SPREADING OF CASEIN AND DERIVATIVES*

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DURING recent years there has been a great revival of interest in surface films, in particular those of proteins. The surface behaviour of casein is of special interest since it can be studied both by the classical spreading method[4,5,6,8] and the new surface ageing technique developed by Doss[2] and independently by McBain and Wilson.[12] In the present work the spreading of casein and some of its derivatives on aqueous substrates has been investigated.

EXPERIMENTAL

1. *Preparation of materials.*—In some of the experiments casein (Hammersten) obtained from Kahlbaum was used. Isodisperse casein obtained by the method of Svedberg, Carpenter and Carpenter[13] was also studied. It was prepared as follows, Hammersten casein was extracted at 40° C. for one hour with two litres of 70% ethyl alcohol containing one c.c. of N hydrochloric acid per litre of alcohol. The extract was centrifuged off from the residue. The soluble portion of the extract was reclaimed by precipitation with dilute sodium hydroxide and separated from the alcohol using the centrifuge. The solid was dried in a vacuum desiccator at laboratory temperature and dissolved in 0·2 N sodium acetate solution.

2. *Formolised casein.*—0·05% solution of casein in 0·2 N sodium acetate was mixed with an equal volume of a 40% solution of formaldehyde. The mixture was allowed to stand for three days.

3. *Deaminised casein.*—Hammersten casein was deaminised according to the method of Dunn and Lewis.[3] 140 c.c. of glacial acetic acid were added drop by drop, with vigorous stirring, to two litres of a 5% suspension of casein in water, the operation lasting two hours. To the suspension, 500 c.c. of 8% sodium nitrite solution were added drop by drop for 1½ hours with vigorous stirring. The mixture was allowed to stand for eighteen hours. The precipitate of the deaminised protein was filtered, washed with hot water until free from nitrite and dried in a desiccator over concentrated

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sulphuric acid at the laboratory temperature. The product was light yellow in colour but had a tendency to turn brown on exposure to light. It was therefore preserved in the dark. A portion of the product was dissolved in 0·5% sodium hydroxide. The alkaline solution was deep red. The nitrogen content of the solid protein was determined by the Kjeldahl method and found to be 14·72% while the Hammersten casein had 15·43%. This value corresponds to what was obtained by Dunn and Lewis.[3]

4. *Sodium metaphosphate.*—This was prepared by heating sodium dihydrogen phosphate in a platinum dish for eight hours at redness. The red-hot dish was carefully quenched in water and the solid metaphosphate stored in a dry bottle.

5. *Trichloracetic acid.*—Acid of C.P. quality was used.

The various buffer solutions were prepared from chemicals of C.P. quality and the quinhydrone electrode was used to measure their pH.

The Technique of Spreading and of the Measurement of Surface Pressure

The essential requirement for the quantitative study of protein films is a reliable and reproducible method for the preparation of films. Since the proteins are soluble in water, there is always a risk of some of the protein entering into the bulk during the spreading process. Several experimenters have described in detail the procedure to be followed for spreading in order to get reliable results. Gorter and Grendel[6] forced the solution through a capillary kept horizontally and close to the surface. Fourt and Schmidt[16] used a micrometer syringe and insisted on the needle being kept just above the surface and in contact with it. Neurath[17] on the other hand, dropped the solution from a height of 2–3 mm. from the surface and found that if the drops of the solution touched the surface before detaching themselves the spreading was incomplete. Hughes and Rideal[10] spread gliadin by placing the solid protein on the surface and obtained more complete spreading than was got by Gorter's method. The success in the former method is due to the fact that the capillary-active substances dissolved at a much slower rate, at the solid-water interface than at the contour in contact with the water-air interface. This method, however, cannot be adopted with casein which does not spread in the solid form. Langmuir and coworkers[11] spread the protein by taking its solution in the form of a band on a metal foil and slowly lowering the foil into the substrate. This technique is elegant as it minimises the penetration of the spreading solution into the substrate in the process of dropping.

In our modification of the Langmuir method, two glass strips about 6″ long and 1″ wide were coated with paraffin, upto 2″ from one end. The strips were handled at the paraffined ends only, thereby preventing the transfer of surface-active material from the hand to the exposed glass. The bare portions were cleaned in warm chromic acid and washed with water. A measured quantity of the protein solution was put on one of the strips and worked into a thin ribbon with the other strip. The strips were then slowly lowered into the surface.

The surface pressure measurements were made by means of a film balance improvised from a Du Nouy tensiometer. The trough was constructed of a beading of glass strips on a glass plate, using high grade paraffin from which traces of surface-active impurities had been removed by heating with activated silica gel. Praffined glass strips were used as barriers. Metal foils, coated with paraffin (in ether solution) prevented the leakage of the film to the other side of the float—a paraffined mica strip. Before each experiment the trough was always tested for contamination. The entire apparatus was enclosed in a cabinet, with glass sides.

Results

1. *Comparison of the two methods of spreading.*—A comparative study was made of the direct dropping method and the modified band method. The results are given in Table I.

Table I

A. Spreading of casein (Hammersten) by dropping method on 0·01 N hydrochloric acid

Time allowed for spreading = 1 minute

Force in dynes per cm.	Expt. 1 Area in sq. metres per mg.	Expt. 2 Area in sq. metres per mg.	Expt. 3 Area in sq. metres per mg.
1	0·70	0·71	0·68
2	0·62	0·61	0·60
3	0·57	0·55	0·55
4	0·55	0·53	0·53
5	0·53	0·50	0·51
6	0·50	0·48	0·49
7	0·48	0·46	0·47
8	0·46	0·44	0·45
9	0·44	0·42	0·43
10	0·42	0·40	0·41

Average limiting area = 0·61 sq. metres per mg.

B. Spreading of Hammersten casein by the modified band method on 0·01 N *hydrochloric acid*

Time allowed for spreading = 1 minute

Force in dynes per cm.	Expt. 1 Area in sq. metres per mg.	Expt. 2 Area in sq. metres per mg.	Expt. 3 Area in sq. metres per mg.
1	1·30	1·25	1·28
2	1·10	1·16	1·17
3	1·05	1·11	1·07
4	0·98	1·01	1·01
5	0·95	0·98	0·96
6	0·92	0·94	0·92
7	0·88	0·90	0·88
8	0·84	0·85	0·84
9	0·79	0·81	0·80
10	0·75	0·77	0·76

Average limiting area = 1·19 sq. metres per mg.

The results show that the modified band method is far superior to the dropping method. Spreading by the former method is found to be quick, one minute being sufficient for complete spreading. The modified band method has therefore been employed in all subsequent work.

Admixture with ethyl alcohol has been employed to enhance spreading.[14] The addition of alcohol to the spreading solution primarily helps by reducing the surface tension so that the liquid spreads more easily on water. Amyl alcohol, known to be more surface-active, can be expected to give better spreading of the protein. It has been tried by us, but contrary to expectation, the limiting area for casein was found to be only 0·47 sq. metres per mg. The cause of this reduction in spreading is not clear. It is probably connected with denaturation, as can be gathered by the behaviour of denatured casein in the following experiment. Casein (Hammersten) was refluxed for an hour with absolute ethyl alcohol and dried for 2 hours at 80°. The product was insoluble in warm 0·2 N sodium acetate and dissolved only in 0·5% alkali. Spread from the alkali solution, the denatured casein was found to have on 0·01 N HCl substrate a limiting area of 0·84 sq. metres per mg. while before denaturation the corresponding value was 1·19.

2. *Effect of neutral salts on spreading of casein.*—The influence of salts (in the substrate) on the spreading of casein was studied. The results are given in Table II.

TABLE II

pH	Substrate	Limiting area in sq. metres per mg.
2·2	1 N Ammonium sulphate in 0·01 N HCl	1·16
2·2	1 N Potassium thiocyanate in 0·01 N HCl	1·19
5·9	0·5 N Barium chloride in distilled water	0·87
5·9	0·01 N ,, ,, ,, ,,	0·88
8·3	1 N ,, ,, 0·005 N borate buffer	0·99
8·3	0·05 N ,, ,, ,, ,,	0·90

The results indicate that salts have no appreciable effect on the spreading of casein on the acid side of the isoelectric point (pH 4·7). On the alkaline side, however, salts do increase the spreading.

3. *Effect of pH on the spreading of casein.*—The effect of pH on the spreading of the protein has been studied using different substrates. The results are given in Table III.

TABLE III

Time allowed in minutes	pH	Substrate	Limiting area in sq. metres per mg.
1	2·3	0·01 N Hydrochloric acid	1·16
30	2·3	0·01 N ,, ,,	1·19
1	1·2	0·1 N ,, ,,	1·22
1	4·7	0·005 N Acetate buffer	0·92
1	5·4	0·005 N ,, ,,	0·84
1	5·9	Distilled water	0·74
30	5·9	,, ,,	0·87
1	6·9	0·007 N Phosphate buffer	0·75
1	8·0	Sodium acetate + alkali	0·62
1	8·3	0·005 M Borate buffer	0·65

The results show that the limiting area diminishes with an increase in the pH. There appears to be an ill-defined minimum in spreading at pH 8·0. It is of interest to note that casein does not attain maximum spreading at its isoeletric point. Casein in this respect radically differs from egg albumin[8,15] and pepsin.[7] Two factors seem to control the limiting area. The extent of spreading itself, is controlled by the charge on the molecules in the film. This factor would lead to minimum spreading at the isoelectric point, for, the charge on the protein at this pH is minimum. At pH values far removed from the isoelectric point, the solubility of the protein is considerable so that the chances of the added liquid going into the bulk of the substrate (instead of spreading) are rendered greater. This factor by itself would cause a maximum spreading at the isoelectric point. At any pH

therefore one has to consider the effect due to both these factors. When the solubility effect becomes predominant there is maximum spreading at the isoelectric point. The increase in film areas on the highly acid side may be caused by the ionisation of the polar groups of film molecules. Ionisation of the end groups brings into play repulsive forces between the molecules and enhances the spreading. With casein the solubility effect seems to be relatively unimportant in acid solutions, but the effect is prominent in neutral and alkaline solutions. This explains why in the spreading of casein on an acid substrate, salts have no effect, while they have a marked effect in the case of a neutral or an alkaline substrate.

4. *Effect of formaldehyde on the spreading of casein.*—The spreading of solutions of casein treated with formaldehyde (for three days) has been studied. The results are given in Table IV.

TABLE IV

pH	Limiting area for the normal casein in sq. metres per mg.	Limiting area for the formolised casein
2·3	1·19	0·60
5·4	0·84	0·52
8·3	0·65	0·43

Formaldehyde therefore diminishes the spreading. The influence of pH on spreading is also less. The decrease in spreading may be due to the formation of methyleneimino-compounds in which two molecules of the protein take part.[1]

5. *Effect of sodium metaphosphate and of trichloracetic acid on spreading.*—These two reagents are known to coagulate proteins in solution. The effect of these two substances has therefore been studied. The results are given in Tables V and VI.

A large reduction in spreading is noticed with the metaphosphate. It is known that metaphosphates form a complex between two amino groups. Complex formation is probably responsible for the lateral contraction of the film. Trichloracetic acid is without any effect on spreading. This may be correlated with the fact that 0·5% trichloracetic acid does not preceipitate casein.

6. *Spreading of deaminised casein.*—The behaviour of deaminised casein is of great interest, as the film on dilute hydrochloric acid (pH 2·0, 3·0 and 4·0) collapses with time. At pH 2·2, the initial area of the film at a

TABLE V

Effect of metaphosphate

pH	% concentration of meta phosphate	Limiting area in sq. metres per mg.
1·2*	0·02	0·60
2·3*	0·02	0·79
2·3*	0·005	0·84
2·3*	0·0002	1·10
4·8 (Acetate buffer)	0·02	0·93

*pH was adjusted by adding hydrochloric acid.

TABLE VI

Effect of trichloracetic acid

H*	% concentration of trichloracetic acid	Limiting area in sq. metres per mg.
2·3	0·5	1·19
2·3	0·05	1·18
2·3	0·005	1·19

*The pH was adjusted by adding the necessary quantity of HCl.

pressure of one dyne per cm. is of the same order as that for Hammersten casein (13,000 sq. cm. per mg.). The limiting area however could not be determined as the film collapsed quickly at higher pressures. No film of deaminised casein could at all be got on distilled water.

7. *Spreading of isodisperse casein.*—Isodisperse casein prepared according to the method of Carpenter[13] is interesting since it has been shown to be a definite chemical individual with a molecular weight of 375,000. Table VII shows the behaviour of this protein on spreading.

TABLE VII

Time allowed for spreading = 1 minute

pH	Area in sq. metres per mg.	Area per molecule in sq. Å units
1·2	1·20	75,000
2·3	1·09	68,000
4·8	0·92	57,000
5·9	0·72	45,000
6·9	0·77	48,000

It is seen from Tables III and VII that isodisperse casein has the same spreading characteristics as the Hammersten casein.

SUMMARY

1. Casein has been spread from its aqueous solutions by different methods and it has been found that the modified band method is the most suitable for the study of protein films.

2. Effect of salts on the spreading of casein has been studied. The results obtained can be explained on the basis that two different factors, solubility and the electric charge of the protein molecule influence spreading.

3. Treatment of the protein with formaldehyde causes a decrease in spreading. Change in pH affects spreading of formolised casein to a smaller degree.

4. Sodium metaphosphate diminishes markedly the spreading of casein. Trichloracetic acid, however, has no effect.

5. Deaminisation of casein alters the spreading properties and gives unstable films on acidulated water. No films can be got on distilled water.

6. The spreading properties of an isodisperse fraction of casein have been studied. The limiting area of this fraction has been found to be of the same order as that of the original material.

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