LXXVIII. THE HYDROLYSIS OF LECITHO-VITELLIN BY PEPSIN AND BY TRYPSIN-KINASE.

BY JANET HENDERSON BLACKWOOD¹
AND GEORGE MACFEAT WISHART.

*From the Hannah Dairy Research Institute, Kirkhill, Ayr, and the
Institute of Physiology, the University of Glasgow.*

(Received February 27th, 1934.)

It has long been recognised that egg-yolk and milk contain somewhat similar proteins. In particular the two phosphoproteins, caseinogen and lecitho-vitellin, show a certain similarity in constitution and properties, though the latter is peculiar in respect of its content of iron and copper. For example Rimington and Kay [1926] found that hydrolysis of caseinogen with 1 % sodium hydroxide liberated all the organically bound phosphorus of the protein as inorganic phosphate. McFarlane [1932], working with lecitho-vitellin, showed that the action of 1 % alkali resulted in the formation of an acid-insoluble material containing 50 % of the original nitrogen, only a trace of phosphorus and no iron, while the acid-soluble products were rich in both iron and phosphorus.

Little information is available with regard to the action of enzymes on lecitho-vitellin. Miescher [1870] observed that an insoluble material separated out from a peptic digest of egg-yolk. This material was named "haematogen" by Bunge [1885], who found that it contained iron in organic combination. The nature of this insoluble product was studied in greater detail by Hugounenq and Morel [1905, 1, 2; 1906] and later by Posternak [1927], whose results indicated that the so-called "haematogen" might not be a pure compound. These investigations were however carried out before the separate preparation of livetin, the second protein of egg-yolk, by Kay and Marshall [1928; see also Plimmer 1908].

The enzymic cleavage of caseinogen has, on the other hand, been extensively investigated. The studies of Rimington [1927, 1, 2], Rimington and Kay [1926] and Holter *et al.* [1932], on the products of enzymic hydrolysis of caseinogen, have thrown considerable light on the structure of the caseinogen molecule. The nature of the enzymic hydrolysis has been further studied by Stirling and Wishart [1932], who observed certain well-defined differences in the digestion of caseinogen by pepsin and by trypsin-kinase. Briefly summarised these differences were as follows:

1. In tryptic digests acid-soluble phosphorus was liberated more rapidly than acid-soluble nitrogen; the reverse held for pepsin.
2. In peptic digests a stage was reached early in digestion (presumably by the liberation of an acid-soluble material containing no phosphorus) in which the residual acid-insoluble material had a N/P ratio of 12.8. Further, the acid-insoluble residue preserved approximately this ratio throughout a prolonged

¹ Carnegie Research Fellow.

period even though it was considerably diminished in amount by further hydrolysis. In the later stages of digestion, however, the N/P ratio fell slowly as a result of the liberation of a nitrogen-rich material. It was suggested that the final acid-insoluble residue might be identical with Linderström-Lang's [Holter *et al.*, 1932] "paranuclein," with a N/P ratio of 8.

3. With trypsin the course of the digestion was entirely different. There was a rapid preferential liberation of acid-soluble phosphorus, so that the N/P ratio of the insoluble residue rose progressively until, under suitable conditions, it reached infinity. At this stage 30 % of the nitrogen still remained acid-insoluble.

On the basis of these findings it was suggested by Stirling and Wishart that trypsin breaks down peptide linkages in proximity to the phosphorus atoms, the resultant phosphorus-containing cleavage products being of small molecular size. On the other hand, the initial peptic rupture occurs at linkages remote from the phosphorus atoms, the phosphorus-containing cleavage products being of much greater molecular dimensions and therefore insoluble in trichloroacetic acid.

It appeared from the results of these experiments that the same method of attack might be usefully employed in elucidating the structure of lecithovitellin.

EXPERIMENTAL.

Preparation of lecitho-vitellin.

The intact yolks of 24 eggs were washed in running water and finally in 0.9 % NaCl until free from egg-white. The yolk membrane was punctured and the contents, without membrane and chalazae, mixed with an equal volume of 10 % NaCl and extracted with an equal volume of ether in a mechanical shaker. Extraction was repeated until the ether was colourless and no qualitative test for lecithin could be obtained. Usually 6 or 7 thorough extractions were required, each with an amount of ether equal to the volume of the egg-yolk solution. The saline solution was poured into 20 volumes of distilled water and, after standing overnight, the supernatant liquid was siphoned off from the white protein precipitate. The precipitate was centrifuged and dissolved in 10 % NaCl; the solution was then filtered and poured into 20 volumes of water. The dissolution and precipitation were repeated, the precipitate being washed well with water before centrifuging for the last time. The protein was extracted with cold 80 % and then 97 % ethyl alcohol for 3 hours each and the residue filtered off, washed with absolute alcohol, ether and finally toluene. The resulting protein was easily friable and passed through a 60-mesh sieve.

Two such preparations of lecitho-vitellin were made, identical procedures being followed. The method given differs in two respects from those recorded in the literature: (1) the use of cold ethyl alcohol in the final lipid extraction; and (2) the addition of toluene after ether as a dehydrating agent (recommended by Van Slyke [1923] for caseinogen). The two products were alike in appearance and general behaviour. Analysis gave the following results:

	I	II
	%	%
Moisture	22.42	23.9
(Nitrogen	15.78	16.78
% dry weight { Phosphorus	0.83	1.03
Ash	2.20	2.38

These show very fair agreement with the analyses of Osborne and Campbell [1900] and Calvery and White [1930].

It is stated that pure vitellin is insoluble in water and almost insoluble in dilute alkali; vitellin in loose combination with lecithin is relatively soluble. Both the above preparations were soluble up to 1 % in approximately $M/40$ KOH. Though insoluble directly in acid, an acid solution could readily be obtained by the addition of acid to the original alkaline solution. The solubility of the preparations at first suggested that they still contained considerable quantities of lecithin, but extraction of the protein with boiling ethyl alcohol showed that the lecithin content could not be more than 2 %.

Experiments were begun with preparation I but unfortunately this did not suffice for all the experiments, and for some of the later experiments the second preparation had to be used. A determination of the N/P ratio of the substrate solution was however made during each experiment, and these figures are recorded in the tables. In evaluating the changes of N/P ratio which occur during the course of each digestion, due consideration must be given to the initial value of this ratio.

Vitellin solutions. In making solutions of the protein for the enzymic hydrolyses we adopted the following method. A weighed quantity (about 2 g.) of vitellin was rubbed up to a smooth paste with a little water, further water was then added to a total volume of about 50 ml.; 2.5 ml. M KOH per g. of vitellin were then added slowly and the rubbing up continued until the preparation, which first became viscous, again liquefied. The total volume was now made up to 75 ml. per g. of vitellin and the solution centrifuged after standing overnight. The supernatant liquid was used for the digestion experiment. The solution so obtained had p_H approximately 9 and, when added to the buffer of p_H 8.8 for the trypsin experiments, did not materially alter the p_H of the latter. For the pepsin experiments a predetermined quantity of N HCl was added to the vitellin solution to bring the p_H approximately to 2 before mixing with the acid buffer. The vitellin, at first precipitated by this acid, redissolved immediately on further addition of acid. Determination of the p_H at various stages during the enzymic hydrolyses showed that it remained well within the optimum range for each enzyme.

By this method we were never able to prepare solutions of higher concentration than about 1 %. This was unfortunate since the nitrogen and phosphorus contents of the enzyme and substrate controls then formed not inconsiderable proportions of the total nitrogen and total phosphorus present. In all digestions substrate and enzyme controls were followed throughout the time course of the experiment, and the tabulated values are net values.

Enzyme solutions. Solutions of B.D.H. trypsin and B.D.H. pepsin were used. The concentration of 0.75 % was chosen after carrying out 2 experiments, one with trypsin and one with pepsin, in which a series of different concentrations of enzyme was allowed to act on the substrate for a period of 2 hours. The results of these experiments are reported in Tables IIIA and IIIB.

Buffer solutions. The buffer solutions consisted of a KCl-borate mixture of p_H 8.8 and a KCl-HCl mixture of p_H 1.8. On occasion these were employed in a concentration 4 times that given in the previous paper in order that the volume saved thereby might be utilised for additional substrate solution (see below).

Procedure.

This was identical with that described in the previous paper [Stirling and Wishart, 1932] except that the digest-tubes on occasion contained 8 ml. of substrate and 1 ml. of the more concentrated buffer instead of 5 ml. and 4 ml. respectively. A large number of experiments were carried out in which the time-

Table IA. *Trypsin-kinase* (7.5 mg. in 10 ml. solution).

		Exp. no.		5		6	
		Total N (mg. per 10 ml. sol.)		11.61		5.68	
		Total P (mg. per 10 ml. sol.)		0.538		0.348	
		Exp. 5		Exp. 6			
		Acid-sol. N		Acid-sol. P		Acid-sol. N	
		Acid-sol. P				Acid-sol. P	
Time		Amount	% of	Amount	% of	Amount	% of
		mg.	total	mg.	total	mg.	total
10 mins.		0.53	4.6	0.059	11.0	0.43	7.6
20 mins.		0.97	8.3	0.095	17.7	0.69	12.1
30 mins.		1.35	11.6	0.119	22.1	0.88	15.5
40 mins.		1.58	13.6	0.126	23.4	1.13	20.0
50 mins.		1.98	17.0	0.149	27.6	1.32	23.2
60 mins.		2.17	18.7	0.173	32.1	1.50	26.4
2 hrs.		3.14	27.0	0.221	41.0	1.95	34.2
3 hrs.		3.91	33.7	0.238	44.2	2.27	39.9
4 hrs.		4.54	39.1	—	—	2.65	46.6
5 hrs.		5.08	43.8	0.255	47.2	2.73	48.0
6 hrs.		5.36	46.2	0.268	49.9	2.98	52.5
9 hrs.		6.72	57.9	0.306	57.0	3.19	56.2
12 hrs.		7.13	61.4	0.335	62.0	3.60	63.3
24 hrs.		8.39	72.3	0.376	70.0	4.62	81.3
						0.239	68.5

Table IB. *Pepsin* (7.5 mg. per 10 ml. solution).

		Exp. no.		8		9	
		Total N (mg. per 10 ml. sol.)		5.31		9.82	
		Total P (mg. per 10 ml. sol.)		0.237		0.460	
		Exp. 8		Exp. 9			
		Acid-sol. N		Acid-sol. P		Acid-sol. N	
		Acid-sol. P				Acid-sol. P	
Time		Amount	% of	Amount	% of	Amount	% of
		mg.	total	mg.	total	mg.	total
10 mins.		1.89	35.6	0.039	16.4	2.46	25.0
20 mins.		2.63	49.6	0.058	24.4	3.55	36.1
30 mins.		2.88	54.4	0.069	29.0	4.35	44.3
40 mins.		3.02	57.0	0.074	31.1	4.78	48.7
50 mins.		3.26	61.5	0.076	31.9	5.17	52.7
60 mins.		3.37	63.6	0.072	30.3	5.44	55.4
2 hrs.		3.44	64.9	0.065	27.3	6.28	63.9
3 hrs.		3.86	72.8	0.058	24.4	6.50	66.2
4 hrs.		3.99	75.2	0.083	34.9	6.84	69.6
5 hrs.		4.12	77.6	0.071	29.8	6.94	70.6
6 hrs.		4.21	79.4	0.063	26.5	7.15	72.8
9 hrs.		4.21	79.4	0.063	26.5	7.41	75.5
* 12 hrs.		4.20	79.2	0.050	21.0	7.64	77.8
24 hrs.		4.40	82.8	0.044	18.5	6.83	69.5
						0.124	27.0

* 15 hrs. in exp. no. 8.

course of the digestion of various concentrations of substrate by 0.75 % enzyme was followed. Of these, 2 experiments for each enzyme are given in Tables I A (trypsin) and I B (pepsin). Graphs are also given for 1 experiment with each enzyme (Figs. 1 and 2). The nitrogen-phosphorus ratios of the acid-soluble and acid-insoluble material at each stage in these experiments are given in Tables II A and II B.

Table II A. *N : P ratios.*

Exp. no.	5		6	
N : P ratio of original substrate	21.6		16.4	
	Exp. 5		Exp. 6	
Time	Acid-sol.	Acid-insol.	Acid-sol.	Acid-insol.
10 mins.	9.1	23.2	7.9	18.0
20 mins.	10.2	24.1	8.1	19.0
30 mins.	11.3	24.6	9.1	19.2
40 mins.	12.6	24.4	10.2	19.2
50 mins.	13.2	24.8	11.5	18.7
60 mins.	12.6	25.8	16.1	16.4
2 hrs.	14.2	26.7	13.0	18.9
3 hrs.	16.5	25.7	14.0	18.4
4 hrs.	—	—	14.1	18.9
5 hrs.	19.9	23.1	14.6	18.4
6 hrs.	20.0	23.2	15.6	17.2
9 hrs.	22.0	21.1	14.8	18.8
12 hrs.	21.2	22.0	16.8	15.6
24 hrs.	22.1	19.9	19.4	9.8

Table II B. *N : P ratios.*

Exp. no.	8		9	
N : P ratio of original substrate	22.4		21.5	
Exp. 8		Exp. 9		
Time	Acid-sol.	Acid-insol.	Acid-sol.	Acid-insol.
10 mins.	48.6	17.1	—	—
20 mins.	45.5	15.0	49.4	16.1
30 mins.	41.7	14.4	50.0	14.6
40 mins.	40.9	14.0	45.1	14.2
50 mins.	43.1	12.7	49.3	13.1
60 mins.	46.8	11.7	48.3	12.6
2 hrs.	52.8	10.8	51.0	10.5
3 hrs.	66.6	8.1	50.9	10.0
4 hrs.	48.0	8.5	52.9	9.0
5 hrs.	58.0	7.4	52.2	8.8
6 hrs.	66.8	6.3	54.5	8.1
9 hrs.	66.8	6.3	57.5	7.3
*12 hrs.	84.0	5.9	60.2	6.5

* 15 hrs. in exp. no. 8.

Tables III A and III B show the results obtained when the same substrate solution was digested for a uniform period of 2 hours with enzyme concentrations varying from 100 mg. to 1 mg. per 10 ml.

In addition to these, a third type of experiment was carried out which has no parallel in the previous paper. The production of an insoluble precipitate, analogous to that named "haematogen," by Bunge, was a striking feature of the peptic digests. We therefore carried out a series of digestions with enzyme concentrations of 100 mg. and 20 mg. of pepsin per 10 ml. in which no trichloro-acetic acid was added, but the insoluble material was quickly filtered off, and the nitrogen and phosphorus contents of the filtrate were examined. Table IV A records the results of this experiment and shows the early formation of this "haematogen" material and its remarkable resistance to peptic hydrolysis. Table IV B records the nitrogen-phosphorus ratios for filtrate and residue in this experiment.

Table IIIA. *Effect of varying trypsin concentration.*

Total N 8.95 mg. per 10 ml.
Total P 0.579 mg. per 10 ml.
N : P ratio of original substrate 15.5
Time of incubation 2 hours.

Trypsin (mg. per 10 ml. sol.)	Acid-sol. N as % of total N	Acid-sol. P as % of total P	N : P ratio	
			Acid-sol. material	Acid-insol. material
50	82.5	61.8	20.6	7.1
25	74.5	58.2	19.8	9.4
10	56.7	40.6	21.6	11.3
5	40.7	38.2	16.5	14.8
2.5	27.2	34.5	14.4	15.9
1	15.1	19.3	12.0	16.3

Table IIIB. *Effect of varying pepsin concentration.*

Total N 11.10 mg. per 10 ml.
Total P 0.577 mg. per 10 ml.
N : P ratio of original substrate 19.2
Time of incubation 2 hours.

Pepsin (mg. per 10 ml. sol.)	Acid-sol. N as % of total N	Acid-sol. P as % of total P	N : P ratio	
			Acid-sol. material	Acid-insol. material
100	77.8	38.1	39.3	6.9
50	77.2	32.2	46.1	6.5
25	74.7	30.7	46.7	7.0
10	69.7	30.1	44.5	8.3
5	61.3	29.2	40.3	10.5
1	34.3	15.9	41.4	15.0

Table IVA.

Exp. no.	10	11
Total N (mg. per 10 ml.)	8.08	8.08
Total P (mg. per 10 ml.)	0.501	0.501
Enzyme conc.	100 mg.	20 mg.

Time	Composition of filtrate			
	% N	% P	% N	% P
1 hr.	86.5	25.5	80.0	19.9
2 hrs.	86.1	27.5	79.3	22.4
3 hrs.	—	—	78.5	22.8
4 hrs.	—	—	79.8	22.8
9 hrs.	—	—	82.7	25.3
24 hrs.	—	—	87.8	27.2

Table IVB. *N : P ratios of filtrate and residue.*

Exp. no.	10	11
N : P ratio of original substrate	16.1	16.1
Enzyme conc.	100 mg.	20 mg.

Time (hrs.)	N : P ratio of			
	Filtrate	Residue	Filtrate	Residue
1	54.5	2.9	64.7	4.0
2	50.3	3.1	57.1	4.3
3	—	—	55.5	4.5
4	—	—	56.3	4.25
9	—	—	52.6	3.7
24	—	—	52.1	2.7

DISCUSSION.

There are certain points of resemblance between the experiments now reported and those recorded by Stirling and Wishart [1932], but the resemblance is not complete—a circumstance which may be ascribed to the apparently greater complexity of the lecitho-vitellin molecule.

As with caseinogen, the first step in the degradation of the protein by either enzyme does not yield products which are completely soluble in trichloroacetic acid. This is evident from the N/P ratios of the earlier stages in Tables II_A and II_B, and it confirms the criticism, made in the previous paper, concerning experiments on the kinetics of enzyme action which are based on the assumption that trichloroacetic acid precipitates only the unattacked substrate.

As with caseinogen there is a preferential liberation of acid-soluble phosphorus in tryptic digests and of acid-soluble nitrogen in peptic hydrolysis. With trypsin however the resemblance is only superficial. With the lower concentrations of caseinogen substrate, all the phosphorus was rendered acid-soluble, so that the nitrogen-phosphorus ratio of the acid-insoluble material gradually rose to infinity. In the present experiments (Table I_A) the preferential liberation of phosphorus by trypsin ceases when about half of the nitrogen and phosphorus have been rendered acid-soluble, and thereafter the nitrogen liberation may even gain on that of the phosphorus. A similar condition is revealed in the results shown in Table III_A, where the time of reaction was kept constant, and the enzyme concentration was varied. With the lower enzyme concentrations more phosphorus than nitrogen was rendered acid-soluble; with concentrations which would liberate about 40 % of the total nitrogen the percentage liberations of nitrogen and phosphorus were equal; while, with the stronger enzyme concentrations, there was a relatively greater liberation of acid-soluble nitrogen.

It is also noteworthy that in none of the experiments with lecitho-vitellin, however prolonged the digestion, or however strong the enzyme concentration, have we been able to secure a liberation of more than about 80 % of the total nitrogen and 70 % of the total phosphorus of the substrate.

The results of Stirling and Wishart [1932] on caseinogen were explicable on the assumption that the phosphorus of caseinogen was concentrated in one relatively small group. The results of the time-course experiments on lecitho-vitellin, together with the maxima of hydrolysis, suggest that such a hypothesis will not hold for lecitho-vitellin, and that there must be at least two phosphoric complexes in the molecule, one having similar properties to that of caseinogen and the other being highly resistant to tryptic hydrolysis.

Turning to the peptic digestions, Tables I_B and III_B show that there is a preferential liberation of acid-soluble nitrogen. In Tables IV_A and IV_B it may be seen that, with an enzyme concentration of 20 mg., the shorter periods of digestion result in the formation of an insoluble material or "haematogen" embodying about 20 % of the original nitrogen and 77 % of the original phosphorus, thus giving a N/P ratio of just over 4. This "haematogen" is obviously resistant to further peptic hydrolysis since it remains unchanged for at least 4 hours in the presence of the weaker enzyme concentration. Indeed, it is only at the ninth and twenty-fourth hours at this enzyme concentration that further degradation of the "haematogen" is observed. The change is however slight and, as Table IV_A shows, affects the nitrogen slightly more than the phosphorus. With the stronger enzyme concentration degradation is more rapid.

Turning now to Exps. 8 and 9 (Tables I_B and II_B), in which the cleavage products were separated by trichloroacetic acid, it appears that "haematogen"

is acid-insoluble—a fact which we have confirmed directly—and that digestion is confined mainly to the non-haematogen portion, proceeding towards the attainment of a N/P ratio in the acid-insoluble material approximating to that of the insoluble material of Tables IV A and B. (The ratios are not identical, but due weight must be given to the fact that in Table IV B the N/P ratio of the original substrate is 16, while in Table II B, it approximates to 21.)

In the digestion of the non-haematogen portion the liberation of the phosphorus of this fraction in acid-soluble form is more rapid than that of the nitrogen of the same fraction. This can be brought out by expressing the amounts of acid-soluble nitrogen and phosphorus, say in Exp. 9 (Table I B), not as percentages of the total nitrogen and phosphorus, but as percentages of the non-haematogen nitrogen and phosphorus, these being taken as 7.64 mg. and 0.13 mg. respectively. The results are as follows:

Experiment No. 9. To show relative rates of liberation of phosphorus and nitrogen from the non-haematogen portion.

Total non-haematogen-nitrogen assumed to be	7.64 mg.	
Total non-haematogen-phosphorus assumed to be	0.130 mg.	
Time	Acid-sol. N as % of total non-haematogen-N	Acid-sol. P as % of total non-haematogen-P
10 mins.	32.2	—
20 mins.	46.4	55.5
30 mins.	56.8	67.0
40 mins.	62.6	81.6
50 mins.	67.6	80.8
60 mins.	71.1	87.0
2 hrs.	82.0	94.8
3 hrs.	85.1	98.5
4 hrs.	89.8	99.1
5 hrs.	91.0	104.0
6 hrs.	93.6	101.0
9 hrs.	97.0	—
12 hrs.	100.0	—
24 hrs.	89.2	—

These observations seem to allow us to envisage at least 4 stages in the peptic digestion of lecitho-vitellin, which, although occurring for the most part simultaneously, may be thus separately described. First, a cleavage of the protein molecule into 2 fractions, one containing 80 % of the original nitrogen and 23 % of the phosphorus, and the other being "haematogen." Second, in the nitrogen-rich fraction, disruption of peptide linkages close to its phosphorus group leads to the rapid liberation of all this phosphorus in acid-soluble form, leaving a residue which is entirely nitrogenous and still of sufficient complexity to be acid-insoluble. The third stage is the disruption of this nitrogenous

residue into acid-soluble fractions. Finally there is the very slow and feeble digestion of the "haematogen" portion of which we have but slight evidence. This series of reactions is shown concisely in the diagram (p. 557).

It is evident that such a conception of the process of peptic digestion requires the presence of at least two widely separated or dissimilar phosphoric complexes in the vitellin molecule. This assumption is in accordance with the observations on tryptic digestion already discussed.

In this connection it is interesting to note that in tryptic digestion only 70 % of the protein-phosphorus could be rendered acid-soluble, whilst in peptic digestion a somewhat similar amount (77 %) of the phosphorus remained acid-insoluble as the "haematogen" fraction and was highly resistant to further peptic digestion. One might be tempted to assume identity between these fractions. Such an assumption seems however to be unwarranted in view of Posternak's [1927] preparation from the digestion of the residue from ether extraction of egg-yolk of a fraction rich in both phosphorus and iron, which is highly resistant to both peptic and tryptic actions.

SUMMARY.

The action of trypsin-kinase and pepsin on lecitho-vitellin has been studied. Products are formed which are not completely soluble in trichloroacetic acid. With pepsin the rate of liberation of acid-soluble nitrogen is greater than that of acid-soluble phosphorus, and the production of "haematogen" with a N/P ratio of 4.25 is a striking feature of these digests.

With trypsin the rate of liberation of acid-soluble phosphorus relative to that of acid-soluble nitrogen has been shown to depend on the enzyme concentration employed. The maximum of hydrolysis is reached, however, at all concentrations at 80 % of the total nitrogen and 70 % of the total phosphorus.

It is considered that the vitellin molecule must contain at least two widely separated or dissimilar phosphoric complexes, one being highly resistant to enzymic attack.

REFERENCES.

- Bunge (1885). *Z. physiol. Chem.* **9**, 49.
Calvery and White (1930). *J. Biol. Chem.* **94**, 635.
Holter, Linderstrøm-Lang and Funder (1932). *Z. physiol. Chem.* **260**, 85.
Hugounenq and Morel (1905, 1). *Compt. Rend. Acad. Sci.* **140**, 1065.
— (1905, 2). *Compt. Rend. Acad. Sci.* **141**, 848.
— (1906). *Compt. Rend. Acad. Sci.* **142**, 805.
Kay and Marshall (1928). *Biochem. J.* **22**, 1264.
Miescher (1870). *Med.-chem. Untersuchungen*, **4**, 502.
McFarlane (1932). *Biochem. J.* **26**, 1061.
Osborne and Campbell (1900). *J. Amer. Chem. Soc.* **22**, 413.
Plimmer (1908). *J. Chem. Soc.* **93**, 1500.
Posternak (1927). *Compt. Rend. Acad. Sci.* **184**, 909.
Rimington (1927, 1). *Biochem. J.* **21**, 204.
— (1927, 2). *Biochem. J.* **21**, 1179, 1187.
Rimington and Kay (1926). *Biochem. J.* **20**, 777.
Stirling and Wishart (1932). *Biochem. J.* **26**, 1989.
Van Slyke (1923). *Proc. World's Dairy Congress*, **2**, 1145.