$$

neglecting the activity coefficient terms of the unneutralised groups. These values are given in table II and are seen to be quite close to the *p*H values at which these caseins were isolated (column 1).

Knowing the values of pK_1 and pK_2, it is also possible to calculate equivalent weights of caseins using the following equations applicable at *p*H values lying below and above the isoionic point respectively.

$$pK_1 = pH - \log\ (C/a - 1), \text{ and}$$
$$pK_2 = pH + \log\ (C/b - 1)$$

where the solution is made up of C equivalents of casein and a equivalents of HCl in the first equation and b equivalents of NaOH in the second equation. Knowing that 0.25 g casein had been dissolved in 25 ml solution, the equivalent weights could be easily calculated. These values for the three acid caseins are given in Table III and are seen to be of the order expected for proteins. Moreoever, both the equations give values fairly close to one another, considering the approximations in deriving the above equations.

Number of ionisable groups: Titration curves of proteins have been used[2] for determining the number of various ionisable groups on the basis that different groups have different dissociation constants and therefore ionise in different regions of *p*H values. Information regarding the *p*H range in which these groups ionise can be obtained from heats of ionisation at different *p*H values. This method has been used by Wyman[5] in the case of oxyhemoglobin of the horse. But since casein unlike oxyhemoglobin contains phosphoric acid groups as well, we can not make use of the information collected by him. It was considered necessary, therefore, to determine heats of ionisation at different *p*H values of the three acid caseins used in these investigations. This was done by determining titration curves at two different temperatures (30° and 40°) and by applying van't Hoff equation

$$Q = 4.579\ T_1T_2\ (pH_2 - pH_1)/T_2\text{-}T_1$$

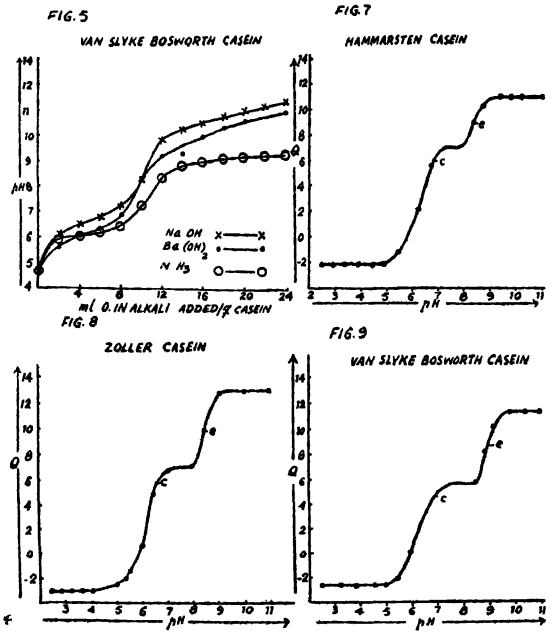
where pH_2 and pH_1 are *p*H values at temperatures T_2 and T_1 respectively.

The values so obtained are plotted in figs. 7, 8, 9 for the three acid caseins where Q is expressed in Kcals. It is seen that in the *p*H range 2.5 to 11.0, the various ionisable groups fall into three main classes, corresponding to three plateaus, in each acid casein. The groups of class I have an average apparent heat of dissociation of about -2000 to -3000 calories and are active in the acid range of titration curve between between *p*H 2.5 and 5.0. The group of class II have an average apparent heat of dissociation of about +6500 to +7000 calories and are active in the middle range viz., *p*H 7 and 8. The groups of class III have an average apparent heat of dissociation above + 10,000 calories and are active in the alkaline range of the titration curve between *p*H 9 and 11.

These three plateaus are separated by two transition regions whose mid-points lie close to *p*H 6.5 and 8.4 respectively. The left-hand transition is completed in about 2 or 2.5 *p*H units while the right-hand transition is completed in about 1 *p*H unit .

The acids of class I are certainly carboxyl and phosphoric (first ionisation) groups which are known to have a small heat of dissociation, never more than $\pm$2,000 or 3,000 calories. These may be considered to be neutralised in the *p*H range starting with the initial *p*H value ($\sim$2.5) to the *p*H at the mid-point of the first transition (*p*H$\sim$6.5). It may

be mentioned that Hipp *et al*[1] on the basis of *p*K values of these groups have also suggested that carboxyl groups and first equivalent of phosphoric acid groups are neutralised in the *p*H range 2.5—6.5.



Figs. 7-9 Heats of ionization of different samples of cow's casein at different *p*H values.

The acids of class II should be imidazole and phosphoric acid (second ionisation) groups. The *p*K value for second ionisation of phosphoric acid (7.2) is very close to that of imidazole group which has been shown (7,8) to lie between 6.0 and 6.7. Therefore, both these groups are expected to have about the same value for heat of ionisation and to be neutralised in the same *p*H range. This range may be taken to lie between the midpoint of the first transition about (*p*H 6.5) to the mid-point of the second transition about (*p*H 8.4). It may be noted that the heat of ionisation of imidazole groups in oxyhemoglobin protein was reported[5] to be about 6200 calories which is very close to our value corresponding to the second plateau in the caseins.

It is also seen that before the approach of the second plateau, there is a definite trend towards a decrease in the slope of the curve as, for example, indicated by the point *c*. We are of the opinion that the imidazole groups, which are relatively stronger acids, are neutralised at this point and that the phosphate (second ionisation) groups are neutralised between the points *c* and *e*. The *p*H values at the point *c* are 6.7, 6.65, 6.7 for the three caseins. It looks probable that the imidazole groups are neutralised in the *p*H range

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6. E. J. Cohn, *Ergel Physiol.*, 1931, **33**, 781.
7. Cohn and Edsill, "Proteins, Amino acids and Peptides", Reinhold Publishing Corporation, New York, 1943, p. 85.
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lying between *p*H 6.5 and 6.7, whereas the second equivalent of phosphoric acid is neutralised between *p*H 6.7 and 8.4.

The rest of the groups left unneutralised at *p*H 8.4 are α-amino, ϵ-amino, phenolic, sulphhydryl and guanidyl groups. These are evidently neutralised in the third and final stage commencing from *p*H 8.4 and going right up to the point the titration could be carried out.

TABLE III

Equivalent weights of acid caseins obtained from their titration curves

	Cow's casein		Buffalo's casein	
Method of preparation	*p*H 4.0	*p*H 6.7	*p*H 4.0	*p*H 6.7
Hammersten	7874	8030	7905	7960
Zoller	9309	9350	9380	9410
Van Slyke-Bosworth	9901	9930	9962	9985

The *p*H ranges in which the various groups are neutralised on the above considerations are summed up below :

*p*H range	Groups neutralised
2 — 6.5	Carboxyl+1. eq. of phosphate
6.5-6.7	Imidazole
6.7-8.4	1 eq. of phosphate
8.4-11.0	α-amino, ϵ-amino, phenolic and sulphydryl.

TABLE IV

Equivalents of various ionic groups present in acid caseins

	Amounts of groups present eq/10^5 g. of casein				
Method of preparation	Carboxyl	Phosphate	Imidazole	α-Amino, ϵ Amino, guanidyl, sulphydryl and phenolic	Total
			Cow's casein		
Hammarsten	93	33	21	130	277
Zoller	101	30	22	133	286
Van Slyke Bosworth	103	29	21	127	280
Analytical values (Gordon)	99	29	20	125	273
			Buffalo's casein		
Hammersten	94	30	20	125	269
Zoller	101	29	21	120	271
Van Slyke Bosworth	102	28	21	115	266

The number of the various acidic groups contained in the three acid caseins, as obtained from the titration curves on the above considerations, are recorded in table IV. The values obtained by Gordon[9] *et al.* from direct chemical analysis of casein isolated from cow milk on the addition of HCl are also included in the table for ready reference. The agreement is fairly close to justify the use of titration curves for the estimation of various ionisable groups in caseins.

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