THE HEAT COAGULATION OF CALCIUM CASEINOGENATE

and

FACTORS AFFECTING THE SOLUBILITY OF MILK POWDERS.

A Thesis submitted to the University of Glasgow in accordance with the Regulations for the Degree of Doctor of Philosophy in the Faculty of Science

by

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CALCIUM CASEINOGENATE

INTRODUCTION.

In view of the great complexity of the chemical structure of protein molecules it is not surprising to find that the solutions of these substances in water and dilute acids and alkalies present many problems both of interest and at times of economic importance. In the past two decades a large number of these problems have been successfully tackled and the chemistry of protein solutions has been to a great extent unravelled, both in their behaviour as colloid systems and in their chemical structure. Among the problems on which much work has been done and yet which cannot be regarded as being altogether solved is the exact nature of the effect of heat on solutions of the proteins. It is, of course, well known that, by heating a dilute solution of certain proteins, such as egg-albumin, some change or series of changes takes place in the structure of the molecule whereby inter alia the stability of the solution as a colloid system is greatly diminished and the protein can be easily coagulated by the addition of small amounts of electrolytes. This change, whose exact nature is still unknown, is termed "denaturation". Although the chemical changes involved in denaturation are small, the process itself is probably irreversible. In this connection it is interesting to note that Sørensen (1925) has shown that small hydrolytic cleavages take place during denaturation; these however he appears to consider as incidental to denaturation rather than

its cause. Denatured proteins are characterised by a "loss of solubility in water and in dilute salt solutions; they dissolve, however, in dilute acids and alkalies giving colloidal solutions which react as if they were of the suspensoid type rather than the emulsoid type characteristic of normal proteins"(Jordan Lloyd (1926)).

In a comprehensive survey of this aspect of protein chemistry W. C. M.Lewis (1931) has cited the work of Chick and Martin (1911), P. S. Lewis (1926) and Cubin (1929) showing that denaturation of egg-albumin and haemoglobin at different temperatures and over a wide range of pH values is a monomolecular reaction and that it is at a minimum at the pH of pure water. While this is no doubt true for many other proteins besides those two mentioned it is not necessarily the case for all. Indeed with regard to caseinogen Lewis states "It is very doubtful whether caseinogen is denaturable, probably it is not. Incidentally the change of caseinogen to casein by means of rennet ..... is of a much more profound character than would be demanded by denaturation alone". Certainly caseinogen is usually regarded as a relatively heat-stable protein. At the same time, in addition to the chemical changes which accompany the heating of caseinogen solutions, to be hereinafter described, certain other alterations occur in the physical properties of the

solutions. For example after heating a neutral 3 per cent. solution of calcium caseinogenate solution at 120°C. for 110 min. it was found that the pH had fallen by about 0.1 unit (at pH 6.50), the relative viscosity as measured in an Ostwald viscometer at 25°C. had also decreased from 1.53 to 1.23, while a similar decrease in the amount of electrolyte required for coagulation at room temperature was also noted.

While caseinogen is probably not denaturable in the generally accepted sense of the term yet fairly drastic heat treatment (such as occurs in the sterilisation of evaporated milk and the manufacture of milk powder) will lead at times to its coagulation. As milk is such a complex colloid system, containing different amounts of electrolytes and non-electrolytes dissolved in the dispersion medium, such coagulation may be, and indeed probably is, not so much due to any structural changes in the protein molecule itself as to changes taking place in the dispersion medium. No attempt appears to have been made so far to study the effect of drastic heat treatment on the protein molecule in solution and to find out the possible causes which produce heat coagulation of the protein per se. It was for this purpose that the present investigation was carried out.

Quite a number of investigators have shown that the caseinogen molecule is relatively easily disrupted by the action

of various agents; this is especially true of the cleavage of phosphorus from the molecule. Thus Rimington and Kay (1926) and Stirling and Wishart (1932) have shown that trypsin-kinase liberates the phosphorus from caseinogen with comparative ease; in two experiments by the latter workers the entire phosphorus content was disrupted in less than one hour. Rimington and Kay have further shown that hydrolysis in 1% alkali at 37°C. also results, after about 30 hours, in complete dephosphorisation of the molecule. In addition Rimington (1927) and more recently Lipmann (1933) and Levene and Hill (1933) each claim to have isolated a phosphopeptone from caseinogen; in the first and third cases this was obtained from a tryptic digest; and in the second by acid (HCl) hydrolysis. Such examples serve to show that the phosphorus grouping in caseinogen is a relatively labile one.

While dealing with the ease of the breakdown of caseinogen it is well to state the views of Linderstrøm-Lang (1928). He considers that caseinogen as normally prepared is heteromolecular, being in reality an association of several very similar proteins, each having a different phosphorus content. These proteins act as a co-precipitation system in all normal reactions in which caseinogen takes part, thus accounting for the constancy of the composition of the protein as generally prepared.

Whether or not this suggestion is correct need not concern

us here directly, since we are dealing with the protein as it occurs in normal cows' milk either as a single molecule or a molecular complex, and the changes observed must be regarded as occurring in the caseinogen system whether or not this system is homomolecular or heteromolecular. For the sake of simplicity, however, the data presented here will be analysed on the assumption that caseinogen is a homomolecular substance.

In view of the work cited above on the ease with which the caseinogen molecule can be disrupted, it was not surprising to find that, when neutral solutions of sodium caseinogenate were heated in closed tubes at 120°C., there was a definite cleavage of phosphorus. It was felt that these preliminary experiments would repay further study. A record of the work so attempted is presented in the following section.

I. THE EFFECT OF HEAT ON THE CLEAVAGE OF PHOSPHORUS FROM CASEINOGEN.

Technique.

Preparation of solutions: Caseinogen "nach Hammarsten" was used throughout. It contained 9% moisture, the nitrogen and phosphorus contents (calculated on dry matter) being 15.08 and 0.83% respectively. Solutions were prepared as follows:-

3.5% sodium caseinogenate: 3.5 g. caseinogen were mixed with sufficient water and 0.1 N NaOH to give, when

diluted to 100 ml., a final concentration of 0.0175 N NaOH.

3.5% calcium caseinogenate: 3.5 g. caseinogen were mixed with sufficient water and solid $Ca(OH)_2$ to give, when diluted to 100 ml., a final concentration of 0.0175N $Ca(OH)_2$.

The solutions were mechanically stirred until the caseinogen was completely dissolved. The final solutions appeared homogeneous, the solutions of sodium caseinogenate being almost clear and those of calcium caseinogenate opalescent but transparent in thin layers. The pH of the solutions lay between 6.5 and 7.0. It may be noted here that, immediately they were immersed in the glycerol bath at 120°, both solutions became very turbid. There was, however, no flocculation, and the solutions, if removed from the bath, remained stable for an indefinite period.

Heating control: The solutions were heated in an open tinned copper bath containing glycerol. The bath was fitted with an efficient stirrer, and the temperature was controlled by a thermionic valve relay in conjunction with a contact thermometer (Baily, Grundy and Barrett, Cambridge). The temperature used throughout the entire series of experiments was 120° and did not vary by more than 0.25°.

As the experiments were carried out above boiling-point it was necessary to use closed tubes for the caseinogen solutions. A very cheap and efficient method was devised. Small heavy-

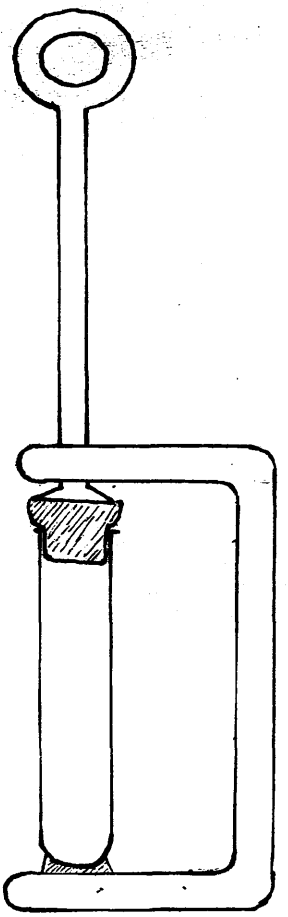

FIG. 1.

walled pyrex tubes were used, each being closed with a rubber stopper. Each tube was fixed tightly in a metal clamp, a small piece of rubber being inserted as a cushion between the bottom of the tube and the clamp (see Fig. 1). This arrangement enabled the observer to shake, invert and remove each tube independently. After removal from the bath the tubes were cooled quickly in running water.

Analysis: Nitrogen determinations were made by the micro-Kjeldahl method, (Pregl (1930)). Phosphorus was estimated by the method of Fiske and Subbarrow (1925).

Experimental procedure: A number of tubes, each containing 5 ml. of 3.5% sodium (or calcium) caseinogenate, were immersed in the glycerol-bath at 120° for periods of from 1 to 5 hours. The tubes were removed from the bath at appropriate intervals, cooled and, where necessary, shaken to break up any coagulum which had been formed. 5 ml. of 10% trichloracetic acid were then added to each tube. After standing for 5 minutes the contents were filtered through a No.1 Whatman filter. Nitrogen and phosphorus determinations were made on aliquot portions of the filtrate. Each experiment was carried through in duplicate.

Total nitrogen and phosphorus determinations were also made on aliquot portions of the original solution.

Results and Discussion.

The amount of acid-soluble nitrogen and phosphorus

TABLE I.

Time of Heating (hours)	Sodium caseinogenate				Calcium caseinogenate			
	Acid-soluble N	% Acid-soluble N	Acid-soluble P	% Acid-soluble P	Acid-soluble N	% Acid-soluble N	Acid-soluble P	% Acid-soluble P
0	3.50	0.7	0.0	0.0	3.64	0.9	0.0	0.0
1	26.2	5.5	13.7	53.9	26.3	6.4	9.1	36.9
2	41.3	8.7	19.6	76.7	46.9	11.5	14.0	56.7
3	81.2	17.2	25.2	98.7	60.9	14.9	17.0	69.1
4	67.6	14.3	24.5	96.0	69.4	17.0	18.7	76.1
5	86.8	18.4	25.6	100.0	89.3	21.9	19.8	80.3
		Total N	471.5			Total N	408	
		Total P	25.5			Total P	24.7	

All amounts expressed in mgs. per 100 ml. of original solution.

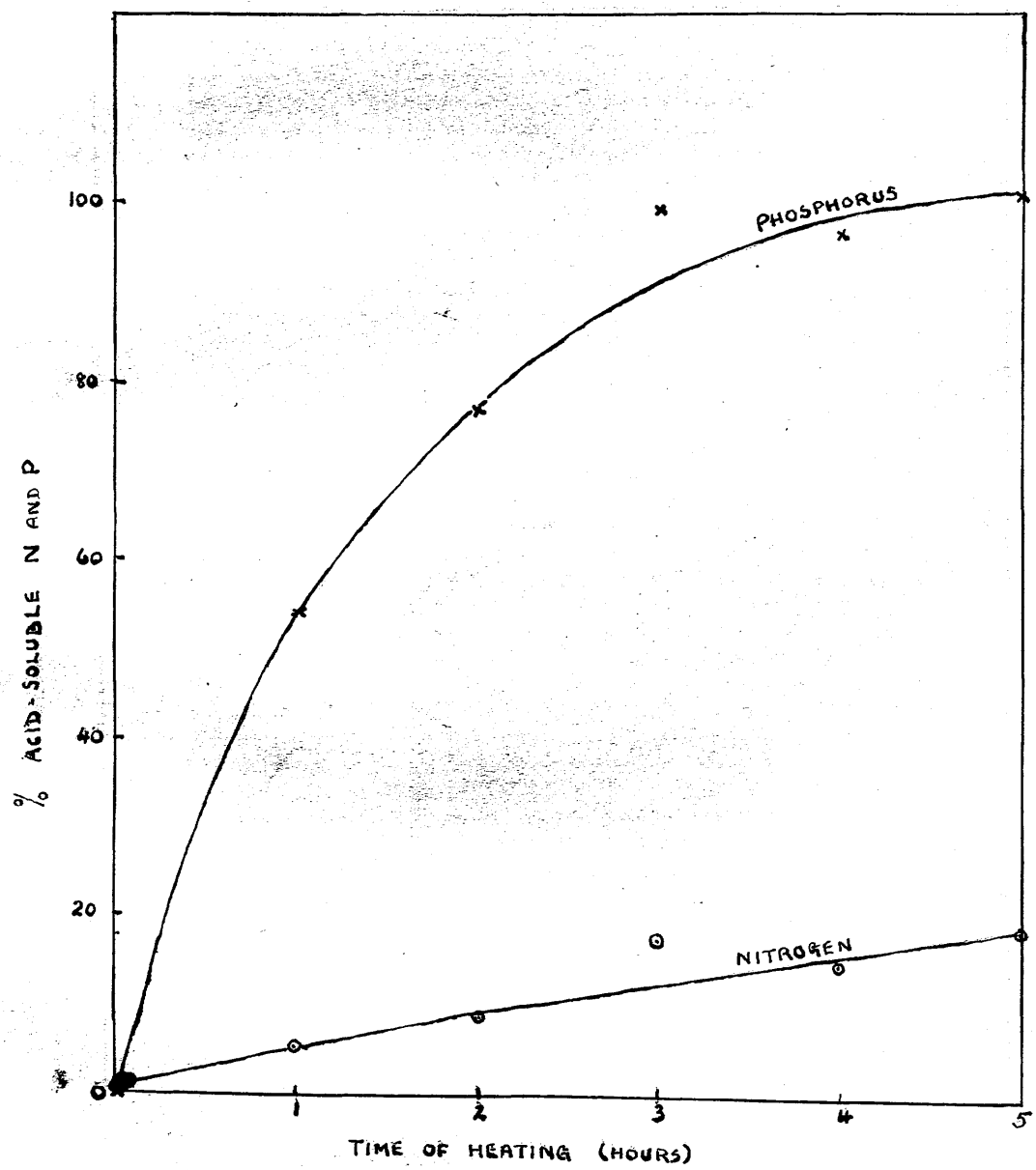
100
80
60
40
20
0
% ACID-SOLUBLE N AND P
PHOSPHORUS
NITROGEN
1
2
3
4
5
TIME OF HEATING (HOURS)

Fig 2.

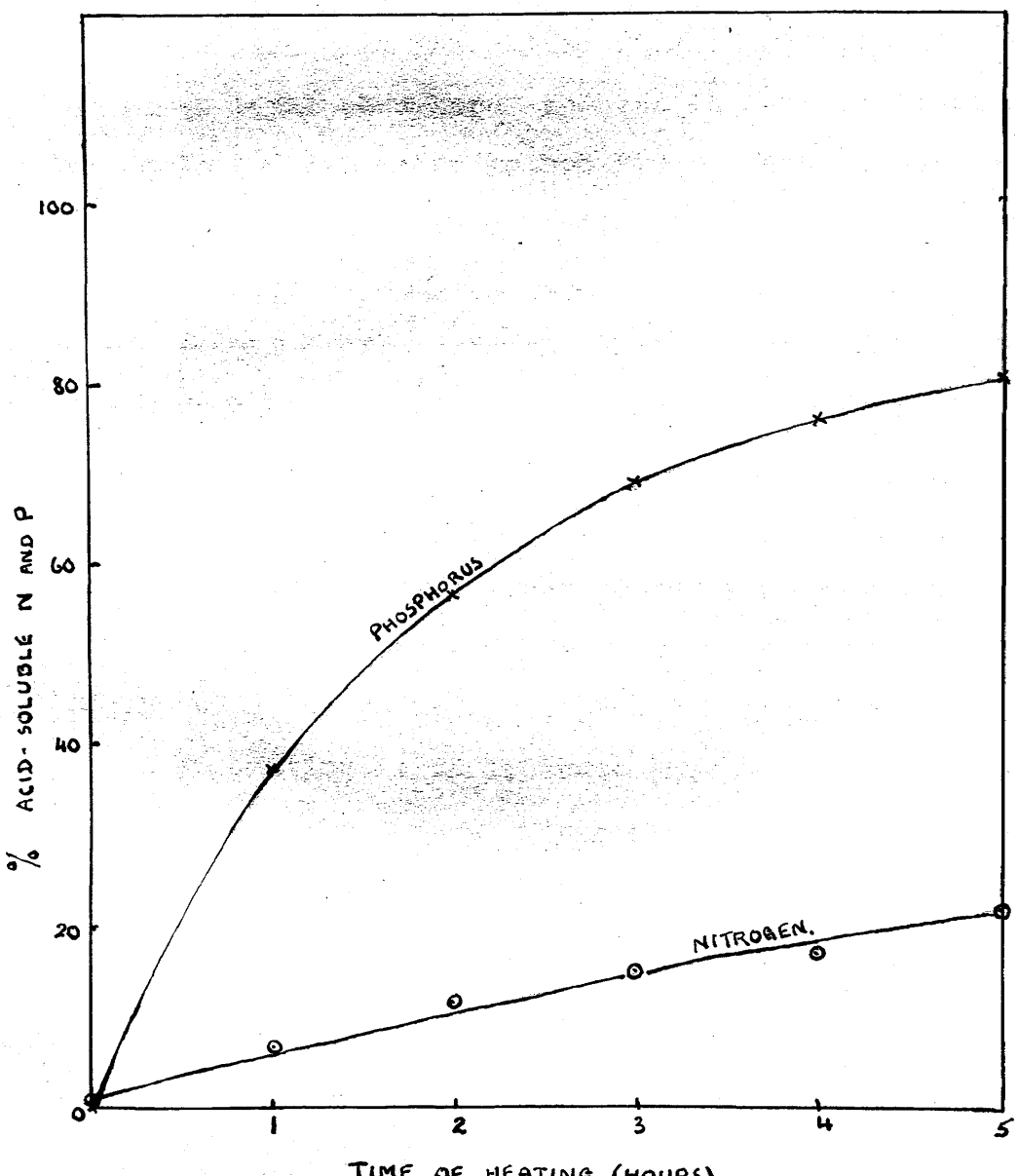
100
80
60
40
20
0
% ACID-SOLUBLE N AND P
PHOSPHORUS
NITROGEN.
1
2
3
4
5
TIME OF HEATING (HOURS)

FIG. 3

liberated from 3.5% solutions of sodium and calcium caseinogenate is shown in Table 1. These values are also shown graphically in Figs. 2 and 3. It should be noted that neither in these experiments nor in later ones was inorganic phosphate determined and that the values given for acid-soluble phosphorus represent total phosphorus soluble in 5% trichloracetic acid. The Fiske and Subbarrow method for the estimation of inorganic phosphate is not always reliable in the presence of phosphopeptone etc., but several approximate determinations by this method gave definite evidence that a large part, if not all, of the phsophorus liberated was present as inorganic phosphate. In addition, the relatively small amount of nitrogen liberated by heat treatment, together with the fact that (as shown in Figs. 2 and 3) nitrogen cleavage takes place at the same rate with both the calcium and the sodium salts, while the rate of phosphorus cleavage differs, provides strong evidence that the effect of heating is to liberate phosphorus as inorganic phosphate.

It is apparent that the general trend of the results is the same for both the sodium and calcium salts, namely a rapid production of acid-soluble phosphorus and a relatively slow production of acid-soluble nitrogen. In addition, there is no evidence from the trend of the graphs to suggest a slowing off in the liberation of nitrogen when the entire phosphorus

of the caseinogen molecule is broken off. This finding is comparable with the results of Rimington and Kay (1926) for mild alkaline hydrolysis. These workers continued to estimate nitrogen after all the phosphorus had become acid-soluble and found no slowing off in the rate of production. They further found that alkaline hydrolysis of caseinogen resulted in the production of a part of the acid-soluble nitrogen in the form of ammonia. The exact figures for the ammonia nitrogen produced expressed as a percentage of the total nitrogen present are not given. It is, however, possible to calculate from their data, on the assumption that the caseinogen sample had originally an N/P ratio of 20:1 that when the entire content of phosphorus had been rendered acid-soluble (after about 25 hours) the ammonia nitrogen is about 3.4 per cent.

In the present experiments, no estimations of free ammonia were made, but the fact that the solutions tended to become slightly more acid rather than alkaline as heating proceeded seems to preclude the possibility of the formation of any considerable quantity of ammonia. It appears probable therefore that the mode of liberation of nitrogen from the caseinogen molecule by heat-treatment differs from that resulting from treatment with alkali. It is further of interest, in view of the suggestion regarding the liberation of inorganic phosphate, to note that Rimington and Kay estimated acid-soluble phos-

phorus as inorganic phosphate by magnesium citrate mixture.

As regards the rate of liberation of acid-soluble phosphorus conditions were not kept sufficiently constant to allow for any definite conclusion, there being invariably a slight increase in the acidity of the solution during heating. At the same time the values obtained for the reaction constant K, in the case of sodium caseinogenate, do correspond fairly closely with a monomolecular reaction, while those obtained from the calcium salt, while not so close, also suggest the probability of a similar order of reaction. Such a finding of course would give weight to the probability of all the phosphorus of the caseinogen molecule being concentrated in one relatively small group in the protein.

There is, however, a marked difference between the curves for the sodium and calcium salts. With the sodium salt 100% of the phsophorus is rendered acid-soluble within 5 hours, whereas with the calcium salt only 80% is rendered acid-soluble in the same period. At first sight it appeared possible that this might be due to the fact that with the calcium salt, a clot is formed within two hours and that in spite of vigorous shaking part of the phosphorus, although rendered acid-soluble, might remain embedded in the clot. Such an explanation would not, however, account for the difference in the amounts of phosphorus which were rendered acid-soluble within the first

hour (i.e. 54 and 37% respectively), during which time no coagulation occurred. Moreover the form for the calcium salt indicates that the reaction is practically complete when only 80% of the phosphorus is in the acid-soluble form. It seems possible that the explanation of this marked difference between the sodium and calcium salts lies in the catalytic effect of the Na ions.

II. THE PREPARATION AND PROPERTIES OF HEAT-DEPHOSPHORISED CASEINOGEN.

Since it had been shown possible to rupture the entire phosphorus content of caseinogen and yet leave in the solution a protein presumably similar in some respects (since there was no evidence of any great degradation), it appeared desirable to prepare a sample of this protein. Rimington and Kay (1926) had obtained such a dephosphorised sample from a 36-hour digest of 1% caseinogen in 1% alkali. The product so obtained was a white powder similar in appearance to, and giving all the colour reactions of, caseinogen but containing only traces of phosphorus. It was decided to endeavour to obtain a sample of this heat-dephosphorised product using in general the method employed by Rimington and Kay. The method finally adopted was as follows.

Preparation of Heat-dephosphorised Caseinogen.

One litre of 3.5% sodium caseinogenate (pH 6.7) was prepared as previously described and placed in a 2 litre flask. The mouth of the flask was covered with a thin sheet of cellophane and the flask heated in an autoclave. In about 30 minutes the pressure had reached 1 atmosphere (121°) and it was retained at this value for 4 hours. A further 30 minutes was allowed for the pressure to fall, and the flask was then removed and cooled in running water. No coagulation occurred during the heating, but a thin skin was formed on the bottom of the flask. The heated solution was definitely pink in colour. The solution was then treated with small quantities of 33% acetic acid until maximum precipitation took place. The gelatinous precipitate was filtered off through linen, well washed with water and redissolved in dilute NaOH. It was then reprecipitated with 33% acetic acid. At this stage the precipitate appeared white, but it soon darkened to brown even though not directly exposed to air. After settling, decanting off the supernatant liquid and thorough washing, the precipitate was again filtered through linen and well drained. It was next transferred to a mortar and ground with absolute alcohol for about an hour. On first contact with alcohol the precipitate became a gummy mass, but with continued grinding it became a friable powder. This was filtered off on a Büchner

funnel, re-extracted with fresh alcohol for another hour, again filtered off and finally allowed to dry in a current of air. The final product was a brownish pink powder. The moisture content (mostly alcohol) was 15%. This could be reduced by treatment with ether to about 10%, but the product so obtained was less easily soluble in alkali: maximum yield, 49%.

Properties of Heat-dephosphorised Caseinogen.

In order to compare the properties of the heat-dephosphorised caseinogen with those of the untreated caseinogen and of Rimington and Kay's alkali-dephosphorised caseinogen, samples of the latter two products were also prepared. The alkali-dephosphorised caseinogen was prepared according to Rimington and Kay's directions. The control sample of caseinogen was prepared from an untreated solution of 3.5% sodium caseinogenate, the methods of precipitation and purification being identical with those used for the dephosphorised products. The nitrogen and phosphorus contents of the three preparations (expressed as % of dry matter) were as follows:-

	N (%)	P (%)
Original (untreated) caseinogen	15.08	0.830
Heat-dephosphorised caseinogen	13.86	0.032
Alkali-dephosphorised caseinogen	13.56	0.016

Two methods were used for differentiating the three products, (a) the determination of their titration curves, and

(b) the determination of their relative rates of coagulation when heated at 120°.

(a) Titration Curves.

Technique: 1% solutions (on dry matter basis) of each product were prepared. For acid-binding capacity the solutions were made up with standard HCl (approximately 0.1 N) of sufficient amount to give an initial pH of 2.0 - 3.0; for base-binding capacity sufficient standard NaOH (approximately 0.1 N) was used to give an initial pH of 8.0 - 9.0. The amount of acid or alkali added was noted. . The method of determining the combining capacity was that of "back-titration" from the initial pH to the region of the isoelectric point. For this purpose known amounts of standard alkali or acid were added to aliquot portions of the original solutions, and the pH was determined after each addition.

For base-binding capacity the pH measurements were made with a glass electrode. For acid-combining capacity a quinhydrone electrode was used in combination with a saturated calomel half-cell. With both methods of determination the accuracy of the measurements was within 0.5 millivolt.

The method of calculation of the equivalents of acid or base bound was as follows:-

Acid-binding: At any given pH, equivalents bound per g. protein = [H] added - [H] found, where

[H] = concentration of acid in equivalents.

The concentration of acid added was obtained directly from the amount of HCl added minus the amount of NaOH added (expressed in equivalents). The concentration of free acid ([H] found) was calculated from the observed pH.

Base-binding: At any given pH, equivalents bound per g. protein = [OH] added - [OH] found, where

[OH] = concentration of alkali in equivalents.

The concentration of alkali added was obtained directly from the amount of NaOH added minus the amount of HCl added (expressed in equivalents). The concentration of free alkali ([OH] found) was calculated from the observed pH.

The ionic product for water was taken as $K_W = 0.74 \times 10^{-14}$ at 18° (Michaelis, 1926). The degree of dissociation, α, was taken as unity throughout the concentrations employed. Hoffmann and Gortner (1925) have pointed out that the degree of dissociation as determined by conductivity methods does not agree with the values obtained by potentiometric methods and suggest the use of the latter in calculating combining capacities. It was found that, up to the concentrations used in the present experiments, this correction was not of sufficient magnitude to be considered necessary.

Results.

The actual amounts of acid and base bound at the various

TABLE II .

Acid-Binding Capacity per gm. Protein.

Untreated Caseinogen		Alkali-Dephosphorised Caseinogen		Heat-Dephosphorised Caseinogen.	
pH	Equiv. bound x 10^5	pH	Equiv. bound x10^5	pH	Equiv.bound x 10^5
2.30	98.9	2.30	110.4	2.83	81.8
2.36	94.3	2.37	106.3	3.04	68.4
2.44	89.3	2.47	103.1	3.14	63.5
2.58	83.6	2.66	91.8	3.18	59.4
2.64	79.3	2.77	91.0	3.32	54.4
2.77	74.0	2.85	75.9	3.37	50.5
2.91	66.3	2.99	68.0	3.49	44.6
3.11	59.1	3.12	58.7	3.60	40.2
3.30	50.0	3.35	48.8	3.72	34.4
3.49	39.9	3.43	38.9	3.81	29.5
3.70	29.3	3.67	29.1	4.03	23.7
4.03	10.1	3.99	17.7	4.15	19.5
4.51	4.1	4.86	7.1	4.36	12.5
				4.76	5.18

TABLE III.

1. Base-binding capacity per gm. protein.

Untreated Caseinogen.		Alkali dephosphorized caseinogen.		Heat-dephosphorized caseinogen.	
p_H	Equiv. bound $\times 10^5$	p_H	Equiv. bound $\times 10^5$	p_H	Equiv. bound $\times 10^5$
9.58	93.9	8.02	107.6	7.80	61.0
9.15	91.9	7.73	103.7	7.16	53.0
8.58	88.6	7.50	99.8	6.89	49.0
8.22	84.8	7.25	95.7	6.79	46.9
7.91	80.8	7.01	91.7	6.67	44.9
7.50	72.8	6.79	87.6	6.57	42.9
7.34	68.7	6.63	83.6	6.49	40.9
7.08	63.7	6.45	79.5	6.34	38.9
6.82	56.7	6.26	75.5	6.24	36.9
6.71	52.6	6.13	71.5	6.16	34.8
6.56	48.6	6.01	67.44	6.08	32.8
6.37	42.5	5.94	63.4	6.02	30.8
6.15	36.5	5.83	59.4	5.95	28.8
6.04	32.4	5.75	55.3	5.89	26.8
5.85	26.4	5.68	51.2	5.79	22.7
5.68	20.3	5.62	47.2	5.70	20.7
5.54	12.2	5.55	43.2	5.63	18.7
5.48	8.2	5.50	39.2	5.57	16.7
5.33	2.1	5.46	35.1	5.48	15.4
4.77	.11	5.41	31.1	5.38	12.6
		5.37	27.1	5.23	10.6
		5.33	23.2	5.03	8.6
		5.29	19.0	4.77	6.6
		5.24	14.9	4.60	4.6
		5.20	10.9	4.44	2.5
		5.13	6.9		
		5.04	2.8		

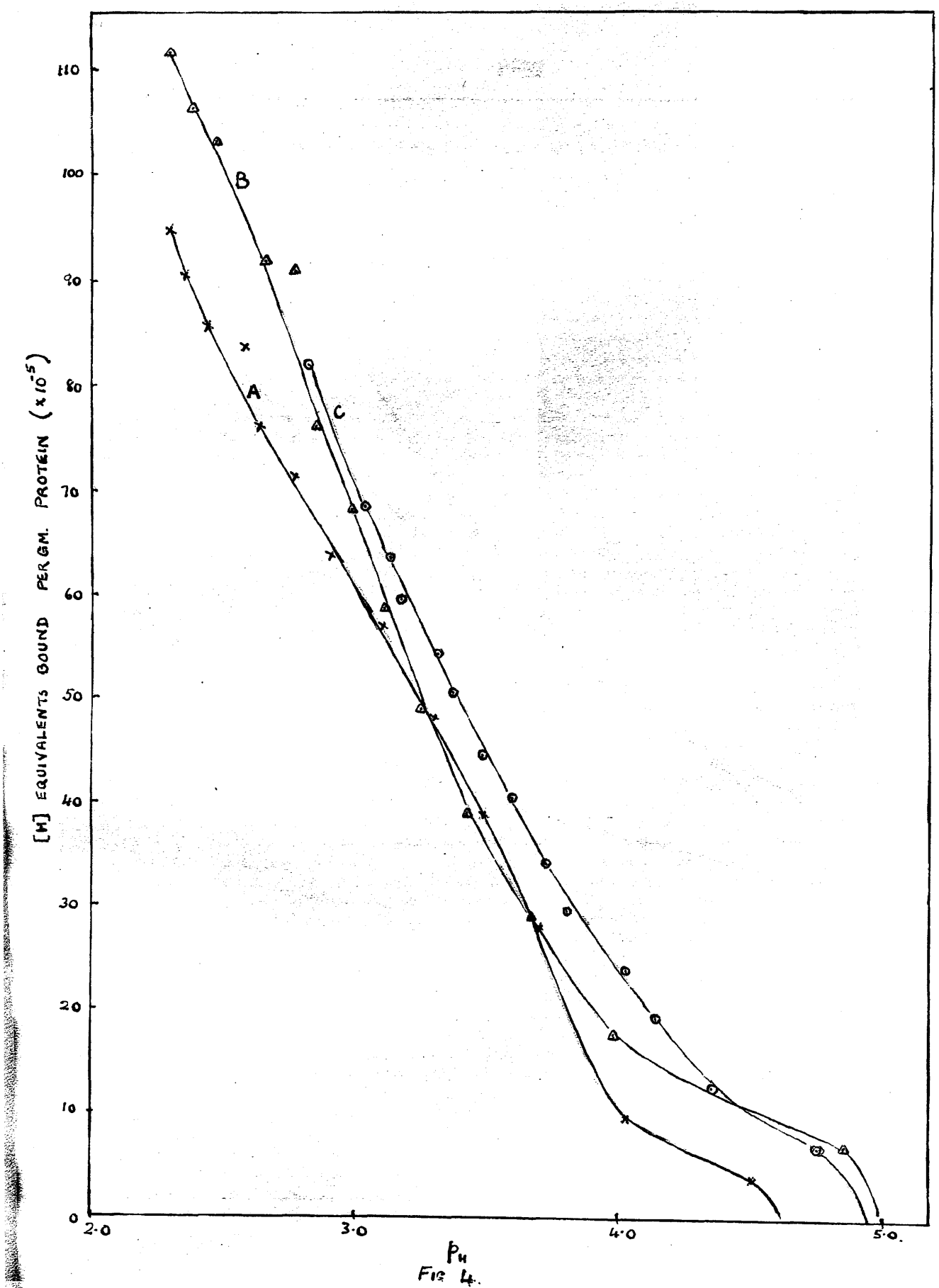
[H] EQUIVALENTS BOUND PER GM. PROTEIN (x10⁻⁵)
110
100
90
80
70
60
50
40
30
20
10
0
2·0
3·0
4·0
5·0
pH
A
B
C

FIG 4.

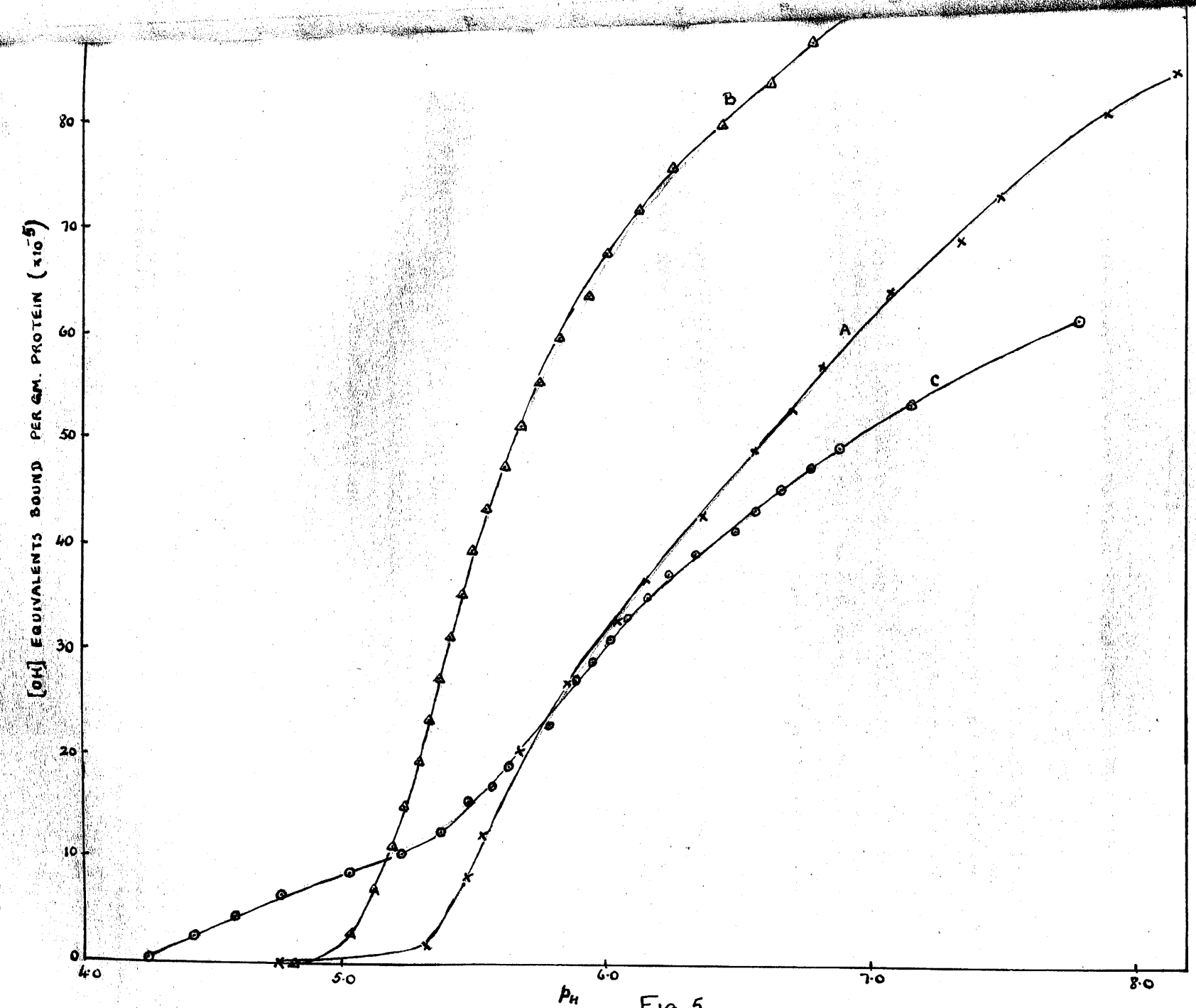
[OH] EQUIVALENTS BOUND PER GM. PROTEIN (×10⁻⁵)
0
10
20
30
40
50
60
70
80
4·0
5·0
6·0
7·0
8·0
pH
A
B
C

FIG. 5

pH values are given in Tables II and III. The titration curves of the three products are shown in Figs. 4 and 5. The curves for the untreated caseinogen coincide fairly closely with those recorded by Pertzoff and Carpenter (1932). It will be seen, however, that both alkaline hydrolysis and heat treatment have effected marked alterations in the base-binding capacity and, to a less extent, in the acid-binding capacity. Moreover, it is of special significance that the curves for the two dephosphorised products differ markedly, the alkali-dephosphorised caseinogen having a higher and the heat-dephosphorised caseinogen a lower base-binding capacity than untreated caseinogen. It has already been noted that the mode of liberation of phosphorus by heat appears to be identical with that caused by alkaline hydrolysis. On the other hand, it has been shown that the mode of cleavage of nitrogen probably differs in the two treatments. It seems likely therefore that the differences in combining capacity are associated with the cleavage of nitrogen rather than of phosphorus.

It should be added that, so far as the two dephosphorised products are concerned, it is not legitimate to consider these as specific proteins of fixed chemical composition, as in the case of caseinogen. Owing to variations in the rate of heating and cooling of the autoclave, as well as to other conditions which cannot be kept exactly constant, two successive samples of the

heat-dephosphorised caseinogen may vary somewhat in composition. The same criticism holds for the alkali-dephosphorised caseinogen. Such variations in composition are, however, unlikely to affect the acid- and base-binding capacities to any serious extent, and it is reasonable to assume that the curves shown in Figs. 4 and 5 represent typical acid- and base-binding capacities of the two types of dephosphorised product.

(b) The Rate of Heat Coagulation.

Technique: The rate of heat coagulation of the calcium salts of each of the three products already described was measured in 3 per cent. solutions. The solutions were prepared as follows.

The requisite amounts of alkali required to give an approximately neutral solution were calculated from the titration curves. The actual quantities of $Ca(OH)_2$ used, per 100 ml. of solution, were 0.061 gm. for the untreated caseinogen, 0.051 gm. for the heat-dephosphorised caseinogen, and 0.093 gm. for the alkali-dephosphorised caseinogen. The pH values of the solutions varied from 6.83 to 6.99.

The solutions were prepared as before by stirring the caseinogen and the solid $Ca(OH)_2$ with about 80 ml. of water from 1 - 2 hours. When the solution of the protein was complete the solutions were centrifuged as before for 15 minutes at

TABLE IV .

All amounts expressed in mgs. per 100 ml. of original solution.

Time of Heating (Hours)	Protein N in solution	% Protein N in solution	Acid-sol. N in solution	% Acid sol.N in solution	Protein N in solution	% Protein N in solution	Acid-Sol. N in solution	% Acid sol.N in solution	Protein N in solution	% Protein N in solution	Acid-Sol. N in solution	% Acid sol.N in solution
0	342.5	100	11.5	3.25	307.5	100	5.5	1.76	344.5	100	9.0	2.55
1	318	92.8	25.5	7.20	101.0	32.8	9.5	3.03	151.5	44.0	39.0	11.03
2	273.5	79.8	35.0	9.89	72.2	23.6	15.0	4.79	146.5	42.5	51.5	14.57
3	76.0	22.2	45.0	12.71	71	23.1	17.0	5.43	114	33.1	61.0	17.26
4	47.5	13.9	46.5	13.14	64	20.8	21.5	6.87	1130.5	37.9	59.0	16.69
5	50.0	14.6	55.0	15.54	62	20.2	27.5	8.80	111.5	33.8	65.5	18.53

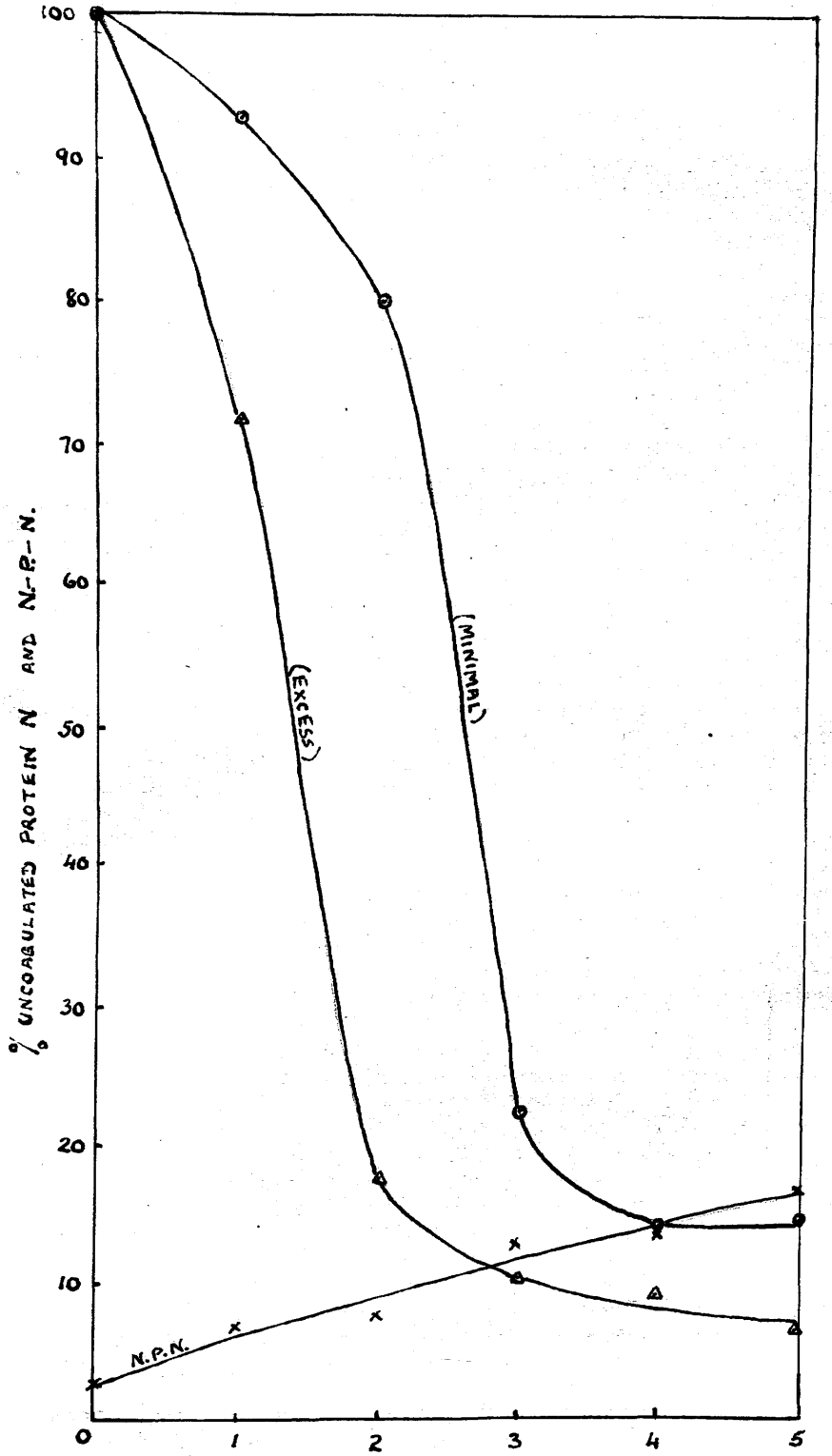
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
% UNCOAGULATED PROTEIN N AND N-P-N.
(EXCESS)
(MINIMAL)
N.P.N.
TIME OF HEATING (HOURS)

FIG. 6.

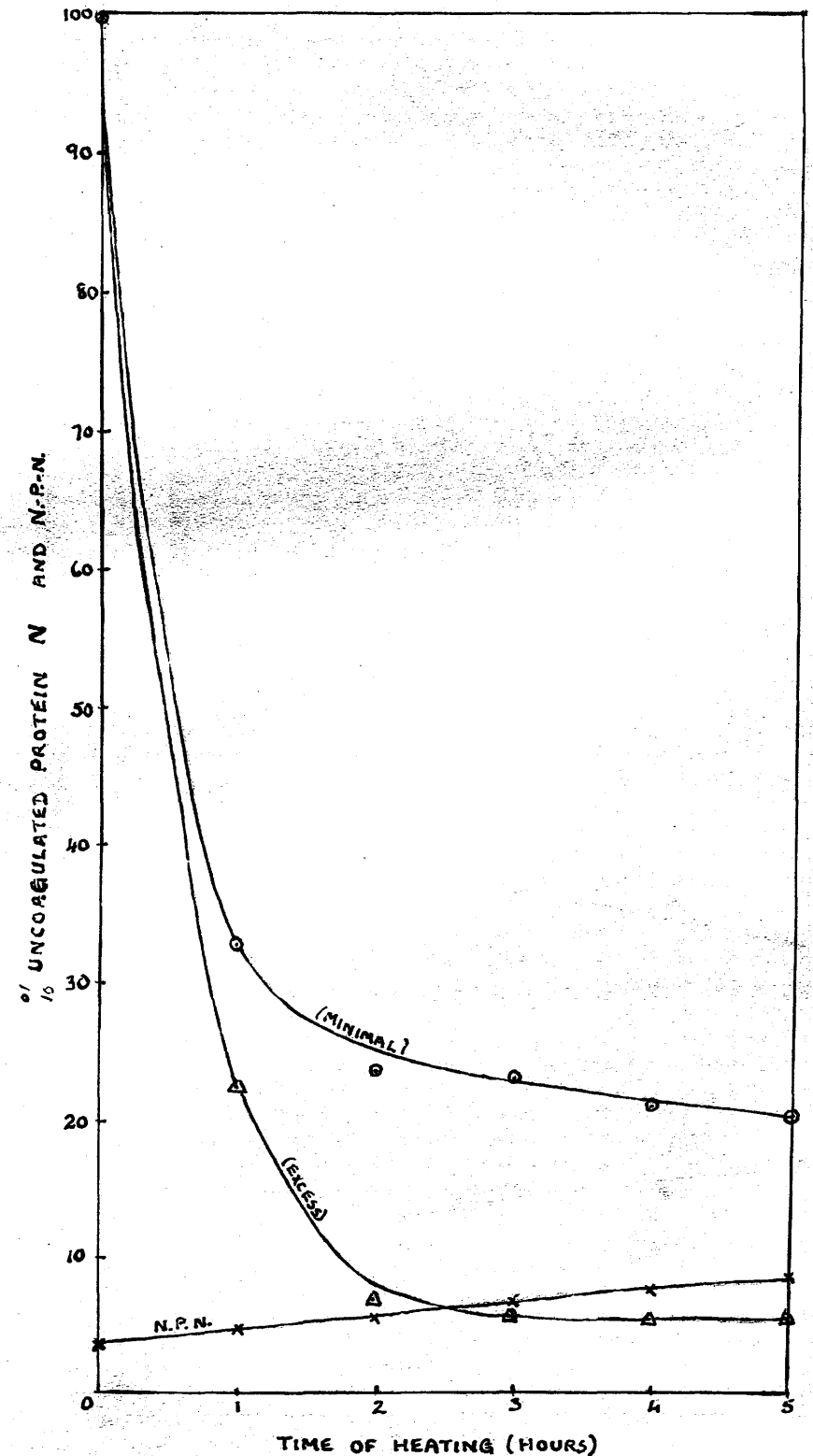
100
90
80
70
60
50
40
30
20
10
0
% UNCOAGULATED PROTEIN N AND N.P.N.
(MINIMAL)
(EXCESS)
N.P.N.
1
2
3
4
5
TIME OF HEATING (HOURS)

FIG 1.

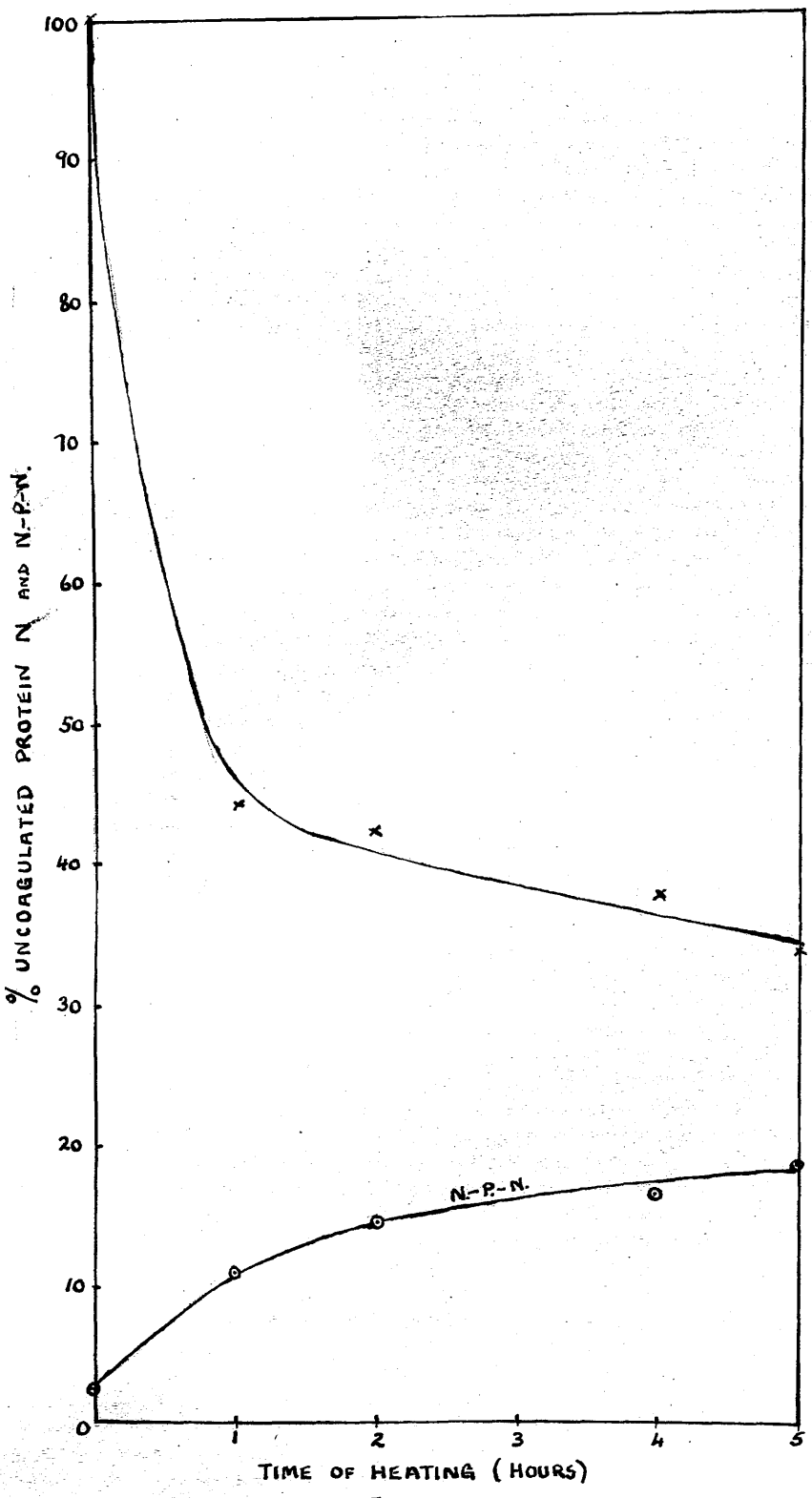
100
90
80
70
60
50
40
30
20
10
0
% UNCOAGULATED PROTEIN N AND N-P-N.
N-P-N.
1
2
3
4
5
TIME OF HEATING (HOURS)

FIG. 8.

3000 r.p.m. In the case of the heat dephosphorised caseinogen there was a considerable quantity of sediment after centrifuging. The presence of this sediment is shown by the smaller original total nitrogen content of the solution. After centrifuging, and decanting off from the sediment the solutions were made up to 100 ml. in standard flasks.

Heating: 7-8 ml. portions of the solutions were transferred to stoppered tubes and the tubes were heated in a glycerol bath at 120°C. (as previously described) for periods of 1-5 hours. After removal from the bath the tubes were cooled in cold running water and the solutions again centrifuged for 5 minutes at 3000 r.p.m. The supernatant liquid was decanted off and aliquot portions of the liquid removed for analysis.

Analysis: Determinations were made of total and acid-soluble nitrogen (i.e. soluble in 5 per cent. trichloracetic acid) the difference representing protein nitrogen. The percentage of uncoagulated protein and acid-soluble nitrogen were calculated from these figures.

Results and Discussion.

The results of these experiments are given in Table IV and are shown graphically in Figs. 6 - 8. (MINIMAL CALCIUM) ^ It will be seen that the dephosphorised products coagulate much more quickly than the untreated caseinogen and that the form of the coagulation curve obtained with the dephosphorised products is

fundamentally different from that of caseinogen. Indeed the coagulation curve of the latter may be said to be made up of two parts; the first a relatively slow process, followed by a very rapid action coinciding with the point of "visible coagulation". Thus there is during the first hour of heating a rapid coagulation of the dephosphorised products, over 70 per cent. of the heat-dephosphorised caseinogen and almost 60 per cent. of the alkali dephosphorised caseinogen being coagulated within this period. Within the same period barely 10 per cent. of the untreated caseinogen is coagulated. After almost 3 hours heating, however, there is a sudden and marked increase in the rate of coagulation of the untreated caseinogen. If reference is made to Fig. 6 it will be seen that this increase coincides with the liberation of 60 to 70 per cent. of the phosphorus, so that, in the part of the curve where coagulation is most rapid we are, in fact, dealing not with caseinogen as generally understood but with a partly dephosphorised product.

The fact that the two dephosphorised products give coagulation curves of similar type and that maximum coagulation occurs in the untreated caseinogen only after it has been partly dephosphorised demonstrates the enhanced heat-sensitivity which is associated with loss of phosphorus from the caseinogen molecule.

III. FURTHER EXPERIMENTS ON HEAT COAGULATION.

This apparently close connection between the rupture of the phosphorus from the caseinogen molecule and the subsequent heat coagulation of the solutions appeared to merit more detailed study. It was decided therefore to carry through another series of experiments on the rate of heat coagulation of calcium caseinogenate solutions at lower temperatures and to determine the simultaneous production of acid-soluble phosphorus and nitrogen. The temperature range chosen was 90° - 115°C. inclusive.

Technique.

The solutions used in this series of experiments were 3 per cent. calcium caseinogenate solutions prepared exactly as before. The amount of $Ca(OH)_2$ used was 0.065 gm. per 100 ml. The amount of sediment after centrifuging was always very small.

As the temperature at which the solutions were maintained was lowered, the time necessary to produce coagulation naturally increased; thus while coagulation occurred after $2\frac{3}{4}$ hours at 115°C., over 40 hours were required at 90°C. The time-intervals between successive tubes being taken from the bath varied therefore throughout the series. Treatment after removal from the bath was the same as previously.

TABLE VI.

All amounts expressed in mgs. per 100 ml. of original solution

Temperature of heating		90°C.						95°C.						100°C.				
Time of heating (hours)		0	9	18	17	36	45	0	8	16	24	32	0	3	6	9	12	
Nitrogen	Total in solution	388	380	347	297	229	98	391	358	275	110	-	392	387	249	284	201	
	Acid - soluble	3.8	12.0	19.6	27.0	33.3	38.6	4.3	14.6	23.9	32.5	38.3	4.90	13.3	21.0	27.8	34.0	
Phosphorus	Total in solution	21.9	20.9	19.6	18.1	15.8	11.1	21.65	20.5	17.15	12.1	-	21.74	21.45	19.30	16.40	13.0	
	Acid - soluble	0.0	3.8	6.7	8.6	10.0	11.2	0.0	5.9	9.2	10.6	11.1	0.0	3.8	6.8	8.9	10.5	

		108°C.					110°C.						115°C.					
		0	2	4	6	8	0	1	2	3	4	5	0	1	2	3	4	5
Nitrogen	Total in solution	392	327	222	105	87	370	365	337	280	208	108	382	375	285	180	112	107
	Acid - soluble	5.60	16.1	25.1	34.0	42.0	4.90	12.10	18.4	24.0	28.8	32.6	4.2	15.6	26.0	34.4	39.7	42.6
Phosphorus	Total in solution	21.7	17.2	14.0	12.55	11.8	20.5	20.1	18.9	17.0	14.5	11.3	21.7	21.2	17.0	11.9	12.3	13.0
	Acid - soluble	0.0	4.70	7.8	10.4	11.6	0.0	3.50	5.80	7.70	9.25	10.6	0.0	5.4	8.5	10.2	11.6	12.3

TABLE V

All amounts expressed as mgs. per 100 ml. of original solution.

Temperature of heating		90°C.						95°C.					100°C.				
Time of heating (hours)		0	10	18	27	35	45	0	8	16	24	30	0	3	6	9	12
Nitrogen	Total in solution	385.7	392.0	319.9	289.8	250.6	98.0	390.6	361.2	275.1	111.3	-	392.0	394.1	338.1	283.5	203.1
	Acid - soluble	4.2	12.32	18.68	27.58	32.76	38.64	4.70	17.50	19.60	30.80	38.36	4.90	13.30	21.70	26.60	34.30
Phosphorus	Total in solutiom	21.85	21.20	18.92	17.38	16.46	11.06	21.82	21.09	16.35	12.10	-	21.74	21.68	19.17	16.41	12.97
	Acid - soluble	0.0	3.88	6.79	8.58	9.85	11.23	0.0	5.66	9.40	10.49	11.08	0.0	4.00	6.67	8.56	10.82

		108°C.					110°C.						115°C.					
		0	2	4	6	8	0	1	2	3	4	5	0	1	2	3	4	5
Nitrogen	Total in solution	392.7	308.0	264.6	84.0	100.1	369.6	366.8	333.2	158.9	208.6	108.5	380.8	382.2	268.1	184.8	108.5	109.2
	Acid - soluble	5.6	16.1	24.8	34.3	41.7	4.9	13.16	16.52	23.94	29.40	38.62	4.20	17.92	23.10	33.60	40.46	42.70
Phosphorus	Total in solution	21.66	17.15	12.92	13.59	11.84	20.51	20.23	18.52	12.51	14.32	11.36	21.68	21.41	16.99	11.86	12.45	12.90
	Acid - soluble	0.0	4.85	7.93	10.46	11.56	0.0	4.12	5.78	8.17	9.19	10.97	0.0	5.45	8.46	10.14	11.73	12.25

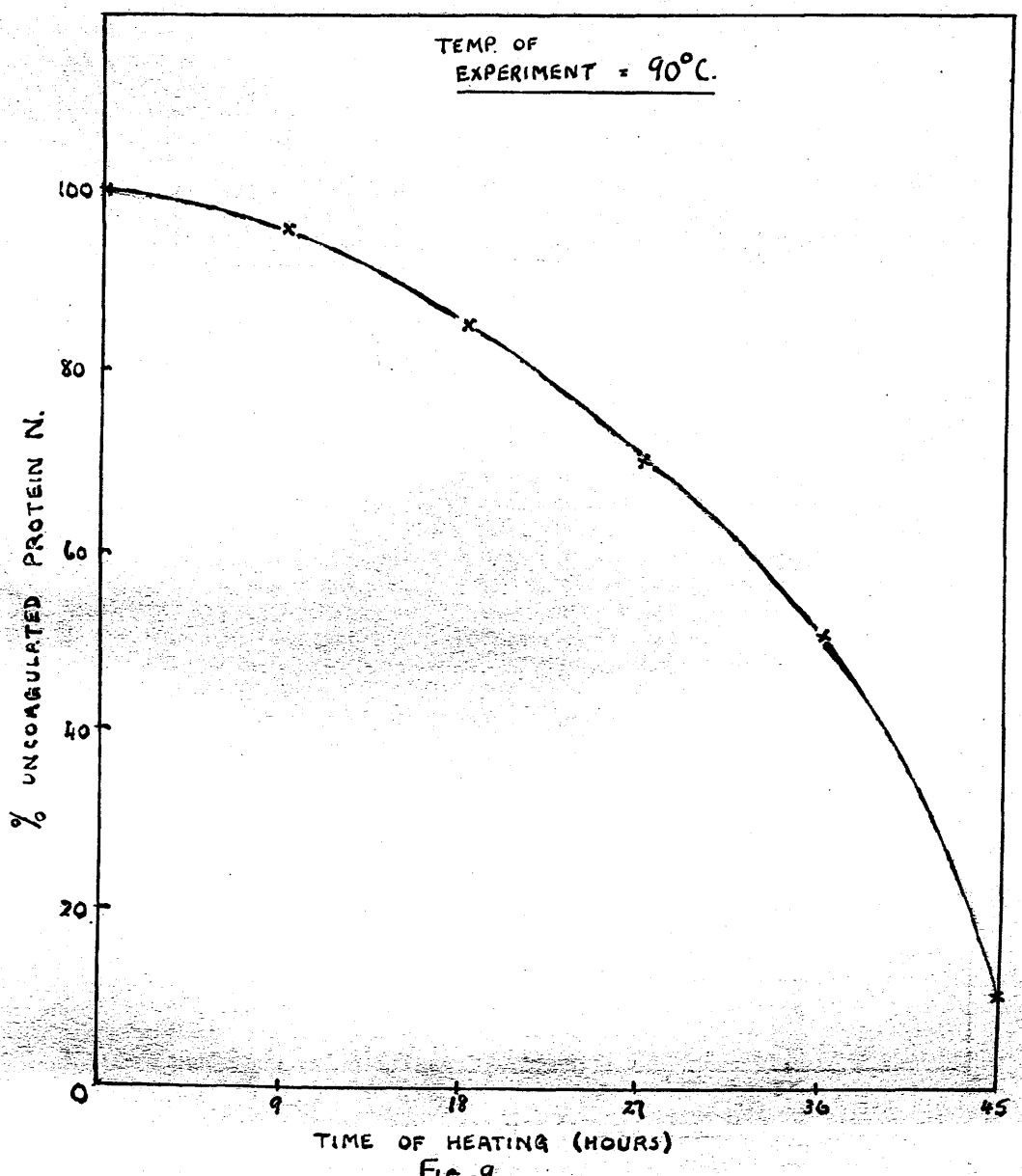
TEMP. OF
EXPERIMENT = 90°C.
100
80
60
40
20
0
% UNCOAGULATED PROTEIN N.
9
18
27
36
45
TIME OF HEATING (HOURS)

FIG. 9.

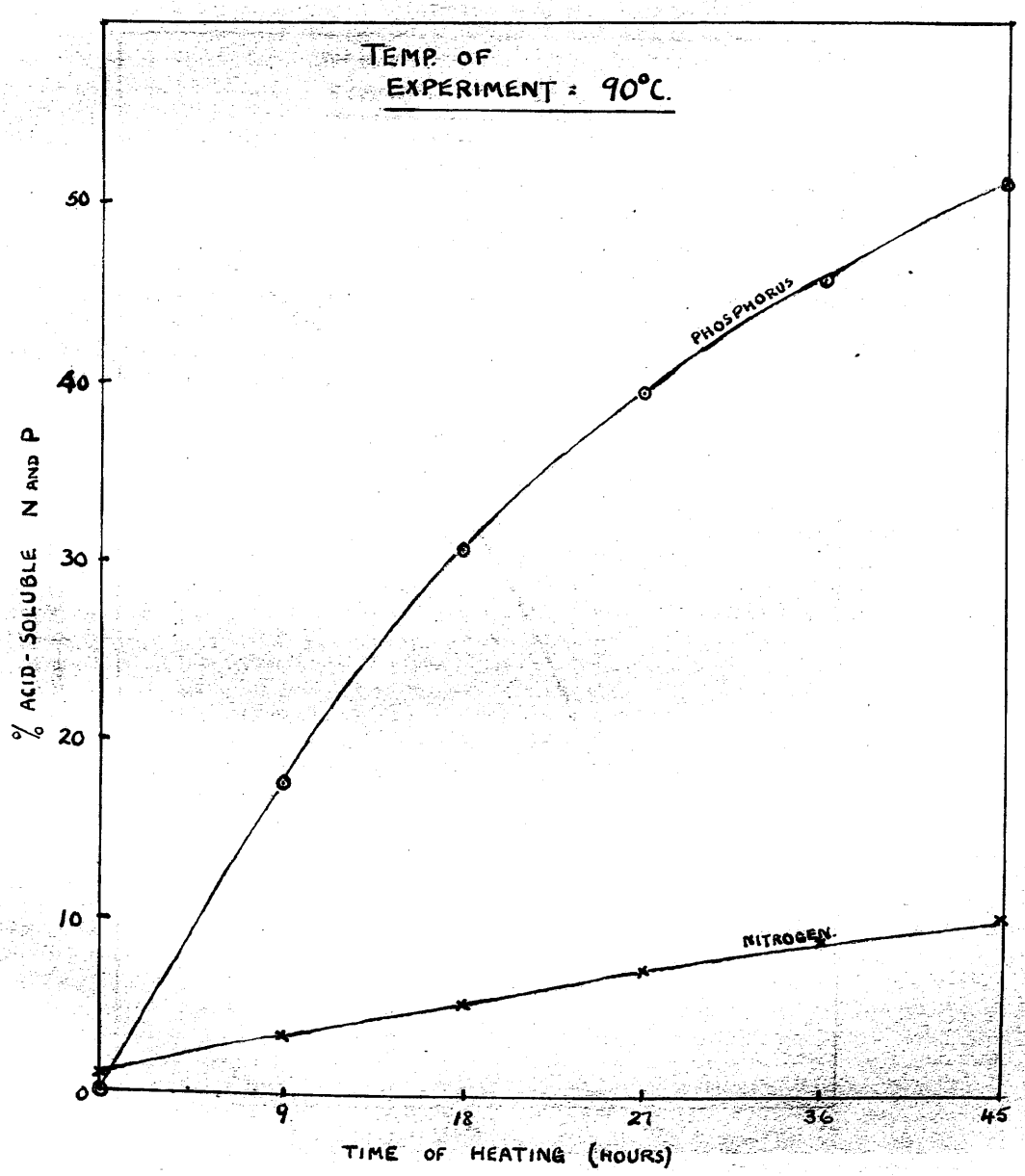
TEMP. OF
EXPERIMENT : 90°C.
50
40
30
20
10
0
% ACID-SOLUBLE N AND P
PHOSPHORUS
NITROGEN.
9
18
27
36
45
TIME OF HEATING (HOURS)

FIG. 10.

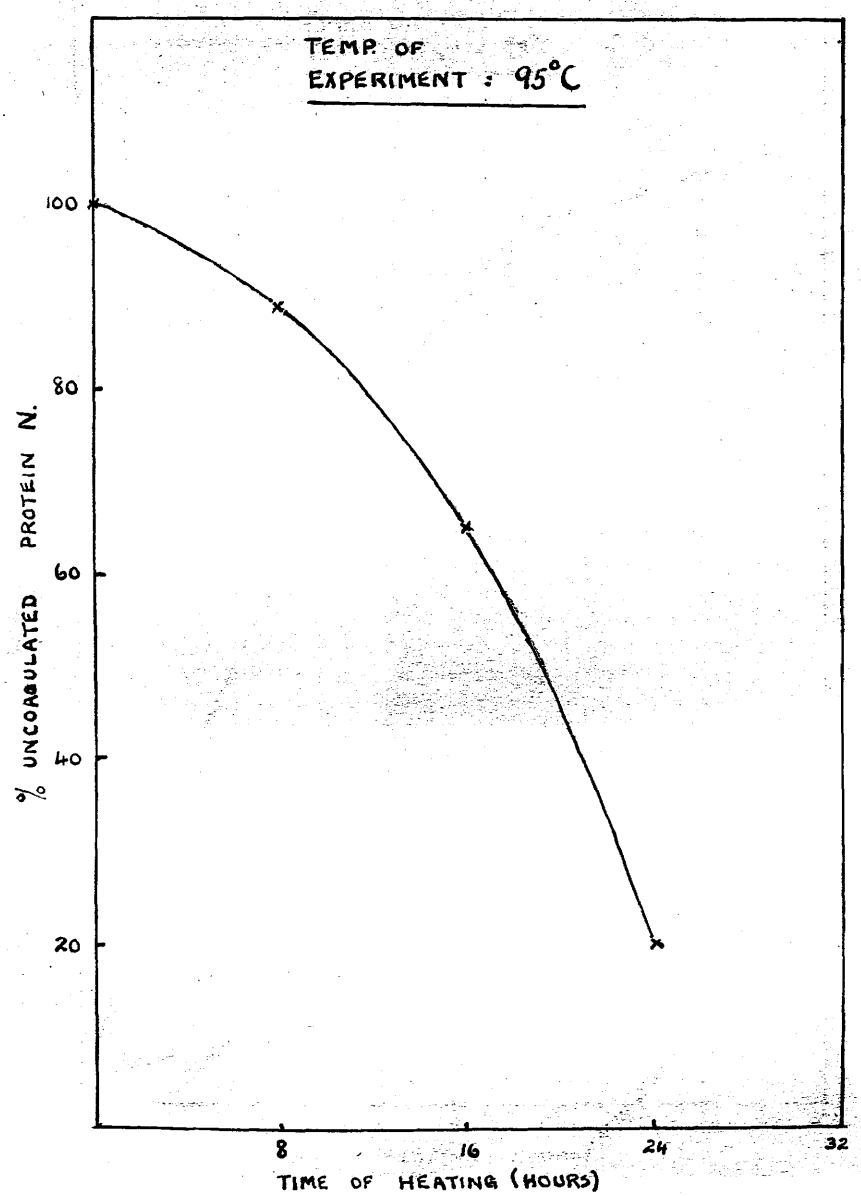
TEMP. OF
EXPERIMENT : 95°C
100
80
60
40
20
% UNCOAGULATED PROTEIN N.
8
16
24
32
TIME OF HEATING (HOURS)

FIG. 11.

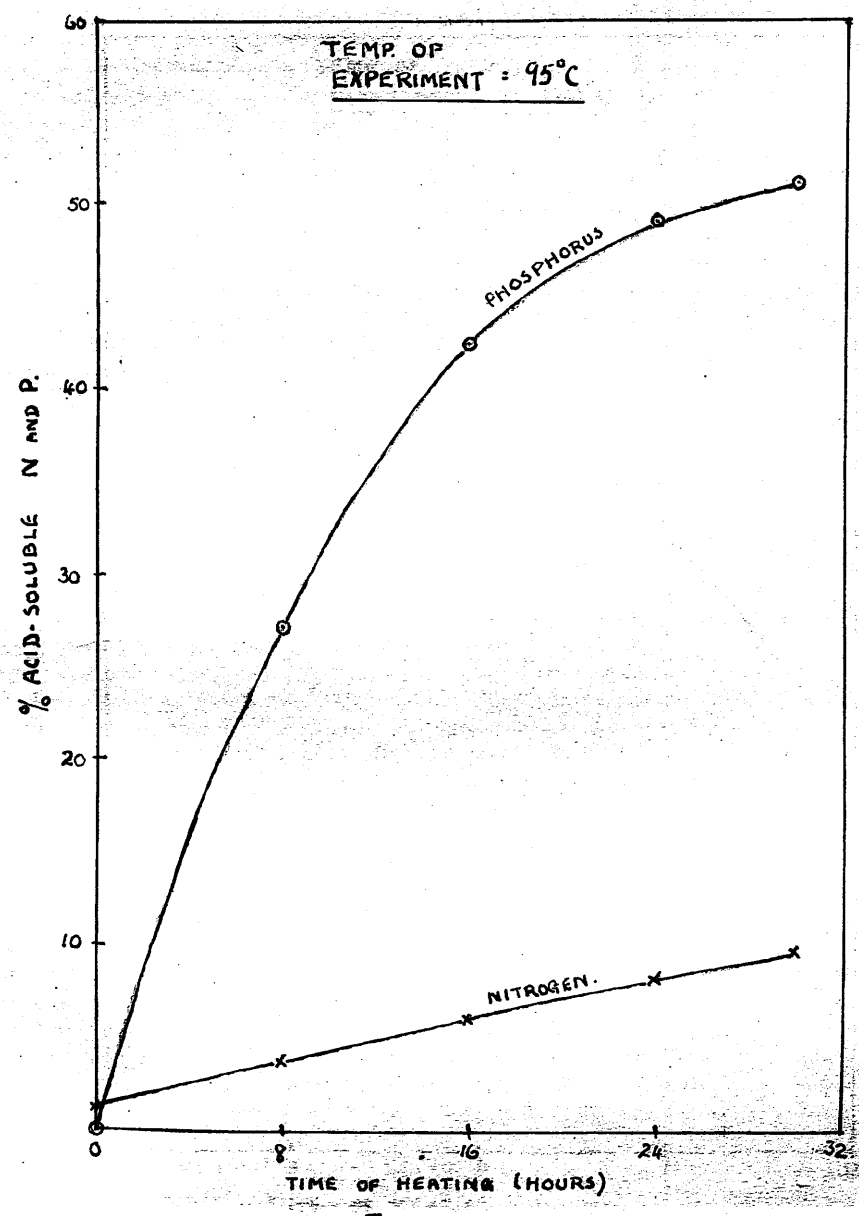
TEMP OF
EXPERIMENT = 95°C
PHOSPHORUS
NITROGEN.
% ACID-SOLUBLE N AND P.
60
50
40
30
20
10
0
8
16
24
32
TIME OF HEATING (HOURS)

FIG. 12.

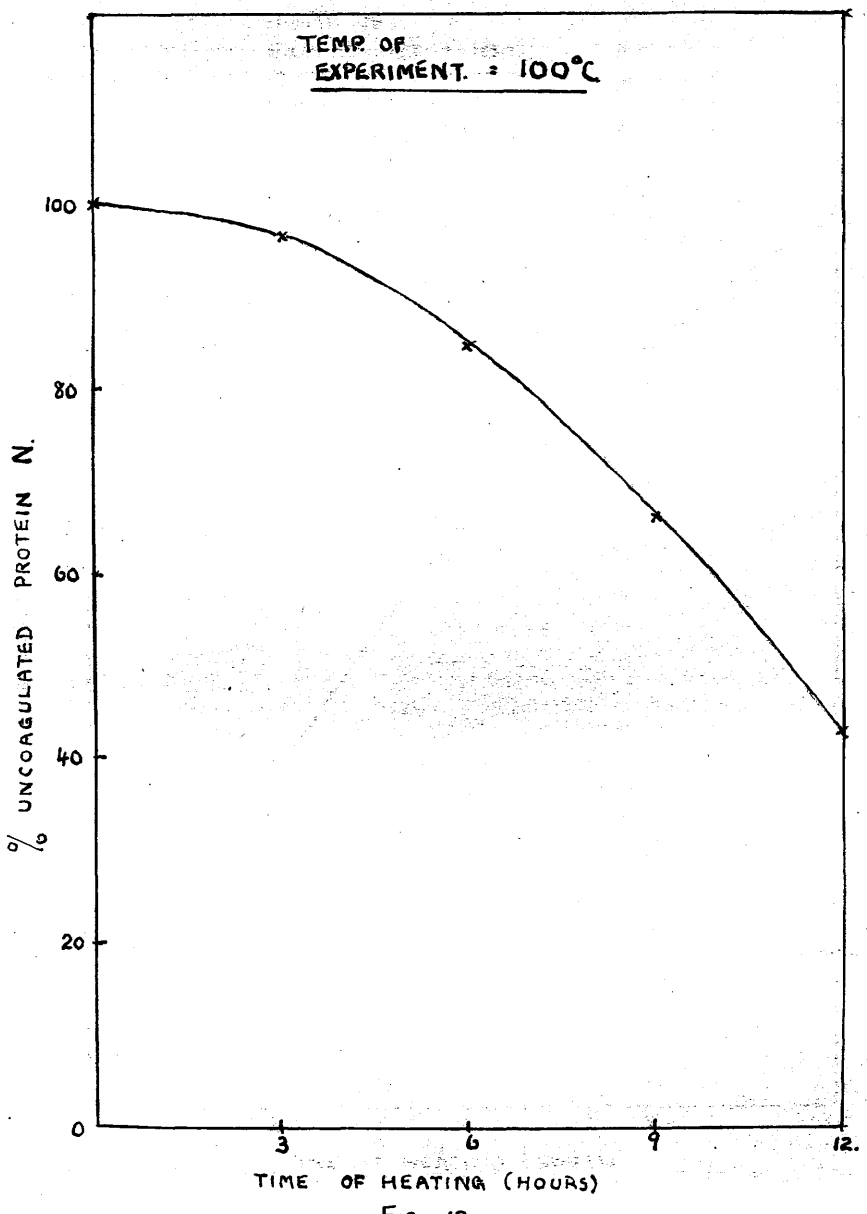
TEMP. OF
EXPERIMENT. = 100°C.
100
80
60
40
20
0
% UNCOAGULATED PROTEIN N.
3
6
9
12.
TIME OF HEATING (HOURS)

FIG. 13.

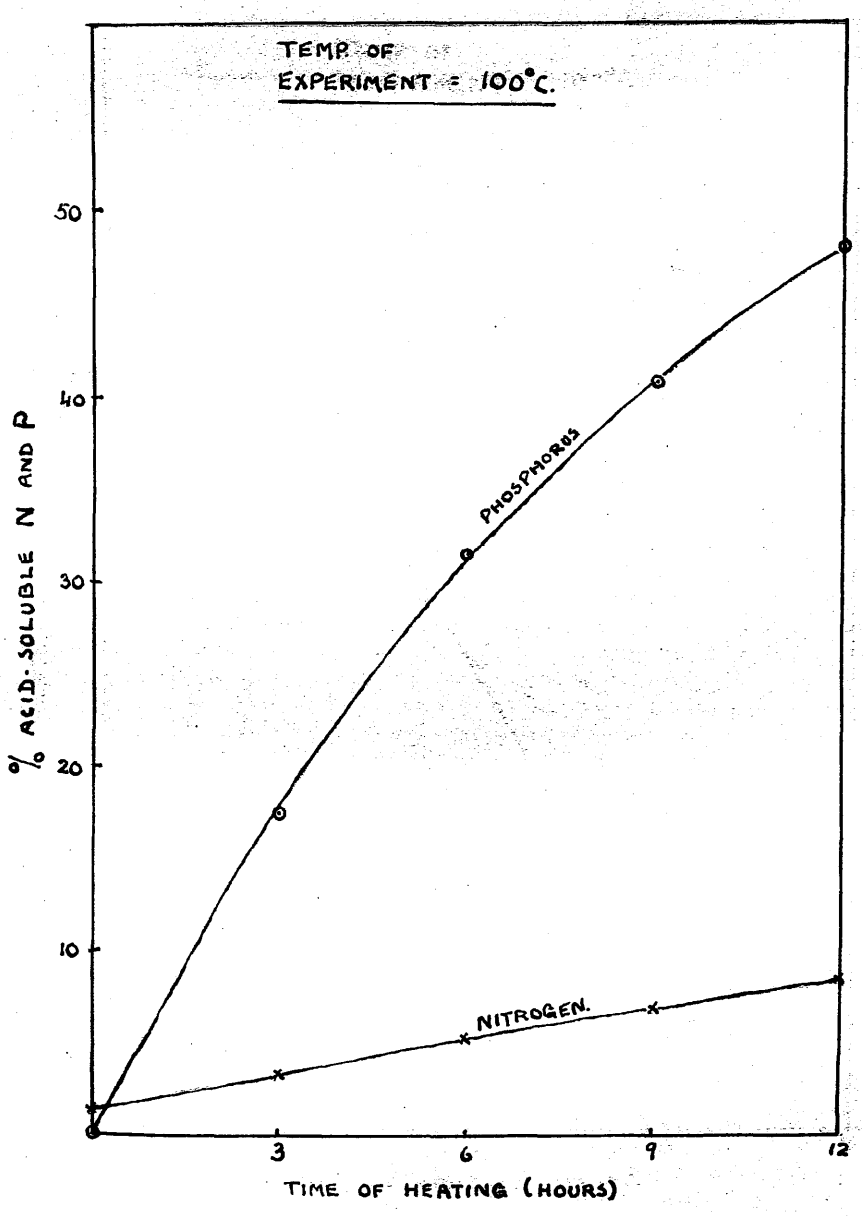
TEMP. OF
EXPERIMENT = 100°C.
50
40
30
20
10
% ACID-SOLUBLE N AND P
PHOSPHORUS
NITROGEN.
3
6
9
12
TIME OF HEATING (HOURS)

FIG. 14.

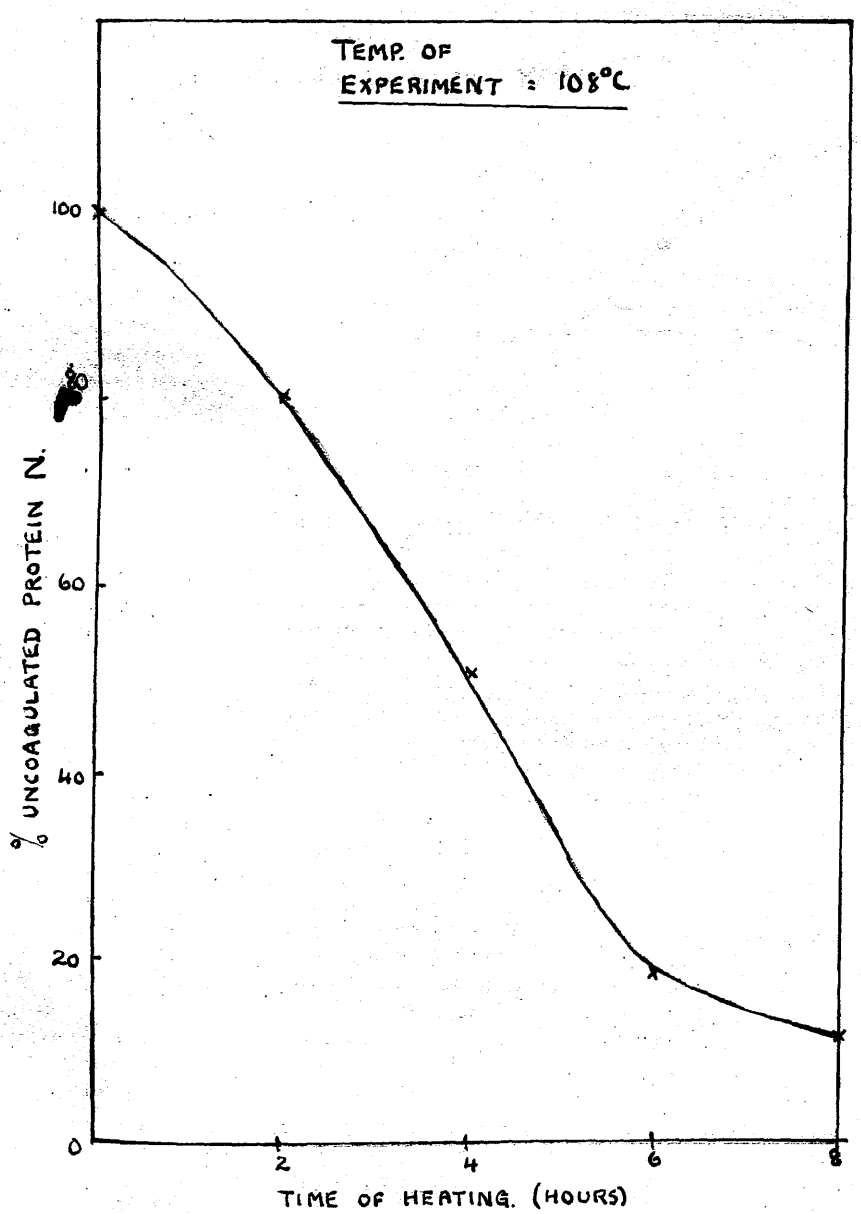
TEMP. OF
EXPERIMENT = 108°C.
100
80
60
40
20
0
% UNCOAGULATED PROTEIN N.
2
4
6
8
TIME OF HEATING. (HOURS)

FIG. 15.

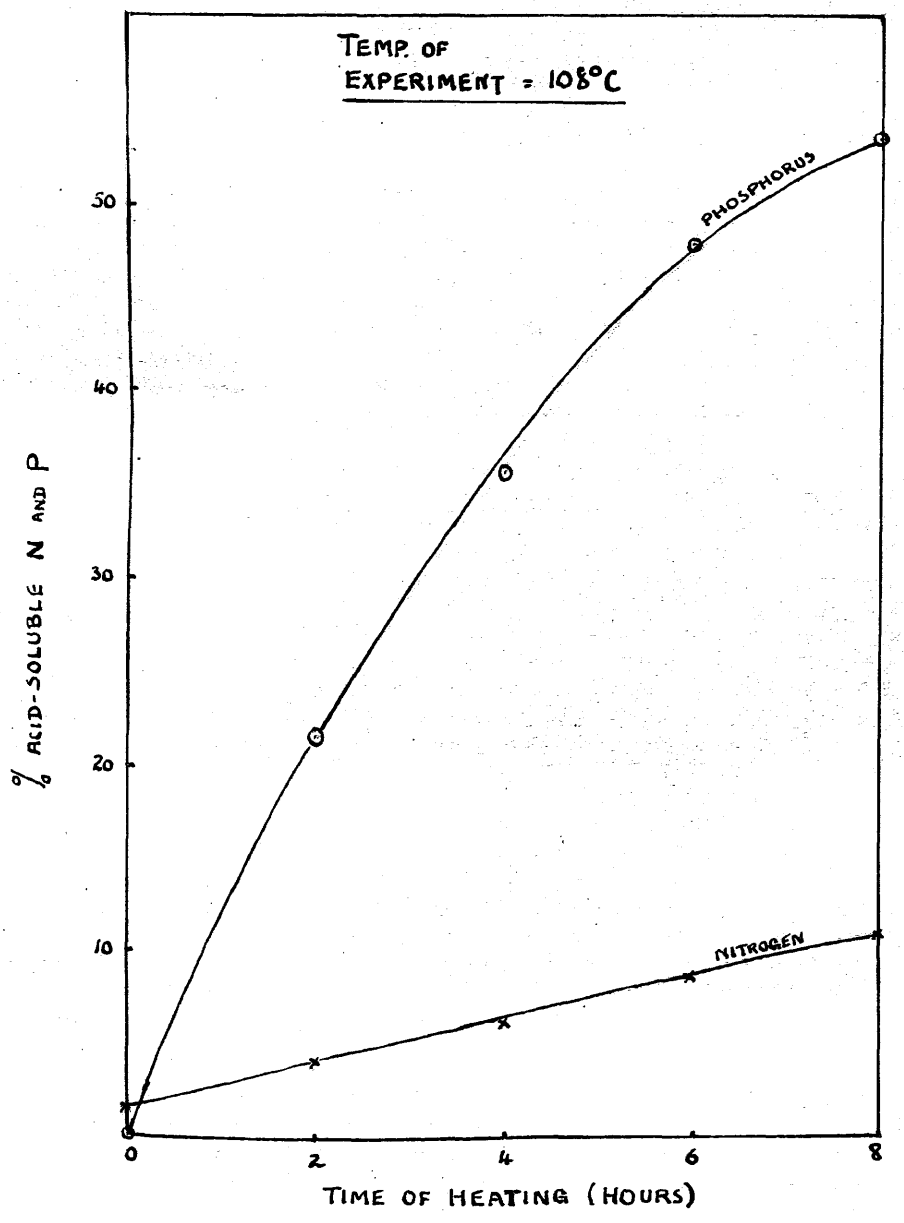
TEMP. OF
EXPERIMENT = 108°C
PHOSPHORUS
NITROGEN
% ACID-SOLUBLE N AND P
50
40
30
20
10
0
2
4
6
8
TIME OF HEATING (HOURS)

FIG. 16

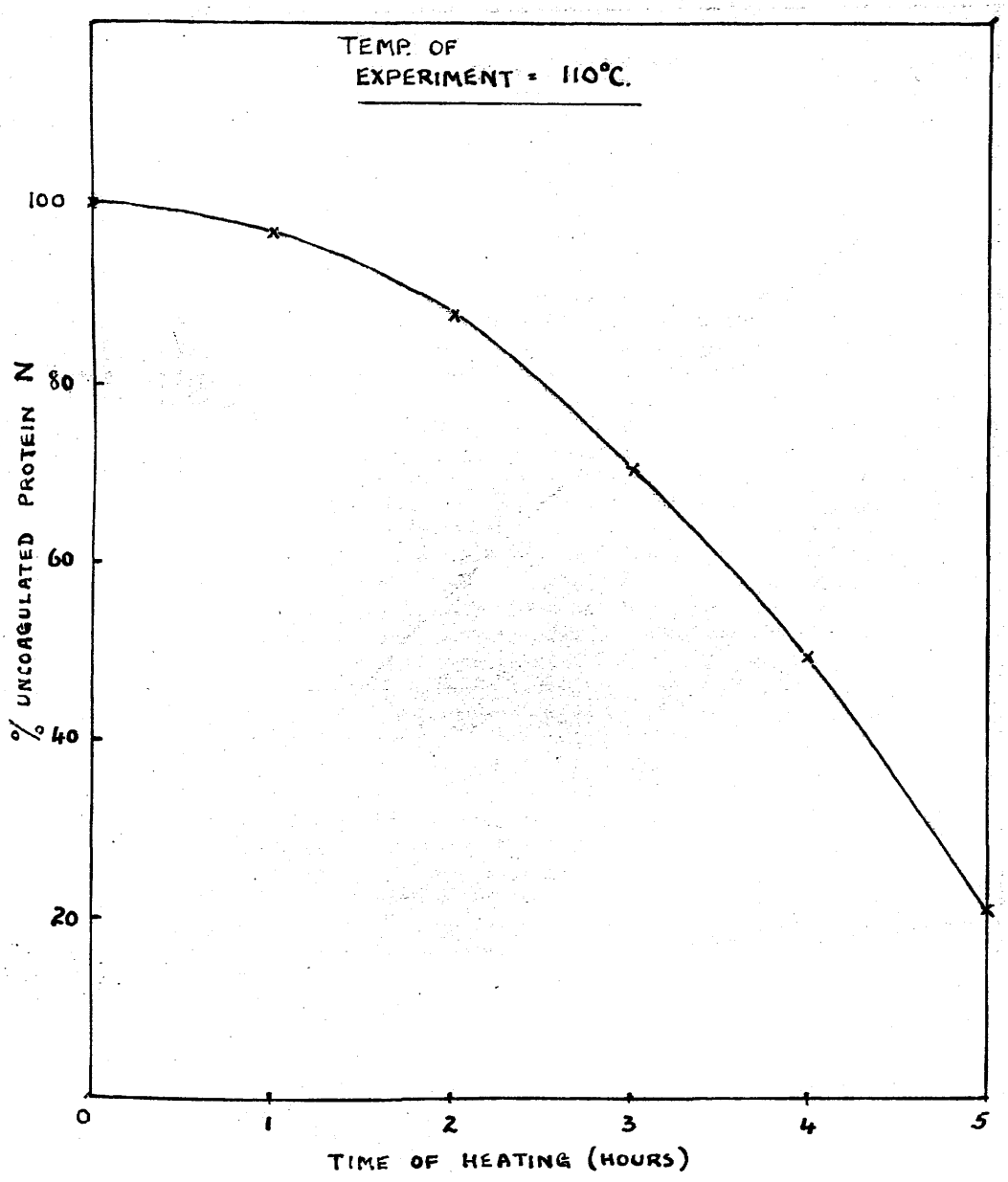
TEMP. OF
EXPERIMENT = 110°C.
100
80
60
40
20
% UNCOAGULATED PROTEIN N
0
1
2
3
4
5
TIME OF HEATING (HOURS)

FIG. 17.

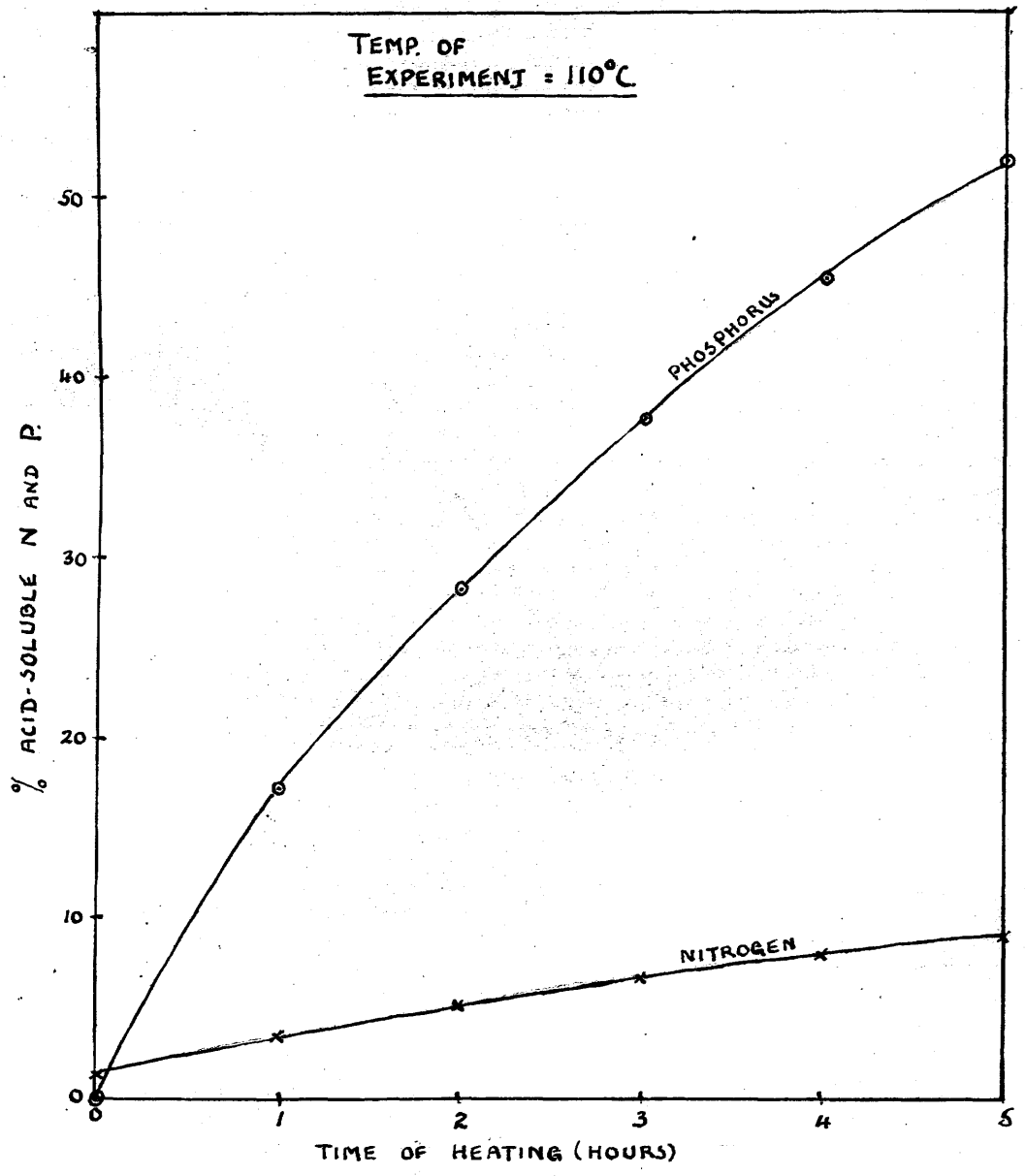
TEMP. OF
EXPERIMENT = 110°C.
% ACID-SOLUBLE N AND P.
50
40
30
20
10
0
PHOSPHORUS
NITROGEN
0
1
2
3
4
5
TIME OF HEATING (HOURS)

FIG. 18.

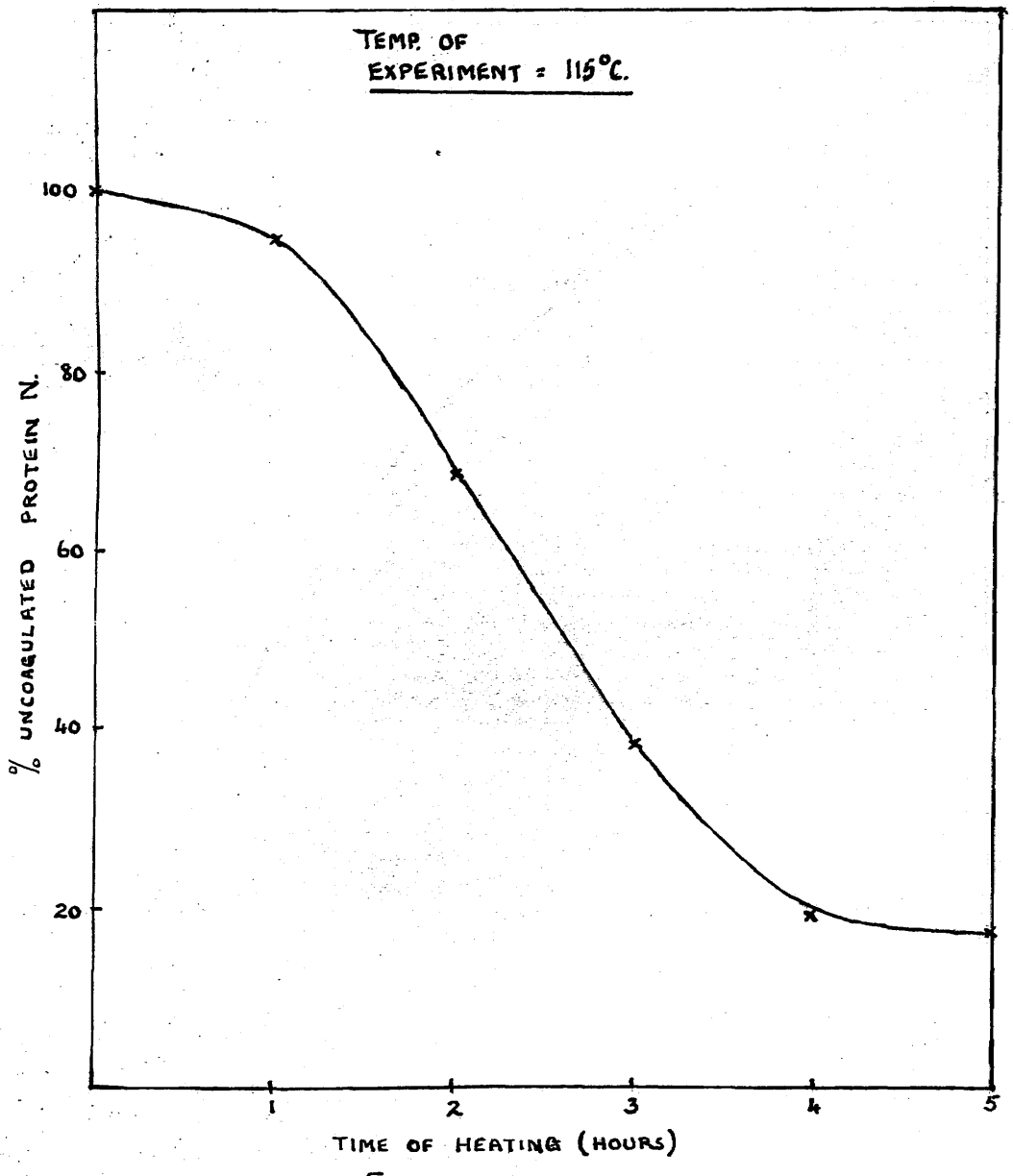
TEMP. OF
EXPERIMENT = 115°C.
100
80
60
40
20
% UNCOAGULATED PROTEIN N.
1
2
3
4
5
TIME OF HEATING (HOURS)

FIG. 19.

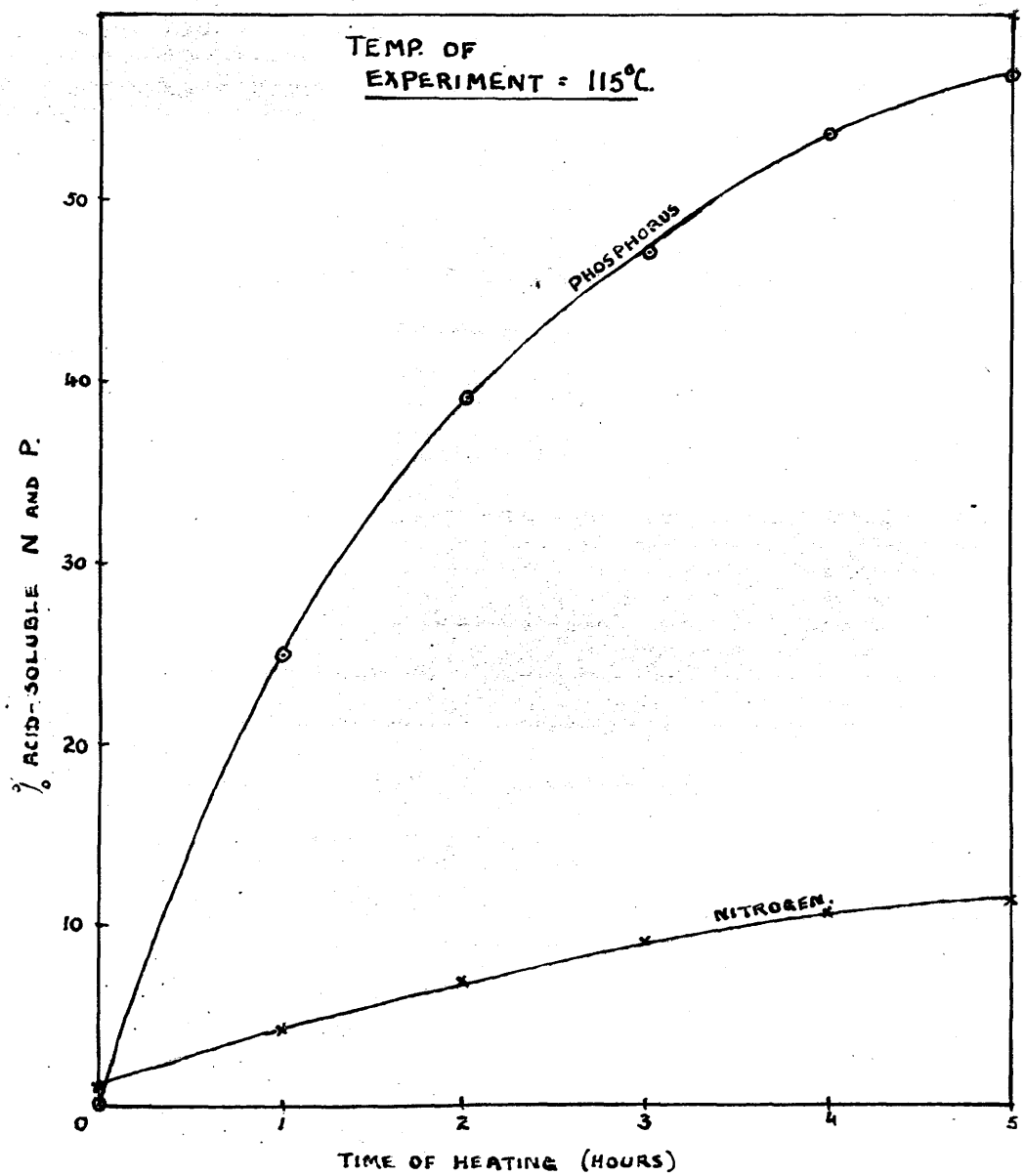
TEMP. OF
EXPERIMENT = 115°C.
PHOSPHORUS
NITROGEN
% ACID-SOLUBLE N AND P.
50
40
30
20
10
0
1
2
3
4
5
TIME OF HEATING (HOURS)

FIG. 20.

Results.

The actual figures obtained for total nitrogen and phosphorus, and acid-soluble nitrogen and phosphorus are given in Table V . These figures were subsequently graphed and a series of curves drawn through the points; definitely anomalous results being omitted in all cases. The values corresponding to the various time intervals were then read off from each curve; from these figures the percentage of total soluble (uncoagulated) nitrogen and acid-soluble nitrogen and phosphorus were calculated. They were also used to obtain the N/P ratios. The results are given in Table VI . In Figs. 9-20 the percentages of total soluble nitrogen and acid-soluble nitrogen and phosphorus are shown graphically.

Discussion.

It is at once evident that the effect of continued heat treatment of calcium caseinogenate solutions at these slightly lower temperatures is similar to that at 120°C. Thus there is a slow liberation of acid-soluble nitrogen and a rapid liberation of acid-soluble phosphorus, but the effect is not so marked over the time-intervals used in these experiments. It will be noted that the amount of acid-soluble phosphorus does not exceed 60 per cent. in any experiment nor does the acid-soluble nitrogen rise above 12 per cent.; at 120°C. the

TABLE VIA.

	Temperature (°C)	90°	95°	100°	108°	110°	115°
Time required to produce: (Measured in hours from commencement of heating)	Visible coagulation	43	23	12	5.5	4.5	2.75
	Log.	1.63	1.36	1.08	0.74	0.65	0.44
	45 per cent. dephosphorization	35.5	18.0	10.5	5.4	3.95	2.7
	Log.	1.55	1.25	1.02	0.73	0.59	0.43
	50 per cent. soluble protein N	36.0	18.6	11.0	4.0	3.95	2.60
	Log.	1.56	1.27	1.04	0.60	0.59	0.41

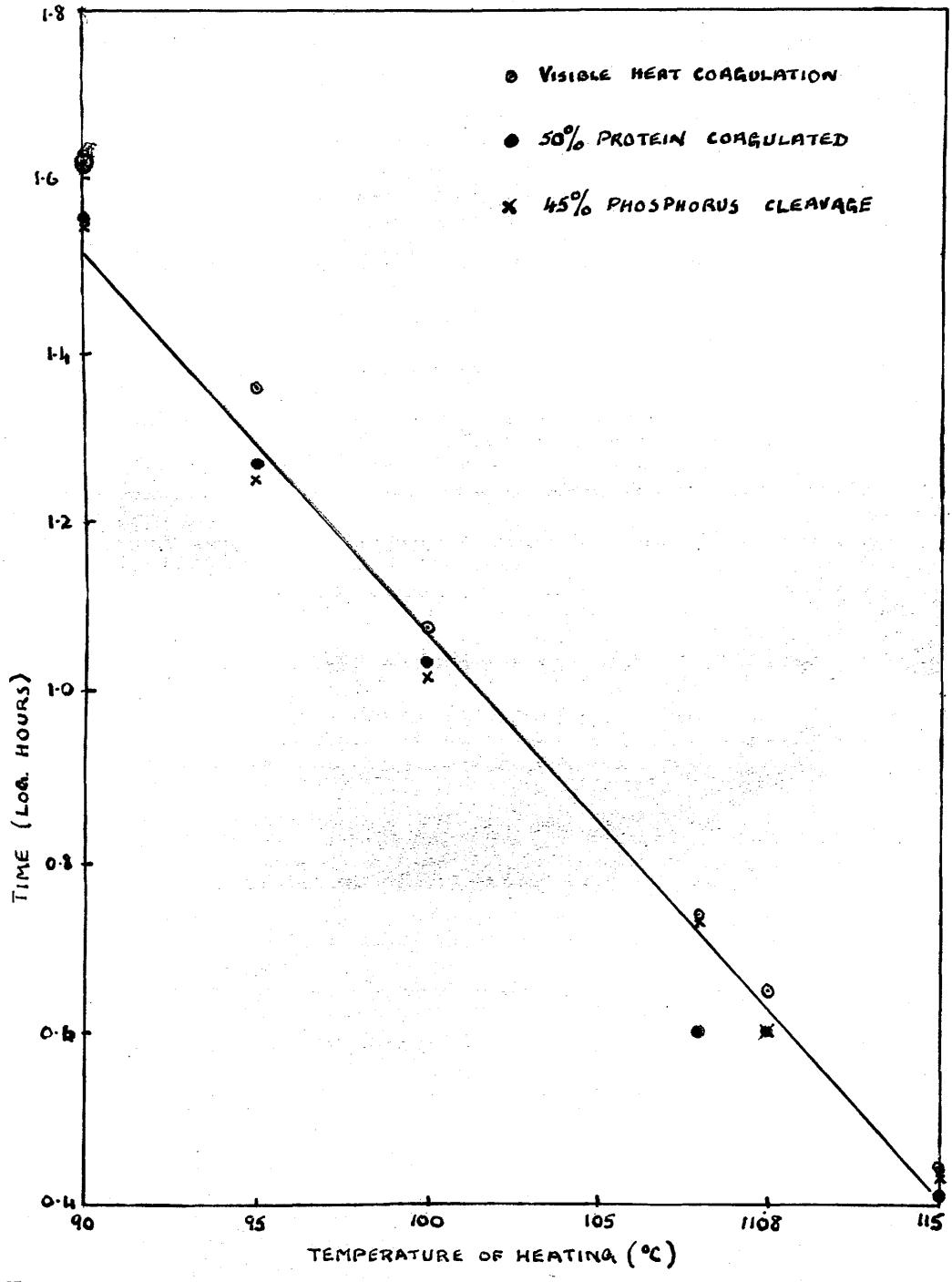

FIG. 21. RELATION BETWEEN PHOSPHORUS CLEAVAGE AND COAGULATION.

corresponding maximum values were 80 and 22 per cent. respectively.

Similarly the trend of the curves showing the progress of coagulation of the protein is similar to that obtained at 120°C. In all cases the amount of protein decreases slowly at first and then more rapidly, although the distinct "S" shape of the curve at 120°C. was not so easily discernible. It should be noted here that after centrifuging these solutions which had been in the bath for some time, a sediment, presumably of coagulated protein, was observed in the bottom of the centrifuge tubes, although visible coagulation had not yet taken place. The presence of this sediment in increasing amounts undoubtedly accounts for the slow preliminary fall in soluble nitrogen previously noted.

It seemed possible, from the figures given in Table VI and the general shape of the curves in Figs. 9 - 20 that the appearance of visible coagulation might coincide roughly with approximately the same percentage dephosphorisation of the protein molecule. Accordingly the time taken to produce visible coagulation, to effect 45 per cent. dephosphorisation and to reduce the soluble protein nitrogen to 50 per cent., all expressed logarithmically in hours, were calculated for each temperature, and plotted against temperature in Fig. 21. and Table VIA. It is evident that at these temperatures, the ap-

TABLE VIII.

Value of the ratio:

$$\frac{\text{Acid-soluble N}}{\text{Acid-soluble P}} \quad (N/P)$$

at the following temperatures.

Time of heating (hours)	90°C.						95°C.						100°C.					
	0	9	18	27	36	45	0	8	16	24	32		0	3	6	9	12	
N/P	-	3.16	2.92	3.14	3.33	3.45	-	2.47	2.60	3.07	3.45		-	3.5	3.09	3.13	3.24	
Time of heating (hours)	108°C						110°C						115°C					
	0	2	4	6	8		0	1	2	3	4	5	0	1	2	3	4	5
N/P	-	3.42	3.22	3.27	3.62		-	3.46	3.17	3.12	3.12	3.08	-	2.89	3.06	3.38	3.43	3.46

Time of heating (hours)	120°C.					
	0	1	2	3	4	5
	-	2.92	3.18	3.60	4.06	4.47

pearance of visible coagulation coincides closely with a reduction of the uncoagulated protein to 50 per cent. and that this takes place when the caseinogen molecule has suffered about 45 per cent. dephosphorisation. The close connection suggested earlier (p.22) appears to be fully warranted.

One further point worth noting is suggested by the N/P ratios of the acid-soluble fractions given in Table VIII. The value of this ratio appears to keep fairly constant so long as the cleavage of phosphorus is taking place fairly rapidly. When this ceases, or becomes slow, as, for example, in the sample heated at 120°C., the N/P ratio tends to rise. It might be suggested therefore from these figures (although the evidence is scanty) that the part of the caseinogen molecule which is preferentially disrupted is that part which contains the phosphorus grouping, and that only when this cleavage has largely been completed does degradation occur in other parts of the molecule.

IIIA. ADDENDUM ON THE DEPHOSPHORISATION OF CASEINOGEN AT LOWER TEMPERATURES:

For various reasons it is sometimes necessary to store milk products, such as evaporated milk (unsweetened condensed milk), for long periods of time at fairly high storage

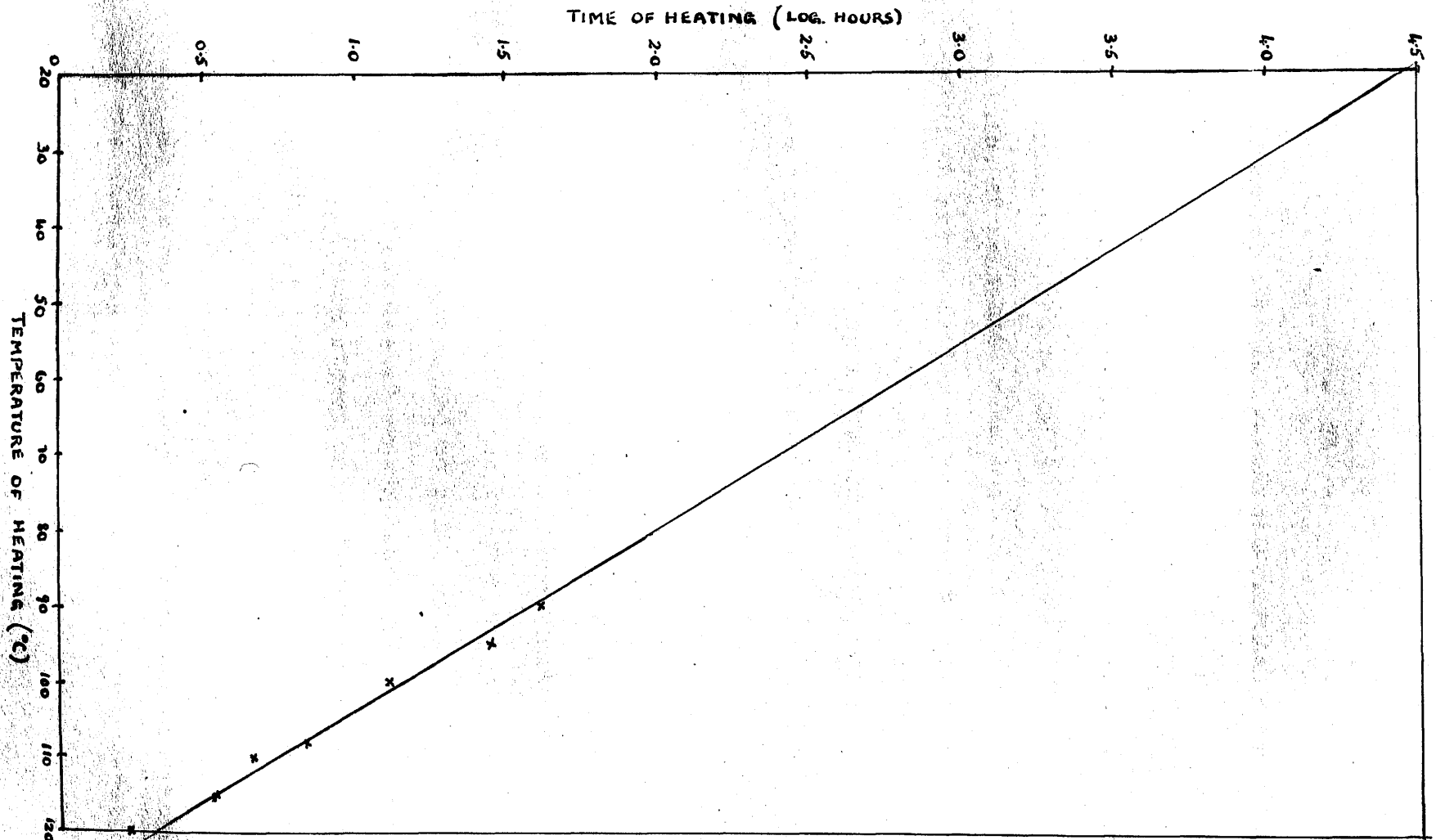

FIG. 22. EXTRAPOLATION OF 50% PHOSPHORUS CLEAVAGE (TIME IN LOG. HOURS) TO STORAGE TEMPERATURES

temperatures. If the dephosphorisation of the caseinogen which proceeded rapidly at 120°C. and more slowly at 90°C. still proceeded at, say, 25°C., such dephosphorisation might result in subsequent coagulation of the solution, thus rendering the product unfit for consumption. In order to obtain, if possible, some idea of the rate of the reaction at 25°C. an extrapolation curve from the available data was drawn. The times expressed logarithmically in hours required to produce 50 per cent. dephosphorisation at each of the temperatures studied was plotted against temperature in Fig. 22 . The points fell, within the limits of experimental error, on a straight line which was extrapolated down to storage temperatures. From this graph the time required to produce 50 per cent. dephosphorisation at 25°C. is 2.4 yrs. which is well beyond the usual time limits of storage.

Since results obtained in pure protein solutions, however, cannot always be applied to milk, it was decided to investigate a number of samples of evaporated milk which were of different ages and had been stored under different conditions to show if any evidence of dephosphorisation could be secured.

Technique.

It was obvious that any change in the phosphorus content of the caseinogen would be reflected by an alteration in the N/P ratio. To obviate any long and tedious isolation of the

TABLE IX.

Effect of storage at different temperatures for different periods of time on the N/P ratio of the caseinogen.

	N/P ratio.						
Freshly prepared samples	18.91	21.31	21.32	19.62			
Stored samples	19.14	21.20	20.90	20.90	18.50	19.90	21.50

protein itself the following method was adopted for determinin the nitrogen and phosphorus content of the caseinogen.

Analysis: A dilution of known concentration (4-5 per cent.) was prepared from the sample of evaporated milk. The total nitrogen and phosphorus of this dilution were determined. Caseinogen was precipitated from another portion of this dilution with acetic acid (A.O.A.C. p.260) and after filtration the non-caseinogen nitrogen and phosphorus were determine in the filtrate. The nitrogen and phosphorus values for the caseinogen were obtained by difference.

Results and Discussion.

The results are given in Table IX . Any small differences in the N/P ratios of the various samples do not appear statistically to be of significance. The conclusion stated above, i.e. that dephosphorisation of caseinogen under the conditions occurring in milk does not occur to any extent under normal times and temperatures of storage, is evidently confirmed.

IV. THE INFLUENCE OF THE CALCIUM SALTS.

It has been pointed out earlier (p. 16) that, as shown in Fig. 5 ,the alkali-binding capacity of heat dephosphorised caseinogen is less than that of unchanged caseinogen within the narrow limits of the pH range used in these experiments.

At pH = 6.9 this difference amounts to 10 x 10^{-5} gm.-equivalent of alkali per 1 gm. protein. It seems reasonable to assume therefore that during heat treatment of a neutral calcium caseinogenate solution the production of dephosphorise caseinogen will be accompanied by a liberation of calcium, either in association with inorganic phosphate, probably partly in colloidal solution, or in combination with some protein degradation products. In either case it seems almost certain that the presence of such a strong coagulating agent in an increasing concentration will undoubtedly accelerate heat coagulation.

In order to obtain experimental data on the subject the following experiments were carried out.

(a) The Effect of Added Calcium Salts.

Technique. Solutions: A 3 per cent. solution of the calcium salt of heat-dephosphorised caseinogen was prepared as in Section II. In this case, however, the amount of $Ca(OH)_2$ used was that required by the same volume of ordinary caseinogenate (i.e. 0.061 gm. $Ca(OH)_2$ per 100 ml. in place of 0.051 gm.). The pH was adjusted to the required value by the addition of small quantities of 0.1 N HCl. The resultant solution can therefore be considered broadly as a solution of calcium caseinogenate in which the entire protein has been

TABLE X .

All amounts expressed in mgs. per 100 ml. of original solution.

Time of Heating (hours)	Untreated caseinogen.				Heat-dephosphorized caseinogen.			
	Protein N in solution	% Protein N in solution.	Acid sol. N in solution	% Acid sol.N in solution	Protein N in solution	% Protein N in solution	Acid sol. N in solution	% Acid sol. N in solution
0	364	100	10.5	2.80	360.5	100	14	3.74
1	258.5	71.03	25.5	6.81	79.5	22.1	18.5	4.83
2	63	17.31	28.5	7.61	24.	6.7	21	5.61
3	35.5	9.75	46.5	12.42	27.5	7.6	25	6.68
4	31.5	8.65	52.5	14.02	20	5.5	29	7.74
5	22.5	6.18	61.5	16.42	20	5.5	30.5	8.14

TABLE X.

All amounts expressed in mgs. per 100 ml. of original solution.

Time of Heating (hours)	Untreated caseinogen.				Heat-dephosphorized caseinogen.			
	Protein N in solution	% Protein N in solution.	Acid sol. N in solution	% Acid sol.N in solution	Protein N in solution	% Protein N in solution	Acid sol. N in solution	% Acid sol. N in solution
0	364	100	10.5	2.80	360.5	100	14	3.74
1	258.5	71.03	25.5	6.81	79.5	22.1	18.5	4.83
2	63	17.31	28.5	7.61	24.	6.7	21	5.61
3	35.5	9.75	46.5	12.42	27.5	7.6	25	6.68
4	31.5	8.65	52.5	14.02	20	5.5	29	7.74
5	22.5	6.18	61.5	16.42	20	5.5	30.5	8.14

dephosphorised but with the original amount of calcium still present.

A similar 3 per cent. solution of calcium caseinogenate was prepared with the addition of a similar extra quantity of $Ca(OH)_2$ and adjusting the pH as before.

Heating, etc.: Heating and methods of analysis were also as in Section II .

Results and Discussion.

The results obtained are given in Table X . Figs. 6 and 7 show the results graphically. (EXCESS CALCIUM)

It is evident that the presence of excess calcium ions accelerates the heat coagulation of both solutions, but the effect is more striking with the untreated product than the treated. The rate of heat coagulation of the untreated caseinogen is, however, still markedly less than that of the dephosphorised product, the relative proportions coagulated at the end of one hour being 30 and 75 per cent. respectively. It will further be noticed that the amount of protein coagulated in both solutions containing excess Ca ions is considerably increased.

It appeared very desirable therefore to obtain evidence on the question of the amount of soluble calcium in the various solutions, especially with a view to obtaining direct evidence of the increase in soluble calcium suggested by the

TABLE XI.

All amounts expressed in mgs. per 100 ml. of original solution.

		90°C.						95°C.						100°C.					
		0	10	18	27	36	45	0	8	16	24	30	0	2.5	5.0	7.5	10	12.5
Nitrogen	Total in solution	406.7	356.3	366.3	269.5	158.2	93.1	395.0	360.5	280.7	95.2	94.5	400.4	387.8	310.8	298.2	145.6	-
	Acid-soluble	5.2	16.0	15.5	17.4	21.7	40.6	4.6	15.8	24.5	25.9	43.4	4.5	9.8	15.8	22.4	23.7	22.4
Calcium	Total in solution	37.1	32.8	33.0	-	-	14.5	35.5	31.9	27.6	12.7	13.5	38.3	37.1	30.3	29.5	24.8	19.5

		105°C.					110°C.						115°C.					
		0	2	4	6	8	0	1	2	3	4	5	0	1	2	3	4	5
Nitrogen	Total in solution	392.5	395.5	287.0	220.5	107.0	379.8	352.8	322.0	194.5	114.0	105	395.5	315.7	115.5	101.5	88.2	109.9
	Acid-soluble	4.2	13.7	20.7	29.4	35.0	5.2	13.9	18.9	23.9	30.8	36.4	4.6	15.5	25.8	34.3	40.7	46.9
Calcium	Total in solution	33.2	32.8	26.5	21.3	15.2	34.4	33.0	29.2	21.4	17.2	15.5	34.6	30.5	15.6	14.2	13.5	17.2

titration curves.

(b) The Amounts of Soluble Calcium in Heated Solutions.

Technique: 3 per cent. solutions of calcium caseinogenate prepared exactly as in the preceding section were subjected to the same heat treatment as before; treatment after removal from the bath was also the same.

Analysis: Total soluble nitrogen, acid-soluble nitrogen and total calcium were determined. Calcium was estimated by McCrudden's method (1909). 4 or 5 ml. of the supernatant liquid were ashed according to the amount of solution available; due to the small amounts present precipitation of the calcium oxalate was made in a centrifuge tube, the precipitate being washed free from ammonium oxalate by repeated washings with water and syphoning off the supernatant liquid after centrifuging for 5 minutes at 2000 r.p.m. The titrations were made with N/50$KMnO_4$.

Results.

The actual figures obtained for the various time-intervals at each temperature are given in Table XI. As before these values were graphed and from the curves so obtained the values corresponding to each time-interval were read off. From these figures the percentage of total soluble (uncoagulated) acid-soluble nitrogen, and total calcium were

TABLE XII

All amounts expressed in mgs. per 100 ml. of original solution

		90°C.						95°C.						100°C.					
		0	9	18	27	36	45	0	8	16	24	32	0	2.5	5	7.5	10	12.5
Nitrogen	Total in solution	403	383	347	268	159	92	395.0	360	280	96	93	400	388	350	282	150	118
	Acid - soluble	5.2	12.2	15.6	17.6	22.0	40.6	4.6	16.2	25.4	34.0	40.9	4.2	11.0	17.0	21.0	23.1	23.9
Calcium	Total in solution	36.8	33.3	32.6	29.4	18.3	14.5	35.0	31.8	27.4	14.0	12.3	38.3	36.6	33.7	29.7	24.9	19.5

		105°C.					110°C.						115°C.					
		0	2	4	6	8	0	1	2	3	4	5	0	1	2	3	4	5
Nitrogen	Total in solution	395	380	315	223	103	380	354	321	195	116	105	396	316	117	102	95	91
	Acid - soluble	4.2	13.8	21.9	29.0	35.0	5.2	12.8	19.4	25.2	31.0	35.7	4.6	15.8	25.6	34.1	41.3	47.0
Calcium	Total in solution	33.1	32.4	27.5	21.6	15.3	34.5	33.0	29.2	21.3	17.2	15.5	34.6	30.3	16.0	14.1	13.6	14.2

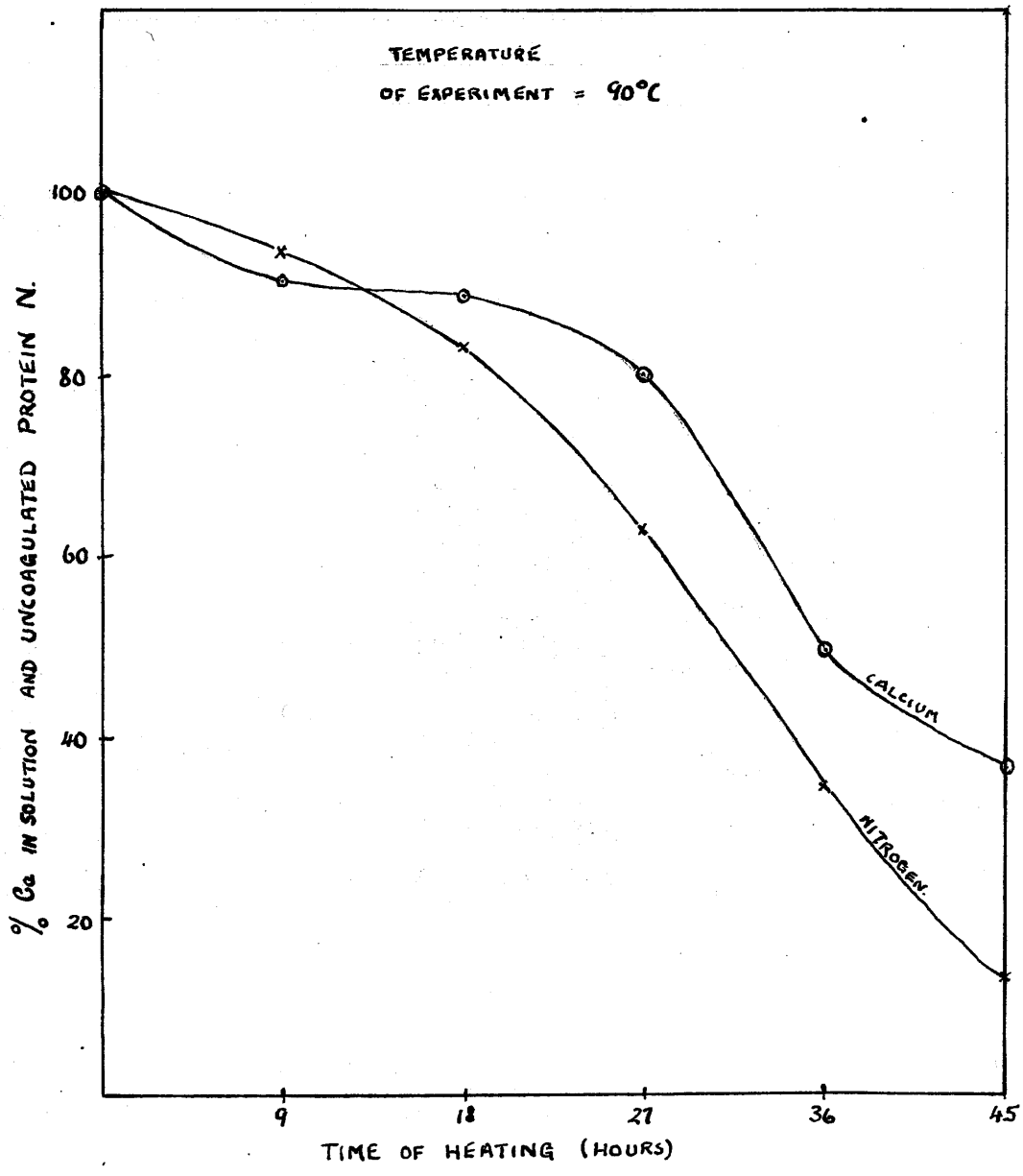
TEMPERATURE
OF EXPERIMENT = 90°C
100
80
60
40
20
% Ca IN SOLUTION AND UNCOAGULATED PROTEIN N.
CALCIUM
NITROGEN.
9
18
27
36
45
TIME OF HEATING (HOURS)

FIG. 23.

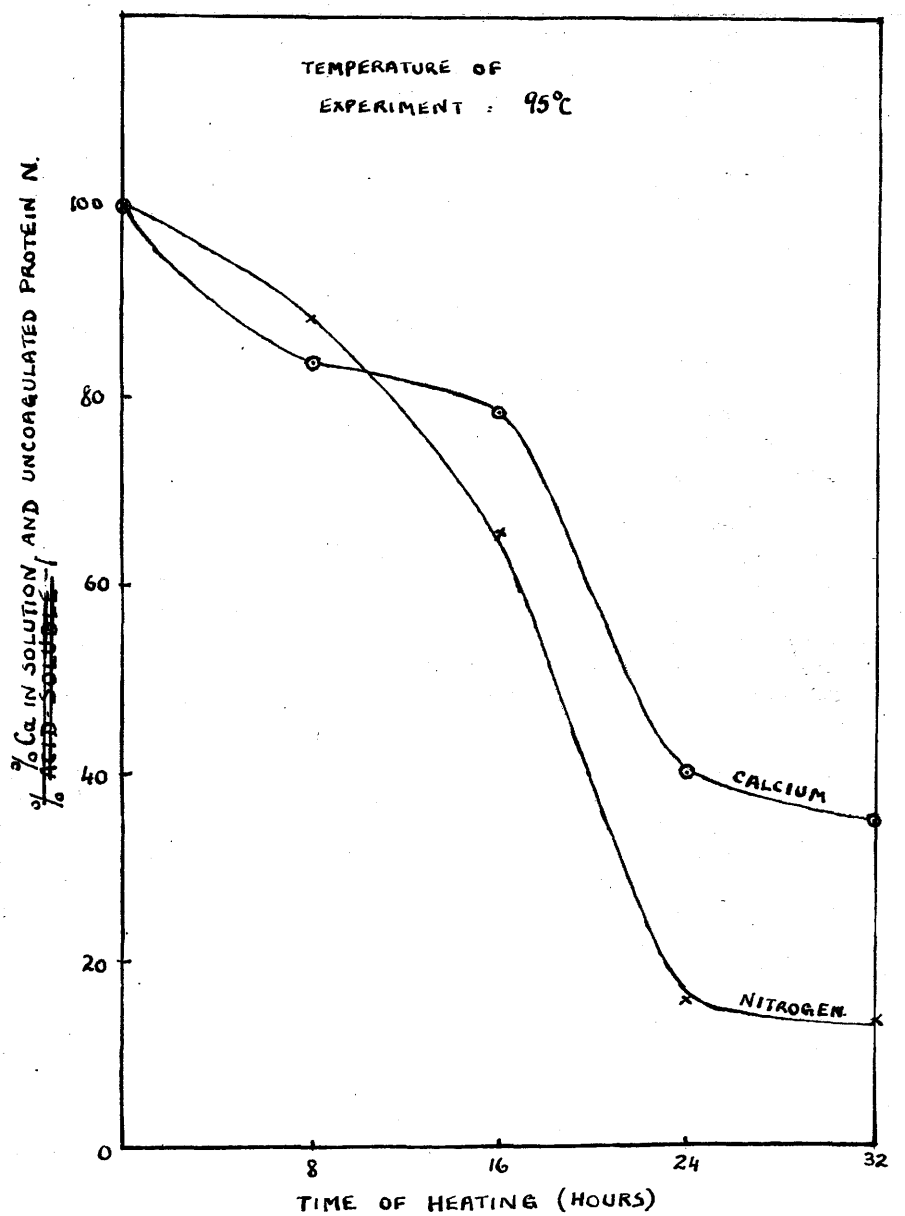
TEMPERATURE OF
EXPERIMENT : 95°C
% Ca IN SOLUTION AND UNCOAGULATED PROTEIN N.
% ACID SOLUBLE
100
80
60
40
20
0
8
16
24
32
CALCIUM
NITROGEN
TIME OF HEATING (HOURS)

FIG 24.

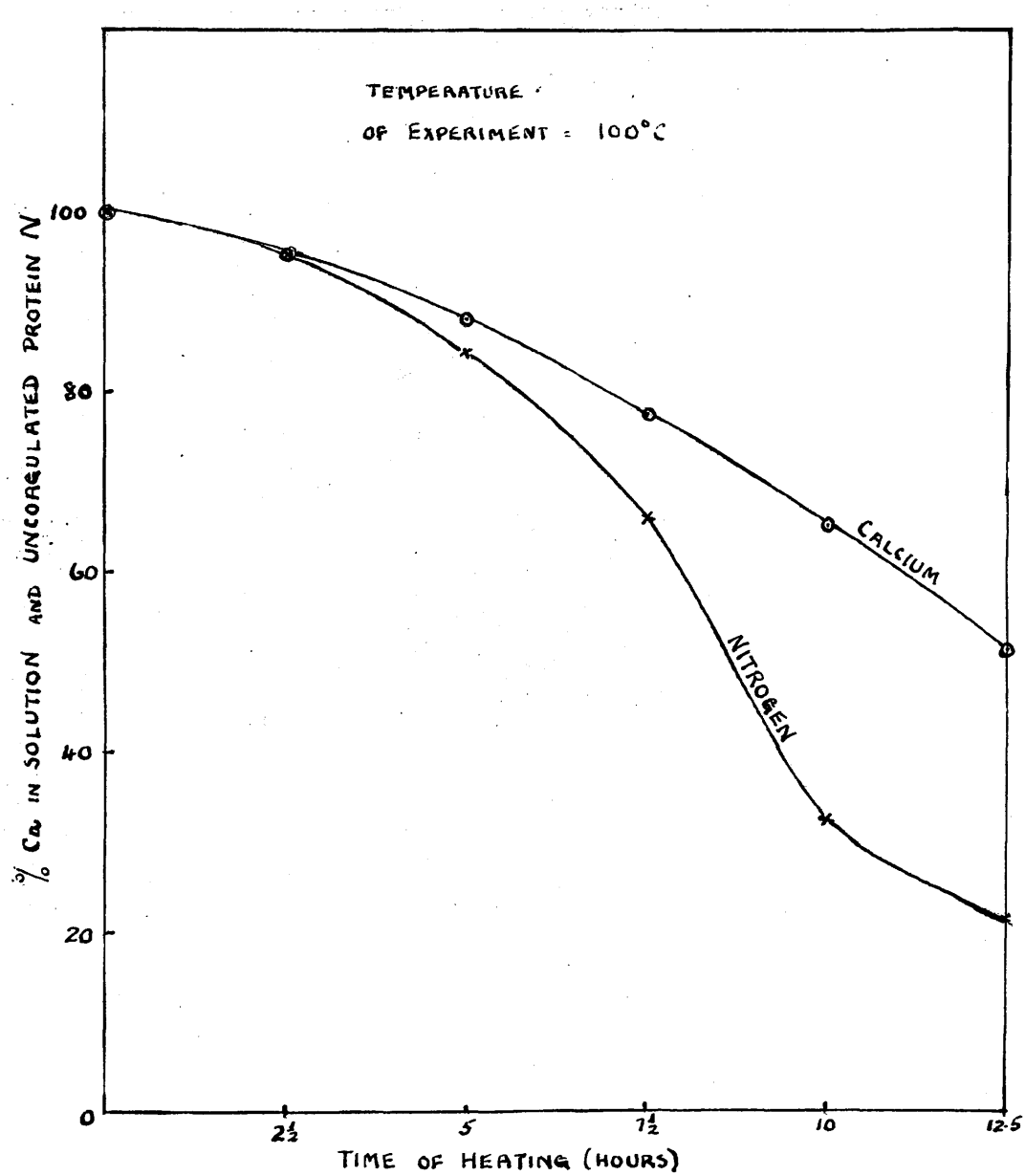
TEMPERATURE OF EXPERIMENT = 100°C
% Ca IN SOLUTION AND UNCORGULATED PROTEIN N
100
80
60
40
20
0
CALCIUM
NITROGEN
2½
5
7½
10
12·5
TIME OF HEATING (HOURS)

FIG. 25

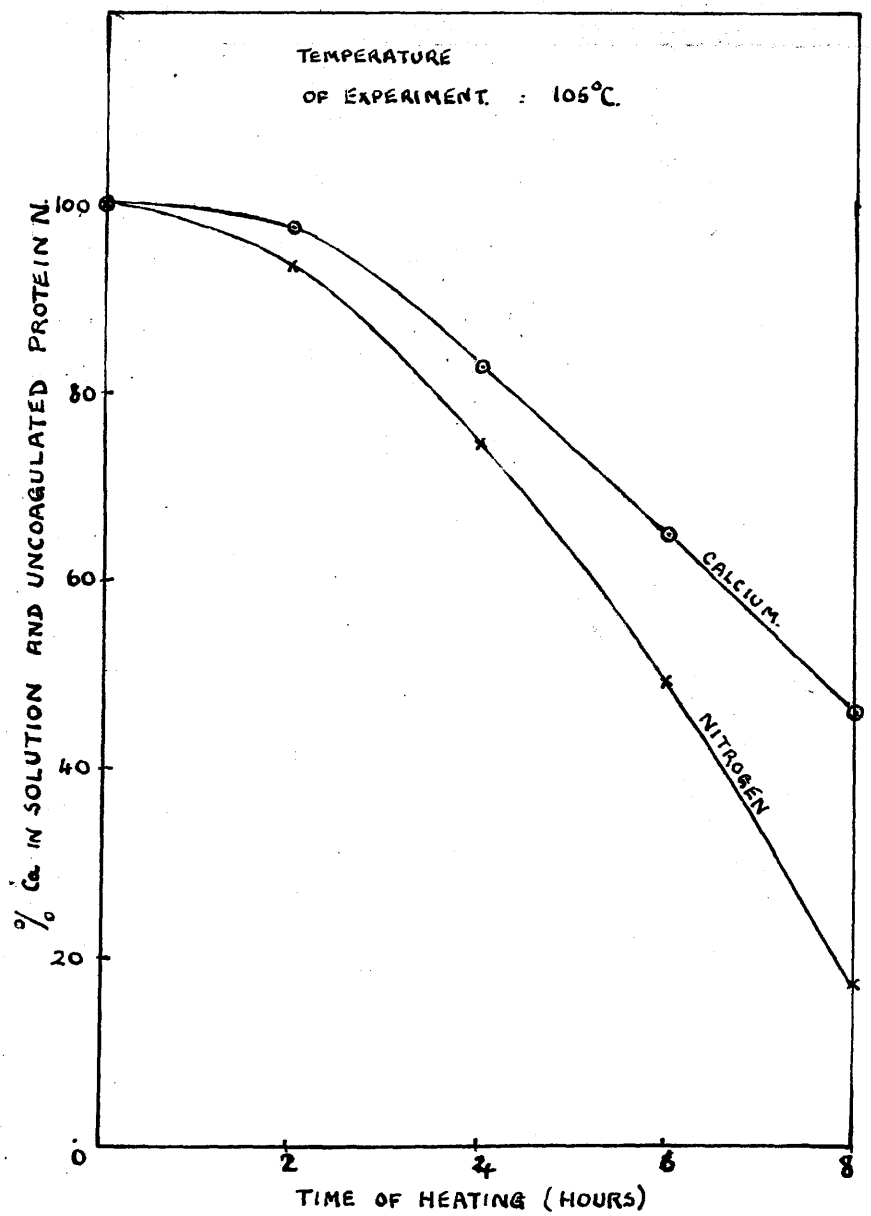
TEMPERATURE
OF EXPERIMENT : 105°C.
% Ca IN SOLUTION AND UNCOAGULATED PROTEIN N.
100
80
60
40
20
0
2
4
6
8
CALCIUM.
NITROGEN
TIME OF HEATING (HOURS)

FIG. 26.

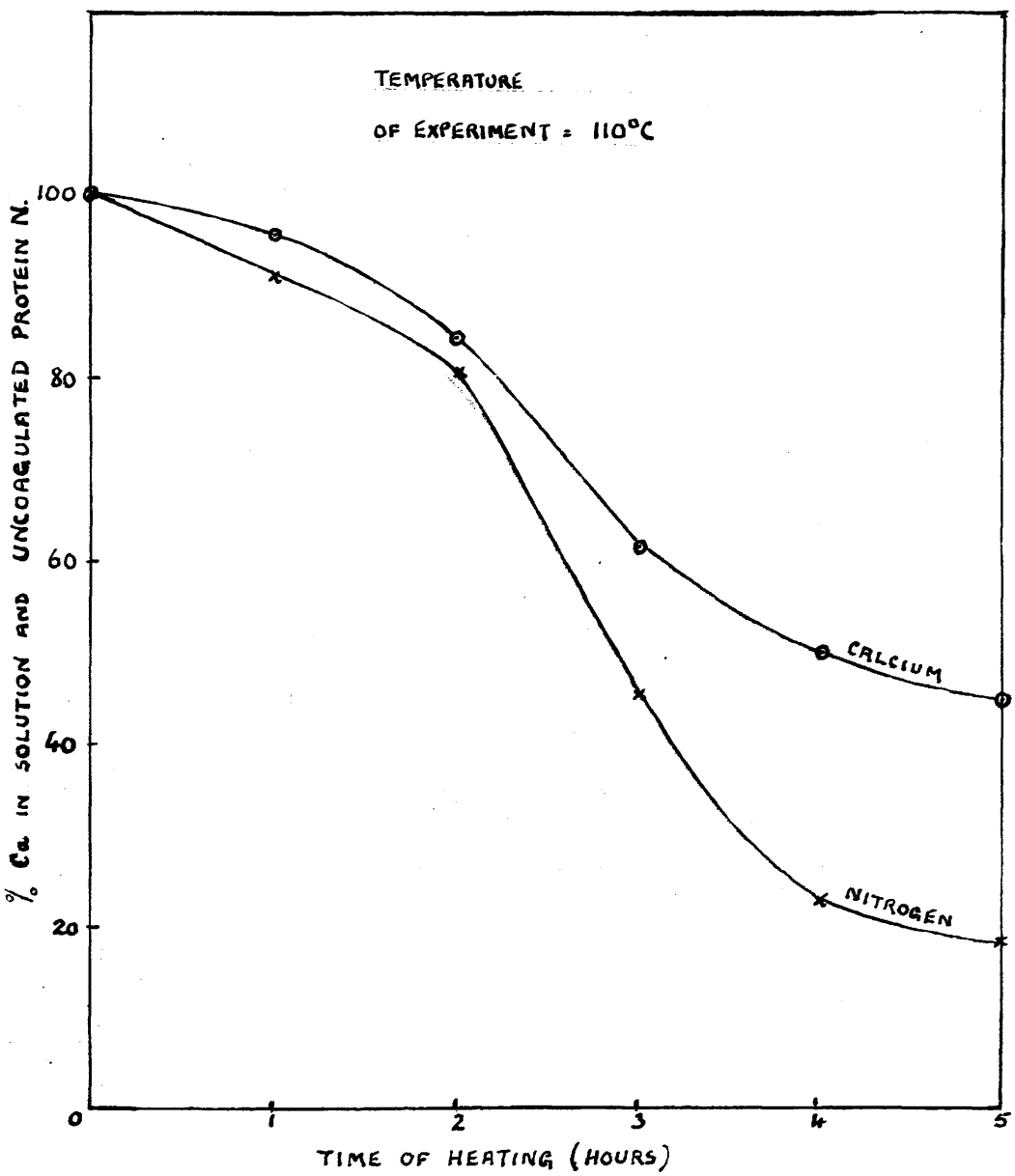
TEMPERATURE
OF EXPERIMENT = 110°C
% Ca IN SOLUTION AND UNCOAGULATED PROTEIN N.
100
80
60
40
20
0
1
2
3
4
5
CALCIUM
NITROGEN
TIME OF HEATING (HOURS)

FIG. 27.

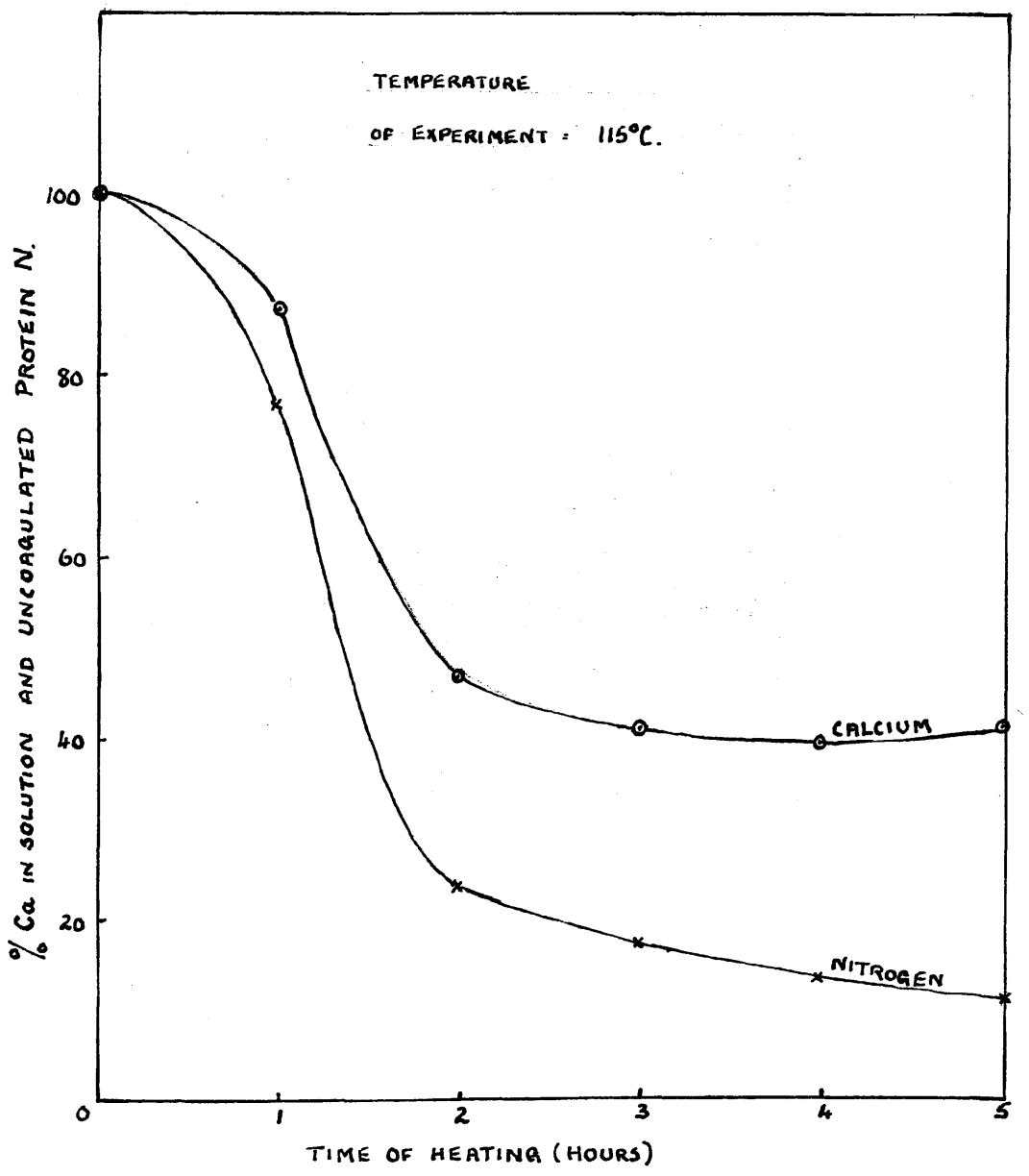
TEMPERATURE
OF EXPERIMENT = 115°C.
100
80
60
40
20
0
% Ca IN SOLUTION AND UNCOAGULATED PROTEIN N.
CALCIUM
NITROGEN
1
2
3
4
5
TIME OF HEATING (HOURS)

FIG. 28°

obtained. They were also used to calculate the N/Ca ratios. The results are given in Table XII, and expressed graphically in Figs. 23 - 28 . As might be expected the values obtained for the various nitrogen fractions are similar to those recorded in the earlier experiments.

Discussion.

It will be noticed that the only figure given for calcium is the value for the total amount present in the solution. A figure for the amount actually in combination with the protein would be of definite interest and would also allow the amount of non-caseinogen calcium to be calculated by difference. Unfortunately the various methods in use for the precipitation of proteins such as caseinogen, e.g. trichloracetic acid, acetic acid and phosphotungstic acid, depend partly on bringing the reaction of the liquid to a pH which is very close to the iso-electric point and so affecting the amount of base bound by the protein. One method which would give some idea of the relative amounts of ionic calcium in a series of solutions is that of dialysis. Even on the (questionable) assumption that only the ionic calcium would dialyse and that no interfering effects due to Donnan equilibria alterations would arise, any calcium present as colloidal calcium phosphate could not be determined. It is, of course, quite impossible that all the calcium present in

TABLE XIII.

Value of the ratio:

$$\frac{\text{Protein N in solution}}{\text{Total Ca in solution}} \quad (N/Ca)$$

at the following temperatures.

	90°C.						95°C.						100°C.					
Time of heating (hours)	0	9	18	27	36	45	0	8	16	24	32	0	2½	5	7½	10	12½	
N/Ca	10.8	11.1	10.2	8.50	7.48	3.55	11.1	10.8	9.32	4.43	4.24	10.3	10.3	9.9	8.8	5.1	4.3	

	105°C					110°C						115°C					
Time of heating (hours)	0	2	4	6	8	0	1	2	3	4	5	0	1	2	3	4	5
N/Ca	11.8	11.3	10.7	9.1	4.44	10.9	10.3	10.3	7.98	4.95	4.47	11.6	9.9	5.7	4.8	3.9	3.1

the solution after several hours heating at, say, 110°C. could be combined with the protein present at the pH of the solution, as (in, for example, the case cited) after 4 hours heating the uncoagulated protein N has fallen to 23 per cent. while the total soluble calcium is still 50 per cent. of its initial value.

The N/Ca ratios given in Table XIII show the progressive relative increase of calcium.

One interesting point which arises is that the increase in amount of calcium in solution after several hours heating is considerably greater than the amount expected from the difference in the base-binding capacities of the ordinary and heat-dephosphorised caseinogen. The source of this increase is obscure. One possibility which might be suggested is that the protein coagulated during the slow heat-coagulation process reverts in part at least to a pseudo-iso-electric form and so liberates the calcium. Evidence on this point is obviously desirable.

Taken as a whole, however, the results fully confirm the suggestion put forward on the basis of the titration curves that the dephosphorisation process is accompanied by the liberation of calcium into the solution.

The further effect of temperature in enhancing the coagulating power of the calcium ions must not be overlooked.

Thus, as is well known, the flocculation of any solution is finally determined by two factors: the probability of collision and the probability of adhesion after collision. Since, in the case under consideration, the collisions of the particles are probably entirely the result of the Brownian movement, any increase in the temperature of the solutions by increasing the rate of Brownian movements will increase the probability of collision. Also the increased speed of the particles will lead to a greater probability of adhesion after collision. In a study by Deacon (1930) of the effect of heat on the coagulation of a copper colloidal solution, evidence is produced that these considerations are fully borne out.

While it is very probable that factors such as these may be of assistance in the process under consideration there seems to be little doubt from the data presented that the main factors underlying the heat coagulation of calcium caseinogenate solutions are

1. The production of a heat-sensitive dephosphorised product, and

2. The simultaneous liberation into the solution of calcium, probably present largely as colloidal calcium phosphate, which when in sufficient concentration at the temperatures of heating produces the phenomenon of visible coagulation.

PART II

FACTORS AFFECTING THE SOLUBILITY OF MILK POWDERS.

INTRODUCTION.

In a very comprehensive study, Wright (1932) has recently investigated the effect of heat treatment on concentrated milk solutions and on dry milk powder, with a view to discovering the factors affecting the solubility of milk powders. (It is well to point out here that the term "solubility" of milk powders and solutions which will be referred to frequently applies only to the protein content of the system. The lactose and the bulk of the inorganic salts exhibit true molecular solubility under the conditions of heat treatment employed in processing. The milk proteins (and, in whole milk, the milk fat) are, however, present in colloidal solution whose stability may readily be affected by heat. The term "solubility" will therefore be used to denote "suspension stability" of the proteins under the prevailing conditions). In order to obtain a sample of milk which would be identical for all the experiments, and which could be obtained readily in different concentrations, a sample of spray-dried separated milk (prepared by the "Milkal" process) was used for the preparation of the solutions throughout the experiments. The sample was over 99.5 per cent. soluble in cold water and easily reconstituted to any desired concentration.

Wright found that insolubility could be readily produced by heating milk solutions of greater concentration than

20 per cent. It was also found possible to correlate the degree of insolubility produced at any given temperature by a known period of heating in a time-temperature-concentration curve. The action appears to be irreversible and the solubility is not affected by the temperature at which the concentrated solution is reconstituted to a standard dilution (2 gm. powder in 20 ml. water). This type of insolubility is termed by Wright "denaturation". While the use of this term, applied to caseinogen, is, in the light of the work recorded in Part I probably not justified, it will be retained and understood in the special meaning given to it here, namely an irreversible protein insolubility.

The effect of heating the milk powder in a dry state was also studied. It was found that heat treatment of the dry powder for different times at temperatures between 100° and 139°C. resulted in the production of another type of protein insolubility. In this case, however, the amount of insoluble protein was found to vary with the temperature of reconstitution to standard dilution. Thus a sample of milk powder which had been heated for 40 hours at 100°C. was 57 per cent. insoluble when reconstituted at 20°C. but only 2 per cent. insoluble when the temperature of reconstitution was raised to 90°C. It is suggested that the cause of this type of insolubility is a dehydration effect such as the

removal of water of imbibition from the protein particles. One further feature which is noted is that after reconstitution at 90°C. it was found impossible to obtain an accurate separation of the caseinogen and albumin globulin fractions. The explanation suggested for this is that after reconstitution at 90°C. for 30 minutes the albumin + globulin really had been denatured. Vigorous stirring of the solution during reconstitution, however, caused some change in the condition of the protein and prevented the flocculation of the denatured particles. In consequence they were still in the supernatant liquid after centrifuging. The addition of acetic acid to coagulate the caseinogen did, however, bring down the bulk of the denatured albumin + globulin with it, and so an incorrect distribution of the fractions was obtained. This point will be referred to later.

A complex system such as milk, and more particularly concentrated milk solutions, where the dispersion medium is not pure water but a solution of lactose containing a mixture of electrolytes cannot be expected to behave towards heat-treatment in the same way as a solution of calcium caseinogenate. Hence the processes which precede and probably influence the heat coagulation of calcium caseinogenate will not necessarily take place in milk solutions. Indeed it seems likely that the cause of coagulation in the latter is to be found in the

dispersion medium and not in any structural changes in the protein during heating.

Wright had limited his observations on the effect of temperature of reconstitution to two extreme temperatures, 20°C. and 90°C. In view of the marked difference in the results obtained at these two temperatures it appeared desirable to extend the investigation to include the influence of intermediate temperatures of reconstitution on the solubility of milk powders.

It should be noted that the methods used for the determination of the solubility of milk powders vary widely as regards both technique and temperature of reconstitution. Marquardt (1920) recommends treating the sample with successive charges of water at 50 - 55°C. centrifuging after each extraction and determining the amount of insoluble residue. Suplee (1923) reconstitutes the milk powder by stirring for 10 minutes in water held at 65°C. and subsequently determines the total protein in the supernatant liquid after centrifuging. On the other hand, Hunziker (1926) records results obtained by reconstituting for 10 minutes at 65°C. and for 5 minutes at 100°C. At the time when these methods were devised, however, the existence of two types of protein insolubility in milk powders was not clearly recognised.

Technique.

In the following experiments the technique employed was made as drastic as possible in order to obtain reliable and reproducible results.

Solutions: 2 gm. of each sample of milk powder were stirred into a smooth paste with 4-5 ml. of water and the volume was made up to 20 ml. This diluted mixture contained in a Pyrex boiling tube was stirred for 30 minutes by means of a small double propellor revolving at 2000 r.p.m.; the boiling tube was kept immersed in a small water-bath at the desired temperature of reconstitution ($\pm$ 1°C.). After stirring, the contents of the tube were transferred to a 25 ml. centrifuge tube and centrifuged for 15 minutes at 3000 r.p.m. The supernatant liquid was removed by a syphon to a collecting tube, care being taken not to disturb the loose upper layer of the sediment.

Analysis: Determinations were made in the supernatant liquid as follows: total soluble nitrogen; caseinogen by Moir's method (1931); albumin globulin by trichloracetic and precipitation of the caseinogen filtrate; and non-protein nitrogen in the filtrate from the trichloracetic acid precipitation; total nitrogen in the original sample was determined on a weighed sample of the powder. All nitrogen determinations were carried out by the Kjeldahl method.

The difference between the total nitrogen originally present and the nitrogen in the supernatant liquid was taken to represent insoluble protein; at any temperature the percentage of insoluble protein being given by the formula

$$\% \text{ insoluble protein} = \frac{U - T}{U} \times 100$$

where U represents the total nitrogen of the sample and T represents the total nitrogen in the supernatant liquid.

The influence of temperature of reconstitution on the solubility of an artificially heated milk powder.

It appeared probable that any alterations produced in the solubility of a milk powder by variations in the temperature of reconstitution would be primarily dependent on the effect of the dry heat treatment to which the powder had been subjected during manufacture. The preliminary experiments were therefore limited to a study of this aspect of solubility.

Technique: A quantity of spray-dried separated milk was heated in thin layers in an electric air oven for 6 hours at 105-110°C. The percentage of insoluble protein in the heated powder as well as in the original powder was then determined after reconstitution. The temperature range over which solubility was measured was 20° to 100°C. inclusive.

TABLE XIV.

	Temperature of Reconstitution (°C)	20°	30°	40°	50°	60°	70°	80°	90°	99°
% Insoluble protein.	A. Heated sample	55.49	44.50	43.64	38.70	30.92	35.99	13.24	6.89	0.0
		58.24	46.38	46.38	30.53	27.17	29.48	14.26	5.92	0.0
	B. Untreated sample.	1.99	-	-	1.68	-	-	-	-	0.0
		1.82	-	-	1.53	-	-	-	-	0.0

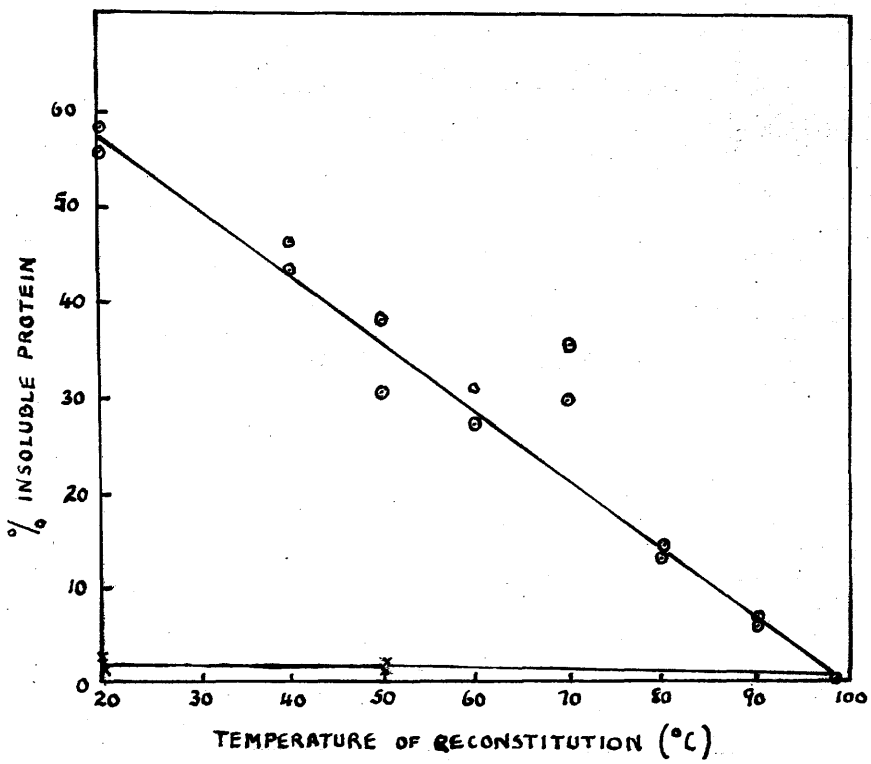
60
50
40
30
20
10
0
% INSOLUBLE PROTEIN
20
30
40
50
60
70
80
90
100
TEMPERATURE OF RECONSTITUTION (°C)

FIG. 29.

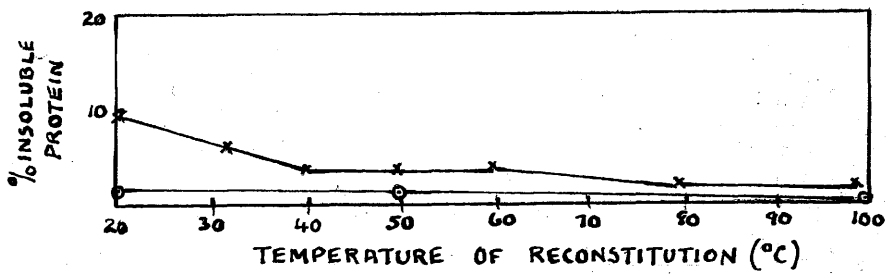
20
10
% INSOLUBLE PROTEIN
20
30
40
50
60
70
80
90
100
TEMPERATURE OF RECONSTITUTION (°C)

FIG. 34.

Results and Discussion.

The results obtained are given in Table XIV . and Fig. 29 It will be seen that the solubility of the unheated sample varies but slightly with the temperature of reconstitution, the percentage falling from under 2 per cent. at 20°C. to zero at 99°C. On the other hand the sample which had been subjected to dry heating showed practically 60 per cent. insolubility at 20°C. but the solubility increased as the temperature of reconstitution was raised until at 100°C. the whole of the protein was soluble.

It was found impossible to obtain close agreement between duplicate determinations on this powder, probably owing to difficulties in sampling, and considerable errors were undoubtedly involved in determining the solubility at some temperatures (see especially the results at 70°C.). Taken as a whole, however, the results indicate that with a constant time of heating the percentage of protein rendered soluble is directly proportional to the temperature of reconstitution as shown by the straight line relationship illustrated in Fig. 29.

One interesting point which should also be noted is that the results show no sign of any production of the "denatured" protein as characterised by its insolubility at any temperature of reconstitution. Such a finding is, of course,

in harmony with Wright's original work.

B. The influence of temperature of reconstitution on the solubility of commercially prepared milk powders.

The above results were obtained on an artificially heated milk powder. The next step was to investigate the influence of temperature of reconstitution on the solubility of commercial milk powders. For this purpose the percentage of insoluble protein in each of the following eight samples was determined after reconstitution at various temperatures between 20° and 100°C.:

Sample I. A commercial brand of roller-dried separated milk powder.

Sample II. A commercial brand of roller-dried whole milk powder containing added lactose.

Sample III. A sample of dried separated milk powder prepared on a single-cylinder model roller drier: steam pressure in roll 25 lb./in.^{2}, time of drying 1 sec.

Sample IV. A sample prepared on the same plant, but from whole milk (4.2 per cent. butterfat).

Sample V. Identical with IV, but prepared from separated milk (0.1 per cent. butterfat).

Sample VI. Identical with IV, but prepared from whole milk to which cream had been added (7.7 per cent. butterfat).

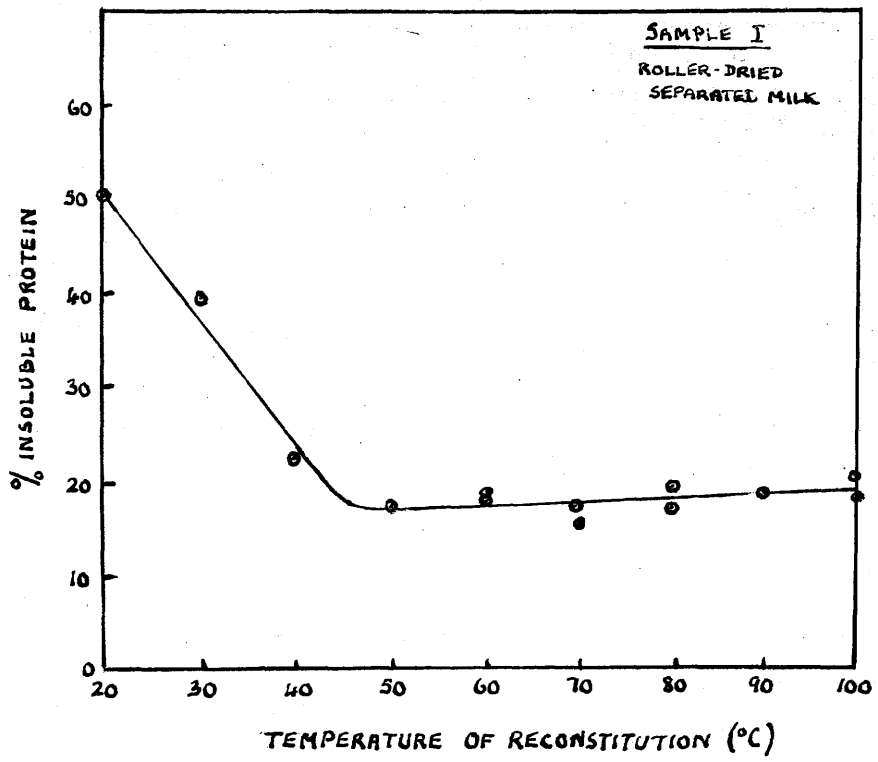
SAMPLE I
ROLLER-DRIED
SEPARATED MILK
% INSOLUBLE PROTEIN
0
10
20
30
40
50
60
20
30
40
50
60
70
80
90
100
TEMPERATURE OF RECONSTITUTION (°C)

FIG. 30

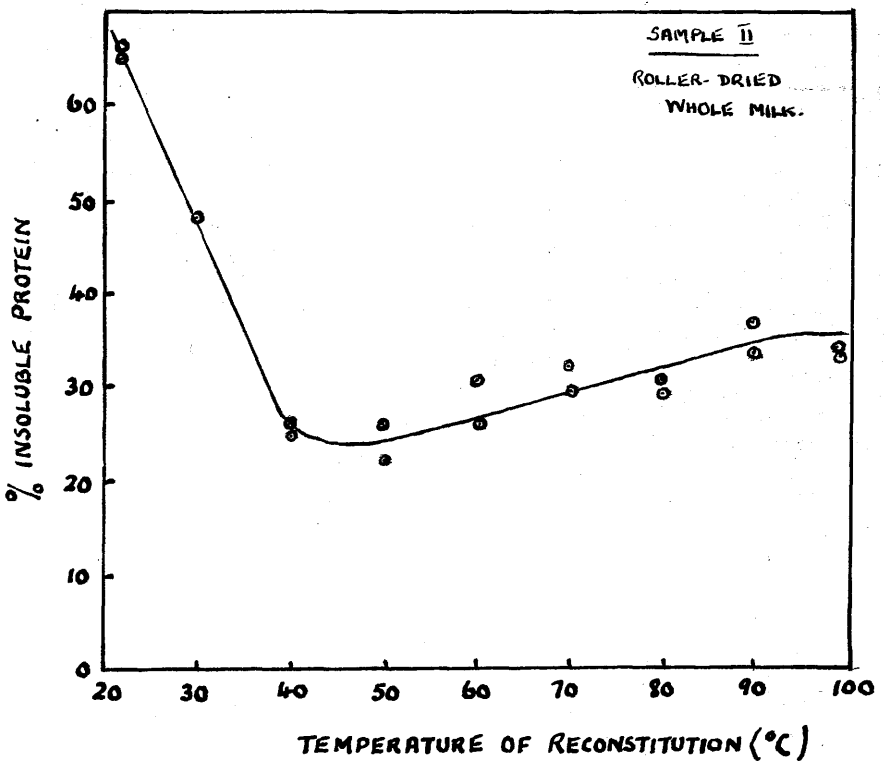
SAMPLE II
ROLLER-DRIED
WHOLE MILK.
% INSOLUBLE PROTEIN
0
10
20
30
40
50
60
20
30
40
50
60
70
80
90
100
TEMPERATURE OF RECONSTITUTION (°C)

FIG. 31.

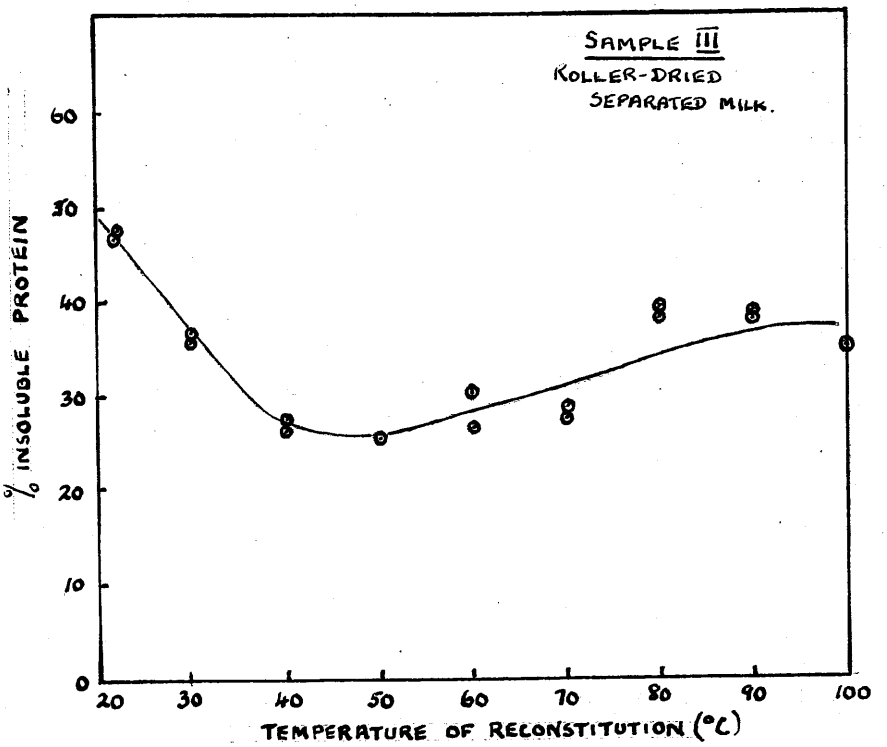
SAMPLE III
ROLLER-DRIED
SEPARATED MILK.
% INSOLUBLE PROTEIN
0
10
20
30
40
50
60
20
30
40
50
60
70
80
90
100
TEMPERATURE OF RECONSTITUTION (°C)

FIG. 32.

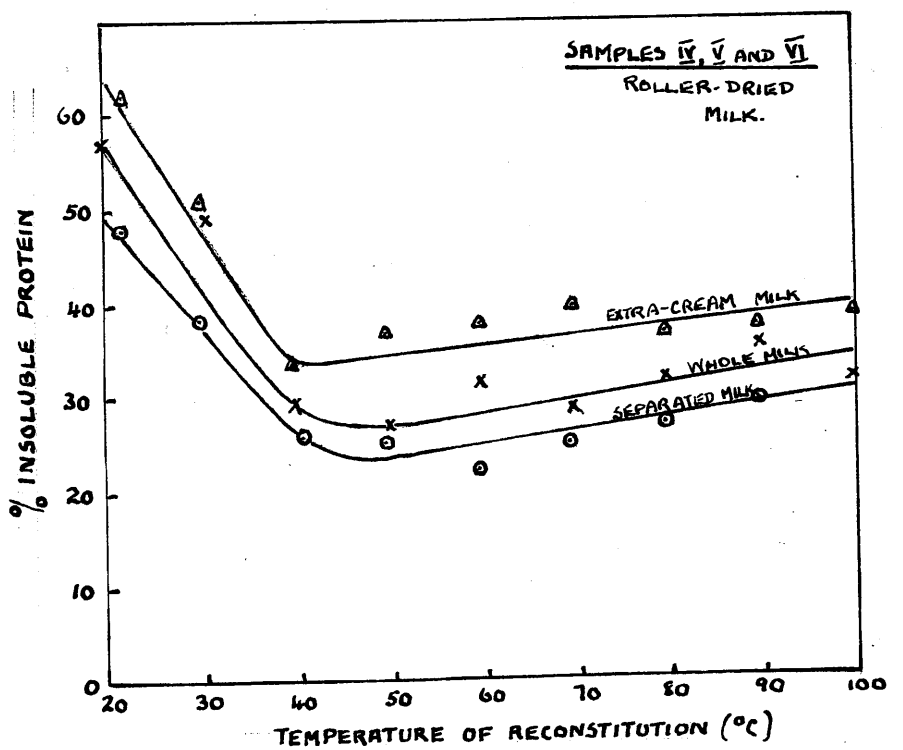
SAMPLES IV, V AND VI
ROLLER-DRIED
MILK.
% INSOLUBLE PROTEIN
0
10
20
30
40
50
60
EXTRA-CREAM MILK
WHOLE MILK
SEPARATED MILK
20
30
40
50
60
70
80
90
100
TEMPERATURE OF RECONSTITUTION (°C)

FIG. 33.

Sample VII. A commercial brand of spray-dried separated milk powder.

Sample VIII. A commercial brand of spray-dried whole milk powder.

The results of the determinations on these samples are shown in Figs. 30 - 34. For convenience they will be discussed under three heads:

(a) There is an increase in the solubility of each roller-dried sample between 20 and 50°C. There is little doubt that this increase is chiefly due to the fact that the milk powders contain a considerable proportion of protein which has been rendered insoluble by dry heating, but which (as shown in the preceding section) will regain its solubility when reconstituted in hot water. With regard to the whole milk powders, however, the increased solubility at the higher temperatures may also be attributed in part to the influence of the fat content of the milk (see (c) below).

(b) Between 50 and 100°C. the roller-dried samples show a more or less marked decrease in solubility, which is best illustrated in Figs.31 and 32. This decreased solubility might very naturally be attributed to the precipitation of albumin by the prolonged reconstitution at the higher temperatures, since Rupp (1913) has shown that the denaturation of albumin commences at 63°C. and is completed instantaneously

at 85°C. Further, in the samples examined the increase in protein insolubility is about 10 per cent., while the percentage of albumin in milk accounts for approximately 12.5 per cent. of the total nitrogen. The experimental facts do not, however, appear to bear out this assumption. Separate determinations of caseinogen, albumin plus globulin, and non-protein nitrogen were made on Sample III after reconstitution at 20, 50 and 100°C. The results showed that between 50 and 100°C. there was an apparent increase in insolubility of caseinogen of 7.80 per cent., while the albumin plus globulin only showed an increased insolubility of 3.68 per cent. The non-protein nitrogen remained constant.

In view of the suggestion made by Wright (1932) and cited previously, these results may be partly due to the inherent difficulty of determining these proteins separately after reconstitution at high temperatures, the increase in caseinogen being apparent and not real. On the other hand it is possible that there is a physical association of some sort between the caseinogen and albumin which is only stable under certain circumstances. In support of this latter suggestion it is significant to note that the two spray-dried samples do not show any decreased solubility after reconstitution at higher temperatures and that the caseinogen of these powders is almost completely soluble. In spray-

dried powders therefore the high percentage of soluble caseinogen may induce mutual stability of the proteins, whereas in the roller-dried powders which contain much less soluble caseinogen the denaturation of the albumin may induce mutual precipitation.

(c) In order to differentiate between the direct effect of heat treatment during drying on the protein solubility, and the indirect effect of the fat content on solubility (for instance, through the influence of the latter on the miscibility of the powder with water), experiments were made with both separated and whole milk powders. It will be seen that the effect of the fat is to depress protein solubility throughout the entire range of temperatures at which the samples were reconstituted. This is best shown in Fig.33, where the solubilities of three powders, all derived from the same bulk of whole milk and all manufactured under similar conditions, are compared. It is also indicated in the comparative solubilities of the two commercially prepared roller-dried samples (I and II) and of the two spray-dried samples (VII and VIII), although direct comparisons are not valid in these instances, the powders being manufactured on different types of plant. From the point of view of the present study, however, the important fact is that the influence of fat content on protein solubility is approximately constant throughout the entire

range of temperatures of reconstitution. The presence of the fat in the powder therefore does not invalidate any conclusions which may be drawn from the comparative solubilities of the protein at different temperatures. The increase in solubility between 20 and 50°C., and the subsequent decrease between 50 and 100°C., occur whether the milk powder is manufactured from separated milk or from whole milk. Appendix I shows the figures obtained for each sample.

Application of the Results.

The results presented in the preceding section show quite definitely that the temperature of reconstitution is an extremely important factor in determining the solubility of a given milk powder. If the results for Sample II are examined it is seen that when the sample is reconstituted at 20°C. the insoluble protein amounts to almost 70 per cent., at 50°C. this value is only 25 per cent., while if the temperature of reconstitution be raised to 100°C. the amount increases to about 35 per cent.

The determination of the solubility of the samples over the wide range of temperatures employed in these experiments effectively demonstrates the presence of both types of protein insolubility. Moreover from the solubility-temperature curve obtained it is possible to gauge the

amount of each type produced in the manufacture of the sample. Thus it appears that the increase in solubility between 20°C. and 50°C. is due to the presence of protein which has been rendered insoluble by overheating in a dry state. In Sample II, for example, the proportion of such protein is 70 per cent. minus 25 per cent., or 45 per cent. of the total protein in the powder. Further, the protein which remains insoluble at 50°C., i.e. 25 per cent., is undoubtedly that fraction which has been rendered insoluble by moist heating during the drying process and which is therefore irreversibly coagulated.

In view of these facts, it is apparent that, using the rather drastic technique employed in the present investigation, the most valuable indication of the extent and nature of the protein insolubility will be obtained by reconstituting a milk powder at 20°C. and 50°C. The values so obtained will enable the observer to gauge the extent of protein insolubility induced by moist and dry heat respectively during manufacture. Such information should prove of real value in indicating what modifications, if any, may be necessary in the thermal conditions of the drying plant. If less drastic methods of reconstitution are employed it might be necessary to raise the latter temperature somewhat but it is doubtful whether a temperature greater than 60°C. could be used without

serious risk of denaturing the albumin.

Some physico-chemical properties of concentrated solutions of milk solids.

In the preceding section the problem of the ultimate cause of the protein insolubility induced by moist heating has not been dealt with. It may be assumed, however, that the production of this type of insoluble protein is really a heat-coagulation phenomenon. If this be so, then it is very probable that the ionised electrolytes in milk, concentrated as they are during the drying process on the rolls, are the agents responsible for the irreversible protein insolubility. Electrolytes have a marked effect on the stability of protein solutions and the effect of the increasing concentration of electrolytes in the highly concentrated solutions of milk solids will undoubtedly influence the stability of the protein system. It therefore appeared desirable to make a detailed study of those physico-chemical properties which are primarily dependent on ionic concentration. The properties investigated were the electrical conductivity, the hydrogen-ion concentration and (as a means of measuring the general osmotic activity of the solutions) the depression of the freezing-point.

Technique.

(a) Preparation of Solutions.

In order to obtain comparable results throughout the entire series of experiments, all solutions of milk solids were made up from the same highly soluble brand of spray-dried separated milk powder. This powder was similar to that used by Wright and was readily and completely soluble in cold water. The method employed in the preparation of the solutions, which were invariably made up in duplicate, was as follows: The requisite quantity of milk powder was weighed into a Pyrex boiling tube, a few millilitres of water were added, and the mixture was stirred with a steel rod into a thick paste. After further dilution the contents of the tube were transferred to a 50 ml. standard flask, and made up to the mark with washings. A drop of caprylic alcohol was added to prevent frothing. This was essential in the highly concentrated solutions where the occlusion of small air bubbles was otherwise liable to lead to serious inaccuracies. By this method homogeneous solutions could be readily prepared up to the highest concentration used in the experiments, i.e. 65 per cent. Throughout this section of the thesis percentage concentrations are expressed as g. solute per 100 g. water and not as g. solute per 100 ml. solution. Although the viscosities of the solutions increased

markedly with the higher concentrations of milk solids, all the solutions were definitely fluid and could be poured from one vessel to another.

Conductivity measurements were made very shortly after preparation of the solutions (within 2 hours); pH and freezing-point determinations were made after standing for 20-24 hours at 3-4°C.

It may be noted here that when solutions containing 34 per cent. or more of milk solids were allowed to stand overnight, a white sediment invariably settled out. On investigation this sediment proved to be lactose. The significance of this fact is dealt with in a later section.

(b) Physico-chemical measurements.

Electrical conductivity: Conductivity measurements were made in a cell consisting of two vertical platinum electrodes, each about 1 cm. square, rigidly sealed into glass tubes, the upper ends of the latter being firmly fitted into the ebonite lid of the cell. The electrodes were heavily coated with platinum black. The readings were made on an Electrolytic 10-metre Bridge Wire (Cambridge Instrument Co., Ltd.) in conjunction with a buzzer and telephone. All measurements were made at 18°C. The cell constant was checked each day, but remained constant at 0.112.

Freezing-point determinations: These were made with a Hortvet cryoscope, fitted with a Beckmann thermometer. Check determinations on distilled water showed a variation of only 0.01°C. throughout the course of the work. In the solutions of low milk solids concentrations considerable supercooling was experienced; for instance, in the 17.8 per cent. solution the degree of supercooling was 1.72°C. While this value greatly exceeds the optimum suggested by Findlay (1923), two successive readings never showed a greater difference than 0.005°C., and in general gave identical values. In solutions containing more than 40 per cent. milk solids the degree of supercooling was smaller, and it continued to decrease up to 65 per cent. milk solids content. At this concentration the degree of supercooling was about 0.30°C.

Hydrogen-ion concentrations: Determinations of the hydrogen-ion concentration were made with the quinhydrone electrode. In the highly concentrated solutions there was always a slight drift, but even with the highest concentration (65 per cent. milk solids) this only amounted to 1.0 millivolt in 5 minutes. A drift of this magnitude would not appreciably alter the results. According to Clark (1928) such drifts in complex solutions are due to secondary reactions after normal equilibrium had been attained. In order to minimise

TABLE XV.

Concentration (gms. per 100 gms. water)	Conductivity mhos. x 10^4		Freezing-point depression (^oC)		p_H at 18°C		C_H d 10^7 (mean value)
	A	B	A	B	A	B	
65.3	79.8	81.3	2.313	2.313	5.97	-	10.72
61.1	75.6	77.0	2.175	2.195	6.01	5.97	9.78
54.0	78.4	77.7	2.098	2.080	6.04	6.03	9.20
46.8	75.6	75.0	1.945	1.953	6.08	6.08	8.32
40.0	74.9	74.9	1.785	1.775	6.12	6.14	7.59
34.0	71.1	71.8	1.650	1.655	6.18	6.18	6.61
28.4	68.7	66.6	1.423	-	6.23	6.23	5.89
23.0	62.0	62.5	1.163	-	6.30	6.30	5.01
17.8	56.7	57.1	0.925	0.920	6.42	6.42	3.80
13.0	47.4	47.4	0.665	0.670	6.48	6.49	3.31
8.6	35.3	35.7	0.445	0.450	6.62	6.64	2.40
4.4	21.3	20.9	0.230	0.225	6.73	6.73	1.86

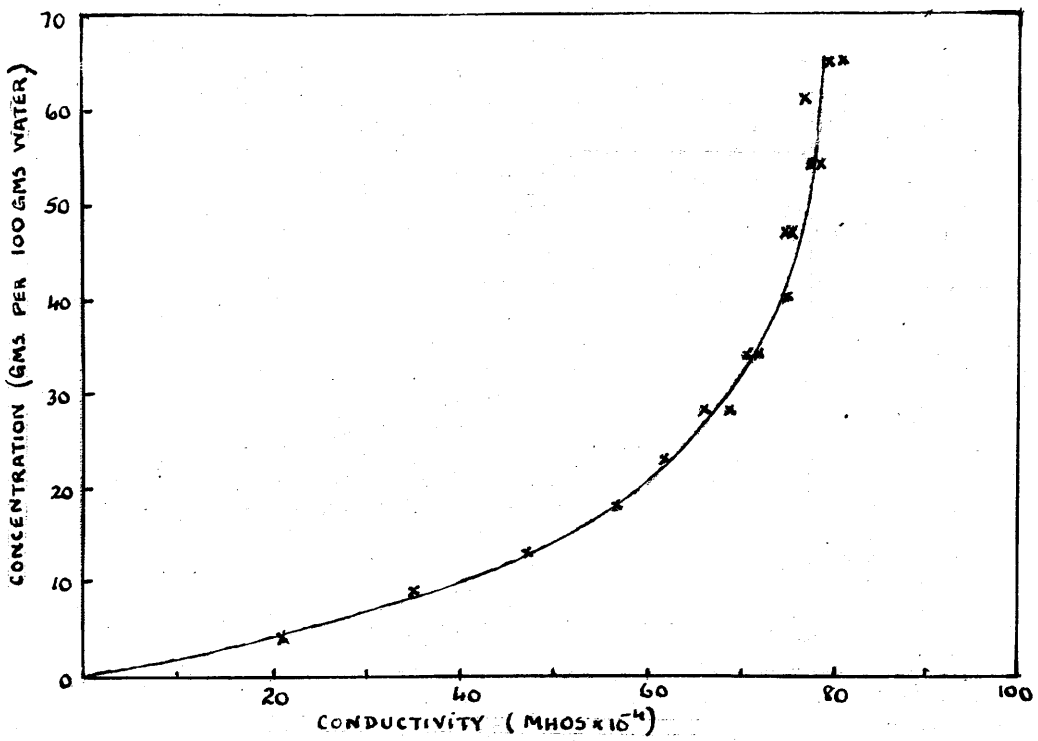
CONCENTRATION (GMS. PER 100 GMS WATER)
0
10
20
30
40
50
60
70
20
40
60
80
100
CONDUCTIVITY (MHOS × 10⁻⁴)

FIG. 35

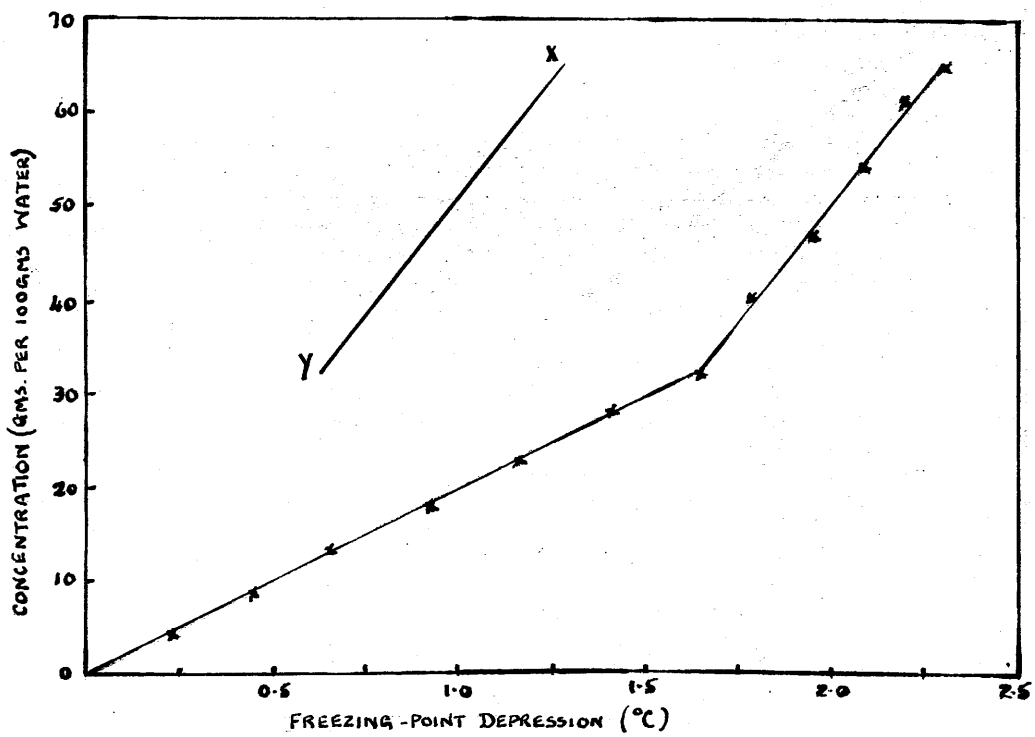
CONCENTRATION (GMS. PER 100 GMS WATER)
0
10
20
30
40
50
60
70
X
Y
0·5
1·0
1·5
2·0
2·5
FREEZING-POINT DEPRESSION (°C)

FIG. 36.

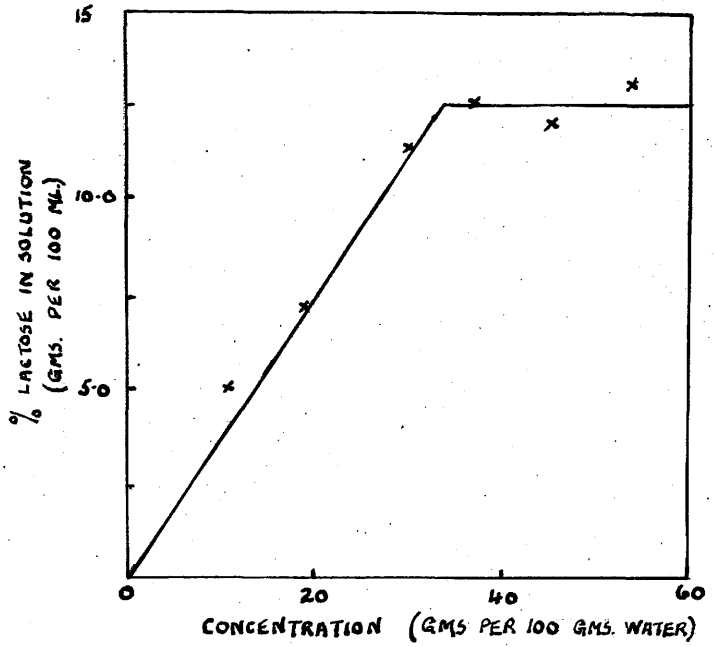
15
10·0
5·0
0
20
40
60
% LACTOSE IN SOLUTION (GMS. PER 100 ML.)
CONCENTRATION (GMS PER 100 GMS. WATER)

FIG. 38.

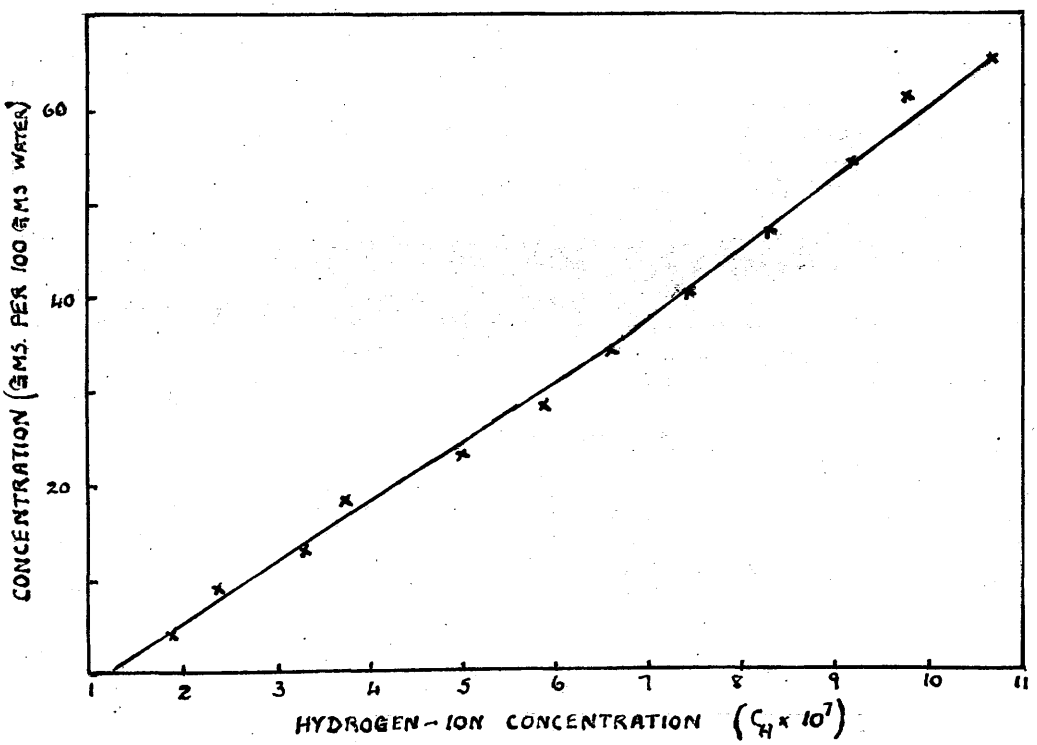
60
40
20
1
2
3
4
5
6
7
8
9
10
11
CONCENTRATION (GMS. PER 100 GMS WATER)
HYDROGEN-ION CONCENTRATION ($C_H \times 10^7$)

FIG. 37.

any small errors arising from these drifts, measurements were always made after the solution had been in contact with the quinhydrone for the same period of time (10 minutes) and check readings were made 5 minutes later. The temperature at which measurements were made was 18°C. (± 1°C.).

Discussion of Results.

The results are shown in Figs. 35 - 37 and Table XV. They will be discussed separately under three heads:-

(a) Electrical Conductivity (Fig. 35).

From the general shape of the conductivity curve it is seen that the relation between conductivity and concentration is not linear, but that there is a definite retardation in the rate of increase of conductivity with increasing concentration of milk-solids. At high concentrations, beyond 40 per cent. milk solids, this becomes so marked that the increase in conductivity is scarcely appreciable. Now, since the degree of ionisation of electrolytes diminishes with increasing concentration, the conductivity-concentration curve will naturally be concave to the conductivity axis. It will be seen from Fig. 35 that the type of curve obtained coincides with that which would be expected theoretically. On the other hand the magnitude of the retardation at the higher concentrations demands some further explanation.

In this connection it might be noted that there is one other property of solutions which might exert a marked influence on the electrical conductivity, i.e. viscosity. No actual measurements of viscosity were made in these experiments but there was no doubt from visual observation that the viscosity of the solutions increased greatly with increasing concentrations of milk-solids. Bateman and Sharp (1928) have shown that the viscosity of normal skim milk (about 10 per cent. milk-solids) is increased by about 3.5 times by evaporation to 25 per cent. milk-solids. Further, Greenbank, Steinbarger, Deysher, and Holm (1927) found that at concentrations of 45-50 per cent. milk solids, skim milk exhibited the properties of plastic flow. It must be concluded, therefore, that the retardation in the rate of increase of the conductivity is partly (and possibly largely) due to the increasing viscosity of the concentrated milk solutions.

The conductivity of a 10 per cent. solution of milk-solids (corresponding to normal skim milk) is shown from Fig.35 to be 40.0×10^{-4} mhos. This value falls outside the general range noted by Rogers (1935) for whole milks, i.e. $45 - 48 \times 10^{-4}$ mhos. The figures quoted by Rogers are presumably based on measurements made at 25°C. while the temperature employed in these experiments was 18°C. An

independent measurement made on a 10 per cent. solution at 25°C. showed a conductivity of 49.0 x 10^{-4} mhos.

(b) Freezing-Point Depression. (Fig. 36).

The curve showing the relation between concentration of milk solids and the depression of the freezing-point consists of two distinct parts, the first extending from 0 to 34% of milk-solids, and the second from 34% upwards. The sharpness of the break in the curve at 34% indicates that at this point there is a definite physical change in some component of the system.

Dealing first with the break at 34% milk solids, it has already been noted (p.47) that above this concentration a white sediment invariably settled from the solutions after standing overnight. On analysis this sediment was found to consist of pure lactose. It therefore appeared possible that a concentration of 34% milk-solids represented the point of maximum solubility of the lactose present in the system under the prevailing conditions. The increase in the freezing-point depression beyond this point would then be due to the remaining osmotically active milk constituents, chiefly dissolved electrolytes. Two procedures were adopted to test this theory.

In the first place determinations were made of the amount of soluble lactose present in varying concentrations

of milk-solids. Solutions containing known quantities of milk-solids were prepared as previously described and were left in an ice-chest at 3°C. overnight. On removal from the ice-chest they were immediately centrifuged for 5 minutes at 3000 r.p.m. The amount of soluble lactose in the upper layer was determined by the volumetric method of Shaffer and Hartmann (1921) after removal of the proteins by precipitation with 10% tungstic acid. The results are shown in Fig. 38 . They show clearly that the maximum solubility of the lactose in solutions of milk-solids at about 0 °C. is reached at a concentration of 34% of the latter, and that beyond this point the solubility remains (within experimental error) constant.

Secondly, the amount of lactose present in a 12% milk-solids solution was calculated from the data used to prepare Fig. 38 . The theoretical depression of the freezing-point due to this amount of lactose was calculated from the formula

$$\Delta(°C) = \frac{100\ g\ K}{ML}$$

where g is the weight in gm. of the solute of molecular weight M dissolved in L gm. of the solvent and K is the molecular depression constant for the solvent. This value was subtracted from the observed depression of the solution containing 12 per cent. milk-solids, the difference represented

the depression caused by the other osmotically active milk constituents. In this way a freezing-point depression curve for these constituents only was drawn. Part of this curve is shown in the ~~Xxxxxxx~~line XY in Fig. 36. It is evident that the slope of the upper part of the freezing-point curve coincides with that of XY. It may, therefore, be safely concluded that the freezing-point depressions given in the top part of the curve are due to the action of electrolytes etc., the amount of lactose in solution remaining constant.

Several workers, notably Newton and Gortner (1922), Newton and Martin (1930) and Briggs (1931) have reported that in solutions of some lyophilic colloids, especially certain proteins, a definite fraction of the water is "bound" by the colloid, "bound" water being defined as water which is no longer capable of acting as a normal solvent. It is obvious that, if any considerable quamtity of "bound" water existed in the higher concentrations of milk solids (containing up to 20 per cent. of protein) it might exert a considerable influence on the physical properties of the solutions, since it would result in the production of abnormally high concentrations of electrolytes. Such increases in concentration of electrolytes (and of other osmotically active constituents of the system) would, however,

be reflected in abnormally high osmotic pressures, and hence in corresponding increases in the freezing-point depression. The linear relationship between concentration of milk-solids and freezing-point depression shown in Fig. 36 indicates that "bound" water is absent from the solutions studied.

As confirmatory evidence on this point seemed desirable, the following experiment was carried out. A 65 per cent. solution of milk-solids was prepared (the exact quantity of water present being known) and the depression of the freezing-point was determined. Sufficient glucose was then dissolved in an aliquot part of this solution to give, with the amount of water present in the aliquot, a 5 per cent. glucose solution. The depression of the freezing-point of this solution was also determined. A further determination of the freezing-point depression was made on a solution containing 5 g. glucose dissolved in 100 ml. pure water. The results were as follows:-

	°C.
Freezing-point depression of 65 per cent. milk-solids solution containing 5 per cent. glucose	2.870
Freezing-point depression of 65 per cent. milk-solids solution alone	2.313
Difference due to dissolved glucose	0.557
Freezing-point depression of 5 per cent. glucose in pure water (duplicate solutions)	0.558 0.555

This result confirms the previous conclusion that, up to concentrations of 65 per cent. milk-solids, there is no evidence of the existence of "bound" water.

(c) Hydrogen-Ion Concentration. (Fig. 37).

In order to determine whether or not a linear relationship exists between the concentration of milk-solids and the hydrogen-ion concentration, the latter was plotted as such and not in pH units. It will be seen that the curve is roughly linear, any increase in the concentration of the milk-solids being accompanied by an equivalent increase in the acidity of the solution. The slight deviation from a straight line relationship is again explainable on the basis of small differences in the degree of ionisation of the electrolytes present occurring as a result of the amount of lactose in the solution becoming constant.

Application of the Results to the Heat-Coagulation of Evaporated Milk and to the Insolubility Induced in Milk Powders.

As was pointed out earlier the object of the preceding experiments was to obtain some idea of the alterations in the ionisation and concentration of the electrolytes and the possible relation of such changes to the stability of the

milk proteins in concentrated solutions of milk-solids.

As regards determinations of electrical conductivity, these failed to furnish any useful information owing to the effect of viscosity on ionic mobility in the more concentrated solutions, any changes in the degree of ionisation being vitiated by the unknown magnitude of the viscosity effect.

In the pH determinations, where this factor was no longer operative, very suggestive results were obtained. Thus, while a 10 per cent. solution (roughly equivalent to normal skim milk) had a pH of about 6.6, the pH of a 30 per cent. solution was 6.20 and in a 65 per cent. solution it had fallen to 5.97. The sensitivity of caseinogen to heat (and electrolyte) coagulation increases towards its iso-electric point and other experiments (unpublished data) have shown that milk solutions of normal solids content may be rapidly coagulated by heating to 120°C. when the pH is on the acid side of 6.0. It will be seen therefore that the concentration of milk solutions (such as takes place in the roller-drying of milk) will inevitably produce conditions which are very favourable to heat coagulation. Hence it may be stated with a fair degree of certainty that the insolubility of milk powders induced during the drying process on the rolls is partly attributable to the increasing concentration of the milk salts.

While the results obtained for the freezing point depressions, on account of the limited solubility of lactose at 0°C. are not of direct value in giving a true picture of the osmotic activity of the solutions, the regularity of the results for the depressions makes it reasonable to assume that a similar relationship will hold for the elevation of the boiling point. Moreover, since at the boiling point complete saturation of solutions of milk solids with lactose cannot be reached, there will be no break in the boiling point curve equivalent to that which is found in the freezing point curve. Scott (1933) has in fact found that in solutions of milk solids up to 50 per cent. and at temperatures between 140° and 150°F. the relation between concentration and boiling point elevation (under reduced pressure) is linear. In view of the fact that in commercial evaporative processes such as roller-drying of milk powders the temperature to which the heated milk is subjected is dependent on its boiling-point, the establishment of a basis on which the latter value can be calculated is of definite practical importance.

APPENDIX.1.

Effect of temperature of reconstitution on the solubility of samples of roller- and spray-dried milks.

Temperature of Reconstitution(°C)		20°	30°	40°	50°	60°	70°	80°	90°	100°
A. Roller dried samples.	I	50.42	39.68 (at 32°)	22.76	17.48	18.88 18.07	21.97	21.00 18.80	23.19	18.27 20.00
	II	65.24 66.22 (at 22°)	48.33 -	26.02 24.77	25.81 22.38	30.92 26.02	32.16 29.70	30.92 29.22	33.64 37.05	33.64 33.88
	III	47.00 48.50 (at 22°)	35.70 37.00	26.40 27.90	25.50 -	26.60 30.10	27.40 28.70	38.00 38.80	37.20 38.30	35.20 -
	IV	57.32 56.82 (at 22°)	50.93 50.50	29.82 28.59	26.39 27.35	30.44 32.10	28.96 28.67	32.00 31.79	33.78 36.95	30.87 31.79
	V	48.19 47.80	38.28 38.28	26.43 25.64 (at 42°)	25.24	22.92 21.78	24.43 24.79	25.72 27.63	30.07 28.03	31.66 30.12
	VI	62.0 - (at 22°)	50.27 47.49 (at 31°)	33.15 33.78	38.13 35.34	37.81 37.70	41.26 37.70	36.24 36.83	38.76 35.62	39.38 36.24
B. Spray dried samples.	VII	1.99 1.82	-	-	1.68 1.53	-	-	-	-	0.0
	VIII	9.41	6.04 (at 32°)	3.61	3.61	3.85	-	1.79	-	1.68 (at 99°)

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